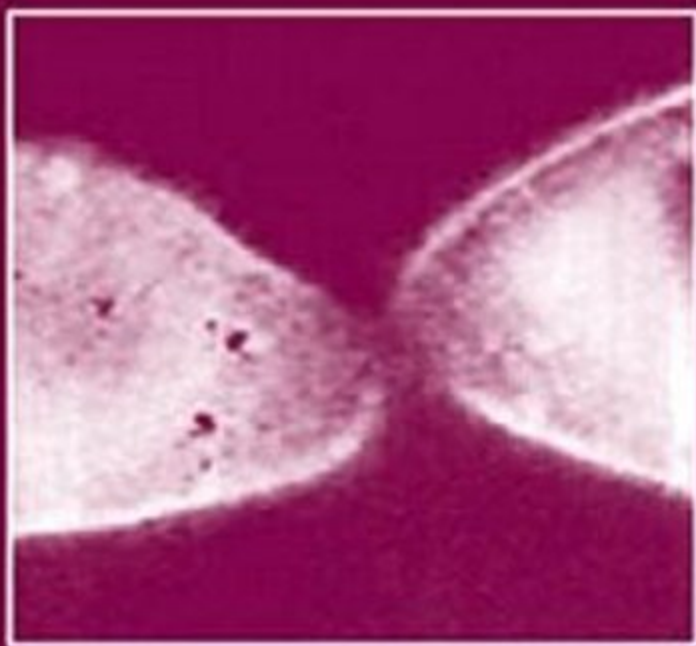


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CELLULAR MECHANISM FOR TARGETING HETEROCHROMATIN FORMATION IN *DROSOPHILA*

Joel C. Eissenberg^{*} and Gunter Reuter[†]

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Abstract

Near the end of their 1990 historical perspective article “60 Years of Mystery,” [Spradling and Karpen \(1990\)](#) observe: “Recent progress in understanding variegation at the molecular level has encouraged some workers to conclude that the heterochromatinization model is essentially correct and that position-effect variegation can now join the mainstream of molecular biology.” In the 18 years since those words were written, heterochromatin and its associated position effects have indeed joined the mainstream of molecular biology. Here, we review the findings that led to our current understanding of heterochromatin formation in *Drosophila* and the mechanistic insights into heterochromatin structural and functional properties gained through molecular genetics and cytology.

Key Words: *Drosophila*, Heterochromatin, Position-effect variegation, Chromatin. © 2009 Elsevier Inc.

1. INTRODUCTION

The term “heterochromatin” was coined by [Heitz \(1928\)](#) as the material he observed in liverwort nuclei that failed to disappear after telophase in the mitotic cell cycle. In subsequent studies ([Zacharias, 1995](#)), Heitz showed that *Drosophila* somatic nuclei contained heterochromatic material resembling what he had seen in plants. He noted the heterochromatic Y chromosome of *Drosophila* and initially characterized the heterochromatin as “genically passive.” Since that time, heterochromatin has been the subject of considerable research and conjecture. The extraordinary cytology afforded by the giant polytene chromosomes of *Drosophila* third-instar-larval salivary glands, combined with the rapid accumulation of cytological aberrations and genetic mutations, made *Drosophila* the organism of choice for the elucidation of heterochromatin properties. Although both euchromatin and heterochromatin are composed of DNA and are packaged into nucleosomes, the sequence composition in heterochromatin and the structural modifications of histones in heterochromatin are distinctive, respectively. Heterochromatin-associated nonhistone proteins have been identified through genetic and biochemical approaches. Much of the success in the molecular dissection of heterochromatin has been the result of genetic screens that identified modifiers of heterochromatin silencing activity.



2. HETEROCHROMATIN DOMAINS IN THE *DROSOPHILA* GENOME

2.1. Cytological heterochromatin in *Drosophila*

2.1.1. Pericentric heterochromatin

2.1.1.1. Pycnotic appearance In *Drosophila*, as in all higher eukaryotes, certain regions of each chromosome do not cyclically change their degree of condensation between interphase and metaphase but remain condensed throughout most of the cell cycle. These regions comprise the heterochromatin of the genome and include substantial amounts of the chromatin surrounding each centromere (pericentric heterochromatin). Regions that show an allocyclic behavior are collectively called euchromatin. During interphase, euchromatin is differentially packed too, and in polytene giant chromosomes of certain larval tissues, this differential condensation of interphase euchromatin is visible as transverse bands of condensed chromatin. This higher-order structure of chromatin and the mechanism of its assembly are by no means understood. The non-DNA moiety of chromatin represents not only a structural component, but is implicated in fundamental regulatory processes. During development, the determined programs of gene activity are stably inherited over mitoses; this cell memory information stored in the chromatin has been called “epigenetic information.” The mechanisms of establishment and maintenance of epigenetic information are now being dissected and the biochemical and genetic tools to detect and manipulate epigenetic marks are becoming available (see the following sections).

2.1.1.2. Under-replication in polytene chromosomes The giant polytene chromosomes of *Drosophila* larval salivary glands (and various other larval tissues as well) are the result of multiple rounds of chromosome replication in the absence of mitosis. The replication products in euchromatin remain paired, giving rise to large bundles of chromatin fibers (Zhimulev *et al.*, 2004). In wild-type polytene chromosomes, both homologs pair. The alternating intervals of chromatin condensation and decondensation along the axis of each chromosome arm result, in the paired polytene chromosome bundles, in the appearance of transverse bands of condensed chromatin alternating with interbands of decondensed chromatin.

Relative to the euchromatin, most of the pericentric heterochromatin in polytene tissues is under-replicated (Gall *et al.*, 1971), giving rise to the amorphous, attenuated appearance of this material. Remarkably, certain loci that reside in pericentric heterochromatin are nonetheless fully polytenized, even as flanking repetitious DNA sequences are severely under-replicated (Berghella and Dimitri, 1996; Zhang and Spradling, 1995).

In addition, the pericentric regions of all the polytene chromosomes coalesce into a single structure called the chromocenter.

Using a combination of mutations in genes encoding the heterochromatin-associated proteins SU(UR) and SU(VAR)3–9 (discussed later in this chapter), Zhimulev and colleagues were able to force the polytenization of pericentric heterochromatin (Andreyeva *et al.*, 2007). Under these conditions, the polytenized heterochromatin forms banded material that resembles euchromatin, permitting *in situ* hybridization mapping of specific genes in formerly heterochromatic regions. Immunolocalization on polytenized heterochromatin showed a number of bands that stained for HP1 and for histone H3 dimethylated at lysine 9 (H3K9me2). Presumably, these sites represent the vestigial staining sites previously observed within the heterochromatic chromocenter in *Su(var)3–9* homozygous mutant chromosomes (Schotta *et al.*, 2002). Interestingly, the remaining HP1 and H3K9me2 show relatively little overlap, suggesting that HP1 is targeted by a distinct mechanism from that used elsewhere in the genome (discussed later in this chapter). While the polytenization of heterochromatin by doubly inactivating SU(UR) and SU(VAR)3–9 creates a high-resolution picture of the DNA organization in this elusive region, caution should certainly be exercised in interpreting the distributions of proteins and chromatin modifications, considering that both SU(UR) and SU(VAR)3–9 are themselves being chromatin modifying proteins. Their loss could have secondary effects on gene expression and chromatin structure.

Heterochromatin protein distribution has mainly been studied in larval salivary gland chromosomes. Some mutations (e.g., *otu*) cause formation of polytene chromosomes in female ovarian nurse cells with morphology similar to salivary gland chromosomes (Heino, 1989; King *et al.*, 1981). Although similar in banding pattern, most of the cytological manifestations of heterochromatin are significantly less pronounced in nurse cell polytene chromosomes (Mal'ceva and Zhimulev, 1993). Interestingly, the heterochromatin proteins HP1, SUUR, and SU(VAR)3–9 are more abundant (Koryakov *et al.*, 2006). For the chromosomal distribution of SU(VAR)3–9, besides chromocentric heterochromatin, more than 200 additional binding sites along the euchromatic arms are detected. In these chromosomes, SU(VAR)3–9 binding only depends on SUUR in autosomes but not in the X chromosomes. Since ovarian nurse cells represent germ line cells, these findings suggest differential organization of heterochromatin in somatic and germ line chromosomes.

2.1.2. Intercalary heterochromatin

The term “intercalary heterochromatin” was first coined to describe certain sites along the euchromatic polytene X chromosome that showed an elevated frequency of breakage in squash preparations, similar to that seen at the heterochromatic base of the X (Kaufmann, 1939). This property has

been generalized to sites on the major autosomal arms as well, and the definition of intercalary heterochromatin has been expanded to include sites that are relatively under-replicated in polytene chromosomes and that undergo a thread-like physical association with the chromocenter termed “ectopic pairing” (Zhimulev, 1998; Zhimulev *et al.*, 2003a). The definition of intercalary heterochromatin, like the definition of heterochromatin itself, is a cytological one. As the genetic dissection of heterochromatin has progressed, and molecular probes for chromosomal proteins have been developed, similarities and distinctions between intercalary and pericentric heterochromatin have emerged (Zhimulev and Belyaeva, 2003).

The SUUR protein controls under-replication of intercalary heterochromatin as well as pericentric heterochromatin in polytene tissue (Belyaeva *et al.*, 1998). Many of these regions contain unique genes (e.g., BX-C and ANT-C) and about 60% of sites of intercalary heterochromatin show binding of PC-G proteins, suggesting that intercalary heterochromatin reflects silenced genes (Belyakin *et al.*, 2005).

2.1.3. Telomeric heterochromatin

The sequence organization of telomeric DNA in *Drosophila* is unusual among eukaryotes. Rather than being composed of monotonous polymers of short repeats synthesized by telomerase, *Drosophila* telomeres are built and maintained by the saltatory addition of copies of the HeT-A and TART non-LTR retrotransposable elements (Biessmann *et al.*, 1990; Cenci *et al.*, 2003a; Danilevskaya *et al.*, 1994; Levis *et al.*, 1993; Young *et al.*, 1983).

The tendency of telomeres to undergo ectopic pairing in polytene chromosomes (Hinton and Atwood, 1941; Kaufmann and Gay, 1969) gives them the impression of being heterochromatic. They are also sites of binding for the heterochromatin-associated protein HP1 (Fanti *et al.*, 1998; James *et al.*, 1989; discussed later in the following sections), which seems to have a role in telomere capping (Fanti *et al.*, 1998) and telomere length regulation (Savitsky *et al.*, 2002). Like the pericentric and intercalary heterochromatin, telomeres are under-replicated in polytene salivary gland chromosomes (George *et al.*, 2006).

As will be discussed further, transgene insertions into subtelomeric regions in *Drosophila* show variegated silencing, but the genetic basis for this silencing appears to be distinct from that of pericentric heterochromatin.

2.1.4. Y chromosome

The Y chromosome of *Drosophila* is entirely heterochromatic in somatic tissue. Like pericentric heterochromatin, the Y is under-replicated in polytene tissue (Gall *et al.*, 1971). Chromosome translocations with breakpoints anywhere in the Y chromosome can cause heterochromatic position-effect silencing, and transposon insertions into the Y also experience heterochromatic silencing (Zhang and Spradling, 1994).

A supernumerary Y chromosome can suppress heterochromatic position-effect silencing (discussed later in this chapter) that occurs in chromosome rearrangements (Gowen and Gay, 1934). This suppression has since been confirmed for all rearrangements that variegate for euchromatic loci and has been generalized into a rule by Morgan and Schultz (1942). Noujdin (1936, 1944) first reported the suppression of PEV by additional heterochromatin of either of the Y chromosome arms. Schultz (1936) extended the analysis of the effect of the Y chromosome aneuploidy on PEV and first described the strong enhancement due to a loss of Y chromosome in XO males. He was also the first to observe that susceptibility of variegation to Y chromosome aneuploidy represents a diagnostic feature for PEV. Since then, the modifier effect of Y chromosome aneuploidy has been used to discriminate between PEV and other cases of variegated gene expression (Gans, 1953; Becker, 1957).

The suppressive effect of additional heterochromatic material can best be understood if the heterochromatic regions in the nucleus are considered to be a sink for limiting quantities of silencing factors. For euchromatic genes experiencing silencing by neighboring heterochromatin, the silencing complexes propagated across the heterochromatic breakpoint would be recruited by additional heterochromatic material in *trans*, depleting them from the variegation-inducing site and thereby relieving silencing. The sequestering effect of additional heterochromatin may account for the curious observation of Cooper (1955) that, in XXYY females and XYYY males having an otherwise wild type chromosomal constitution, the adult eye becomes variegated, with large patches of bleached pigmentation. This effect could be explained if the consequence of such a large amount of additional heterochromatin was the misregulation of normally heterochromatic genes (such as *light*) even within their normal chromosomal context.

Taken together, the genetics and cytology of the Y chromosome in *Drosophila* argue that it is an example of heterochromatin. Since the Y chromosome is required in males for fertility and is extensively decondensed and transcribed in primary spermatocytes (Bonaccorsi *et al.*, 1988), it is probably best regarded as facultative heterochromatin: heterochromatic in somatic tissue but euchromatic in the germ line.

2.2. Genetic properties of heterochromatin in *Drosophila*

2.2.1. Gene silencing: Heterochromatic position-effect variegation

Beginning with Muller (1930), chromosome rearrangements that break within pericentric heterochromatin, the Y chromosome or the fourth chromosome have been associated with variegated gene silencing, a phenomenon called “position-effect variegation” (Spofford, 1976).

The term “position effect” was first used by Sturtevant (1925) and Dobzhansky (1932, 1936) to designate effects on gene action that were clearly dependent on a new position in the chromosome complement.

In this early period, the experimental work of geneticists on position effects was aimed at learning about chromosome organization and regulation of gene action. [Lewis \(1950\)](#) first classified the different phenomena of position effects into the relatively rare cases of a stable type and the more frequently observed variegated type position effects. For variegated type position effects, it was first shown by [Schultz \(1936\)](#) that the new position of a gene adjacent to heterochromatic regions is the cause for the variegated expression. Such rearrangements were first described by [Muller \(1930\)](#) in his classical experiments demonstrating the mutagenic action of X-rays, and referred to by him as “eversporting displacements,” “sport” being an early term referring to mutants. Between 1930 and 1940, all of the defining characteristics of PEV were described. [Morgan and Schultz \(1942\)](#) first defined a series of general rules which hold true for the cases of PEV he had studied. In the three classical reviews on PEV ([Baker, 1968](#); [Lewis, 1950](#); [Spofford, 1976](#)), these main rules have been further refined on the basis of a large amount of data then available.

2.2.2. Silencing requires the placement of the silenced locus in *cis* to silencing heterochromatin

This rule, according to [Baker \(1968\)](#), consists of demonstrating a wild-type function of the variegating gene after its restoration from the rearrangement back to a normal chromosome via crossover. This was shown by [Dubinin and Sidorov \(1935\)](#), [Panshin \(1935\)](#), and [Judd \(1955\)](#) in *Drosophila melanogaster*. Additional proof for the *cis* dominance came from revertant analysis ([Griffen and Stone, 1940](#); [Grüneberg, 1937](#); [Kaufmann, 1942](#); [Panshin, 1938](#)). Reversion of the variegated phenotype was found to be accompanied by reversion of the rearrangement or by relocation of the affected locus into a new position.

An apparent exception to this rule is the dominant variegation seen at the *brown* locus (discussed later in this chapter). In this case, a wild-type *brown* allele on the homologous chromosome is inactivated by a variegating rearrangement. It appears, however, that the underlying basis for the dominant variegation is the conjunction of two mechanisms: (1) the establishment of a heterochromatic domain as a result of chromosome rearrangement and (2) an unusual sensitivity of the *brown* regulatory machinery to pairing interactions which, in the case of a paired homolog associated with a heterochromatic breakpoint, results in *trans*-silencing. The mechanistic basis for pairing-dependent inactivation is unknown, but since inactivation is coupled to a mechanism which does fulfill the rules of PEV, the exceptional nature of this phenomenon is more apparent than real.

2.2.3. Additional dosage of heterochromatin titrates silencing

As discussed above, increasing the dose of the Y chromosome, X chromosome heterochromatin or the fourth chromosome all have the effect of suppressing position-effect variegation.

Interdependence between the amount of heterochromatin and the dosage of factors controlling heterochromatin formation is supported by identification of mutations displaying heterochromatin-sensitive lethality. Altogether, mutations for three genes (*Su(var)2-1*, *Su(var)3-3* and *bonus*) have been identified which display recessive lethal interaction with additional heterochromatin, such as an extra copy of the Y chromosome. The mutations are homozygous viable in XX females and XO males but semilethal in XY males and almost completely lethal if an additional Y chromosome (XXY females or XYY males) is present (Beckstead *et al.*, 2005; Reuter *et al.*, 1982b). The *Su(var)3-3* gene encodes the *Drosophila* homolog of the mammalian histone H3 lysine 4 demethylase LSD1 and was shown to control the balance between euchromatin and heterochromatin in early *Drosophila* embryogenesis (Rudolph *et al.*, 2007).

2.2.4. The silencing effect of heterochromatin diminishes with distance from nearby heterochromatin

The polar effect of inactivation for PEV was discovered as a consequence of extensive cytogenetic analyses by Demerec and coworkers (Demerec, 1940, 1941; Demerec and Slizynska, 1937) using different *white*- and *Notch*-variegating rearrangements. In the inversion *In(1)N²⁶⁴⁻⁵²*, variegation was observed for five linked genes located within a ca. 50-band interval adjacent to the heterochromatic breakpoint. Heterochromatin appears to propagate for a variable distance into euchromatin and cause the variegated expression of genes in PEV (Demerec, 1941; Prokofieva-Belgovskaya, 1939, 1941, 1947). Prokofieva-Belgovskaya (1947) summarized her results of a thorough cytological analysis of several different position-effect rearrangements (*sc⁸*, *w^{m5}*, *rst³*, and *w^{m4}*), finding that gene inactivation in all these rearrangements could be correlated with a visible heterochromatinization of the euchromatic regions immediately adjacent to the rearrangement breakpoint. She also studied the frequency of heterochromatinization under the influence of different modifiers of PEV like the presence of an additional Y chromosome, temperature of development, parental origin of the rearrangement, and age of parents. Most of these studies were performed with *In(1)sc⁸*, a rearrangement with visible variegation for the *yellow*, *achaete*, and *scute* genes, which had been already extensively used for a study of different PEV modifier effects by Noujdin (1944).

Using a series of secondary rearrangements derived from X-irradiation of *In(1LR)pn2a*, Gvozdev and colleagues (Tolchkov *et al.*, 2000) showed that the amount of heterochromatin at the breakpoint has a quantitative effect on variegation. Importantly, though, the presence of a centromere within the heterochromatic block had a stronger effect than a much larger block of paracentric heterochromatin lacking the centromere. This suggests that sequence composition of heterochromatin plays an important role in the induction and/or propagation of silencing heterochromatin. In addition

to the rules described earlier, there are a number of frequently (though not universally) observed properties associated with heterochromatic silencing.

2.2.5. The clonal nature of PEV

The clonal nature of gene silencing in PEV was suggested by mitotic twin spot analysis and comparisons to pattern formation during development of the *Drosophila* eye anlage. These studies were performed by [Becker \(1957\)](#) and [Baker \(1967\)](#) for the eye anlage of *D. melanogaster* and *D. virilis*, respectively. Comparison of clonal patches marked by X-ray-induced mitotic crossing-over induced at different developmental stages with the variegation pattern of different *white*-variegating rearrangements suggested that inactivation of the *white* gene is based on cell lineage and determined by the end of first larval instar, when about 20 presumptive eye cells are present ([Baker, 1967](#); [Becker, 1961, 1966](#)). [Janning \(1970, 1971\)](#) induced twin spots in the eye anlage at different times during development and analyzed their overlap with PEV induced mutant white spots in a *Dp(1;3)w^{m264-58}* background. Because clones induced at the end of the first larval instar overlap with the white PEV spots, the time of *white* inactivation by heterochromatin was inferred to be clonally decided at this time during development of the eye anlage.

An outstanding question in chromatin biology is whether levels of a histone modifier must be maintained continuously to set levels of gene expression or whether modifier levels initiate an epigenetic mark that is stable to changes in modifier levels later in development. Reuter and colleagues tested whether reduced SU(VAR)3–9 is required continuously in the *Drosophila* eye to set PEV levels. Clonal analysis with *Su(var)3–9* null mutations suggests that SU(VAR)3–9 controls heterochromatin formation by dimethylation of histone H3 at lysine 9 (H3K9me2) during early embryonic development ([Rudolph et al., 2007](#); [Fig. 1.1](#)). *Su+ / Su+* clones

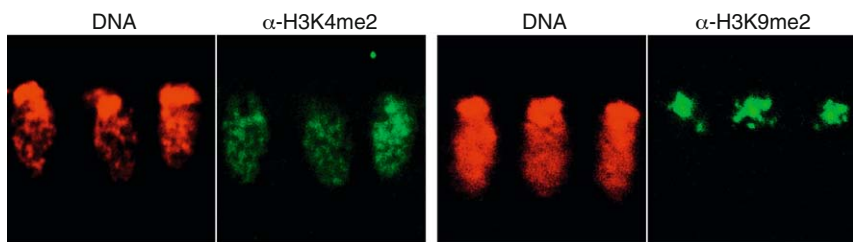


Figure 1.1 Establishment of the euchromatin-associated (H3K4me2) and heterochromatin (H3K9me2) histone methylation marks first occurs during early embryogenesis at the syncytial blastoderm stage when nuclei show an apico-basal polarity (“Rabl configuration”). Euchromatin, identified by diffuse H3K4me2 staining, is located towards the lower basal side of the nuclei. DAPI (“DNA”; red) intensely stains the pericentric heterochromatin at the upper apical pole, which is enriched in histone H3K9me2 methylation mark. Photo provided by Thomas Rudolph.

were induced in *Su(var)3-9/+* heterozygotes by the flipase/FRT mitotic recombination system. Although two wild type copies of the *Su(var)3-9* gene are present in the *Su+/Su+* clones, *white* gene silencing in the *w^{m4}* PEV rearrangement is only restored in large clones induced early in development. Reversion of the suppressor effect in late-induced clones does not occur with additional cell divisions, suggesting that after early establishment of heterochromatin, the chromatin state established by reduced *Su(var)3-9* dosage is stably maintained throughout consecutive development. This contrasts with another chromatin modifier, the histone H3 lysine 4 trimethylase TRITHORAX, which is required continuously to maintain HOX gene activity in *Drosophila* (Ingham, 1985). Thus, SU(VAR)3-9 functions in heterochromatin as part of a mechanism of epigenetic memory.

2.2.6. Paternal and parental effects

Although a basic analysis of paternal and maternal effects in PEV had been reported by Noujdin (1944), the analysis of such effects was mainly extended within a period (1950–1970) when PEV was only studied by few scientists. Genetic analysis of most basic characteristics of PEV was already advanced and experimental work was more focused on modifying factors. A maternal suppressor effect of an extra Y was first reported by Noujdin (1944) for the *In(1)sc⁸* rearrangement. The *sc⁸* homozygous female offspring showed a ca. eightfold reduction in variegation for *yellow* and *acheate* if additional Y chromosome material was present in the mother. Spofford (1976) reported a similar maternal suppressor effect of an additional Y chromosome for *Dp(1;3)N²⁶⁴⁻⁵⁸* and a similar effect was reported for the *white*-variegating rearrangement *Dp(1;3)w^{vc}* (Khesin and Bashkirov, 1978). Noujdin (1944) also reported a paternal suppressor effect of additional Y chromosome material for *In(1)y^{3P}* variegating for *yellow*. In other variegating rearrangements of *D. melanogaster*, no significant maternal or paternal effects of additional Y chromosome material have been reported. Schneider (1962) found a maternal suppressor effect of an additional Y chromosome in one of the six *peach*-variegating rearrangement of *D. virilis* studied.

Parental source of the rearrangement can also affect significantly the extent of variegation in the offspring. Such effects were described for *Dp(1;3)N²⁶⁴⁻⁵⁸* by Spofford (1959), and for *In(1)sc⁸* by Prokofieva-Belgovskaya (1947). In the case of *Dp(1;3)N²⁶⁴⁻⁵⁸*, variegation was more enhanced if the rearrangement was maternal in origin, whereas in *sc⁸*, heterochromatinization was enhanced with a paternal origin of the rearrangement.

A stable paternal effect was observed in crosses with several *Enhancer of variegation* [*E(var)*] mutations (Dorn et al., 1993). In a cross of *w^{m4}/w^{m4}+/+* females to *w^{m4}/Y E(var)/+* males the *w^{m4}/Y+/+* offspring males show enhanced *white* variegation, although the enhancer mutation is not present. In a series of crosses, it was demonstrated that the Y chromosome has

acquired a stable ability to enhance *white* variegation in w^{m4} independent of the genetic background. Once acquired, the enhancer effect of the Y chromosome is maintained through consecutive generations. The molecular basis for this Y chromosome imprinting effect is unknown.

2.2.7. Temperature sensitivity of PEV

As a rule, in *Drosophila*, low temperature of development enhances variegation of euchromatic genes subject to PEV, whereas a higher temperature shows a suppressor effect. This was first described for *white* variegating rearrangements by [Gowen and Gay \(1934\)](#). The effect of temperature on PEV extended to the polar effect of genetic inactivation as well as cytological condensation ([Hartmann-Goldstein, 1967](#); [Rudkin, 1965](#)). The temperature effect can be understood in the general context of thermal effects on protein folding if one imagines silencing as mediated by a proteinaceous complex: high temperature would weaken such a complex, while lower temperatures would stabilize it. There are consistent differences in the strength of the modifying effect of temperature on PEV when different rearrangements are compared. This might be due to differences in the genetic and molecular nature of the heterochromatin inducing PEV. Temperature-sensitive periods were mapped in order to determine the developmental stage(s) when gene inactivation in PEV takes place. In the rearrangement studied, the major temperature sensitive period was found during the first two days of puparium formation ([Becker, 1961](#); [Chen, 1948](#); [Hartmann-Goldstein, 1967](#); [Schultz, 1956](#)). Possible tissue-specific differences were indicated by results of a study of temperature-sensitive periods for PEV in salivary glands and Malpighian tubules ([Hartmann-Goldstein, 1967](#)).

By inspection of the giant polytene chromosomes of *Drosophila* larval salivary glands taken from PEV lines, the cytological site of the variegating locus loses its banded, fully polytenized euchromatic appearance in some nuclei, and to take on the disorganized, densely staining, attenuated appearance of heterochromatin ([Caspersson and Schultz, 1938](#); [Henikoff, 1981](#); [Kornher and Kauffman, 1986](#); [Prokofyeva-Belgovskaya, 1939](#)). Moreover, this structural dimorphism is sensitive to temperature ([Belyaeva and Zhimulev, 1991](#); [Prokofyeva-Belgovskaya 1947](#); [Schultz, 1941](#); [Zhimulev et al. 1988](#)) and to genetic modifiers of PEV ([Belyaeva and Zhimulev, 1991](#); [Reuter et al., 1982a](#); [Zhimulev et al., 1988](#)). In at least one case, the morphologically heterochromatic variegating locus recruits the normally heterochromatin-associated protein HP1 ([Belyaeva et al., 1993](#)). Thus, the cytological compaction of a variegating locus is taken to be a morphological manifestation of the silencing mechanism.

The chromosome structural changes that correlate with genetic silencing imply that the mechanism operates at the level of transcription. Indeed, a reduction in transcription of a variegating locus has been demonstrated by

in vivo pulse-labeling (Henikoff, 1981) and by quantitative mRNA blot hybridization (Henikoff and Dreesen, 1989; Kornher and Kauffman, 1986; Rushlow *et al.*, 1984; Wallrath and Elgin, 1995). While position-dependent changes in RNA turnover rates cannot rigorously be excluded in any of these cases, the simplest interpretation is that the silencing mechanism acts at the level of transcript synthesis. Central to the notion that epigenetic changes underlie the phenomenon of PEV is that no irreversible genetic change explains the silencing effect (although covalent changes in DNA structure may be a concomitant of the silencing mechanism in some cases). Thus, demonstrating that silencing can be efficiently reversed represents strong evidence that the mechanistic basis of silencing does not require the mutation or deletion of variegating genes.

Two recent studies bear on this point. Lu *et al.* (1996) used a ubiquitously inducible reporter for PEV to investigate the developmental progression of PEV silencing (see further for details on this system). They found that silencing of an *HSP70-lacZ* reporter was extensive in the third instar precursors of the adult eye (the eye imaginal discs) but was dramatically relaxed in the adult eye. This extensive relaxation of silencing during differentiation is difficult to explain as an efficient reversal of mutational inactivation. In a separate study, Ahmad and Golic (1996) used a *white* reporter flanked by the FRT recombination sites, subject to PEV silencing. When they mobilized somatic DNA excision with a heat shock-inducible FLP “flippase” activity, they found red-pigmented facets among the white sectors of the adult eye. This result suggests that when the *white* reporter escapes the chromosomal context by FLP-catalyzed excision and circularization as an episome, it recovers full function. Here too, such efficient recovery of function is best explained by a model of chromosome-dependent epigenetic silencing.

Interest in the phenomenon of heterochromatic PEV has intensified in the last 20 years because it has proven to be such a useful tool for the structural and functional dissection of heterochromatin and euchromatin (Eissenberg, 1989; Eissenberg and Wallrath, 2003; Grigliatti, 1991; Reuter and Spierer, 1992). As modifiers of PEV have been cloned and characterized, the vast majority have proven to encode chromosomal proteins or their modifiers. Many of these are evolutionarily conserved, arguing for a conserved mechanism for heterochromatin assembly and maintenance.

2.2.8. Centromere activity

In *Drosophila*, the centromeres of chromosomes are diffuse structures. The *Drosophila* X centromeric region, the best characterized of the *Drosophila* centromeres, consists of interspersed blocks of unique sequence and repetitive DNA (Le *et al.*, 1995; Murphy and Karpen, 1995). Using a series of deletions that remove blocks of pericentric X chromosome heterochromatin, Karpen *et al.* (1996) found evidence that centric heterochromatin

contains multiple pairing elements that are required for the proper alignment of achiasmate chromosomes in meiosis I in *Drosophila* females. Genetic screens for factors influencing the fidelity of meiotic chromosome transmission identified a number of genes previously implicated in heterochromatic position-effect variegation (Hari *et al.*, 2001; Le *et al.*, 2004).

Surprisingly, the pattern of histone modifications in the putative centromeric chromatin is distinct from those associated with pericentric heterochromatin (Sullivan and Karpen, 2004). Presumably, such differences reflect the distinctive role of centromeric chromatin in the formation of the kinetochore and the recruitment of microtubules during mitosis.

2.2.9. Suppression of meiotic recombination

The frequency of meiotic recombination is very low within the pericentric heterochromatin of *Drosophila* (Brown, 1940; Muller and Painter, 1932). Furthermore, the frequency of recombination relative to physical distance is reduced in the euchromatin near the pericentric heterochromatin and the telomeres of the major chromosome arms (Ashburner, 1989). A mechanistic link between the inhibition of meiotic recombination in heterochromatin and the position-effect silencing of gene expression observed at heterochromatic breakpoints is suggested by the observation that several mutations that cause dominant suppression of heterochromatic position-effect variegation (discussed later in this chapter) enhance recombination in pericentric heterochromatin (Westphal and Reuter, 2002). Thus, the mechanism that silences euchromatic gene as a result of position effects also interferes with normal meiotic recombination.

2.2.10. Suppressor of under-replication

The *Suppressor of Underreplication* [*Su(UR)*] locus was discovered serendipitously in a stock carrying an X-ray induced X chromosome rearrangement (Belyaeva *et al.*, 1998). The original *Su(UR)* allele is a semidominant, maternal-effect enhancer of polytenization of pericentric and intercalary heterochromatin in larval salivary gland and ovarian polytene chromosomes. Flies homozygous for *Su(UR)* mutation are viable, and the enhancement of polytenization of heterochromatin is more extreme. The protein encoded by the *Su(UR)* locus contains an AT hook domain and homology to the ATPase domain of SWI2/SNF2 family chromatin remodeling proteins (Makunin *et al.*, 2002). Outside of these motifs, though, there are no obvious homologs of *SU(UR)* in other organisms. In salivary gland polytene chromosomes, the *SU(UR)* protein is concentrated in the chromocenter and at sites of intercalary heterochromatin on the euchromatic arms.

Overexpression of *SU(UR)* protein in transgenic larvae results in a striking, temperature-sensitive distension, or swelling of the chromocenter and intercalary heterochromatin sites (Zhimulev *et al.*, 2003a). These swellings, unlike classical polytene chromosome puffs, are not a consequence of

transcription, since they do not accumulate tritiated uridine. EM micrographs reveal lacunae within these swellings, and at the light microscope level, the swellings are largely devoid of SU(UR) protein while still staining strongly for DNA.

In flies that are doubly mutant for *Su(UR)* and *Su(var)3-9* (which encodes a histone H3 lysine 9 methyltransferase; discussed later in this chapter), a further polytenization of the pericentric heterochromatin of the third chromosome occurs beyond that seen in *Su(UR)* mutants alone (Andreyeva *et al.*, 2007). Surprisingly, double mutation for *Su(UR)* and the gene encoding HP1 (discussed below) does not have the same effect, suggesting that the phenotype of the *Su(UR)*-*Su(var)3-9* double mutant is not simply due to the loss of HP1 binding to the methyl mark normally created by the *Su(var)3-9* gene product.

2.3. Biochemical properties of heterochromatin

Several studies have tested the accessibility of DNA at variegating loci to nucleolytic attack or enzymatic modification; most yielded results suggesting little or no structural difference between a silenced locus and its euchromatic counterpart (Hayashi *et al.*, 1990; Locke and McDermid, 1993; Schloßherr *et al.*, 1994; Wines *et al.*, 1996). However, the relatively low resolution of the measurements in these cases, together with a lack of detailed structural information concerning the euchromatic structure of the variegating locus argue for caution in the interpretation of these experiments. When the conditions of high resolution measurements and prior knowledge of the gene involved were met; however, a close correlation between silencing and DNA packaging was observed (Wallrath and Elgin, 1995).

Wallrath and Elgin (1995) employed a transposon bearing the *hsp26* promoter to analyze the structural consequences of heterochromatic inactivation. The choice of the *hsp26* promoter was especially apposite in this case, since the chromatin structure of this promoter at its normal chromosomal position at position 67B has been characterized in detail (Cartwright and Elgin, 1986; Thomas and Elgin, 1988) and since the *in vivo* structural requirements for its chromosomal architecture and activity have been extensively investigated (Lu *et al.*, 1992, 1993). Transgene insertions showing variegation of a linked *Hsp70*-mini-*white* reporter were selected and characterized as to their insertion sites: 4 were insertions in pericentric heterochromatin, 9 were insertions at telomeric sites, and 18 were found at various positions throughout the fourth chromosome. Only for the pericentric and fourth chromosome inserts did inclusion of the dominant suppressor *Su(var)2-1⁰¹* or reduced HP1 levels result in suppression of *white* variegation; none of the inserts at the telomeres of the second or third chromosome responded to these modifiers. For selected lines, suppression of *hsp26*-driven transcription was confirmed by quantitative

Northern blotting. It was possible to distinguish transgene transcription from the endogenous *hsp26* gene transcription because the transgene promoter directs transcription of a barley cDNA fragment not found in flies. For all pericentric insertion lines tested, transcripts of the barley sequence were reduced, and this reduction was reversed for all but one line when HP1 dose was reduced. Again, though, variegating transgene insertions in the telomeres showed levels of barley transcripts comparable to euchromatic controls, and this expression was either not affected or slightly reduced by reduced HP1 dosage.

Nuclei were prepared from non-heat-shocked transgenic third instar larvae and the sensitivity to digestion with *Xba* I, which cleaves within each of two DNase I hypersensitive sites in the *hsp26* promoter, was measured by Southern blot hybridization using a transgene-specific probe. With the digestion efficiency obtained using a euchromatic insertion arbitrarily set as 100% accessibility, it was found that all variegating inserts showed significantly reduced accessibility, even a telomeric insert on the second chromosome which showed no effect on *hsp26*-mediated transcript levels. In this and a more recent study (Sun *et al.*, 2001), micrococcal nuclease digests revealed a more regular nucleosomal ladder at the silenced loci compared to their euchromatic counterparts, suggesting a more ordered chromatin structure underlies the nuclease resistance.

One reservation concerning the interpretation of nuclease sensitivity studies is that, of necessity, suspensions of free nuclei must be used, since nucleases cannot penetrate the plasma membrane. Thus, one cannot be certain that no rearrangement of chromatin structure occurs during the preparation of nuclei. To circumvent this objection, the accessibility of the DNA in silenced loci to modification by *E. coli* Dam methyltransferase in transformed flies has been tested (Boivin and Dura, 1998). Since methylation is occurring in intact cells, this differential accessibility is the *in vivo* state. Adenine methylation is tolerated well in flies (Boivin and Dura, 1998; Wines *et al.*, 1996); a maximum of 50% methylation overall is observed. Boivin and Dura (1998) used transgenic flies expressing Dam methyltransferase under an *Hsp70* promoter, and assayed for methylation based on sensitivity of the purified DNA to digestion with a methylation-sensitive restriction endonuclease. Under these conditions, the efficiency of methylation is the same for a euchromatic locus independent of its transcriptional state. In contrast, *white* DNA sequences in a classical PEV reporter as well as in transgene reporters subject to heterochromatic PEV showed reduced methylation compared to euchromatic DNA controls. Methylation in 5' and 3' sequences was similar at a given locus, suggesting that the chromatin differences were not restricted to the promoter.

The pycnotic appearance of heterochromatin, the relative resistance of heterochromatic DNA to exogenous nuclease digestion (Wallrath and Elgin, 1995) and endogenously expressed DNA methylase (Boivin and

Dura, 1998), and the relatively ordered nucleosome arrays that package heterochromatic DNA (Sun *et al.*, 2001) together suggest a model in which heterochromatin silencing is imposed by occlusion of DNA binding sites for transcription activators and/or RNA polymerase. Chromatin footprinting analysis supports this model (Cryderman *et al.*, 1999a).

In some cases, though, transcription factors with high affinity for their DNA target sites can compete successfully with heterochromatin to prevent the establishment of silencing (Ahmad and Henikoff, 2001; Eissenberg, 2001a). Furthermore, there are a number of genes in *Drosophila* that reside in pericentric heterochromatin and require a heterochromatin context in order to function (Eissenberg and Hilliker, 2000; Rossi *et al.*, 2007; Yasuhara and Wakimoto, 2006). Thus, heterochromatin does not present an impenetrable barrier to DNA binding proteins, but would seem to shift the binding equilibrium away from the bound state.



3. DNA SEQUENCES THAT TARGET HETEROCHROMATIN

Unlike mammals and plants, *Drosophila* has little DNA methylation, and whatever cytosine methylation exists has no discernible role in gene regulation. In respect to the DNA sequence composition of heterochromatin of *Drosophila*, however, there are characteristics that distinguish it from the sequence composition of euchromatin.

3.1. Pericentric DNA

Drosophila is the first organism for which a large part of the heterochromatic sequence was successfully mapped, assembled, and finished (Hoskin *et al.*, 2007; Smith *et al.*, 2007). The studies revealed that the constitutive heterochromatin of *Drosophila*, like constitutive heterochromatin in other animals and plants, is highly enriched in middle repetitive and satellite DNA. Within complex repeats, though, islands of highly conserved genes are found. Altogether, more than 230 protein-coding genes were detected which are also found in other *Drosophila* species. Altogether, 77% of the heterochromatic sequences are repetitive or transposable element (TE) sequences. Frequently, nests of TE elements that are fragmented, interdigitated, and transposed into one another are found. Almost 900 such repeat nests could be defined. Specific TE elements have been implicated in gene silencing by heterochromatin (discussed later in this chapter).

There is no conserved sequence feature shared by eukaryotic heterochromatin. Between different species and even different strains of *Drosophila*, the amount of heterochromatin and satellite compositions vary widely (Bosco *et al.*, 2007; Gall *et al.*, 1971; Halfer, 1981; Kuhn and Sene, 2005;

Lohe and Brutlag, 1986; Schweber, 1974). These studies suggest that variation in heterochromatic satellite DNA contributes significantly to genome size evolution. Such evolutionary diversity may reflect competition between centromeres for success in the egg, as has been proposed for the centromeric satellite DNA (Henikoff *et al.*, 2001; Malik and Henikoff, 2002). But regardless of the evolutionary basis, the extraordinary diversity of DNA sequences that underlie pericentric heterochromatin suggests that the mechanism of heterochromatin formation requires factors besides an underlying consensus DNA sequence.

The heterochromatin-associated protein HP1 binds DNA *in vitro* (Perrini *et al.*, 2004; Zhao *et al.*, 2000). Crosslinking studies suggest that direct binding of HP1 to telomeric DNA could target HP1 to these chromosomal sites (Perrini *et al.*, 2004). In an attempt to infer a preferred DNA sequence target site for HP1 binding, Greil *et al.* (2003) compared the nonrepetitive sequences targeted by HP1 in Kc cultured cells. They found that AT-rich motifs, consisting of stretches of adenosines or stretches of thymidines were enriched at these sites. Previous *in vitro* experiments showed that there is no strong DNA sequence preference for purified recombinant HP1 (Zhao *et al.*, 2000). However, *Drosophila* HP1 is multiply phosphorylated (Eissenberg *et al.*, 1994; Zhao and Eissenberg, 1999; Zhao *et al.*, 2001), so either posttranslational modification or association with other factors could confer sequence-preferential binding *in vivo*.

3.2. The fourth chromosome

The fourth chromosome in *D. melanogaster* is by far the shortest autosome, representing ca. 3.5% of the genome and estimated to be ca. 5 Mb in length (Locke and McDermid, 1993). The fourth chromosome shares some attributes of heterochromatin, notably that it undergoes no detectable meiotic recombination (Bridges, 1935; Hochman, 1976), replicates late in the cell cycle (Barigozzi *et al.*, 1966), is relatively enriched in middle repetitive DNA (Locke *et al.*, 1999; Miklos *et al.*, 1988), is associated with rearrangements that induce heterochromatic position effects, and is enriched in the heterochromatin-associated protein HP1 (discussed below) and histone H3 dimethylated at lysine 9 (de Wit *et al.*, 2007; Greil *et al.*, 2003; James *et al.*, 1989; Schotta *et al.*, 2002). Interestingly, however, the enzyme that methylates lysine 9 of histone H3 in pericentric heterochromatin, and that is required for silencing of genes mislocalized to pericentric heterochromatin (see below) is not required for methylation of lysine 9 of histone H3 (H3K9) on the fourth chromosome (Schotta *et al.*, 2002). Instead, the *Drosophila* SETDB1 protein is required for H3K9 dimethylation on chromosome four and for silencing of transgene reporters on the fourth (Seum *et al.*, 2007).

Genetic analysis suggests that one or more TE families mediate heterochromatin-like transgene silencing on the *Drosophila* fourth chromosome.

Using the expression of *white*-marked transposons as reporters and a series of nested deletions, [Sun et al. \(2004\)](#) implicated nearby copies of the 1360/*hoppe* transposon as sources for transgene silencing on the fourth chromosome. The silencing depended on the dosage of the heterochromatin-associated protein HP1. At other chromosomal sites, members of the 1360/*hoppe* transposon family recruit high levels of HP1, although the levels appear to depend on whether the transposon is in chromosome regions enriched for repetitious DNA ([de Wit et al., 2005, 2007](#)). Short RNAs corresponding to both sense and antisense strands of 1360/*hoppe* elements are detectable in Kc cultured cells ([Haynes et al., 2006](#)), suggesting that RNA interference (RNAi) is part of the mechanism of 1360/*hoppe*-dependent silencing. Indeed, mutations in genes encoding subunits of the *Drosophila* RNAi machinery partially relieve silencing associated with 1360/*hoppe* elements ([Pal-Bhadra et al., 2004](#)). In a direct test of the ability of 1360/*hoppe* elements to impose position-effect silencing, [Haynes et al. \(2006\)](#) cloned a copy of 1360/*hoppe* adjacent to a mini-*white* transposon reporter. Of 22 independent insertions spanning both major autosomes, only one insert showed variegated silencing. This insert is located near the base of the left arm of the second chromosome at a transposon-rich site, and the silencing is sensitive to HP1 dosage and mutation in *Su(var)3-9*, mutation in genes encoding components of the *Drosophila* RNAi mechanism. While these results clearly show a requirement for chromosomal positioning near natural heterochromatin in the silencing mechanism, the authors show that deletion of the 1360/*hoppe* sequence at this insertion site partially relieves silencing. Thus, 1360/*hoppe* elements appear unable to independently target heterochromatin formation to ectopic sites, but can cooperate with nearby repeat elements to enhance or spread heterochromatin. This model for cooperative interactions to target heterochromatin assembly fits well with tethering experiments showing that tethering HP1 to a transgene reporter can target heterochromatic silencing only when the transgene insertion is in a repeat-rich region ([Seum et al., 2001](#)).

3.3. Transposon arrays and ectopic heterochromatin

In *Drosophila*, the introduction of multiple transgenes carrying the same marker generally results in dosage effect for the expression of the marker. In certain cases, however, the multiplication of transgenes leads to silencing. The first report of this paradoxical phenomenon involved transposons marked with mini-*white*; local transposition of the transposon results in transposon arrays, some of which result in variegated *white* expression ([Dorer and Henikoff, 1994, 1997](#)). HP1 is found at the site of transgene arrays in polytene chromosomes ([Fanti et al., 1998](#)). Interestingly, lower amount of HP1 are present at arrays at which no silencing is detectable, suggesting that HP1 binding alone is not sufficient for the silencing.

X-irradiation of stocks carrying variegating mini-*white* transposon arrays resulted in lines showing enhanced or suppressed variegation (Dorer and Henikoff, 1997). Lines showing enhanced variegation had chromosome rearrangements that placed the transgene closer to pericentric heterochromatin, and recombining the transgene back to its original position suppressed the variegation. Transgene array silencing is not limited to mini-*white* transposons. Arrays composed of transposons marked with *brown* also show variegation (Sabl and Henikoff, 1996), but in this case, only when the array is located very close to pericentric heterochromatin. Thus, mini-*white* transposons seem more prone to array-induced heterochromatin formation than are transposons marked with *brown*.

On the other hand, a transposon carrying a *Prat-brown* fusion transgene appears refractory to array induced silencing. Arrays of up to 320 kb at a euchromatic insertion site show no evidence of silencing (Clark *et al.*, 1998). Thus, like other transposons in the *Drosophila* genome, P-elements can promote heterochromatin assembly, but not all P-elements do so. These differences could be explained by the differences in protein complexes assembled on different transgenes, differences in promoter strength of the transgenes, the presence of elements that inhibit heterochromatin assembly, or some combination of factors.

3.4. Spreading of heterochromatin at rearrangement breakpoints

In 1988, Tartof and colleagues proposed a mass-action model for the assembly and propagation of heterochromatin based on the genetics of heterochromatin position-effect silencing in *Drosophila* (Locke *et al.*, 1988). This model was designed to explain the genetic observation that several genes each appeared to have dosage-dependent effects on heterochromatic silencing, implying that multiple rate-limiting factors for heterochromatin exist simultaneously. The model also incorporated the idea that spreading of heterochromatic silencing appears to occur from a heterochromatic breakpoint. There are three general features of this model: (1) that heterochromatin consists of nucleoprotein complexes whose integrity depends on a multiplicity of interactions, and that multiple complex subunits can each be limiting for the *cis*-spreading of the complex; (2) that such complexes normally are targeted to one or more “initiation sites” in the DNA of pericentric heterochromatin and spread in *cis* from these sites in a cooperative fashion; and (3) that spreading is normally contained by termination sites and that position-effects arise when chromosome rearrangements permit spreading into a normally euchromatic region. Considerable molecular and genetic data support all three elements of this model.

Spreading of heterochromatin has been demonstrated directly by chromatin immunoprecipitation analysis in the *w^{m4}* PEV rearrangement

(Rudolph *et al.*, 2007). Here, spreading of the heterochromatic dimethylated H3K9 (H3K9me2) histone methylation mark, a histone modification normally enriched in pericentric heterochromatin (discussed later in this chapter) into the euchromatic *white* gene region has been demonstrated. Spreading of H3K9me2 clearly depends on dosage of the histone H3K9 methyltransferase SU(VAR)3–9 (discussed later in this chapter) and the function of the histone H3 lysine 4 (H3K4) demethylase SU(VAR)3–3 (DmLSD1).

The mechanism that normally constrains heterochromatin spreading in higher eukaryotes is poorly understood. One contributor could be the abundant DNA binding protein GAGA factor. GAGA factor was shown to interact with the FACT complex, which facilitates nucleosome remodeling essential for maintenance of Hox gene expression (Shimajima *et al.*, 2003). The GAGA factor–FACT complex is also involved in control of heterochromatin spreading in PEV by facilitating replacement of H3K9-methylated histones by the unmethylated histone variant H3.3 (Fig. 1.2). This function counteracts heterochromatin spreading and could explain at the molecular level why GAGA factor mutations are enhancers of PEV (Nakayama *et al.*, 2008).

An overly literal reading of the model would infer that heterochromatin complexes can only be initiated within canonical pericentric heterochromatin and must spread continuously from initiation sites within these regions in a crystallization-like process without interruption to silence genes in nearby euchromatin. However, the mass-action model is also completely consistent with the idea that abnormal proximity of euchromatin to heterochromatin enforced by rearrangements results in the stochastic

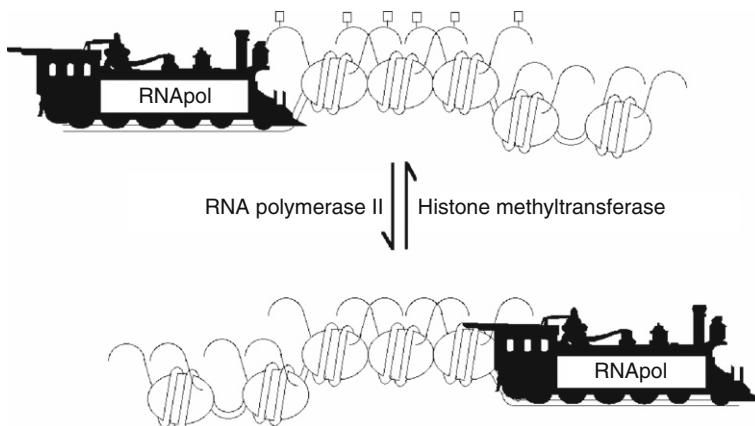


Figure 1.2 Remodeling of methylated histone H3 by RNA Polymerase II passage. As RNA Polymerase II traverses a chromatin domain containing methylated histone H3 (□, the nucleosomes in its path are evicted from the DNA). As chromatin is reassembled in the wake of the Polymerase, unmethylated histone H3.3 is used, leaving a domain of unmarked chromatin. Redrawn from Eissenberg and Elgin (2005).

colonization of euchromatin by heterochromatin at latent, cryptic “initiation” sites in euchromatin such as TE or repetitive sequences. Since key factors that assemble heterochromatin by mass action are diffusible, they could spread in *cis* or in *trans*, either continuously or discontinuously.

4. HISTONE MODIFICATIONS AND HETEROCHROMATIN TARGETING

4.1. Heterochromatin-associated chromatin marks

The idea that covalent histone modifications could regulate transcription was first advanced by Vincent Allfrey and colleagues (Allfrey *et al.*, 1964; Pogo *et al.*, 1966). In the past 15 years, this inference has gained robust support, as the tools for the genetic dissection and cytological characterization of chromatin modifications have been developed. The current model driving experiments on histone modifications is the “histone code” hypothesis, which posits that combinations of covalent histone modifications partition chromosomes into distinct functional domains (Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 1993, 2002). In many cases, the key observations that have led to our current understanding of histone modifications in heterochromatin were first made in *Drosophila*.

The different histone H4 isoforms acetylated at lysines 5, 8, 12, or 16 were shown to have distinct genomic distributions in *Drosophila* polytene chromosomes (Turner *et al.*, 1992). H4 isoforms acetylated at lysines 5 or 8 are found widely dispersed throughout the euchromatic arms, but only in low amounts in the chromocenter heterochromatin. In contrast, the isoform acetylated at lysine 12 is significantly enriched in the chromocenter and in bands along the polytene fourth chromosome, a pattern highly reminiscent of HP1 distribution. Subsequent genome-wide analysis using chromatin immunoprecipitation and cDNA microarrays confirmed that hyperacetylated histone H3 and H4 isoforms are enriched in the transcription units of active genes in *Drosophila* (Schübeler *et al.*, 2004).

H3K9me2 is found at high concentrations throughout the pericentric heterochromatin, along the fourth chromosome and at dispersed euchromatic sites in *Drosophila* polytene chromosomes, while H3K9me3 has a more restricted distribution within pericentric heterochromatin (Cowell *et al.*, 2002; Ebert *et al.*, 2004; Jacobs *et al.*, 2001; Schotta *et al.*, 2002; Fig. 1.3). The SU(VAR)3–9 protein is required for most of the H3K9me2 in pericentric heterochromatin (Ebert *et al.*, 2004; Schotta *et al.*, 2002), while the SETDB1 protein is required for most of the H3K9me2 on the fourth chromosome (Seum *et al.*, 2007). Histone H4 trimethylated at lysine 20 (H4K20me3) is also found at high concentrations in the pericentric heterochromatin in *Drosophila*, although it is also

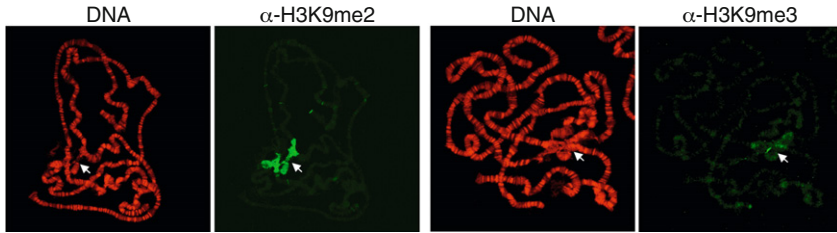


Figure 1.3 In salivary gland nuclei, all pericentric heterochromatin coalesces into a structure called the chromocenter (arrow) that stains strongly for H3K9me2 (left), a characteristic histone methylation mark of heterochromatin in *Drosophila*. In *Drosophila*, only a low amount of H3K9me3 within the core of chromocenter heterochromatin is detected in polytene chromosomes (right). Photo provided by Anja Ebert.

widespread in euchromatin (Schotta *et al.*, 2004). In larvae lacking SU (VAR)3–9, the H3K9 dimethylase (see below), the heterochromatic concentration of H4K20me3 is markedly reduced. Strikingly, loss of HP1, a heterochromatin-associate protein that binds the H3K9me2 methyl mark (see below) results in loss of the H4K20me3 mark from both heterochromatin and euchromatin. A mutation in the gene encoding the H4K20 trimethylase, *Suv4–20*, is a dominant suppressor of heterochromatic position-effect variegation (Schotta *et al.*, 2004; but see also Sakaguchi *et al.*, 2008), suggesting that the H4K20me3 methyl mark plays a role in the establishment or maintenance of heterochromatin.

4.2. Proteins that bind heterochromatin-associated marks

Methylated H3K9 is specifically recognized by HP1-family proteins in mammal and *Drosophila* (Bannister *et al.*, 2001; Jacobs and Khorasanizadeh, 2002; Lachner *et al.*, 2001; Nielsen *et al.*, 2002; Peters *et al.*, 2002). In a genome-location analysis, dimethH3K9 was associated with loci that were also associated with hypoacetylated histones in human cells (Miao and Natarajan, 2005). HP1 colocalizes with H3K9me2 in *Drosophila* and fission yeast (Jacobs *et al.*, 2001; Noma *et al.*, 2001), and loss of methylation causes a dramatic reduction of HP1 in heterochromatin in mammalian, yeast, and *Drosophila* cells (Ebert *et al.*, 2004; Lachner *et al.*, 2001; Nakayama *et al.*, 2001; Schotta *et al.*, 2002). Targeted H3K9 methylase represses a mammalian gene *in vivo* (Snowden *et al.*, 2002), although it is not clear that the repression is by the same mechanism as that operating in heterochromatin. An HP1 chromo domain mutation that ablates PEV silencing in *Drosophila* (Platero *et al.*, 1995) also interferes with the ability of HP1 to bind H3K9me2 *in vitro* (Jacobs *et al.*, 2001), strengthening the mechanistic connection between HP1–nucleosome interactions and HP1-mediated repression. However, evidence suggests that other HP1–histone

and HP1–DNA interactions could contribute to HP1 targeting in chromosomes (Eskeland *et al.*, 2007; Perrini *et al.*, 2004; Zhao *et al.*, 2000).

5. NONHISTONE PROTEINS AND HETEROCHROMATIN TARGETING

5.1. Heterochromatin protein 1

One of the first genes encoding a heterochromatin-associated protein to be cloned was the *Drosophila* Heterochromatin Protein 1 (HP1; James and Elgin, 1986). It was identified as a band on SDS-PAGE among proteins from embryo nuclei that remained complexed with DNA in 0.25 M potassium thiocyanate, but were solubilized by 1 M potassium thiocyanate. This band was used to immunize mice, and a monoclonal antibody generated from these mice immunolocalized the antigen primarily to the heterochromatic chromocenter of fixed salivary gland polytene chromosomes (James and Elgin, 1986; James *et al.*, 1989). The antibody was used to screen a λ gt11 expression library, resulting in the cloning of HP1 cDNA (James and Elgin, 1986).

The *in situ* hybridization of this cDNA clone to cytological region 29A on the second chromosome coincided with the map location of a genetic suppressor of heterochromatic position-effect silencing termed *Su(var)205* (Sinclair *et al.*, 1983), and molecular characterization of alleles from multiple independent screens established HP1 as a dose-dependent modifier of heterochromatin silencing (Eissenberg and Hartnett, 1993; Eissenberg *et al.*, 1990, 1992). Subsequent work shows that HP1 family proteins are found in fission yeast, animals and plants, though not in bacteria or budding yeast.

In all higher eukaryotes, at least one HP1 family protein shows a preferential localization to pericentric heterochromatin. In cases where they have been tested, these HP1 family proteins have been implicated in silencing in certain assays; all three mammalian HP1 homologs target heterochromatin when expressed in *Drosophila*, and one isoform promotes heterochromatin silencing in flies (Ma *et al.*, 2001). In other contexts, though, HP1 is required for normal transcription. It has long been known that several genes map to pericentric heterochromatin in *Drosophila*. In some cases, the normal expression of these genes has been shown to require HP1 (Lu *et al.*, 2000) and the chromatin containing such genes is enriched in HP1 (de Wit *et al.*, 2005, 2007; Greil *et al.*, 2003).

Evidence from immunolocalization of *Drosophila* HP1 on polytene chromosomes (Fanti *et al.*, 2003; James *et al.*, 1989) and from DamID genomic localization in cultured Kc cells (de Wit *et al.*, 2005, 2007; Greil

et al., 2003) shows that HP1 is enriched at certain euchromatic loci. Prominent among these in polytene chromosomes is the cytological interval 31 in the middle of the left arm of the second chromosome, a region subsequently shown to contain genes that are HP1-repressed in third instar larvae (Hwang *et al.*, 2001). At least one polytene chromosome puff site also shows significant staining, suggesting that transcriptionally active chromatin could accumulate significant amounts of HP1. This observation was extended by Piacentini *et al.* (2003), who found HP1 associated with heat shock puffs as well. HP1 is probably not targeted by directly binding to nascent RNA, since HP1 binding in polytene chromosomes is not significantly affected by RNase digestion (Piacentini *et al.*, 2003) although some studies suggest RNA contributes to HP1 binding (Muchardt *et al.*, 2002).

The first immunolocalization of HP1 in polytene chromosomes also revealed significant concentrations of HP1 at telomeres (James *et al.*, 1989). Although telomeres are associated with transgene silencing in *Drosophila* (Cryderman *et al.*, 1999b; Golubovsky *et al.*, 2001; Hazelrigg *et al.*, 1984; Karpen and Spradling, 1992; Levis *et al.*, 1993; Marin *et al.*, 2000; Wallrath and Elgin, 1995), the telomeric silencing mechanism in *Drosophila* is, for the most part, genetically distinct from centromeric silencing (Donaldson *et al.*, 2002; Mason *et al.*, 2004). Mutations in *tefu*, the *Drosophila* ATM homologue, reduce the amount of HP1 at telomeres and cause a recessive suppression of telomeric silencing (Oikemus *et al.*, 2004), suggesting an indirect mechanistic link between HP1 binding at telomeres and telomeric silencing.

Mutations in the *Drosophila* HP1-encoding gene *Su(var)2-5* are recessive lethal. The lethal period was mapped to the third larval instar by temperature shift experiments using a *Hsp70*-HP1 transgene (Eissenberg and Hartnett, 1993) and by using larval cuticle markers (Lu *et al.*, 2000). Loss of heterochromatic silencing and reduced expression of heterochromatic genes were noted in larvae approaching the lethal period (Lu *et al.*, 2000), suggesting a failure of HP1-dependent gene regulation could contribute to lethality. However, adult flies rescued by HP1 expression in midlarval development show eye and wing defects suggesting defects in imaginal disc cell proliferation and behavioral defects consistent with CNS defects (Eissenberg and Hartnett, 1993). Detailed examination of neuroblasts in HP1 mutant larvae revealed extensive telomeric fusions (Fanti *et al.*, 1998; Perrini *et al.*, 2004). These fusions could result in proliferation defects in the dividing cells of the imaginal disks and CNS, and suggest that HP1 also plays an essential role in telomere capping in *Drosophila*.

In biochemical fractionation, HP1 is associated with the telomeric HOAP protein (Badugu *et al.*, 2003; Shareef *et al.*, 2003). HOAP is encoded by the *caravaggio* locus, and mutations in *caravaggio* cause telomere fusions in larval neuroblasts that resemble those seen in HP1 mutant neuroblasts (Cenci *et al.*, 2003b). Thus, HOAP and HP1 are thought to be essential

components of the *Drosophila* telomere capping complex, although it is unknown whether HP1 is targeted to telomeres by HOAP itself or through a distinct mechanism (Cenci *et al.*, 2003b). HOAP is also enriched in the pericentric heterochromatin, and *caravaggio* mutations dominantly suppress heterochromatic silencing (Badugu *et al.*, 2003).

In situ measurements of HP1 family proteins in mammalian cells using fluorescence recovery after photobleaching (FRAP) indicate that much of nuclear HP1 is remarkably dynamic (Cheutin *et al.*, 2003; Festenstein *et al.*, 2003). In heterochromatic regions, most HP1 turns over within 60–200 s. This observation is consistent with the mass action model of Locke *et al.* (1988), which invokes a dynamic equilibrium between dissociated and assembled heterochromatic subunits. However, the original protocol used to identify HP1 in *Drosophila* demonstrates that a fraction of HP1 is sufficiently tightly bound to chromatin in embryos to resist extraction by moderate concentrations of chaotropic agents (James and Elgin, 1986). FRAP studies in transgenic *ex vivo* T cells indicate that ca. 30% of heterochromatic HP1 is immobile (Festenstein *et al.*, 2003), while in Hep-2 cells this represents ca. 5% of HP1 in heterochromatin (Schmiedeberg *et al.*, 2004). Perhaps this relatively immobile fraction corresponds to the tightly bound HP1 in *Drosophila*, and could account for the stability of heterochromatic silencing.

Drosophila HP1 was the first characterized protein with a chromo domain motif (Eissenberg, 2001b; Eissenberg and Khorasanizadeh, 2005). The HP1 chromo domain is located in the N-terminal half of all HP1 family proteins (Eissenberg and Elgin, 2000). Subsequent sequence comparisons identified a second chromo domain motif in the C-terminal half of HP1 family proteins, called the “chromo shadow domain” (Aasland and Stewart, 1995; Epstein *et al.*, 1992; Koonin *et al.*, 1995). The HP1 chromo domain is sufficient to target heterochromatin *in vivo*, and a point mutation in the *Drosophila* HP1 chromo domain inactivates the ability of the protein to contribute to heterochromatin silencing (Platero *et al.*, 1995). Structural studies revealed that the chromo domain is a high-affinity binding site di- and tri-methylated lysine 9 of histone H3 (Jacobs and Khorasanizadeh, 2002; Nielsen *et al.*, 2002). The chromo shadow domain is also capable of targeting heterochromatin *in vivo* (Powers and Eissenberg, 1993). Structural studies show that the HP1 family chromo shadow domain is a self-association motif (Brasher *et al.*, 2000; Cowieson *et al.*, 2000). Self-association through the chromo shadow domain could explain the heterochromatin-targeting ability of this domain in a nucleus containing endogenous HP1. Deletions that remove part or all of the *Drosophila* HP1 chromo shadow domain also abolish silencing activity (Eissenberg and Hartnett, 1993; Eissenberg *et al.*, 1992).

Multiple mechanisms could target HP1 to distinct chromosomal sites directly or by recruiting one of the four known histone H3K9

methylase orthologs in *Drosophila* [SU(VAR)3–9, G9A, SETDB1, and EU-HMTase 1], resulting in HP1 binding. Loss of SU(VAR)3–9 leads to a dramatic reduction in HP1 levels in pericentric heterochromatin (Schotta *et al.*, 2002), while loss of SETDB1 leads to a dramatic reduction of HP1 at nontelomeric sites in euchromatin and along the euchromatic fourth chromosome (Seum *et al.*, 2007). However, *in vitro* studies indicate that HP1 can bind to nucleosomes lacking tails, and that nonhistone proteins may contribute to HP1 binding to methylated chromatin (Eskeland *et al.*, 2007; Zhao *et al.*, 2000).

An RNAi mechanism is required for HP1-mediated silencing in fission yeast, and one study suggests that this mechanism may also occur in flies (Pal-Bhadra *et al.*, 2004; Verdel *et al.*, 2004; Volpe *et al.*, 2002). Since transposon transcripts are an important biological target for RNAi, transposons could mediate heterochromatin formation through an RNAi mechanism. In *Drosophila*, a study mapping HP1 binding sites in >3 Mb of DNA using the DamID method found 17 regions of significant HP1 binding, all but one of which were TE or other repeated elements (Sun *et al.*, 2003). This is consistent with a role for 1360/*hoppel* transposons in mediating HP1-dependent silencing on the fourth chromosome (see above). Using an array containing over 6200 cDNA fragments, Greil *et al.* (2003) found significant HP1 concentrations in pericentric and subtelomeric sequences in chromatin from the Kc cell line. Thus, repetitious DNA, rather than any particular DNA sequence, may be an important sequence determinant in heterochromatin formation.

5.2. Su(var)3–7

SU(VAR)3–7 was the second heterochromatin-associated protein to be cloned in *Drosophila* (Reuter *et al.*, 1990). Its gene product, SU(VAR)3–7, is a seven zinc finger protein that binds DNA *in vitro* (Cléard and Spierer, 2001; Cléard *et al.*, 1995), binds HP1 and is enriched in pericentric heterochromatin (Cléard *et al.*, 1997; Delattre *et al.*, 2000). Like HP1, it is essential (Seum *et al.*, 2002) and it is a dosage dependent modifier of heterochromatic position-effect variegation (Reuter *et al.*, 1990). Importantly, however, HP1 binding to the chromocenter does not require SU(VAR)3–7 (Spierer *et al.*, 2005).

When SU(VAR)3–7 is overexpressed from a heat shock inducible transgene, the protein binds extensively to all the euchromatic arms in polytene chromosomes (Delattre *et al.*, 2004). Ectopic SU(VAR)3–7 binding is accompanied by increased euchromatic levels of dimethH3K9 and this increased dimethH3K9 depends on the heterochromatic H3K9 methylase SU(VAR)3–9 (see below). Additionally, SU(VAR)3–7 overexpression results in increased euchromatic accumulation of HP1, which also depends on SU(VAR)3–9. These findings point to model in which SU(VAR)3–7

recruits the H3K9 methylase SU(VAR)3–9, which dimethylates H3K9, resulting in ectopic HP1 binding (Delattre *et al.*, 2004).

Unlike HP1, however, SU(VAR)3–7 does not belong to an evolutionarily conserved family of heterochromatin-associated proteins. Either the SU(VAR)3–7:HP1 interaction and the role of SU(VAR)3–7 in H3K9 dimethylation is a specialized adaptation of *Drosophila* or the homologous zinc finger protein that fulfills this role in other organisms has diverged so much as to be unrecognizable.

5.3. Su(var)3–9

Su(var)3–9 was first identified in *Drosophila* as a gene whose mutations cause strong dominant suppression of heterochromatic silencing (Tschiersch *et al.*, 1994). The protein product of this gene contains a chromo domain motif—a binding motif for methylated lysine—and a SET domain, the catalytic domain of histone methylases.

Biochemical and yeast two-hybrid protein analysis demonstrate that the heterochromatin-associated protein SU(VAR)3–9 interacts directly with HP1 (Aagaard *et al.*, 1999; Schotta *et al.*, 2002). This interaction is consistent with the colocalization of HP1 and SU(VAR)3–9 proteins in the *Drosophila* pericentric heterochromatin (Fig. 1.4). Studies in *Drosophila*, fission yeast, and mammals demonstrate that SU(VAR)3–9 family proteins are H3K9 methylating enzymes (Czermin *et al.*, 2001; Ebert *et al.*, 2004; Eskeland *et al.*, 2004; Nakayama *et al.*, 2001; Rea *et al.*, 2000; Schotta *et al.*, 2002) and that HP1 family proteins bind specifically to the H3K9me2 methyl mark (Bannister *et al.*, 2001; Jacobs and Khorasanizadeh, 2002; Jacobs *et al.*, 2001; Lachner *et al.*, 2001; Nielsen *et al.*, 2002). These findings have coalesced around an attractive cascade model for HP1 family protein recruitment and the assembly and spreading of HP1-dependent heterochromatin: (1) SU(VAR)3–9 proteins are recruited to specific chromosomal sites (by an unknown mechanism) and through its HP1-binding activity, recruits HP1; (2) SU(VAR)3–9 methylates H3K9 residues on nearby nucleosomes; (3) additional HP1 binds to the newly methylated nucleosome; (4) more SU

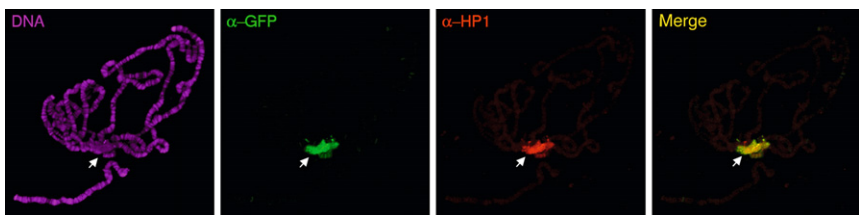


Figure 1.4 The SU(VAR)3–9 and HP1 proteins colocalize in chromocenter heterochromatin. Photo provided by Anja Ebert.

(VAR)3–9 protein is recruited by the additional HP1; and (5) the additional SU(VAR)3–9 protein methylates adjacent nucleosomes, which creates new HP1 binding sites, etc. Loss of HP1 results in increased H3K9 dimethylation in the euchromatin of polytene chromosomes, consistent with the model that SU(VAR)3–9:HP1 interaction constrains SU(VAR)3–9 histone methylating activity to heterochromatin (Ebert *et al.*, 2004).

A mutation in the SU(VAR)3–9 SET domain, *pitkin*^D, is a strong dominant enhancer of heterochromatic position effect (Ebert *et al.*, 2004; Kuhfittig *et al.*, 2001). *pitkin*^D behaves as a hypermorphic allele of *Su(var)3–9*, and results in increased euchromatic H3K9me2 (Ebert *et al.*, 2004). Gene silencing in heterochromatic rearrangements is suppressed by *Su(var)3–9*, *Su(var)2–5* (encodes HP1), *Suv4–20*, and *Su(var)3–1* (encodes the JIL–1 protein kinase) mutations, reflecting a silencing pathway initiated by SU(VAR)3–9-dependent H3K9 dimethylation, a mark recognized by HP1 which anchors SU(VAR)3–9 to heterochromatin and recruits the H4K20me3-catalyzing SUV4–20 histone methyltransferase (Schotta *et al.*, 2002, 2004).

Spreading of heterochromatic H3K9 methylation into euchromatin is normally inhibited by the JIL–1 kinase (Ebert *et al.*, 2006; Lerach *et al.*, 2006; Zhang *et al.*, 2006), which phosphorylates serine 10 of histone H3 (H3S10) in euchromatin. Surprisingly, loss-of-function mutations in JIL–1 suppress PEV of *white* in the classical variegating rearrangement *w^{m4}*, but enhance PEV of *white* when the gene is inserted within pericentric heterochromatin on a P-element transposon (Bao *et al.*, 2007). The suppression of *w^{m4}* could be explained by ectopic relocalization of SU(VAR)3–9 and HP1 proteins to euchromatin from pericentric heterochromatin, reducing the ability of the remaining heterochromatic complexes to spread across the *w^{m4}* breakpoint and silence *white* at a distance. The enhancement of *white* transposon silencing could reflect the local loss of H3S10 phosphorylation at the *white* and the local shift in the euchromatin–heterochromatin balance in favor of heterochromatin. The mechanism by which H3S10 phosphorylation antagonizes heterochromatin appears to involve competition with SU(VAR)3–9, since the lethality associated with loss of JIL–1 can be partially rescued by loss of SU(VAR)3–9 (Deng *et al.*, 2007). Taken together, these results point to a role of JIL–1 and/or H3K10 phosphorylation in the spatial restriction of heterochromatin in the nucleus.

5.4. Origin recognition complex

Studies in budding yeast have implicated components of replication origins in the establishment and maintenance of silencing at the silent mating type cassettes (Laurenson and Rine, 1992). Since some of the key proteins involved in yeast silencing have no clear homologs in *Drosophila*, and since budding yeast lacks both cytologically visible heterochromatin and homologs of some of the key players implicated in *Drosophila*

heterochromatin (e.g., HP1, SU(VAR)3–9, methylation of H3K9, the RNAi mechanism, etc.), it is not clear that replication origins in *Drosophila* play an analogous role in heterochromatic silencing. Antibody to the *Drosophila* origin recognition complex subunit Orc2 shows significant concentration in apical regions of interphase nuclei and in pericentromeric regions of metaphase chromosomes at all stages of syncytial embryos, irrespective of whether cytologically visible heterochromatin is present (Pak *et al.*, 1997). Upon cellularization, embryonic Orc2 is primarily detectable in extrachromosomal material at the metaphase plate in anaphase cells, but shows preferential localization to pericentric heterochromatin in metaphase chromosomes of SL2 cultured cells (Pak *et al.*, 1997). In the giant polytene chromosomes of larval salivary glands, little or no selective association with pericentric heterochromatin by immunostaining of polytene chromosomes is evident (Pak *et al.*, 1997), suggesting that interaction with HP1 in the heterochromatin of these nuclei is limited at best. Interestingly, orc-containing protein complexes copurify with HP1 and HOAP, both of which are found at telomeres and act to prevent telomere fusions in mitotic cells (discussed above). Thus, although origin recognition complexes appear to be enriched in the pericentric regions of mitotically cycling chromosomes, there is no compelling evidence that replication origins are mechanistically involved in heterochromatin assembly in *Drosophila*.

5.5. Cohesins and heterochromatin

In fission yeast, interaction between cohesin and the HP1 family protein Swi6 is important for the establishment of sister chromatid cohesion in centromeric heterochromatin (Bernard *et al.*, 2001; Nonaka *et al.*, 2002). However, cohesins and HP1 show little or no colocalization in *Drosophila* polytene chromosomes, particularly in the heterochromatic chromocenter (Gause *et al.*, 2008). Moreover, mitotic chromosomes from larvae lacking HP1 show no significant loss of sister chromatid cohesion or defects in polytene chromosome organization (Fanti *et al.*, 1998), while loss of cohesin function leads to cohesion defects in mitotic cells (Gause *et al.*, 2008) and altered polytene chromosome morphology (Dorsett *et al.*, 2005). Thus, the evidence from *Drosophila* suggests that interactions between cohesin and HP1-family proteins in heterochromatin are not an evolutionarily conserved feature of sister chromatid cohesion.

5.6. Artificial targeting proteins and ectopic heterochromatin

Studies in *Drosophila* have demonstrated that heterochromatin-like properties can be targeted to euchromatic sites by tethering HP1 protein. Seum *et al.* (2001) tethered HP1, expressed as a Gal4-HP1 fusion protein, to transgene reporters inserted at euchromatic sites. One of six transgene

insertion sites showed GAL4-HP1-dependent variegated silencing; the silencing required the GAL4 binding site and was suppressed by reducing dosage of *Su(var)3-7*, a gene that encodes a heterochromatin-associated protein and silencing modifier (discussed earlier). It was also associated with ectopic pairing to sites of intercalary heterochromatin in polytene chromosomes, another hallmark of heterochromatin. To explain the exceptional behavior of the transposon that did show silencing, the authors note that this transgene was inserted into a copy of the micropia retrotransposon, and near a cluster of middle repetitive elements that are mostly found in centric heterochromatin. Thus, like the case of the 1360/*hoppe* element and fourth chromosome position effects,^{***} ectopic heterochromatin formation with tethered HP1 appears to require a chromosomal context containing nearby transposons and/or middle repetitive DNA. Importantly, transgenes that do not show silencing still bind the GAL4-HP1 fusion protein, as was seen previously for *white* transgene arrays (Fanti *et al.*, 1998). This is consistent with a heterochromatin mass action model in which subthreshold amounts of heterochromatic factors assemble at non-silenced arrays, and a conversion to silencing results from juxtaposition with other chromosomal regions that attain sufficient concentrations of factors to establish stable silencing.

Somewhat different results are obtained when HP1 is tethered as a HP1-lacI fusion protein to tandem arrays of 4–256 lacI binding sites (Li *et al.*, 2003). For 25/26 euchromatic transgene insertion sites, tethering of HP1 resulted in reduced expression of the mini-*white* transgene marker and a large fraction of polytene chromosomes showed ectopic fibers linking the transgene sites to other chromosomal loci, as is seen with intercalary heterochromatin. However, in no case was variegated silencing typical of heterochromatic position effect observed. Also in contrast to classical heterochromatic position effect, no significant accumulation of H3K9me2 was observed even though SU(VAR)3-9 is recruited to the loci where HP1 is tethered. Furthermore, the repression imposed by tethered HP1 was insensitive to loss of SU(VAR)3-9 protein, suggesting that the mechanism of HP1-mediated repression in these tethered HP1 transgenes differs in significant respects from heterochromatin silencing.

In follow-up studies, the effects of tethering HP1 to 256-copy lac I arrays on transgene chromatin structure was investigated (Danzer and Wallrath, 2004). Consistent with previous reports on the effects of classical heterochromatin silencing on transgene chromatin structure (Sun *et al.*, 2001; Wallrath and Elgin, 1995), the repression associated with HP1-lac I arrays is associated with ordered nucleosome arrays and increased resistance to restriction endonuclease cleavage. Surprisingly, the repression of an Hsp 26 heat shock promoter located on one side of the lac I array was independent of SU(VAR)3-9, while repression of an Hsp70 heat shock promoter on the other side of the array is completely relieved in the absence

of SU(VAR)3–9. Taken together, the results of HP1 tethering experiments suggest that HP1 targeting alone is insufficient to nucleate heterochromatin formation, but that HP1 can impose transcriptional repression and modify chromatin by multiple mechanisms.

6. NUCLEAR ASSOCIATIONS AND HETEROCHROMATIN IN *DROSOPHILA*

6.1. *Trans*-inactivation

Most examples of heterochromatic position-effect silencing are recessive; the function of an unrearranged allele masks the silencing of the rearranged allele. A notable exception to this is dominant variegation for the *brown* [*bw*] locus. Rearrangements variegating for the *brown* [*bw*] eye pigment gene cause dominant inactivation of the wild type *bw* allele in *trans* to heterochromatin (Slatis, 1955), a phenomenon termed “*trans*-inactivation” (Henikoff and Dreesen, 1989).

In the case of *brown*^{Dominant} (*bw*^D), a 1–2 Mb block of heterochromatic satellite sequence has been inserted into the *brown* (*bw*) locus. However, in contrast to null alleles of *brown*, which are recessive, the *bw*^D allele causes variegation of a wild type *bw* allele in *trans*. The silenced *bw* allele acquires neither the condensed cytological appearance of heterochromatin nor recruits detectable amounts of HP1 in polytene chromosomes (Belyaeva *et al.*, 1997). This suggests that dominant silencing of *bw* allele in *trans* by *bw*^D occurs by a distinct mechanism from other forms of heterochromatic silencing. Detailed confocal microscopic analysis, combined with immunofluorescence, showed that silencing by *bw*^D is correlated with association with pericentric heterochromatin (Csink and Henikoff, 1996). The idea that dragging a euchromatic locus into proximity with pericentric heterochromatin could silence that locus is consistent with a mass action model of chromatin assembly. However, it seems unlikely that physical proximity alone would be sufficient, since the nearby essential genes *chrw* (ca. 6 kb downstream of *bw*) and *wmd* (ca. 6 kb upstream of *bw*) would otherwise be inactivated. Indeed, a later study demonstrated that heterochromatic associations are compatible with an active *brown* allele (Sass and Henikoff, 1999). Thus, proximity to a domain of heterochromatin alone is not sufficient to confer heterochromatic silencing; such nuclear associations may be a concomitant of, but not causative of, heterochromatic silencing.

Trans-inactivation is not limited to the *brown* locus in *Drosophila*. Martin-Morris *et al.* (1997) and Dorer and Henikoff (1997) described *white* transgenes that variegate due to *cis*-inactivation by pericentric heterochromatin and that can impose heterochromatic silencing on a transgene located at a homologous position in *trans*. Moreover, they present evidence suggesting

that a neighboring essential gene is silenced in *trans* due to spreading of heterochromatic silencing from the *trans*-inactivated allele. These remarkable findings provide particularly clear and dramatic examples of position effects, reflecting the ability of local concentrations of heterochromatic factors to drive the establishment of ectopic heterochromatin domains on nearby loci, either in *cis* or in *trans*.

6.2. Heterochromatin associations

Several studies in yeast strongly implicate regions around the nuclear membrane in promoting transcriptional silencing (Andrulis *et al.*, 1998; Gartenberg *et al.*, 2004; Gasser *et al.*, 1998; Hediger *et al.*, 2002; Taddei *et al.*, 2004). The relationship between silencing and proximity to the nuclear membrane is not a simple one; however, the nuclear pore complex, imbedded in the nuclear membrane, has been implicated in transcriptional activity (Casolari *et al.*, 2004; Schmid *et al.*, 2006). Live-cell measurements have also shown that genes move to the nuclear envelope upon transcriptional activation (Cabal *et al.*, 2006; Drubin *et al.*, 2006; Taddei *et al.*, 2006).

In a rigorous study comparing nuclear associations and heterochromatic silencing on a cell-by-cell basis for three different variegating loci, Harmon and Sedat (2005) found that in the nuclei of cells in which the variegating gene was silenced, the association of the silenced locus with heterochromatin was significantly more intimate than in neighboring cells of the same tissue in which the locus escaped silencing. Oddly, however, they found that in mitotically cycling, undifferentiated cells where silencing of one of the rearrangements occurs in all cells, there was no difference in the extent of associations between these cells and differentiated cells in the same tissue, where variegated activation occurs. This suggests that the associations have no necessary functional relationship to silencing.

In mammals, correlations have also been noted between transcriptionally silent loci and blocks of heterochromatin (Brown *et al.*, 1997). The timing of such associations, however, suggests that they are not an obligatory part of the silencing mechanism (Brown *et al.*, 1999). The inactive X in mammalian females is associated with the nucleolus in mid-to-late S phase (Zhang *et al.*, 2007). An ectopic X inactivation center can target an autosome to the nucleolus, and deletion of the gene encoding the Xist noncoding RNA results in loss of nucleolar targeting (Zhang *et al.*, 2007). Such interactions suggest a physical affinity of certain types of chromatin, but the consequences for gene expression of these interactions may depend on additional factors besides physical proximity.

Taken together, the data concerning gene silencing and nuclear positioning are most consistent with a model in which associations between specific silent loci and certain nuclear regions are correlative but not causative. A simple way to think about this is that certain protein complexes that

promote silencing are recruited to silenced genes, that these complexes are shared with other chromosomal regions (including, in some cases, heterochromatin), and that aggregation between distant chromosomal regions may be facilitated by the affinities of shared complex components for one another. By this model, the association of a variegating gene with heterochromatin is a byproduct of gene silencing. Though a reinforcing or stabilizing role for heterochromatic associations is not excluded by this model, the targeting of heterochromatin to a silenced locus does not appear to require heterochromatic associations.

7. SUMMARY AND PERSPECTIVES

What has *Drosophila* taught us about heterochromatin? Heterochromatic position-effect silencing was first described in *Drosophila* (Muller, 1930) and genetic screens for modifiers of such position effects uncovered key factors in heterochromatin assembly (Dorn *et al.*, 1986; Sinclair *et al.*, 1983; Wustmann *et al.*, 1989). Many of these factors are structurally and functionally conserved.

The findings in *Drosophila* have pointed to a mechanism of heterochromatin assembly in which multiple factors contribute to silencing and in which cooperative protein complex assembly plays an important role. Transgene studies in *Drosophila* have emphasized the role both of specific sequences and proteins, and the cooperativity of interactions that are required to target heterochromatin assembly. As neatly summarized by Yasuhara and Wakimoto (2008): "... it is not repetitive sequences *per se* that specifies heterochromatin but the physical proximity of multiple repetitive sequences of a certain type. . . [suggesting] versatility in the activities of different types of heterochromatin-enriched repetitive DNA sequences and modified histones and. . . the importance of chromosomal context."

Cloning and characterization of heterochromatic DNA and genetic modifiers of PEV in *Drosophila* allowed the study of heterochromatin to join the mainstream of molecular biology. Versatile genetics and extraordinary cytology account for the important role *Drosophila* has played in our understanding of heterochromatin and its formation. With the advent of genome sequencing, chromatin immunoprecipitation and microarray analysis, the advantage of a high-resolution genome-wide map afforded by polytene chromosome cytology has been eclipsed. The genetic toolkit of *Schizosaccharomyces pombe* has also come to complement the genetics of *Drosophila* in this field. To the extent that *Drosophila* will continue to offer unique insights into heterochromatin assembly and function, this will be primarily as a model metazoan. The role, if any, of heterochromatin in development, differentiation, cell signaling and aging is unclear.

The genetic dissection of each of these processes is advancing rapidly using *Drosophila*, and numerous examples of control at the level of chromatin have already been uncovered, including HP1 and heterochromatic histone marks. Many of the mechanisms used to establish heterochromatin in *Drosophila* are likely to underlie mechanisms of growth, development, and aging in *Drosophila* and other animals.

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IMPORT OF NUCLEAR-ENCODED MITOCHONDRIAL PROTEINS: A COTRANSLATIONAL PERSPECTIVE

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Abstract

A growing amount of evidence suggests that the cytosolic translation of nuclear-encoded mitochondrial proteins and their subsequent import into mitochondria are tightly coupled in a process termed cotranslational import. In addition to the original posttranslational view of mitochondrial protein import, early literature also provides both *in vitro* and *in vivo* experimental evidence supporting the simultaneous existence of a cotranslational protein-import mechanism in mitochondria. Recent investigations have started to reveal the cotranslational import mechanism which is initiated by transporting either a translation complex or a translationally competent mRNA encoding a mitochondrial protein to the mitochondrial surface. The intracellular localization of mRNA to the mitochondrial surface has emerged as the latest addition to our understanding of mitochondrial biogenesis. It is mediated by targeting elements within the mRNA molecule in association with potential mRNA-binding proteins.

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1. INTRODUCTION

Mitochondria are unique organelles in eukaryotic cells, which supply cells with energy by generating ATP from metabolic fuels through oxidative phosphorylation. Mitochondria also harbor numerous essential metabolic pathways, such as heme synthesis, purine synthesis, and synthesis of Fe-S clusters. Among eukaryotic cells, mitochondria are highly conserved in terms of their morphology and protein composition. The organelles are enclosed by two membranes which divide the interior into the matrix and an intermembrane space surrounded by the inner and outer membranes, respectively. Mitochondria have their own genetic system which encodes only a tiny proportion of mitochondrial proteins while the vast majority of mitochondrial proteins are encoded by the nuclear genome and synthesized in the cytosol prior to their import into the organelles. Therefore, the process of mitochondrial protein import is vital to the biogenesis and functional attributes of the organelles. One of the main interests in mitochondrial research is focused on the mechanism(s) of protein import into the organelles. This review will highlight the roles of posttranslational and cotranslational mechanisms of protein trafficking to mitochondria.

2. MITOCHONDRIAL ASSEMBLY AND PROTEIN IMPORT

Mitochondrial biogenesis is a complex process which requires the expression of hundreds of nuclear genes as well as the transport of newly synthesized proteins to their correct locations. Since the mitochondrial genome is limited by its capacity for encoding only a small fraction of the mitochondrial proteins, one of the important tasks in mitochondrial biogenesis is the trafficking of these nuclear-encoded and cytosolically synthesized preproteins to the organelle. Protein trafficking through mitochondrial membranes is carried out by several translocation processes consisting of multisubunit proteins, collectively referred to as the mitochondrial protein import apparatus. Both inner and outer mitochondrial membranes contain multisubunit import machineries which are structurally and functionally distinct from each other. To date, a single complex has been described as the only entry point for initial recognition and translocation

of nuclear-encoded mitochondrial proteins through the mitochondrial outer membrane, named the TOM (translocase of the outer membrane) complex (Truscott *et al.*, 2003). After crossing the outer membrane, pre-proteins are guided by either one of the two TIM (translocase of the inner membrane) complexes, known as the TIM23 and TIM22 complexes, depending on the nature of the targeting signal (Truscott *et al.*, 2003). The TIM23 complex interacts with proteins with an N-terminal presequence while the TIM22 complex is required only for the import of inner membrane proteins with internal targeting signals. It is noteworthy that without the involvement of the inner membrane, the outer membrane is very limited in its capacity for protein translocation. The major driving force for protein translocation through the mitochondrial membranes is provided by the membrane potential across the inner membrane. In addition, the ATP-dependent matrix heat shock protein 70 (mtHsp70) along with Tim44 are needed for pulling presequence-containing proteins into the matrix through the Tim23 complex (Truscott *et al.*, 2003). The mitochondrial import apparatus has been extensively studied over the decades to resolve its structural composition as well as the functions of its components. Despite the advances in understanding protein translocation through the mitochondrial membranes (Chacinska *et al.*, 2005), a major question remains as to how these precursor proteins in the cytoplasm are so efficiently recognized by the mitochondrial import apparatus.



3. MITOCHONDRIAL PROTEIN IMPORT: POSTTRANSLATIONAL OR COTRANSLATIONAL?

Given that the bulk of the mitochondrial proteins are encoded by nuclear genes and synthesized on cytosolic ribosomes, one of the earliest and most debated issues relating to mitochondrial protein import is whether protein import into mitochondria occurs posttranslationally or cotranslationally (Fig. 2.1). Most of our current knowledge of the mitochondrial protein import mechanisms has been established through widely used *in vitro* systems that intrinsically favor posttranslational import. As a result, the mitochondrial protein import pathway has been considered posttranslational since two independent studies in the 1970s demonstrated that mitochondrial proteins synthesized in *in vitro* translation systems were able to be imported posttranslationally into mitochondria. One study showed that radio-labeled mitochondrial proteins synthesized in a cell-free homogenate of *Neurospora crassa* were successfully imported into the organelles made translationally inactive by chloramphenicol (Harmey *et al.*, 1977). The second study demonstrated the *in vitro* synthesis of the yeast mitochondrial F₁-ATPase consisting of five nonidentical subunits in a reticulocyte lysate

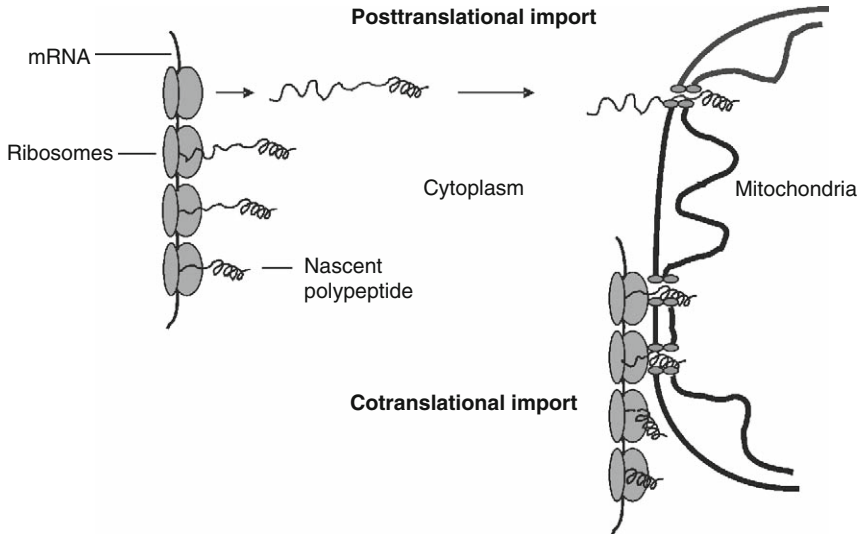


Figure 2.1 Schematic presentation of posttranslational and cotranslational protein import into mitochondria. In the case of posttranslational import, the translation of nuclear-encoded proteins comes to a completion before the full-length precursor proteins encounter the mitochondrial import apparatus. During cotranslational import, precursor proteins are synthesized in the proximity of mitochondria and the mitochondrial import of the nascent peptides takes place while translation is still in progress. Adapted from [Lithgow \(2000\)](#).

and its translocation into isolated mitochondria ([Maccacchini et al., 1979](#)). The case for posttranslational import has been further consolidated over time by a number of reports revealing the necessity for cytosolic chaperones and/or factors in maintaining the import competence of the mitochondrial precursor proteins prior to their translocation through the import apparatus ([Becker et al., 1992](#); [Komiya et al., 1996](#); [Mihara and Omura, 1996](#); [Terada et al., 1995](#)). Posttranslational import was also demonstrated *in vivo* by showing that after the dissipation of the mitochondrial membrane potential using the uncoupler carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP), accumulated mitochondrial precursor proteins *in vivo* in the cytosol can be imported into mitochondria following the reestablishment of the membrane potential ([Reid and Schatz, 1982](#)). *In vivo* evidence for posttranslational protein import into mitochondria also comes from the demonstration that chimaeric fusion proteins can be accumulated *in vivo* as two-membrane-spanning intermediates resulting from a stably folded C-terminal domain trapped outside mitochondria ([Schulke et al., 1997](#); [Wienhues et al., 1991](#)). This indicates the presence of the full-length precursor protein in the cytosol prior to completion of the import process.

On the other hand, several kinds of evidence supporting cotranslational protein import into the mitochondria *in vivo* have been reported, beginning in the 1970s. The first of these came from [Kellems and Butow \(1972\)](#) showing a tight association of cytosolic polysomes with the surface of isolated mitochondria in yeast following cycloheximide treatment to arrest translation. The quantity of ribosomes bound on the mitochondrial surface was shown to depend on the metabolic state of the cells ([Kellems and Butow, 1974](#)), indicating a functional coupling between ribosome binding and the metabolic demands of the cell. Based on electron micrographs, mitochondrion-bound ribosomes appeared to cluster at localized regions of the outer membrane where the inner and the outer membranes are in close contact, suggesting a possible interaction with the mitochondrial import apparatus ([Kellems et al., 1975](#)). Since mitochondrion-bound polysomes isolated from cycloheximide-treated cells were also enriched in mRNA for many mitochondrial proteins, cotranslational import was thus proposed for at least a subset of mitochondrial proteins ([Ades and Butow, 1980](#); [Suissa and Schatz, 1982](#)). Further studies showed that the ribosome-mitochondrial interaction is enhanced by the ribosome-bound nascent peptide chain, involving GTP hydrolysis and protease-sensitive proteins on the mitochondrial surface ([Crowley and Payne, 1998](#); [MacKenzie and Payne, 2004](#)).

Cotranslational import into mitochondria also excludes any significant pool of full length mitochondrial preproteins in the cytosol awaiting import. Consistent with this, the import of the bulk of radio-labeled mitochondrial proteins was instantly inhibited in both *in vitro* and *in vivo* by cycloheximide-induced translational arrest, suggesting that the translation and import of mitochondrial proteins are tightly coupled ([Fujiki and Verner, 1991, 1993](#)). Furthermore, the antifolate compound, methotrexate, which can bind only the folded conformation of dihydrofolate reductase (DHFR), inhibited the posttranslational import of cytochrome oxidase subunit IV-fusion protein (COXIV-DHFR) *in vitro* by preventing its unfolding; but it had little inhibitory effect on the import of COXIV-DHFR *in vivo* ([Fujiki and Verner, 1993](#)). However, [Wienhues et al. \(1991\)](#) reported a partial inhibition of *in vivo* import of DHFR fused to yeast cytochrome *b*₂ using the DHFR inhibitor aminopterin. This indicates that most but not all of the fusion protein was imported cotranslationally into mitochondrial and thus not accessible to aminopterin in the cytosol.

A mutational study with the mitochondrial leader sequence showed that mutations in the leader sequence of aldehyde dehydrogenase caused degradation of the leader peptide and cytosolic accumulation of EGFP alone ([Ni et al., 1999](#)). The results suggest that the import under the control of the native leader peptide must be fast enough to avoid cytosolic degradation of the leader peptide as well as the folding of EGFP into an import-incompetent structure, indicating a cotranslational import. By exploiting the fact that endoplasmic reticulum (ER) targeting can be accomplished by a C-terminal

ER targeting signal (Abell *et al.*, 2004; Kutay *et al.*, 1995), Ni *et al.* (1999) showed that an EGFP fusion with a mitochondrial targeting signal at the N-terminus and an ER targeting signal at the C-terminus was exclusively localized to mitochondria. This result suggests that mitochondrial import of the fusion protein began before translation of the C-terminal ER-targeting signal had occurred. Using the same dual targeting approach, these investigators also obtained evidence that the import of many endogenous mitochondrial proteins is also cotranslational (Mukhopadhyay *et al.*, 2004).

In addition, it is clear that some mitochondrial proteins cannot be imported posttranslationally *in vitro* because of their rapid folding. For example, the yeast enzyme fumarase can only be imported cotranslationally into the mitochondria (Knox *et al.*, 1998; Stein *et al.*, 1994). In *in vitro* import systems, the precursor of fumarase becomes trapped in the import channel after the processing of the N-terminal end, staying outside the channel in a folded conformation. *In vivo* this processed and folded polypeptide is released from the channel and remains active in the cytosol, so that the enzyme is found in both the cytosolic and mitochondrial compartments. The fumarase in the mitochondrial compartment is likely to have not had the opportunity to fold before import. Interestingly, the fumarase precursor was found to be efficiently imported into mitochondria *in vitro* in a coupled translation-import reaction. Similarly, the yeast major adenylate kinase can only be imported into mitochondria cotranslationally because stable folding of the protein in the cytosol prevents import (Strobel *et al.*, 2002). The kinase can only be imported into mitochondria if it reaches a mitochondrial surface during its synthesis; otherwise, it folds and remains in the cytosol in an enzymatically active conformation.

The latest evidence for cotranslational import of proteins into the mitochondria came from a recent *in vivo* study of the import of GFP fusion proteins into mitochondria in *Dictyostelium discoideum* (Ahmed *et al.*, 2006). Using both mitochondrially targeted and nontargeted GFP fusion proteins, these authors observed a novel phenomenon for mitochondrially targeted proteins, import-associated translational inhibition, in which the rate of translation is limited by mitochondrial import. This demonstrated a functional coupling between import and translation *in vivo*, implying a physical association between the mRNA and mitochondrial surface, which was demonstrated directly by northern blotting. Interestingly, the translation of otherwise identical fusions with a second reporter protein, aequorin, was unaffected by whether or not the protein was imported into the mitochondria and the mRNA was not bound to the mitochondrial surface. These observations supported the view that some proteins are imported cotranslationally *in vivo*, while others are not. Although this study involved highly expressed mRNAs encoding a normally nonmitochondrial protein, GFP, such a novel *in vivo* regulatory mechanism could possibly exist *in vivo* for some native mitochondrial proteins (Ahmed *et al.*, 2006).

Given the dynamic nature of the intracellular environment, the location of protein synthesis can also play a vital role in defining whether the mitochondrial protein import will be cotranslational or posttranslational. Under physiological conditions, any translationally active mRNA could encounter mitochondria by chance and thereby initiate a cotranslational import. In this case, the relative kinetics of translation, translocation of the polysome to the mitochondrial surface, and import could determine the extent to which translation and import operate in close proximity at a particular time. However, the evidence, so far, suggests that the selection of cotranslational or posttranslational import for any given protein appears not to be random but to be consistently dependent on which protein is being imported. Overall, it can be concluded that both post and cotranslational import pathways are functionally active *in vivo*, depending on the protein and the physiological consequences for the cell. The following sections of this review will highlight our current understanding of the mechanism for cotranslational protein import into mitochondria.

3.1. Cotranslational protein import into mitochondria: Two possible mechanisms

Research over the last 10 years has begun to reveal the underlying mechanism(s) of cotranslational protein import into mitochondria. Coupling between mitochondrial protein synthesis and import can be initiated by targeting to the mitochondrial surface either (1) a nascent peptide destined for import or (2) a corresponding translationally active mRNA. A few cytosolic factors have already been reported that bind mitochondrial nascent peptides in the cytoplasm and facilitate import (Cartwright *et al.*, 1997; George *et al.*, 1998) (see the following sections). However, whether the nascent peptides are transported to the mitochondrial surface cotranslationally or posttranslationally is not firmly established yet *in vivo*. On the other hand, the sorting of the mRNA itself to the mitochondrial surface seems to be more convincing for the cotranslational import mechanism at least for some mitochondrial proteins—the recruitment of some mRNAs to the vicinity of mitochondria is necessary for the import of corresponding proteins to the organelle (Corral-Debrinski *et al.*, 2000; Margeot *et al.*, 2002) and mRNA-binding proteins are functionally important in mitochondrial import (Gratzer *et al.*, 2000; Zoladek *et al.*, 1995).

3.1.1. Targeting of the translation complex by ribosome-associated nascent polypeptides

A cotranslational translocation pathway is already well-established for secreted proteins as their translation and translocation through the membrane of the ER are tightly coupled processes (Martoglio and Dobberstein, 1996). During cotranslational protein import into the ER, a soluble

targeting factor, the signal recognition particle (SRP), acts as a shuttle between the cytosol and the ER membrane. Once a nascent secretory protein emerges from the ribosome, SRP binds the amino terminal signal peptide and subsequently inhibits its synthesis until the ribosome docks on the ER membrane. The signal peptide-bound SRP eventually mediates the targeting of the ribosome to the ER by interacting with a SRP-receptor at the ER membrane (Rapoport, 1992; Walter *et al.*, 1984). Even though some proteins could be imported cotranslationally into the mitochondria if their translation was not complete before import began, unlike the targeting of secreted proteins to the ER, there is no known mechanism for the cotranslational translocation of the mitochondrial precursor proteins to the organelle. Since a targeting factor comparable to the SRP is yet to be discovered in the mitochondrial protein import pathway, it is not yet clear how a mitochondrial nascent peptide could be recognized in the cytosol and then be delivered to the mitochondrial surface.

Nonetheless, several lines of evidence have already supported the existence of potential cytosolic factors involved in targeting ribosome-associated nascent peptides to the mitochondrial surface. The nascent polypeptide-associated complex (NAC) was purified as a heterodimeric protein from mammalian cell extract that interacts with various nascent chains (Wiedmann *et al.*, 1994). The primary function of the NAC has been suggested as a protective shield for short nascent polypeptides from inappropriate interactions with cytosolic proteins since in its absence the SRP was shown to cross-react with and target proteins nonspecifically to the ER. A potential role for the NAC in mitochondrial protein import was demonstrated by the finding that NAC-deficient yeast mutants show defects in targeting reporter proteins to the mitochondria (George *et al.*, 1998). The mitochondrial import of both fumarase and malate dehydrogenase were also decreased in the yeast mutants while the import of the majority of endogenous mitochondrial proteins was unaffected (George *et al.*, 2002). Interestingly, an *in vitro* association of the NAC with the mitochondria through the nascent chains suggests a possible involvement of the complex in cotranslational import (Funfschilling and Rospert, 1999). In addition, a decreased level of mitochondria-bound ribosomes in yeast mutants lacking NAC also suggests a possible role for the NAC complex in ribosome binding to the mitochondria and cotranslational import (George *et al.*, 2002).

A novel cytosolic protein, Mft52, identified in yeast cells was shown to be crucial for targeting reporter proteins into mitochondria (Cartwright *et al.*, 1997). Mft52 binds mitochondrial targeting sequences through a two-domain structure which has a sequence homology to the yeast mitochondrial import proteins, Tom20 and Tom22. It has been suggested that interaction of the NAC with the nascent mitochondrial peptides is followed by the Mft52 binding to the mitochondrial presequences and that Mft52 can thereby act as a shuttle for mitochondrial proteins between the cytosol and

the mitochondria (Fig. 2.2). Since yeast mutants lacking both NAC and Mft52 were defective not only in targeting reporter proteins to mitochondria but also in mitochondrial morphology, these proteins appear to be jointly essential for targeting endogenous proteins into mitochondria and thus for mitochondrial biogenesis (George *et al.*, 1998).

Molecular chaperones play a major role in mediating correct folding as well as preventing misfolding and aggregation of polypeptides *in vivo*. Mitochondrial precursor proteins have long been envisaged to be held in an extended conformation by 70 kDa heat shock cognate proteins (Hsc70) in the cytosol so that the precursors remain import-competent prior to translocation through the mitochondrial membranes. Another cytosolic chaperone, named mitochondrial import stimulating factor (MSF), selectively binds the presequence of mitochondrial precursor proteins and remains bound until the precursors encounter the mitochondrial import apparatus (Komiya *et al.*, 1994). The interaction of molecular chaperones with mitochondrial precursor proteins is mostly viewed as being part of a posttranslational import mechanism, while cotranslationally imported proteins are predicted to require less assistance from the cytosol. Interestingly, the

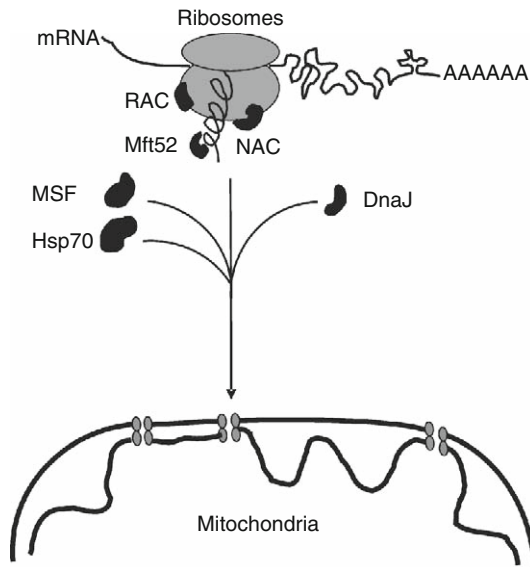


Figure 2.2 Targeting of the nascent peptide to the vicinity of the mitochondria. The NAC protects the nascent peptides from any nonspecific interactions in the cytosol and helps achieve a secondary structure so that the targeting signal becomes accessible to Mft52. Bound to the ribosomes, the RAC can also have a chaperone-like effect on nascent peptides. In addition, other molecular chaperones, such as MSF, Hsp70, and DnaJ, can assist the efficient transfer of the nascent peptides to the mitochondrial import apparatus (Adapted from George *et al.*, 1998 and Lithgow *et al.*, 1997).

interaction of cytosolic chaperones with nascent mitochondrial peptides occurs cotranslationally and could participate in either a posttranslational import mechanism or in transport of the nascent polypeptide to the mitochondrial surface for cotranslational import.

The cotranslational interaction of cytosolic chaperones with ribosome-bound nascent polypeptides was first reported in HeLa cells using two different approaches (Beckmann *et al.*, 1990). Following puromycin-induced premature release from ribosomes *in vivo*, nascent polypeptides were found associated with cytosolic forms of Hsp70. Ribosome-bound [³⁵S] Met-labeled nascent polypeptides were also found in a complex with Hsp70 *in vivo* in pulse-labeled HeLa cells. Another study demonstrated that the bacterial chaperone protein DnaJ binds cotranslationally to firefly luciferase and chloramphenicol acetyltransferase *in vitro* (Hendrick *et al.*, 1993). Interestingly, DnaJ binding appeared to arrest protein folding as well as import into microsomes and mitochondria until its functional partner DnaK (a member of Hsp70 family) and its nucleotide exchange factor, GrpE, were added to the *in vitro* reaction. The cotranslational interaction of DnaJ was proposed to play an important role in protecting nascent peptides from aggregation as well as facilitating their productive folding in cooperation with DnaK. In addition, several other studies demonstrated the association of a number of components of the chaperone machinery with translating ribosomes or nascent peptides (Frydman *et al.*, 1994; Pfund *et al.*, 1998; Thulasiraman *et al.*, 1999; Zhong and Arndt, 1993). Cotranslational interaction between a molecular chaperone and a nascent peptide does not necessarily imply cotranslational import as an end result, but a possible role for any of these chaperones in mediating or assisting cotranslational import can not be ruled out (Fig. 2.2).

A direct involvement of molecular chaperones in stimulating cotranslational import of ribosome-bound nascent peptides into mitochondria was revealed with the identification of a ribosome-associated complex (RAC) in yeast, composed of yeast (Gautschi *et al.*, 2001) (Fig. 2.2). The RAC was characterized as a heterodimer composed of the DnaJ homolog zuotin and the DnaK homolog Ssz1p/Pdr13p in a stable 1:1 complex. Bound to the ribosomes through the zuotin subunit, RAC has been considered to have a chaperone-like effect on nascent peptides and a stimulatory effect on cotranslational protein import into mitochondria *in vitro*. RAC was discovered in an attempt to identify factors sharing the NAC-like ability to stimulate mitochondrial protein import as the yeast mutants lacking NAC did not show any significant defect in growth. Surprisingly, the mutants lacking both NAC and RAC didn't show any significant phenotype, suggesting either that factors involved in cotranslational import into mitochondria are highly diverse and redundant, or that cotranslational import is not essential for mitochondrial biogenesis and function.

3.1.2. Targeting of the translation complex by mRNA

While most proteins are localized to their destination on the basis of signals in the peptide sequence, the transport of mRNA has been recognized over the past 20 years as an additional means for localizing some cytoplasmic proteins. Since the first observation of the uneven distribution of actin transcripts in the ascidian embryo in 1983 (Jeffery *et al.*, 1983), more than 90 localized mRNAs have been reported, so far, in a variety of systems. Most of these were observed in oocytes and early embryos, particularly in *Drosophila melanogaster* oocytes or embryos and *Xenopus laevis* oocytes.

Even though mRNA localization has been well documented over the years, the mechanism for mRNA transport and targeting is not yet well understood. The localization of mRNA depends on specific *cis*-acting sequence elements, called “zipcodes,” that generally lie within the 3′-untranslated region (3′-UTR) of mRNAs (Singer, 1993). Since there is no sequence homology among the zipcodes identified so far, a number of studies indicated that the role of a zipcode depends on its predicted stem-loop conformation rather than on its primary sequence (Chartrand *et al.*, 1999; Gonzalez *et al.*, 1999; Serano and Cohen, 1995). The function of the zipcodes is mediated by RNA-binding proteins most of which are yet to be identified. One of the well-characterized candidates is Staufen, a double-stranded RNA-binding protein, which is essential for localizing maternal RNAs in the anterior cytoplasm of *Drosophila* eggs (St. Johnston *et al.*, 1991).

Since several studies demonstrated that cytoskeletal inhibitors could effectively block mRNA localization, it has been proposed that cytoplasmic mRNAs are transported in the form of large ribonucleoprotein (RNP) complexes in association with cytoskeletal filaments and motor proteins (Bassell *et al.*, 1999; Wilhelm and Vale, 1993). The involvement of cytoskeletal proteins in mRNA localization was further confirmed by the identification of a microtubule-dependent motor required for the transport of *oskar* mRNA and Staufen protein to the posterior end of *Drosophila* oocytes (Brendza *et al.*, 2000). The overall mechanism for mRNA transport is currently viewed as a three-step process (Wilhelm and Vale, 1993). The first step is the interaction of the localized mRNA with mRNA-binding proteins that results in the formation of an RNP complex. The RNP complex then associates with cytoskeletal proteins to initiate active transport. Finally, mRNA is delivered to its destination where it becomes anchored to maintain its localized distribution.

Based on a number of reports over the past 10 years, the transport of mRNA has been regarded as an alternative means for targeting some proteins to the mitochondria (Fig. 2.3). The first evidence came from yeast mutants defective in the *MOD5* gene which encodes a t-RNA processing isoenzyme found in mitochondria, in the nucleus, and in the

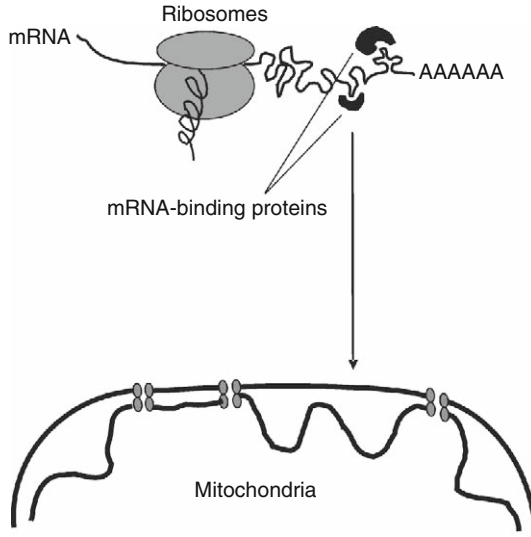


Figure 2.3 The localization of the mRNAs encoding a mitochondrial precursor protein to the vicinity of the mitochondria. Signals in the mRNA molecules can direct the transport of mRNA to the mitochondrial surface, mediated by mRNA-binding proteins.

cytoplasm (Zoladek *et al.*, 1995). Among the four *mdp* mutants characterized as defective in delivering a precursor form of the mitochondrial t-RNA processing enzyme, *mdp3* cells appeared to carry a mutation in a putative mRNA-binding protein, Pan1p, indicating a potential role for mRNA-binding proteins in mitochondrial import. The same study also mapped the defect in *mdp2* cells to a mutation in the cytoskeletal protein verprolin and showed that the defect was suppressed by overexpressing the *ACT1* gene encoding actin. This revealed a role for the cytoskeleton in mitochondrial import. Interestingly, an independent study subsequently demonstrated a functional interaction between the mRNA-binding protein, Pan1p and the actin cytoskeleton in yeast (Tang and Cai, 1996).

Another yeast mutant strain, *mts1*, was identified in which the targeting defect of a mutant β -subunit of the mitochondrial ATPase was suppressed, supposedly with a modified presequence so that import was restored to wild-type levels (Ellis and Reid, 1993). Surprisingly, *mts1* cells were found subsequently to carry a mutation in a known mRNA-binding protein, Npl3p, which acts as a shuttle for trafficking proteins and RNA between the nucleus and the cytoplasm in yeast (Gratzer *et al.*, 2000). The suppression of the targeting defect in *mts1* cells was found to be due to a mutation in the mRNA-binding domain of Npl3p which resulted in a prolonged cytosolic interaction of the mutant Npl3p with mRNA. This increased

the stability of the mRNA, providing more time to encounter the mitochondrial import apparatus so that import was restored to the wild-type level (Gratzer *et al.*, 2000). These observations suggest that specific mRNA-binding proteins as well as cytoskeletal motors exist in the cytoplasm for the potential localization of transcripts to the vicinity of the mitochondria.

Direct observation of localized mRNAs encoding mitochondrial proteins was also reported in a number of systems. Using a high-resolution *in situ* hybridization technique in rat hepatocytes, the mRNA encoding the β -subunit of the mitochondrial F_1 -ATPase complex was found nearby the mitochondria (Egea *et al.*, 1997). The localized β - F_1 -ATPase mRNA was shown to be translationally active, suggesting its potential relevance for the biogenesis of the F_1 -ATPase complex (Ricart *et al.*, 1997). Using a genomic approach to analyze the cytosolic as well as mitochondrion-bound polyosomes in yeast cells, it has been shown that a significant proportion of mRNAs encoding mitochondrial proteins was associated with mitochondrial surface (Marc *et al.*, 2002). Based on the analysis of the mitochondrion-bound polyosomes as well as a method to visualize RNA in living cells (Beach *et al.*, 1999), the mitochondrial localization of *ATM1* mRNA encoding an ATP-binding cassette (ABC) transporter of the mitochondrial inner membrane was found to be mediated by either the 3'-UTR or the presequence coding region (Corral-Debrinski *et al.*, 2000). A recent study has demonstrated that an mRNA-binding protein, Puf3p, which belongs to a family of RNA-binding proteins called Pumilio-Fbf (Puf) proteins, is involved in transporting a subset of mRNAs encoding mitochondrial proteins to the mitochondrial surface (Saint-Georges *et al.*, 2008). Interestingly, Puf3p selectively binds mRNAs that encode proteins destined for only mitochondria and plays an essential role in targeting the encoded proteins into the in mitochondria.

The sorting of mRNA to the vicinity of mitochondria is also physiologically significant as it seems crucial for the functions of the corresponding protein. In yeast cells, *ATP2* mRNA (encoding the β -subunit of the mitochondrial F_1 -ATP synthase, respiratory chain complex V) normally associates with the mitochondria, while the *ALDH1* (aldehyde dehydrogenase) mRNA does not. The replacement of the 3'-UTR of *ATP2* with that of *ALDH1* resulted in a respiratory dysfunction (Margeot *et al.*, 2002). The respiratory dysfunction in the mutated cells was also correlated with the observations that the altered *ATP2* mRNA was enriched on free cytosolic polyosomes and the mitochondrial import of the translated protein was also extensively diminished. The same study also reproduced a similar defect in yeast cells when the 3'-UTR of *ATM1* was replaced with that of *ALDH1*, indicating a significant role of the 3'-UTRs in targeting mRNA to the mitochondrial surface and in mitochondrial import.

A mitochondrial targeting function of 3'-UTRs was supported by another study involving *OXA1*, encoding a protein which is involved in

the insertion of inner mitochondrial membrane proteins. *OXA1* mRNA was found to be associated with mitochondria in both yeast and human cells (Sylvestre *et al.*, 2003). Interestingly, the expression of human *OXA1* in yeast cells didn't alter the usual localization of the mRNA and a reporter mRNA was efficiently localized to the mitochondria in yeast cells using the 3'-UTR of human *OXA1* mRNA. The respiratory function of the yeast *oxa1⁻* mutant cells was also rescued with human full-length *OXA1* gene but not with the truncated form lacking the 3'-UTR. This indicates that the functional role of the 3'-UTR in targeting mRNA to the mitochondria is evolutionarily conserved.

3.2. Properties of structural elements within the coding region of mRNA

What might be the defining feature of a *cis*-acting targeting signal within mRNA molecules? Since the targeting sequences exhibit no distinctive sequence identity but they are predicted to form characteristic stem-loop structures, it has been suggested that a stem-loop structure is recognized by cytosolic mRNA-binding proteins, most of which are still unknown (Serano and Cohen, 1995; St. Johnston, 1995). Even though almost every single mRNA targeting element reported so far, regardless of the subcellular targeting specificity, has been detected within the 3'-UTRs of the molecules, a couple of studies revealed the presence of such *cis*-acting elements in the coding region of the molecule and these elements can vary in structural motifs which could potentially interact with different mRNA-binding proteins (Chartrand *et al.*, 1999; Gonzalez *et al.*, 1999). The sorting of *ASH1* mRNA to the budding tip of *Saccharomyces cerevisiae* was initially shown to be dependent on its 3'-UTR. Surprisingly, the replacement of the 3'-UTR of the molecule had no significant effect on its usual localization and a further analysis resolved the mystery with the identification of three further *cis*-acting elements in the coding region. This finding also showed that the *cis*-acting sequences can vary in their structural motifs which could potentially interact with different mRNA-binding proteins. Based on the redundant localization of the targeting elements in the *ASH1* mRNA, it can also be suggested that such targeting signals could potentially be present elsewhere in other molecules.

A recent observation suggests that similar targeting element(s) could exist serendipitously within the coding sequences of other molecules (Ahmed *et al.*, 2006). Using two reporter proteins, GFP and aequorin, it was found that only GFP mRNA associated with the mitochondrial surface. A comparison of predicted secondary structures showed that three predicted stem-loops in the GFP mRNA exhibit striking similarities with a well-known stem-loop structure of yeast ATP2 mRNA already known for its role in mitochondrial targeting. Thus, the GFP mRNA may mimic in up to

three places the secondary structure of a conserved, long stem-loop that directs mRNA to the mitochondrial surface. No similar structures were identified in aequorin mRNA which did not associate with the mitochondrial surface (Ahmed *et al.*, 2006).

4. CONCLUSIONS AND PERSPECTIVES

Unlike protein targeting to the ER which is mediated by the SRP (Martoglio and Dobberstein, 1996; Rapoport, 1992; Walter *et al.*, 1984), there is no established mechanism for the mitochondrial protein import pathway that must be followed by all mitochondrial preproteins (Beddoe and Lithgow, 2002; Lithgow, 2000). Earlier studies with mitochondrial targeting signals demonstrated that an N-terminal targeting signal is usually sufficient to target any passenger protein to the mitochondria, indicating that the mature part of the protein has no role in the protein targeting process into the organelle (Neupert, 1997). However, a potential role for the mature part of a precursor protein in the mitochondrial protein import pathway has been suggested by a number of reports. Firstly, the binding of the precursor form of F_0 -ATPase subunit 9 to the mitochondrial receptors was found to be carried out by the hydrophobic mature part of the protein (Pfanner *et al.*, 1987). Secondly, following *in vitro* translation in wheat germ extract, the subunit F_{Ad} of ATPase as well as a fusion construct with its mature portion (lacking the prepeptide) could be imported into mitochondria but not the fusion construct with just its presequence. This indicates that the import in this case is dependent on the mature part rather than on the presequence (Dessi *et al.*, 2003). Thirdly, in yeast mutants deficient for Mft52, a novel cytosolic protein involved in targeting precursor proteins to the mitochondria, the import of fusion proteins containing N-terminal mitochondrial targeting signals was inhibited while the import of native mitochondrial proteins was unaffected, suggesting the possibility for the presence of additional targeting information in the mature part of the native proteins (Cartwright *et al.*, 1997). Fourthly, an *in vitro* study of the targeting signals known to have dual targeting ability demonstrated that the specificity of import into mitochondria and chloroplasts was dependent on the passenger proteins (Chew and Whelan, 2003). Finally, the discovery of import-associated translational inhibition and mitochondrial association of the mRNA for one only of two reporter proteins showed that mitochondrial protein import in *Dictyostelium* can be cotranslational or postranslational depending on the nature of the reporter protein (Ahmed *et al.*, 2006).

Due to the biochemical diversity of precursor proteins, a wide range of cytosolic chaperones as well as other factors have been identified which are essential for the import of a variety of mitochondrial proteins (Beddoe and

Lithgow, 2002; Lithgow *et al.*, 1997). Despite the identification of a diverse range of cytosolic factors involved in mitochondrial protein import, the mechanisms for trafficking proteins to the organelle surface are still largely unresolved. The diversity in transport pathways is mainly revealed by the fact that the deletion of individual cytosolic factors affects only a fraction of total mitochondrial proteins, suggesting versatility in trafficking mechanisms (Cartwright *et al.*, 1997; Gautschi *et al.*, 2001). Interestingly, the functions of some factors are confined to fusion proteins designed for targeting reporter proteins rather than the vast majority of native mitochondrial proteins. Apart from the distinctive role of the individual cytosolic factors identified so far, a direct interaction of these factors with the mitochondrial import apparatus is yet to be shown, with the exception of one recent report showing Tom70 to be a docking point for the cytosolic chaperones Hsp70 and Hsp90 (Young *et al.*, 2003). Although both cotranslational and posttranslational import pathways exist *in vivo* (Marc *et al.*, 2002), the cytosolic factors involved in either mediating the distinction between these two pathways or regulating each pathway remain to be clearly distinguished.

The isolation and characterization of endogenous mitochondrial transcripts associated with the mitochondrial surface would shed light on the physiological role of cotranslational protein import into the mitochondria. The structural analysis of such transcripts on the mitochondrial surface might reveal common mRNA targeting motifs within the sequences. One of the future goals could be to investigate the physiological significance of such *cis*-acting targeting motifs in mitochondrial protein import and functions by creating deleted versions of the transcripts without the motifs. It will also be interesting to see whether or not such *cis*-acting targeting motifs are sufficient to target any passenger mRNA to the mitochondrial surface. An associated goal could also be focused on whether “import-associated translational inhibition” we observed is physiologically relevant to and used as a means of regulating the expression of some native mitochondrial proteins.

As mentioned earlier, very little is currently known about the mRNA-binding proteins responsible for trafficking mRNA to the mitochondrial surface. Thus far, only three mRNA-binding proteins, Pan1p, Npl3p, and Puf3p, have been identified in yeast which could potentially play a role in targeting mRNA to the mitochondria (Gratzer *et al.*, 2000; Saint-Georges *et al.*, 2008; Zoladek *et al.*, 1995). In order to clarify the mechanism for mRNA transport to the mitochondria, the mRNA targeting motifs could provide useful tools in future studies to discover relevant mRNA-binding proteins. Apart from the mRNA-binding proteins, future study could also focus on other potential binding partners such as cytoskeletal elements and motor proteins which were previously shown to be an integral part of the RNP complexes in the cytoplasm (Bassell *et al.*, 1999; Wilhelm and Vale, 1993).

The identification of such potential mRNA-associated proteins would allow us to define their specific roles in mRNA transport as well as to understand the largely unresolved mechanisms underlying the trafficking of proteins to the mitochondria.

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GIANT SILICEOUS SPICULES FROM THE DEEP-SEA GLASS SPONGE *MONORHAPHIS CHUNI*

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Abstract

Only 13 years after realizing, during a repair of a telegraph cable pulled out from the deep sea, that the depth of the ocean is plentifully populated with a highly diverse fauna and flora, the Challenger expedition (1873–1876) treasured up a rich collection of vitreous sponges (Hexactinellida). They had been described by Schulze and represent the phylogenetically oldest class of

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siliceous sponges (phylum Porifera); they are eye-catching because of their distinct body plan, which relies on a filigree skeleton. It is constructed by an array of morphologically determined elements, the spicules. Soon after, during the German Deep Sea Expedition “Valdivia” (1898–1899), Schulze could describe the largest siliceous hexactinellid sponge on Earth, the up to 3-m high *Monorhaphis chuni*, which develops the equally largest bio-silica structure, the giant basal spicules (3 m × 10 mm). Using these spicules as a model, basic knowledge on the morphology, formation, and development of the skeletal elements could be achieved. They are formed by a proteinaceous scaffold (composed of a 27-kDa protein), which mediates the formation of the siliceous lamellae, into which the proteins are encased. The high number of 800 of 5–10 μm thick lamellae is concentrically arranged around the axial canal. The silica matrix is composed of almost pure silicon oxide, providing it with unusually optophysical properties, which are superior to those of man-made waveguides. Experiments might suggest that the spicules function *in vivo* as a nonocular photoreception system. In addition, the spicules have exceptional mechanical properties, combining mechanical stability with strength and stiffness. Like demosponges, also the hexactinellids synthesize their silica enzymatically, via the enzyme silicatein (27-kDa protein). It is suggested that these basic insights will surely contribute to a further applied utilization and exploration of silica in bio-material/biomedical science.

Key Words: Sponge, Porifera, Hexactinellida, Spicule, Giant basal spicule, Silicatein, Biomaterial science. © 2009 Elsevier Inc.

1. INTRODUCTION

Sponges are the most simple, multicellular animals which are grouped to the phylum Porifera according to Grant (Poriphera; Grant, 1833). Grant (1833) described these sessile, marine animals just to be built of soft, spongy (amorphously shaped) material. Later, with the discovery of the glass sponges (class Hexactinellida; Schmidt, 1870) this view changed dramatically; from then on they were qualified as the “most strongly individualized, radially symmetrical” entities (Hyman, 1940). From their discovery, the hexactinellids had been appraised as “the most characteristic inhabitants of the great depths, which rival” with the second class of Porifera, the demosponges, “in beauty” (Campbell, 1876). It is the skeleton that supports their thin network of living tissues, the delicate scaffold of siliceous spicules, some of which may be fused together by secondary silica deposition to form a rigid framework (Leys *et al.*, 2007). The Hexactinellida together with the Demospongiae form a common taxonomic unit, comprising the siliceous sponges. Their skeletons are built of silica, that is deposited in the form of

amorphous opal ($\text{SiO}_2 \cdot n\text{H}_2\text{O}$), and constructs a variety of distinct types of structures, termed spicules. The result was very surprising that, according to molecular data of genes which encode receptors and signal transduction molecules (Kruse *et al.*, 1997, 1998; Müller *et al.*, 2004), the Hexactinellida could be established as the phylogenetically oldest class of the Porifera. Based on the earlier discovery that the Porifera share one common ancestor with the other metazoans, the Urmetazoa (Müller *et al.*, 1995, 1998), it was recognized that these animals represent the oldest, still extant metazoan taxon. Even more, the emergence of these animals could be calculated back to 650–665 million years ago (Ma), a number that was confirmed by fossils records (Reitner and Wörheide, 2002). Hence, the Porifera must have lived already prior to the Ediacaran–Cambrian boundary, 542 Ma, and thus may contribute with their elucidated genetic toolkit (Müller *et al.*, 2004), as sketched by Pilcher (2005), to the understanding of the Ediacaran soft-bodied biota as well. It was the evolutionary novelty, the formation of a skeleton, that contributed significantly to the radiation of the animals in the late Proterozoic (Knoll, 2003) and the construction of the metazoan body plan (Müller, 2005). Later in evolution, after the Ediacaran period (Schütze *et al.*, 1999), the third class of Porifera appeared, the Calcarea, which comprises a calcium–carbonate skeleton.

The evolution of the sponges, as “living fossils” (Müller, 1998), proceeded in between two major “snowball earth events”, the Sturtian glaciation (710–680 Ma) and the Varanger–Marinoan ice ages (605–585 Ma), when the Earth was covered by continuous ice layers (Hoffmann and Schrag, 2002). During these periods most metazoan species went extinct, perhaps more than 85%, with the exception of sponges (Hoffman *et al.*, 1998). The primordial earth surface consisted initially of insoluble silicates, carbonates, and to a small extent also of phosphates. During the silicate weathering–carbonate precipitation cycle, occurring prior or simultaneously with the glaciations, a dissolution of these surface rocks composed of insoluble silicates (CaSiO_3) resulted in formation of soluble calcium carbonate (CaCO_3) and soluble silica (SiO_2), under consumption of atmospheric CO_2 (Walker, 2003). As a consequence, soluble minerals leached into the waters of the rivers, lakes, and oceans. There, the minerals were again reprecipitated into new minerals, as part of the sedimentary rocks.

The hexactinellid sponges are characterized by siliceous spicules that display hexactinic, triaxonic (cubic) symmetries, or morphologies derived by reduction from the basic building plans of the spicules. The body shapes of these highly structured animals are less variable and more structured than those found in Demospongiae. As an example, *Hyalonema sieboldi* might be mentioned (Müller *et al.*, 2006b). Like all hexactinellids this species comprises a skeleton composed of megascleres and microscleres. Those two types of spicules have been further sub-classified according to their sizes and forms. The oldest fossil sponge spicules have been excavated in

Australia, China, and Mongolia (>540 Ma) and have been assigned to the Hexactinellida (Müller *et al.*, 2007d). The species *Solactiniella plumata*, which had been discovered in Sansha, Hunan province (Early Cambrian; China; Steiner *et al.*, 1993, 1994), has been grouped to the hexactinellids, the oldest sponge group. In both, the lower and the upper levels of the Niutitang formation more or less completely preserved sponge fossils have been discovered (Steiner *et al.*, 1993). It is important to mention that the sponges found at the Sansha section definitely occur at lower levels than the famous, diverse sponge fauna at Chengjiang prior to the Ediacara (Li *et al.*, 1998). Their spicules are mainly monaxones (size around 5 mm), reduced forms of the hexactin megascleres. Only a few spicules are also definite “crosses”, the evidence of the triaxones from hexactinellids. It should be mentioned that high resolution scanning electron microscopic analyses, coupled with energy dispersive X-ray, identified those spicules of *S. plumata* as to be composed of the original amorphous silica (unpublished data). In contrast, the characteristic fossil hexactin spicules from hexactinellids, found in the Xinjiang Province from the Talimu basin (Carrera and Rigby, 1999; Zhang *et al.*, 2000), and dated back to the Ordovician (510–445 Ma), have not conserved their silica state but have been diagenetically converted to calcite microcrystals.

The Hexactinellida have been divided into two main lineages, the Amphidiscophora and the Hexasterophora (Reiswig, 2006). The Amphidiscophora comprise amphidisc microscleres, which have dumbbell-shaped microscleres showing at their two ends umbel-like expansions. The morphology of the species follows a funnel to cup pattern that achieves the stability of the body by pinular pentactines, and rarely by hexactins, while the fixation to the substrate is maintained by basalia (monactines). It is the variation in the basalia that gives the Amphidiscophora their distinguished morphology. The basalia can be bundled together, or even balled together. The most outstanding species of this order are *Monorhaphis* and *Hyalonema* due to their sizes. The second order of hexactinellids is represented by the Hexasterophora that comprise a rigid dictyonal framework originating from simple hexactins. The body plan features typically a branching and anastomosing form with terminal oscular plates. The best known example is *Euplectella aspergillum*.

It is amazing to perceive empirically the variations by which the sponges form their spicules. Both taxa of the Porifera, the siliceous Hexactinellida and Demospongiae as well as the Calcarea, comprise spicules which apparently have the same basic construction plan. They present structures that follow distinct, species-specific morphogenic programs. The major symmetry axes of megascleres determine the difference between the Hexactinellida, which are monaxons (reduced forms) and triaxons, and the Demospongiae, which are monaxons (reduced forms) and tetraxons. Besides the morphogenetic activity which is induced by the silica matrix

of the spicules (see [Section 5](#)), the interlocked or joined spicules form a solid and flexible skeleton (the constructive scaffold) of inorganic poly-silica which is solidified by the organic cement spongin. It remains enigmatic by which genetic program this complex skeleton is initiated, run, and maintained. We adopt the view that the formation of the spicules, their morphology, is the primary origin of the skeleton, while the spongin cement is secondary. It is hoped that this review will provide the basis for a molecular/cell biological understanding of spicule formation in Hexactinellida, taking the giant basal spicule from *Monorhaphis* as the model structural element.

2. MONORHAPHIS

The giant basal spicules from *Monorhaphis* are in the focus of this review, since they represent the largest bio-silica structure on Earth and allow exemplarily investigations on the formation of the sponge spicules on the different morphological levels. Furthermore, they provide the blueprints for nature-inspired biotechnological applications in the field of bio-optics and biomedicine

2.1. Discovery

The nineteenth century marks the beginning of the deep sea research, when it became overt that this region of our planet is so densely populated ([Murray and Hjort, 1912](#)). During the repair of a telegraph cable laid along the bed of the Mediterranean in 1860, which was brought up from a depth of 2000 m and was found to be covered with mollusks, worms, and bryozoa, it became evident that the deep sea presents a cornucopia of “exotic” species. Already in 1861 [Bocage and Barboza \(1864\)](#) described the first *Hyalonema* species. In the following years an armada of expeditions was sent off to explore the biotic and abiotic world of the deep sea, with the Challenger Expeditions (1870 and 1872) as the most famous and pioneering ones. The major results were published in the series “Report of the Scientific Results of the Voyage of the H.M.S. Challenger during the years 1873–1876.” One complete volume in this series was already devoted to the Hexactinellida; the material collected during this expedition had been prepared and analyzed by [Schulze \(1887\)](#). This author was primarily and initially focused on the species *E. aspergillum*, but finally gave a first comprehensive classification of the different hexactinellids, known at that time. In this compilation, [Schulze \(1887\)](#) did not concentrate primarily on the cytological, structural, and functional aspects of the spicules but on taxonomy. However, with this opus he founded the basis for his intriguing

description of the hexactinellids, with *Monorhaphis* in the center, collected during the German Deep Sea Expedition “Valdivia,” during the years 1898–1899 (Schulze, 1904). Schulze did not participate on that voyage, but the Chief of the Expedition, Chun (1900), gave in his first summary a photograph of a *Monorhaphis* specimen collected from a depth of 1644 m off the coast of East Africa (Somalia basin). This specimen had an estimated size of 3 m and was surrounding one equally long siliceous spicule (Pfahlnadel), which became one of the most lionized collected object of that expedition (Fig. 3.1D). The spicule was surrounded by stony corals. Because of their size, and the depth from which the specimens had been collected, no complete spicule had been found. Using the giant basal spicules from this expedition, Schulze (1904) provided a detailed description of their morphology and their development. The data given there, with their scientific accuracy, must be the reference also for a present day review.

2.2. Organism

Three species of the Monorhaphididae have been described: *Monorhaphis chuni* (Schulze, 1904), *Monorhaphis dives* (Schulze, 1904), and *Monorhaphis intermedia* (Li, 1987). These sponges are distributed in the Indo–West Pacific region and are found in depths between 516 and 1920 m (Tabachnick, 2002). For most studies described here *M. intermedia* specimens, dredged from 800 m in the Okinawa Trough, were used which had been provided by Prof. J. Li (Marine Biological Museum of Institute of Oceanography, Chinese Academy of Sciences, Qingdao). According to the recent view these three forms can be summarized under the authentic species name *M. chuni* (Tabachnick, 2002). *Monorhaphis* inhabits muddy substrata and is fixed there by a single giant basal spicule. Only photographs taken from the natural environment by Roux *et al.* (1991) are available (Fig. 3.1F). Young specimens have been imagined to comprise a continuous body, as has been sketched by Schulze (1904); one giant basal spicule anchors the specimen to the substratum and carries the cylindrical body (Fig. 3.1A). The cylindrical/oval body of *Monorhaphis* is interspersed with many atrial openings which are located along one side (Fig. 3.1C and D). Through these openings the regular choanosomal skeleton consisting of 14 siliceous spicule types can be observed. The diameter of the body reaches 12 cm in the larger specimens. During growth the specimens elongate together with an extension of their giant basal spicules (Fig. 3.1B). From the different size fragments of *Monorhaphis* collected during the Valdivia Expedition (Schulze, 1904), and during the expeditions organized by the Institute of Oceanography (Qingdao), this schematic growth scheme has been deduced. Older specimens apparently lose the basal portions of their soft body and expose the bare giant basal spicule (Fig. 3.1E).

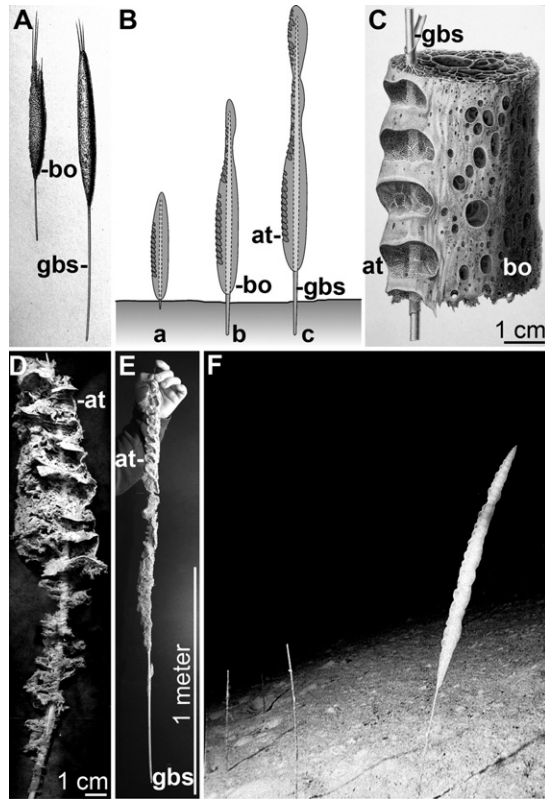


Figure 3.1 *Monorhaphis* specimens. (A) Young specimens are anchored to the muddy substratum by one single giant basal spicule (gbs). The body (bo) surrounds the spicule as a continuous, round cylinder. (B) Schematic representation of the growth phases of the sessile animal with their gbs which anchors them to the substratum and holds their surrounding soft body (bo). The characteristic habitus displays linearly arranged large atrial openings (at) of ~2 cm in diameter. With growth the soft body dies off in the basal region and exposes the bare giant basal spicule (a–c). (C) Part of the body (bo) with its atrial openings (at). The body surface is interspersed with ingestion openings allowing a continuous water flow through canals in the interior which open into oscules that are centralized in atrial openings, the sieve-plates. (D) Original photograph of a *M. chuni* specimen, collected during the Valdivia expedition. (E) A dried specimen of the “moderate” size of 120 cm showing that the basal body had fallen off from the giant basal spicule (gbs). The apical body comprises the serially arranged atrial openings (at). (F) *Monorhaphis* in their natural soft bottom habitat of bathyal slopes off New Caledonia. The specimens live at a depth of 800–1000 m (Roux *et al.*, 1991). In this region, the sponge occurs at a population density of 1–2 individuals per square meter. The animals reach sizes of around 1 m in length. (A, C, D) modified after Schulze (1904); (F) modified after Müller *et al.* (2007c).

2.3. Spicule diversity

Like all other hexactinellids, also *Monorhaphis* possesses microscleres (<0.1 mm) as well as megascleres ($0.2\text{--}30$ mm⁻³ m). Within the oblong, laterally compressed body of *Monorhaphis* (choanosomal body), which is arranged around the single giant basal spicule, 14 further siliceous spicule types with lengths ranging from a few micrometers to 50 mm are found (Müller *et al.*, 2007c; Schulze, 1904; Tabachnick, 2002). The likewise large comitalia (around 60 mm) support the basal habitus of this species. These spicules stabilize the tissue through which particulate food is filtrated via the aqueous canal system of the animal. The choanosomal body comprises mainly triactines (tauactines), diactines, and amphidiscs. The hexactin spicules of the choanosome with their six nonbranched rays are arranged perpendicular to one another; this represents the archetype of hexactinellid scleres. In addition, macramphidiscs are present that are composed of dumb-bell-shaped diactin microscleres. These scleres are restricted to the order Amphidiscophora. Finally, dermal pinular pentactin spicules (Fig. 3.2B and E) exist that are aligned along the tauactines that frame the atrial openings, the sieve-plates (Fig. 3.2A). These tauactines form the grille of the atrial openings and are armed by pentactines (Fig. 3.2B, D and E) allowing a mechanical sealing of the body towards the aqueous environment. The ending of a dermal pinule, a hexact spicule with four basal rays and one perpendicular ray that is ornamented by a set of oblique spines, is shown in Fig. 3.2F. In the sub-surface region mes-amphidiscs are found that are characteristic dumb-bell-shaped microscleres. The size of a mes-amphidiscs is ~ 100 μm (Fig. 3.2C).

No recent data on the formation of the spicules found in the choanosomal body are available.

3. GIANT BASAL SPICULES: MORPHOLOGY—CHEMISTRY—MECHANICS

3.1. Morphology

3.1.1. Protein scaffold

Very likely due to the relatively small number of giant basal spicules collected so far, their detailed analyses started only recently. Surprisingly, it was found that the siliceous lamellae, composing the spicules from *Monorhaphis*, contain not only a bio-silica matrix, but also a proteinaceous scaffold. Initially Schulze (1904) proposed that the proteinaceous material surrounds the lamellae as fibers (Fig. 3.3A). This conclusion based on light microscopic inspections. However, recent studies with high resolution scanning electron microscopy (HR-SEM) demonstrated that the protein

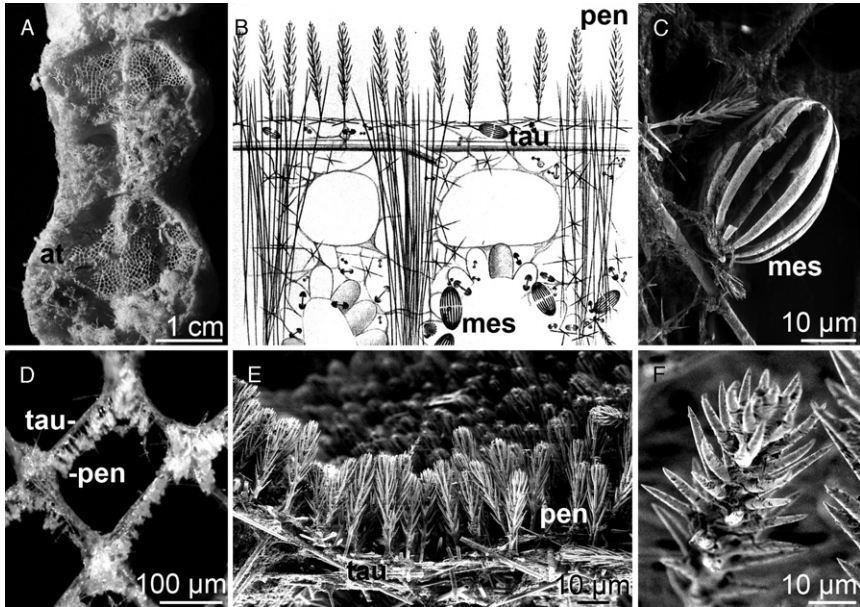


Figure 3.2 Spicules from the *Monorhaphis* choanosomal body. (A) View at a dried specimen displaying atrial openings (at). (B) Lateral view to an atrial opening (scheme). The borders are constructed by tauactines (tau) and are armed by pentactines (pen). In the sub-dermal region microscleres of the mes-amphidiscs (mes) type exist that display the characteristic dumbbell-shape. (C) A mes-amphidisc; HR-SEM. (D) Grilles forming the atrial openings are composed of tauactines (tau), framing of lattices, on which the pentactines (pen) are arranged in a phalanx. (E) HR-SEM image of the lattice of a grille. The pentactines (pen) are oriented towards the exterior of the body thus forming a mechanical and relative sealing of the atrial opening. (F) Tip of a dermal pentactine pinule, HR-SEM. (B) Modified after [Schulze \(1904\)](#).

is localized inside the lamellae. [Schulze \(1904\)](#) already recognized the morphological distinction of two zones within the spicules, the axial cylinder and the lamellar outer region ([Fig. 3.3A](#)).

For our initial light microscopic experiments ([Müller et al., 2008e](#)), loosely attached organic material was removed from the comitalia by ultrasonication. A distinction between the axial cylinder and the surrounding lamellar zone is possible even by light microscopic inspection in a cross section, using Nomarsky DIC (differential interference contrast) imaging ([Fig. 3.3B](#)). The axial cylinder occupies about half of the diameter of the comitalia, usually 150 µm. Furthermore, the spicules were subjected to hydrofluoric acid (HF) dissolution; after about 1 min of exposure to HF the lamellar zone begins to dissolve. Dissolution starts from cracks in the spicule, primarily following the gaps between the lamellae thus forming saw-tooth edges ([Fig. 3.3A and B](#)). During this process the lamellar zone can be distinguished from the axial cylinder by the protruding ends of dissolving

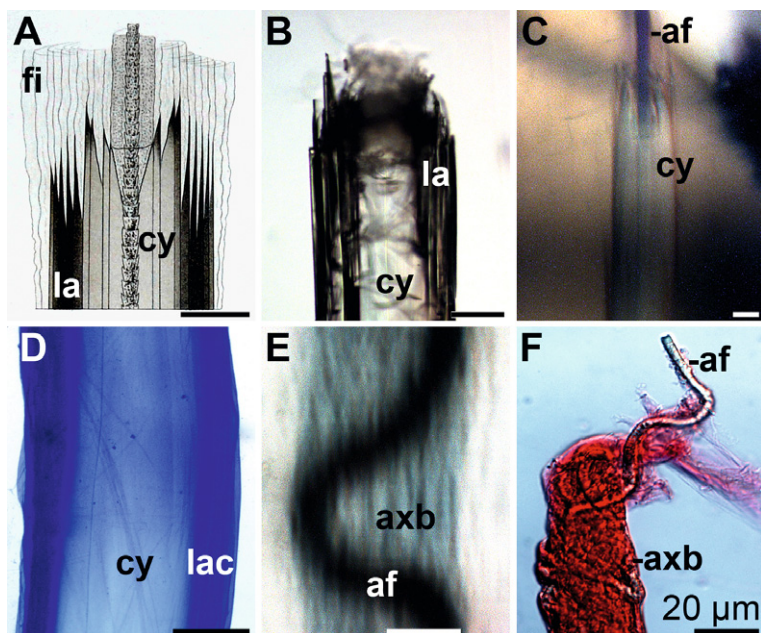


Figure 3.3 Stepwise dissolution of the silica shell around the axial filament of the spicule (comitalia) by treatment with HF solution; light microscopic analysis. (A) First outline of the proteinaceous filaments released by the giant basal spicules (Schulze, 1904). It has been proposed that the filaments (fi) originate from the inter-lamellar space. The axial cylinder (cy) in the center is seen. (B) Initial phase of the dissolution of the silica by HF. Solubilization of the lamellar zone (la) starts after 1–3 min, leaving behind proteinaceous filaments/clumps. The axial cylinder (cy) can be distinguished within the spicule by Nomarsky imaging. (C) After complete dissolution of the lamellar zone (treatment for 30 min) the axial cylinder (cy) which surrounds the axial canal/axial filament (af) is exposed. (D) The lamellar coating (lac), which is released from the lamellar zone after 8 min, surrounds the axial cylinder (cy), and is stained with Coomassie brilliant blue. (E) Inside of the lamellar coating, a second proteinaceous axial barrel (axb) is released from the axial cylinder, which becomes likewise stained with Coomassie Brilliant blue. This barrel originated from the axial cylinder and surrounds the axial filament (af). (F) The axial barrel (axb), completely freed of silica after an HF-treatment of 90 min, can be stained positive with Sirius Red. Inside of this structure the axial filament (af) can be identified, which is not stained by Sirius Red. All size bars: 20 μm .

individual lamellae (Fig. 3.3B). Finally, after an exposure time of 30 min, the intact axial cylinder remains (Fig. 3.3C). The dissolution of the axial cylinder proceeds from the periphery without revealing individual lamellae. The siliceous material in the axial cylinder is completely dissolved after 90 min (Fig. 3.3E). Application of different dyes allows visualization of the proteinaceous components in the two zones surrounding the axial canal. Addition of Coomassie brilliant blue to the HF solution immediately stains the proteins released from the lamellar zone (Fig. 3.3D). During the initial phase of

dissolution a proteinaceous coating (lamellar coating) is uncovered which remains only transiently intact (Fig. 3.3D) until the organized sheets disintegrate to irregular clumps/aggregates. Prior to this disintegration, the lamellar coating blisters. A second tube/sheath is formed from proteins of the axial cylinder. This structure is termed here axial barrel (Fig. 3.3E). In contrast, to the lamellar coating the axial barrel is composed of individual rope-like filaments (Fig. 3.3E). These can be stained besides by Coomassie Brilliant blue also with Sirius Red; the latter stain does not color proteins from the lamellar zone or from the axial canal (Fig. 3.3F). In addition, the axial barrel can be stained with the fluorescent dye Rhodamine 123.

Next we applied the HR-SEM technique to detect/identify the proteinaceous scaffold. For these studies siliceous lamellae were exposed only for a short period to HF (5 min). The images revealed that the stacked lamellae lost most of their siliceous components and exposed their proteinaceous matrix (Fig. 3.4H–J). The siliceous surfaces of the lamellae remained; they were held up by palisade-like pillar structures, to be interpreted as proteinaceous material. The individual pillars have a diameter of 0.1–0.2 μm and a length of 5–10 μm . Hence, these pillars traverse the previously silica-filled lamella. The proteinaceous material obtained was characterized by NaDodSO₄-PAGE (see Section 3.5.1) as one distinct protein with an apparent size of ~ 27 kDa.

3.1.2. Silica matrix

The spicules are formed from an inorganic silica layer/mantel and an organic scaffold. The silica mantel is formed from individual lamellae that had been analyzed mainly by SEM. The description proceeds from the millimeter to the nanometer scale.

A diagonal SEM analysis of a broken comitalia (large spicules existing in the body around the atrial openings) shows the fracture surface disclosing already the lamellar-wise organization of the silica mantel (Fig. 3.4A). In the unbroken state the lamellae are arranged perfectly concentrically around the central axial cylinder (Fig. 3.4B). If the comitalia or the giant basal spicules are broken the central cylinder remains almost intact, while the peripheral lamellar zone undergoes fracturing into concentric piles of chipped lamellae (see Section 3.3).

3.1.2.1. Millimeter scale The basic microscopic architecture of the giant basal spicules (up to 3 m long) is identical with that of the also large comitalia (~ 60 mm) that are found in the choanosomal skeleton of the body. Hence, these two types of spicules have been used for the studies reviewed here. The spicules are, due to their composite texture and structure, distinguished from other bio-silica structures by an unusual mechanical stability with respect to strength, flexibility, and toughness. As reported in Section 3.3 the spicules break sequentially by first splintering off the outer lamellae.

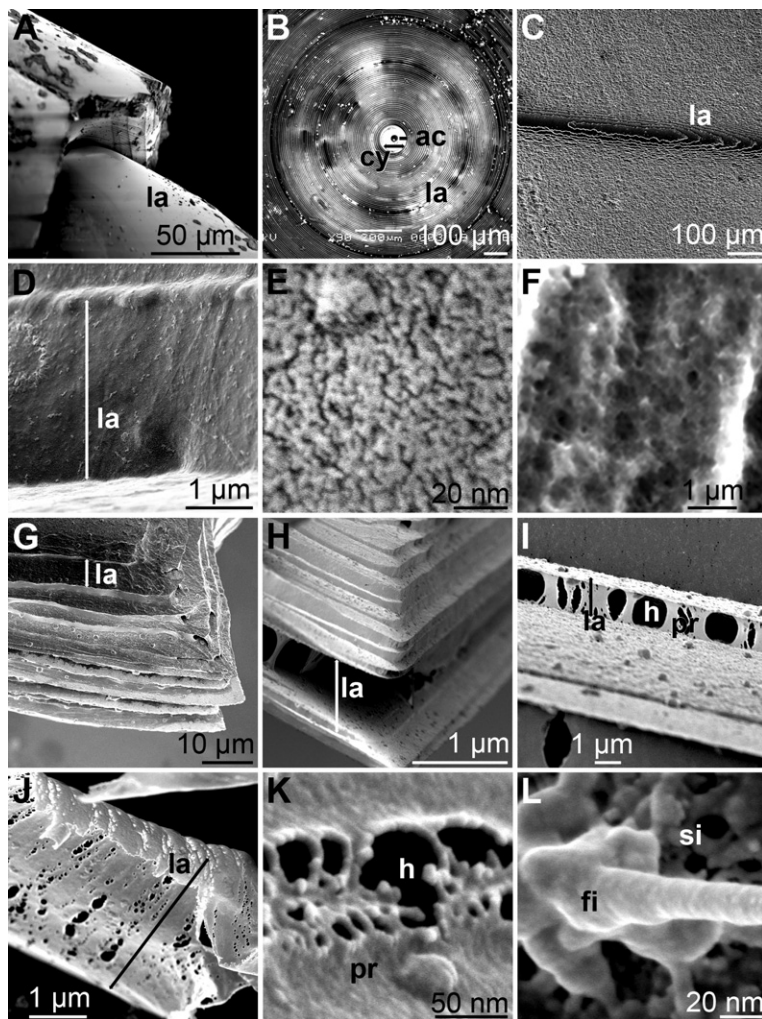


Figure 3.4 Structural architecture of the giant basal spicules from *Monorhaphis*. (A) Lateral view showing the concentric organization of the silica layers in a diagonal view. Two piled lamellae (la) are shown. (B) Structural arrangement of the lamellae; cross section. In the center of the spicule the axial canal (ac) is surrounded by the axial cylinder (cy), a region formed from electron-dense homogeneous silica. The third and major part of the spicules is composed of the outer 300–800 concentrically arranged lamellae (la). (C) A longitudinal cut through the spicule shows the foliate, lamellar (la) piling of the layers within a spicule. (D) A cross breaking showing the solid dimension of a lamella (la). (E) At high magnification small ball like structures that construct the lamellae can be resolved. (F) A rapid exposure of a cross break of a spicule to HF shows removal of the inorganic scaffold and results in clefts. Appearance of a pile of lamellae before (G) and after an extensive exposure to HF vapor (H); an organic matrix within the lamellae (la) can be visualized. (I) At higher magnification the proteinaceous

3.1.2.2. Micrometer scale Studies, recently published, have been performed by HR-SEM (Wang *et al.*, 2007; Müller *et al.*, 2007c). A diagonal SEM analysis of a broken spicule shows the fracture surface, displaying the concentrically arranged lamellae (Fig. 3.4A). Cross sections show a structural division of the spicules into three zones. First, in center of the spicules lies the axial canal, a structural unit that is often circular in hexactinellids (Pisera, 2003; Sandford, 2003; Uriz, 2006; Uriz *et al.*, 2003); however, towards the tips of the spicules the axial canal changes its profile and appears square. The axial canal is surrounded by a region of electron-dense homogeneous silica constituting the axial cylinder of a diameter of 100–150 μm . The third and major part of the spicules is composed of 300–800 regularly and concentrically arranged lamellae (each 3–10 μm thick; Fig. 3.4B). The inter-lamellar space of the spicules does surprisingly not constitute a continuous open slit (Müller *et al.*, 2007c). It is in average 0.1–0.2 μm wide and is composed of fusion zones and open holes; apparently the fusion zones allow a continuum between two silica lamellae. The lamellar organization of the layers within the spicule can also be demonstrated by a longitudinal cut through a spicule (Fig. 3.4C); here, the perfect intertwining stacks of lamellae are evident. A closer lateral view of a cross break shows the solid dimension of a lamella (Fig. 3.4D). At an even more powerful magnification small ball like structures in the nm range can be resolved (Fig. 3.4E).

3.1.2.3.. Nanometer scale Studies, to obtain an insight into the structural organization of the spicules at the nm scale, can be obtained by partial and limited dissolution of the silica using HF with the limitations described (Simpson *et al.*, 1985). A rapid dissolution results in the removal of the inorganic scaffold (Fig. 3.4F), while a less intense exposure of the spicules to HF vapor releases the organic matrix from within the lamellae. Gentle exposure of cross breaks of the spicules (Fig. 3.4G) to HF vapor results in the dissolution of the silica material under release of the organic component of the lamellae (Fig. 3.4H and I; Müller *et al.*, 2008b,e). The proteinaceous palisade-like scaffold is uncovered; it is composed of fibrous structures, into which holes formed by interconnecting fibers are interspersed (Fig. 3.4J and K). Under avoidance of shear forces during the HF treatment the complete proteinaceous layer of one lamella can be obtained (Fig. 3.4J). At higher magnification it becomes overt that the rim of each hole is

palisade-like scaffold (pr) within a lamellae (la) is uncovered; it is composed of fibrous structures. These are interspersed with holes (h). (J) Under avoidance of shear forces during the HF treatment a complete proteinaceous layer of one lamella (la) can be prepared. (K) The proteinaceous palisade-like scaffold protein (pr) is composed of fibers that are interconnected and leave open holes (h) whose rims are reinforced. (L) A protein fiber (fi) that is attaches to the silica scaffold (si) is shown; no banding pattern is visible. HR-SEM.

reinforced by densely arranged 10–15 nm large spheres (Fig. 3.4K). The protein fibers that are attaching the silica scaffold do not contain any banding pattern reminiscent of collagen (Fig. 3.4L).

3.2. Chemical composition

As a first approach to understand the chemical composition of the bio-silica within the giant basal spicule, polished thin sections were prepared and chosen for electron-probe microanalysis (EPMA or electron microprobe), using a JEOL 8200 instrument. The operating conditions of the electron microprobe were, 20 kV accelerating voltage, a spot size of 2 μm , and a 20 nA current. For the analysis a polished cross section through a giant basal spicule that had been surrounded by corals, was used (Fig. 3.5D). This sample retained its lamellar morphology. X-ray mapping of elements with an electron microprobe indicated that the high concentration of Si (continuous area) exists throughout the spicule (Fig. 3.5C) while in its surrounding only small spots are more intensely colored, reflecting the presence of smaller spicules in that region. This finding has implications for the understanding of the growth pattern of the *Monorhaphis* individuals, since it indicates that the giant basal spicule had been, in its initial growth phase, surrounded by sponge tissue prior to the bare/uncovered appearance of the spicule, usually found in the dredged samples. For the identification of the calcareous corals mapping of Ca was performed (Fig. 3.5E). The image shows that spicules are totally free of Ca, while higher levels of the element can be traced, as expected, in the surrounding region of the epibiotically growing corals (Fig. 3.5E).

The gross chemical composition of sponge spicules has been described for both Demospongiae and Hexactinellida in general (see Sandford, 2003) and also for *Monorhaphis* in particular. Already Schulze (1904) determined that, other than Si minerals (96%), only trace amounts of Na and K contribute to the inorganic material in measurable amounts. This composition was later confirmed by Levi *et al.* (1989) and Sandford (2003). Based on microprobe analyses experimental evidence has been presented indicating that Si is uniformly distributed throughout the silica shell of the spicules, whereas Na and K are not (Levi *et al.*, 1989; Müller *et al.*, 2007c; Fig. 3.5C). Higher levels of K have been measured (around 1 wt%) in the central part of the spicules, whereas the amount dropped considerably (≈ 0.4 wt%) at the surface. The inverse is true for the distribution of Na; here, the level was almost negligible at the center (≈ 0.03 wt%) but increased towards the surface to ≈ 0.4 wt%. However, recent studies using the Laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS), allowing the simultaneous determination of 40 elements at detection limits as low as nanograms per gram and at 120 μm spots, revealed an almost uniform distribution of the elements (Müller *et al.*, 2008b). For those studies giant

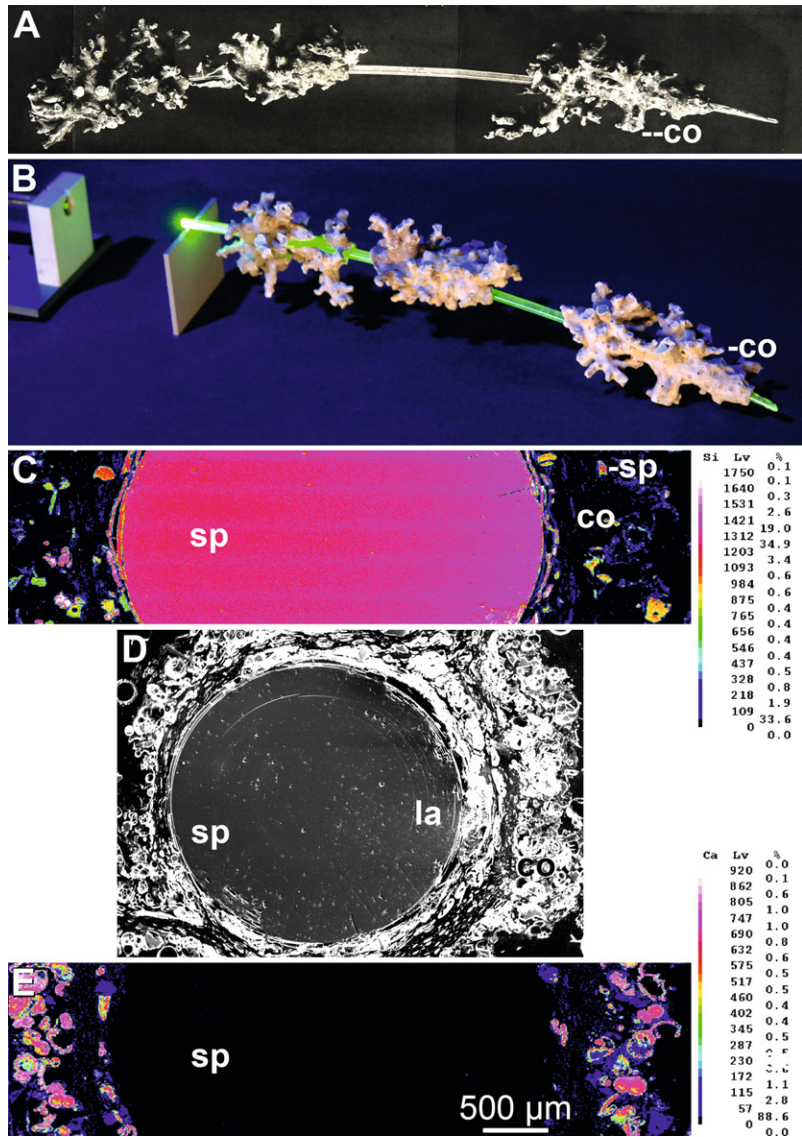


Figure 3.5 Light optical properties and chemical analysis of the giant basal spicule. (A) Original image from the spicule, collected 1898/1899 at a depth of 1079 m off the coast of Somalia (06° 18.800' N—049° 32.500' E) (Museum für Naturkunde Berlin, Germany; ZMB Por 12700). The spicule is surrounded by stony corals (co). (B) This spicule was used as an optical waveguide. The silica spicule is an excellent waveguide, while the calcareous material of the corals (co) is nontransparent. (C–E) Electron microprobe analysis of a giant basal spicule. (D) Polished cross section through the spicule (sp) showing the lamellar-wise organization (la) at the outer region. The spicule is surrounded by fused polyps of the coral (co); SEM analysis (backscatter image). X-ray maps of elements (C and E) of the area shown in D. If the counts of elemental characteristic X-ray increase, the concentrations of the elements increase from blue via green to yellow and finally to red. Elemental mapping is given for the elements Si (C) and Ca (E).

basal spicules with a diameter of ~ 7 mm had been systematically and completely analyzed (Müller *et al.*, 2008b). Si had been chosen as an internal standard for the LA-ICP-MS analysis and a SiO_2 content of 86% (wt) was accepted (remaining: 4.6% of protein and 9% of water). It is of prime interest that the contribution of the trace elements to the total inorganic components in the spicules is less than 0.005-fold with respect to Si. This implies that the quality of biosilica in the spicules is in the range of quartz grade with respect to the low concentrations of the elements other than silicon and oxygen. These trace elements are split as follows; among the monovalent counterions, Na^+ contributes to 86% (wt) [0.21% (wt) with respect to total inorganic material in the biosilica] and among the divalent ions, Ca^{2+} to 12% (wt) [0.03% (wt)]. All other 35 remaining elements contribute with $<2\%$ (wt) only unimportantly to the inorganic composition of the trace elements in biosilica. The impact of this finding becomes even more meaningful in comparison with the element composition of seawater. Referring to natural seawater Na and Cl are dominant there with 32.4% and 58.5% (solid material), respectively. Mg contributes 3.9%, Ca 1.2%, and Si only 0.006%. The other significant outcome of the LA-ICP-MS analysis was the finding that the distribution of the elements across the axis of the spicule does not change considerably (Müller *et al.*, 2008b).

3.3. Mechanical properties

From studies with *Monorhaphis*, Levi and coworkers (Levi *et al.*, 1989) suggested that the layered structure of the spicules has a “beneficial” effect on the mechanical properties of the spicules. Also inspired by these findings, the concept of natural composite material in rigid biological systems was born and fundamentally outlined by Mayer (2005). The organic phase controls energy dissipation especially in systems that are interspersed by very thin organic layers. In continuation of this topic, Mayer *et al.* (2005) proposed from their load–displacement studies that in *Euplectella* breakage of spicules follows a telescope-like pattern. In more recent studies, and as outlined in the previous sections, we could demonstrate that the proteinaceous matrix of the *Monorhaphis* spicules (the giant basal spicules and the comitalia) is not evenly distributed throughout the inorganic shell around the axial canal. In fact, morphological/structural zones can be distinguished; the axial cylinder and the lamellar zone (see Section 3.1). After having described the morphology of the giant basal spicules of *Monorhaphis*, applying modern electron microscopic techniques (Müller *et al.*, 2007c), we demonstrated, that the layers setting up the lamellar zone are composed of one major protein (size: ~ 27 kDa). Based on its binding property to labeled E-64 this ~ 27 -kDa molecule could be characterized as a protease, a (silica-tein-related) polypeptide. The enzymatic parameters for this activity are given below (Section 3.5.1).

Based on the morphological construction and the composite nature of the giant basal spicules from *Monorhaphis* we studied by load–displacement experiments if these properties provide them with an exceptional mechanical stability (Müller *et al.*, 2008e and Müller, submitted). The pattern of fractures within the spicules was correlated with the organization of the lamellar zone and the axial cylinder, since both areas are characterized by different bioorganic/inorganic hybrid compositions (Müller *et al.*, 2007c). A sample from an unbroken spicule was subjected to a classical bend test (Fig. 3.6C).

A representative load–displacement curve showed several characteristic points (Fig. 3.6E). In order to obtain a first insight into the role of the protein scaffold within the silica matrix, two samples of spicules were used. First, a spicule that remained untreated, not heated, and, second, a spicule that had been exposed to 600 °C (for 20 min) prior to the study in the 3-point bending system. The respective spicule sample was fixed in a 3-point bending system, selecting a fixture with a 20-mm span and an articulated center loading rod at a crosshead rate of 0.2 mm/min (Müller *et al.*, 2008e). The diameters of the tested spicules ranged from 2.1 to 2.6 mm. A sample from an unbroken, nontreated spicule was subjected to the classical bend test. The resulting representative load–displacement curve showed several characteristic breaking points (Fig. 3.6E; nontreated). The linear increase reflects the elastic response of the outer lamellar layers of the spicule; at point A (230 N) the first fiber crack occurs. The following deflection does not drop to zero but is fixed according to the viscoelastic view (Mayer *et al.*, 2005) (and/or due to breaking of lamellar packets) by the more central lamellar layers at 115 N. The increment of the load until the second cracking event (point B) reaches a value of 140 N. The subsequent displacement of the curve reaches almost the same value as that after the first crack (115 N); the elasticity of the spicule reaches 130 N until the third crack at C occurs. Subsequently, a broad range of load is recorded, D and the following, which reflects the random cracking of the different lamellae within the axial cylinder.

A partially cracked spicule was inspected by SEM analysis (Fig. 3.6D). The 2.5-mm thick spicule displays several fracture sites, which are marked in Fig. 3.6D; all cracks traverse several lamellae. In the center of the spicule the axial cylinder shows no distinct linear fracture zone. At higher magnification of the peripheral lamellar zone it can be seen that the individual silica layers (comprising microcracks), which are between 3 and 8 μm thick, are separated from each other by small (0.1–0.2 μm) interlamellar gaps (Müller *et al.*, 2008e). The determination of the thickness of the each lamella within one spicule was determined by using a “nanopositioning and nanomeasuring (NPM) machine” (to be published; Fig. 3.6B). Giant basal spicules were cross fractured and the sizes of the lamellae were precisely determined. The thickness of the first, more centrally located layers measure $\sim 7 \mu\text{m}$, while the remaining, outer, lamellae fall in a size range of 4.5 μm . Unlike in

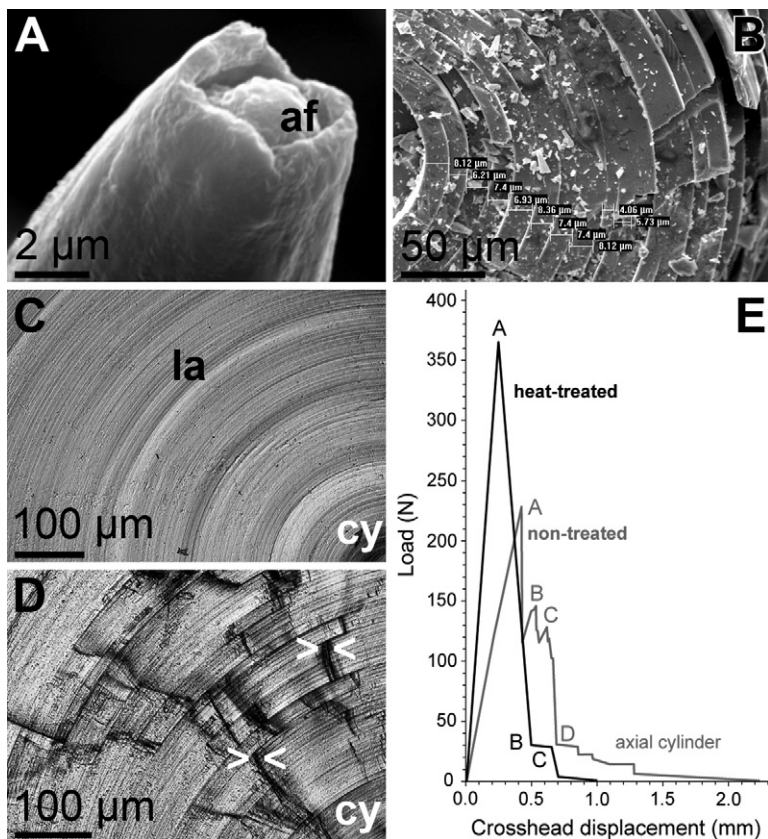


Figure 3.6 Morphology and mechanical stability of the giant basal spicules from *Monorhaphis*. SEM (A and B) and fluorescence microscopic analyses (C and D). (A) An open tip of a tautacin spicule. The spicules of hexactinellids display a quadratic opening, representing the axial canal which harbors the axial filament (af) (after Müller *et al.*, 2007c). (B) Determination of the dimensions of individual lamellae, composing the giant basal spicules. The measurements have been performed with an NPM machine. The thickness in the more centrally existing lamellae varies around 7.5 μm , while that of the more peripheral lamellae were determined to be around 5 μm . (C) Fluorescence microscopic image of cross section. The sample was beamed under a light microscope with fluorescent light of an emission maximum near 490 nm. The central axial cylinder (cy) and the lamellar outer region (la) are marked. (D) A partially fractured spicule was cut and inspected. It becomes apparent that the fractured lamellae have microcracks (>) which are displaced. (E) Force displacement relationship of giant basal spicules, measured in a 3-point bending assay. Records from two representative experiments with nontreated (black curve) and heat-treated (600 $^{\circ}\text{C}$; 20 min—grey curve) spicules are shown. In the nontreated sample the first cracking event is reflected by the linear increase (termed “elastic response”). The first crack (A) is followed by the deflection; two additional breakage events (B and C) are recorded before a random cracking of the remaining lamellae within the axial cylinder occurs. The final breakage (D) can be ascribed to the flexible fracture of the axial filament. In contrast, the heat-treated spicule shows in the bend test only one dominant cracking event (A) which is followed by minor fractures (B and C).

demosponges the tips of immature giant spicules of *Monorhaphis* are not completely covered by silica but show a square opening with a diameter of 2–3 μm (Fig. 3.6A). In most cases, the orifice, which represents the axial canal of the spicules, is not filled with any visible material. Tips of open spicules which still contain the axial filament are only rarely seen (Fig. 3.6A). These observations support and expand earlier experiments by Mayer and colleagues (Sarikaya *et al.*, 2001; Mayer, 2005; Mayer *et al.*, 2005) and Morse and colleagues (Weaver *et al.*, 2007).

As mentioned, consecutively recordable elastic responses of the spicules could be resolved, which are caused by cracks of distinct lamellar piles. By this property, the spicules acquire an unusually high stability. We attribute this property, to combine mechanical stability, strength, and stiffness, to the existence of organic molecules, here the $\sim 27\text{-kDa}$ protein, existing within the inorganic rigid bio-silica material. The inner organic axial barrel, stabilizing the axial cylinder, is composed of rope-like filaments and provides the spicules with a mechanical quality, which is more flexible and less rigid. It might be stressed that, based on our studies, we did not obtain conclusive data that between the individual lamellae of the spicules any organic layer exists. Therefore, we attribute the assumed viscoelastic and/or energy dissipation properties not to a possible organic interphase between the lamellae but to the proteins within them.

In order to support the conclusion that it is the protein composition within the bio-silica which provides the lamellae with high stiffness and considerable flexibility, spicules had been studied with the same method, however after a pretreatment at 600 °C for 20 min in a tube oven in order to denature the protein. Very much in contrast to the study with nontreated spicules is the crosshead displacement record obtained with those heat-treated spicules; Fig. 3.6E. The initial resistance of the spicules is much higher in comparison with the nontreated controls. A force of 365 N is needed to break the spicule (at A), which undergoes an almost complete burst. A deflection occurs which is only slightly retarded at 30 N, and followed by a final very low resistance at 5–0 N (D). These data demonstrate that the heat-treated spicules have lost the characteristic properties of the natural spicules, to comprise stiffness, flexibility, and toughness.

The step-wise breakage of an untreated giant spicule (from *H. sieboldi*; diameter of 0.8 mm) had been recorded (not shown); the spicule was inserted into a traction device between two moving rubber wheels and continuous pulling was achieved by a motor. If the breaking process is recorded with a high-speed camera the first crack of the outer lamellae is not visible even if the spicule has been completely bent by one circumvolution, leaving a diameter of the circular, wrapped around spicule of 4.5 cm. However, after pulling the circle down to 4 cm the first set of the outer lamellae blasts off. Then another period of flexible bending proceeds until the second breakage point is reached. Again, a further flexible bending

phase takes place until a third spalling of the lamellae occurs, followed by a final fracture of the spicule. In comparison, the breakage of a quartz-glass fiber of a similar diameter has been recorded. As expected, the glass fiber cannot even be bent to a large circle but cracks almost immediately after an inflection of about 30° .

3.4. Optophysical properties

Sponges can react fast to physical stimulation from the environment with contraction or expansion. Morphological and cellular structures for such responses are conceivable based on the complex cell-cell and the diverse cell-matrix interaction systems in sponges that have already been detected (see Müller *et al.*, 2004). These observations could imply that the coordinate reactions are governed by a nerve system. However, until now no nerve fibers or synapses could be identified in sponges. However, our previous studies showed that the siliceous demosponges *Suberites domuncula* and *Geodia cydonium* contain and express genes coding for neuronal molecules, for example, a metabotropic glutamate/GABA (γ -aminobutyric acid)-like receptor (Perović *et al.*, 1999). But, in spite of intense molecular biological studies and the availability of more than 15,000 expressed sequence tags (EST) sequences in our EST database (Chevreux *et al.*, 2004), no sequence/partial sequence was found to indicate the existence of a nerve system, for example, of synaptic or axonal molecules. Therefore, it seemed advisable to us to change the direction of our studies towards an investigation of a different signaling system that may permit sponge cells to communicate. To define such a system, the most suitable stimuli would be optical stimuli, since they can be used for qualitative and quantitative exposure of the animals.

It is well established that sponge embryos and adult sponges react to light (Mackie, 1979; Pavans de Ceccatty, 1974). More recent studies even demonstrated that sponges can discriminate between different wavelengths (Leys and Degnan, 2001; Leys *et al.*, 2002), the strongest response is seen to blue light around 440 nm and a secondary peak at 600 nm (Leys *et al.*, 2002). So far all attempts to identify photosensory cells in sponges failed. Therefore, the question was raised, if noncellular structures, like spicules, might function as biological receiver for light energy. This direction appeared to be tempting, since the hexactinellids live in a depth of more than 1000 m (Wyville Thomson, 1874); however, the proof of such a photosensory system would require the demonstration that the spicules are efficient light waveguides and the animals must have the potential to generate light. The first prerequisite was already discovered to a great extent by the findings that spicules from sponges can transmit light (Aizenberg *et al.*, 2004; Cattaneo-Vietti *et al.*, 1996). In these elegant studies, the hexactinellids *Rossella racovitzae* and *E. aspergillum* were used. For our previous studies

(Müller *et al.*, 2006b), we had chosen the hexactinellid *H. sieboldi* which possesses—in contrast to other taxa hitherto used—long-stalk spicules. We could demonstrate that these large spicules from the root-tuft of *H. sieboldi* transmit light with high efficiency. Surprisingly, however, the blue light with a wavelength between 400 and 600 nm is filtered out. Based on this finding we suggested that the spicules from *H. sieboldi* act as optical absorbent in a novel photoreception.

Even more suitable for such optophysical studies than the 4–12 cm long stalk spicules (basalia) of *H. sieboldi* are the longer *Monorhaphis* giant basal spicules. Using those spicules, comparable and more extensive studies have been published by Wang *et al.* (2007). The giant spicules (*Monorhaphis*) were exposed at the end with the wider diameter to a white light source with a spectrum ranging from 400 nm to >1600 nm (Fig. 3.7A and B). The light of a halogen lamp was coupled to a multimode fiber with an adapted collimator, which delivered a nearly parallel beam of ≈ 4 mm in diameter behind the exit aperture (Müller *et al.*, 2006b). The transmitted light was collected with a collimator into a second multimode fiber for analysis within an optical spectrum analyzer. Even with the naked eye, the optical waveguide properties of the giant basal spicule can be recorded (Fig. 3.7B). During the passage of the light a distinct white-to-red color gradient along the spicules of glass sponges is seen, suggesting a (partial) scattering of the light. In a closer view, it becomes obvious that the guided light in the paraxial region of the spicule has a bright yellow color in contrast to the light quality at the outer surface (Fig. 3.7C). The spectrum of the output of the light source was measured between 400 nm and >1600 nm. A distinct cut-off of the wavelengths below 600 nm and above 1400 nm could be measured (Fig. 3.7D), while the light transmission between these borders was only slightly/gradually reduced. Hence, the spicules act as optical fibers (like a high pass filter) cutting off the light of wavelengths below about 600 nm from transmission by more than two orders. A similar cut-off of the spicule is observed in the infrared wavelength range above 1400 nm. Here light transmission is blocked like a low pass filter. Three weaker absorption minima are observed on the overall profile at the centre wavelengths at around ~ 960 nm and ~ 1150 nm. These absorption wavelength regions match the molecular absorption lines of water (970 nm and 1150/1190 nm, respectively; Braun and Smirnov, 1993).

Stimulated by these results we hypothesize that in sponges the spicules might be involved in the transmission of information in a way analogous to the nerve system of higher Metazoa. Surely in a depth of 1000 m, where the glass sponges live, no sun light can be perceived. This implies that sponges have to rely on an own light generation system(s), which probably emits light at wavelengths below 550 nm, as most marine organisms do (Hastings, 1996). In line with this assumption we screened a cDNA library from

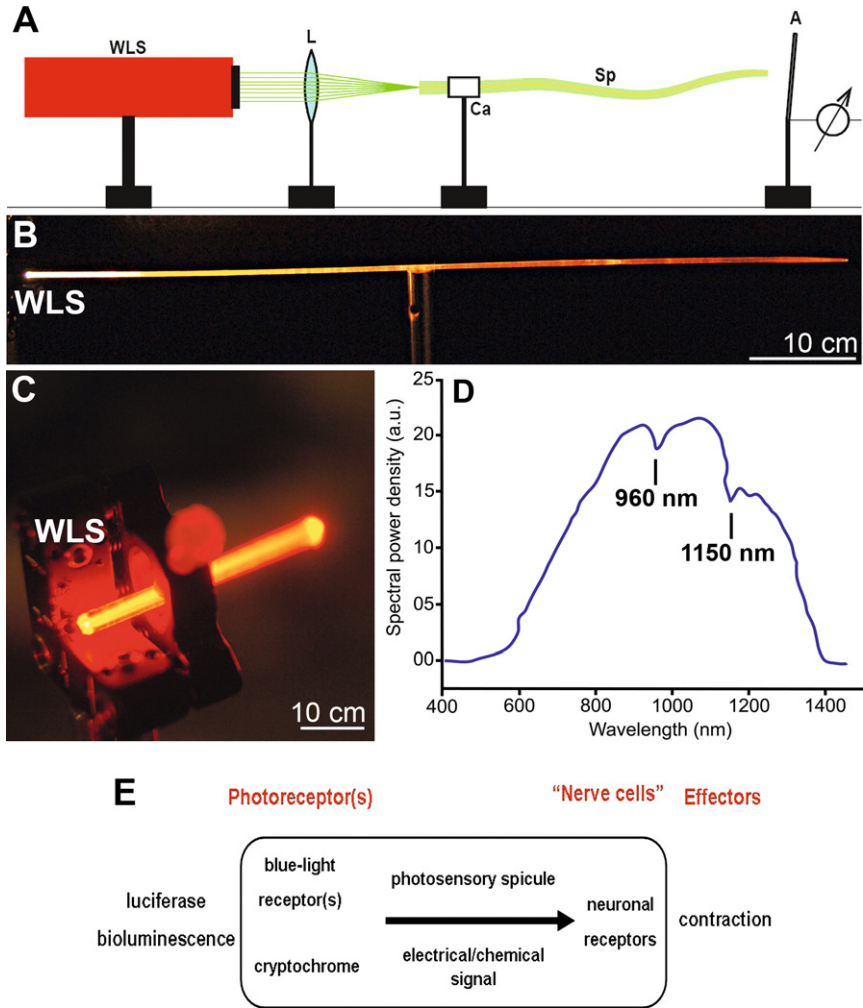


Figure 3.7 Optophysical properties of the giant basal spicules from *Monorhaphis*. (A) Optical unit (scheme). The white light source (WLS) is focused through a biconvex lens (L) onto one end of the spicule (Sp), which is immobilized within a canula (Ca). The emitted light is recorded by an optical spectrum analyzer (A). (B) A 80-cm long spicule had been immobilized and illuminated with white light (WLS); during the course through the fiber the color changes from white to red. (C) Higher magnification, showing the optical unit with the white light source (WLS) and the bright yellow light that is concentrated within the axial cylinder. (D) Transmission properties of sponge spicules for white light. The curve shows the spectrum and the relative intensities of the transmitted light through the spicules, as a function of the wavelengths. The spicules act as sharp high (600 nm) and low pass filters (1400 nm). Within the visible part of the spectrum transmitted by the fiber two less pronounced absorption wavelengths were found at around ~960 nm and ~1150 nm; these weak absorption ranges match the known molecular absorption lines of water (970 nm and 1150/1190 nm, respectively).

S. domuncula successfully for the existence of a luciferase, which we also recently cloned and expressed from this sponge (Müller *et al.*, 2009). In addition, we screened in hexactinellids for genes which might be indicative for the existence of a further potential nonocular dermal photoreception system. The presence of such a system had been thoroughly studied in mollusks, for example, *Lymnaea stagnalis* (Yoshida, 1979); it had been verified that the sensitivity to light stimuli is not restricted to the eyes. Detailed electrophysiological studies with *L. stagnalis* revealed that light evokes stimulation of nonocular dermal photosensitive responses in the foot (Chono *et al.*, 2002). Transferring this view to our system, it will be a major task to identify in the future proteinaceous coupling systems between spicules and cells, which allow the passage of optical signals to sensory cells. It should be mentioned here that the characteristic eye lens proteins, $\beta\gamma$ -crystallins, have been identified in the demosponge *G. cydonium* (Krasko *et al.*, 1997).

As a first approach, a cryptochrome (CRY) sequence from the hexactinellid sponge *Aphrocallistes vastus*, that comprises high sequence similarity to genes encoding (6–4) photolyases and related proteins, has already been identified (Wang *et al.*, 2007). Earlier, functional studies showed that this gene codes in *S. domuncula* for a photolyase-related protein (Schröder *et al.*, 2003a). Based on sequence similarities, the DNA photolyase from *A. vastus* has been classified together with the chryptochromes, which include blue-light receptors, into a single DNA photolyase/chryptochrome protein family (Wang *et al.*, 2007). Taking this experimental finding, together with the demonstration of the luciferase in *S. domuncula*, we propose that sponges are provided with an unusual, (perhaps) unrecognized photoreception system (Fig. 3.7E). We postulate that sponges coordinate their sensory reception systems not on the basis of a protein-controlled nervous network alone, but primarily by a controlled interaction of nerve-cell related sensory molecules with inorganic siliceous spicular rails. Since sponges are provided with the genetic machinery to express luciferase enzymes and also a photolyase/chryptochrome molecule, the optical fibers (spicules) might guide and convert the light, via a chemical/photoelectric reaction, into electric signals. The subsequent amplification system which translates the electric signals into the nervous transmission system in sponges as well as to other metazoan phyla might be mediated by similar biological amplifiers/receptors (Fig. 3.7E).

(E) Proposed (nonocular) photoreception system in sponges. It is anticipated that in sponges light is generated by bioluminescence (e.g., the luciferase system) which is bundled by the silica-based fibers. In addition, a cryptochrome protein exists which might be involved in the reception of light. Within the blue light range a photoelectric/chemical reaction takes place, converting the light into electric impulses. The amplification of the electric impulses occurs in “nerve cells” in sponges.

3.5. Biochemical properties and molecular biological basis

3.5.1. Biochemistry

The routine procedure to isolate the proteins from bio-silica in diatoms or sponge structures is their dissolution in HF. However, as demonstrated in sponges, during this acid treatment the biological function of proteins, especially of silicatein changes (see Müller *et al.*, 2007a; Schröder *et al.*, 2006). During the HF treatment silicatein undergoes slow de-phosphorylation (Müller *et al.*, 2006a), a modification which can be avoided by grinding the spicules in a glycerol containing buffer (Schröder *et al.*, 2006). Since the bio-silica material of the giant basal spicules is stony hard we applied a limited HF-treatment for the release the protein(s). However, even though the dissolution process was terminated immediately after the solubilization of the spicules, a size range of the lowest molecular weight protein band between 24 and 27 kDa (sodium dodecyl sulphate polyacrylamide gel electrophoresis, NaDodSO₄-PAGE) had been obtained by extraction of giant basal spicules from *Monorhaphis* (Müller *et al.*, 2007a,c).

As outlined above the giant basal spicules contain in their outer lamellae a protein which is (apparently) different from that present in the axial canal. However until now we failed to demonstrate by sodium dodecyl sulphate polyacrylamide gel electrophoresis (NaDodSO₄-PAGE) any difference in the mobility of the 24–27-kDa polypeptides. A recently introduced alternative method (Ehrlich *et al.*, 2006), which is basically the same procedure described before (Schulze, 1904), dissolves the bio-silica material with alkaline solutions (2.5 M sodium hydroxide, pH = 13.0) for a period of 14 days at 37 °C. To our experience this procedure is not superior, if at all, to the HF-treatment (buffered 2 M HF/8 M NH₄F, pH 5; overnight at room temperature) used by the group of Morse (Shimizu *et al.*, 1998) and us (Müller *et al.*, 2005). For the biochemical studies, performed by us, only giant basal spicules, which had been stored in a museum, for at least 10 years could be investigated. Freshly collected material is presently not available. These spicules are covered with a collagen net (Müller *et al.*, 2007c) that could be removed by mechanical treatment (sonication).

If mechanically cleaned spicules are used for protein extraction, both a high molecular weight protein of a size of 70 kDa and a low molecular weight species of ~24–27 kDa (Müller *et al.*, 2008a) can be resolved by one-dimensional gel electrophoresis. If such a sample is subjected to two-dimensional gel electrophoresis (first isoelectric focusing and then size separation) the proteins are separated into acidic and basic sets of protein. To mention here, these proteins had been subjected to extensive electrospray ionization (ESI)-mass spectrometry (MS). In none of the fragments which we had analyzed, a single sequence with the characteristic collagen triplet (Gly-X-Y) could be identified, excluding that the 70-kDa protein(s) are collagen, as suspected (Ehrlich and Worch, 2007).

We focused our work especially on the identification of the function of the 24–27-kDa protein. The size of this polypeptide matches the silicatein polypeptide, identified in demosponges (see Müller *et al.*, 2007b). After having suspected in earlier works (Müller *et al.*, 2007c; Wang *et al.*, 2007) that this protein is likely to be a silicatein-related protein, a first experimental support came from experiments that the 27-kDa proteins cross-reacted with anti-silicatein antibodies. Furthermore—and more importantly—the extract comprised proteolytic activity, like that obtained from the demosponge *S. domuncula*. Even more, that sample could be inhibited with the specific cysteine proteinase inhibitor E-64 (Müller *et al.*, 2008a). If a protein extract was prepared from lamellae, only one protein band of a size of 27 kDa was seen (Müller *et al.*, 2008a). Again, this protein was found to cross-react with antibodies raised against *S. domuncula* silicatein (Müller *et al.*, 2005).

After having successfully achieved the elucidation of the kinetic parameters for the (recombinant) silicatein from *S. domuncula* (Müller *et al.*, 2008c) comparable studies could be performed also with the silicatein-related protein from *Monorhaphis*. The Michaelis constant (K_m) for the *S. domuncula* silicatein was determined to be 22.7 μM . The turnover value (molecules of converted substrate per enzyme molecule per second) was 5.2. In continuation with this study the kinetic parameters of the proteolytic activity of the 27-kDa polypeptide from the lamellae of *Monorhaphis* were determined as well, using the substrate Z-Phe-Arg-AMC (Wang *et al.*, 2008). The reaction velocity was determined to be (almost) linear between 2 and 12 μM of the substrate Z-Phe-Arg-AMC. By varying the substrate concentrations, between 5 μM and 20 μM the K_m value for this substrate was determined with 15.4 μM and a corresponding $V_{\max}$ (maximal velocity) with $961.5 \times 10^{-4} \mu\text{M}/\text{min}$.

A further, second organic component could be identified from spicules of *Monorhaphis*. Analyses with spicules from *S. domuncula* revealed that the silicatein molecules, in the extracellular space, are in close association with galectin molecules. These two molecules arrange organic cylinders around the growing spicules (Schröder *et al.*, 2006). Like in the studies with *S. domuncula*, we analyzed also the *Monorhaphis* spicule extract for lectin activity (Wang *et al.*, 2007). A standard hemagglutination assay with erythrocytes from horse blood was applied to screen in extracts from giant basal spicules for the potential existence of lectin. The results revealed that at concentrations above 2.5 $\mu\text{g}/\text{ml}$ a clear agglutination was detectable. As a control, that the agglutination is based on lectin/protein activity, the *Monorhaphis* samples were heated (95 °C for 5 min); these samples subsequently lost the agglutination activity.

3.5.2. Cloning of the hexactinellid silicatein cDNA

With the exception of sponges the other bio-silica-forming organisms, for example, diatoms, foraminifera, etc., produce this inorganic polymer nonenzymatically (see Sumerel and Morse, 2003). The respective enzyme,

silicatein, forms like in *Monorhaphis* gigantic bio-silica structures up to 3 m. The enzyme is concentrated in the organic axial filament of $\sim 0.5\text{--}2\ \mu\text{m}$ in diameter (Bütschli, 1901). After cloning, it became obvious that silicatein belongs to the class of the cathepsins (Cha *et al.*, 1999; Krasko *et al.*, 2000; Shimizu *et al.*, 1998), with cathepsin L as the most prominent member. Cathepsin L is an endopeptidase which cleaves peptide bonds and occurs in lysosomes as well as extracellularly as secreted enzymes (see Mort, 2002). Like most lysosomal enzymes the cathepsins belong to the family of papain-like peptidases that are characterized by the catalytic triad of cysteine (Cys), histidine (His), and asparagine (Asn). Cathepsins are synthesized as proenzymes by ribosomes at the endoplasmic reticulum and undergo maturation steps, release of the signal peptide, activation through limited, autocatalytic proteolysis, and post-translational modification through phosphorylation and glycosylation (see Mort, 2002). Members of cathepsin L are found only in Metazoa (Mort, 2002); more distantly related cysteine proteases have been identified in protozoans or plants (Nakamura *et al.*, 1997).

The silicateins are distinguished from the cathepsins by the exchange of the first amino acid (aa) residue in the catalytic triad, Cys by Ser (Shimizu *et al.*, 1998). In addition, the silicateins are distinguished from the cathepsins by the presence of a Ser stretch that precedes the second aa in the catalytic triad, His. This region was termed “conventional” Ser cluster (Müller *et al.*, 2008d). It has been proposed that serine increases the nucleophilicity during the nucleophilic attack at the silicon atom in the synthetic substrate of silicatein, the tetraethoxysilane (Sumerel and Morse, 2003). In recent years, we cloned a series of silicateins (cDNAs and genes) from marine sponges (*S. domuncula*, *T. lyncurium*, and *G. cydonium*) and freshwater sponges occurring in Lake Baikal (e.g., *Lubomirskia baicalensis*) or in lakes of the Tuva region (Russia/Mongolia), *Ephydatia tuva* (Lake Chagytai) and *Ephydatia altaiensis* (Lake Tore-Khol). Based on these data a comprehensive phylogenetic analysis could be performed (Müller *et al.*, 2007b). Surprisingly, it was found that the silicateins from freshwater sponges, for example, *L. baicalensis*, occur in several isoforms with sizes of 23 kDa, 24 kDa, and 26 kDa. During the course of these studies it could also be demonstrated that silicatein not only mediates polymerization of silicate, but also displays proteolytic activity which is specific for cathepsin L enzymes (Müller *et al.*, 2007b). After alignment of those sequences it could be deduced that the silicateins together with the cathepsin L sequence originate from the papain sequence, existing in *Arabidopsis thaliana*. It could also be established that during the evolution of the papain gene to the silicatein- and the cathepsin L-genes the intron exon borders remained conserved. However, their numbers changed. At first, from plants to protostomians (e.g., the shrimp *Penaeus vannamei*) three introns exist in the coding region for the mature cathepsin L genes. The number of introns decreases to zero in the compressed genomes of *Drosophila melanogaster* and *Caenorhabditis elegans*.

In contrast, the number of introns in this region increases in human to four, and in sponges even to five. In this context it might be noted that a similar trend was described earlier for the stress activated protein kinases p38 and JNK (Müller *et al.*, 2002).

We found (see Section 3.5.1) that also hexactinellids contain an enzymatic activity similar to that identified for demosponges. However, in spite of greatest efforts it had not been possible to clone from *Monorhaphis* (order Amphidiscophora (Hexactinellida)) the cDNA encoding silicatein, since no DNA/mRNA could be isolated from material stored in the different museums. Therefore, we have used freshly collected tissue from the species *Crateromorpha meyeri*, grouped to the second hexactinellid order, Hexasterophora, to identify and sequence the cDNA coding for silicatein (Müller *et al.*, 2008d). The open reading frame (ORF) of the *C. meyeri* sequence spans the mature silicatein- α (Müller *et al.*, 2003), that region which has also previously been used for the preparation of recombinant silicatein from *S. domuncula* (Müller *et al.*, 2003). The ORF was deduced from the cDNA sequence obtained; the catalytic triad aa characteristic of silicateins Ser (aa 22), His (aa 161), and Asn (aa 181), were present at positions that matched exactly those described for the known silicateins of Demospongiae, *S. domuncula* (silicatein- α) and *L. baicalensis* (silicatein- α 3) (Müller *et al.*, 2007b); Fig. 3.8A. New in this hexactinellid sequence is the finding that besides of the “conventional” Ser cluster, which is found also in *C. meyeri* around the aa 155 and 158, this hexactinellid polypeptide comprises a second Ser cluster, termed “hexactinellid-specific” Ser stretch of five residues. It exists in this hexactinellid sequence within the region aa 169 to aa 173. The hexactinellid silicatein comprises the peptidase-C1 papain family cysteine protease domain and reveals the highest sequence similarity to the silicateins- β from *Tethya aurantium* and *S. domuncula* with the high expect-value (E) of $8e^{-58}$. The relationships to the silicateins from the freshwater sponge *L. baicalensis* are somewhat lower ($E = 2e^{-47}$). These sequences have been aligned and compared with the cathepsin sequence of the hexactinellid *A. vastus* (Wiens *et al.*, 2006) to highlight the high sequence similarity between the silicateins and cathepsin and the difference in the first aa of the catalytic triad. A phylogenetic analysis of the *C. meyeri* silicatein was performed by including related silicateins, the hexactinellid cathepsins from *A. vastus* and *C. meyeri*, as well as the *A. thaliana* papain, as a founding member of the cysteine protease family (Fig. 3.8B). The *C. meyeri* silicatein forms a branch with silicatein- β from *T. aurantium*, within the silicateins.

Model prediction of the *C. meyeri* silicatein protein was performed applying the comparative modeling process/procedure described (Müller *et al.*, 2007a, 2008d) together with the silicateins from *S. domuncula* and *L. baicalensis*. We found that all aa residues of the silicatein models (from *C. meyeri*, *S. domuncula* and *L. baicalensis*) reside in the allowed regions of the Ramachandran areas (Ramachandran *et al.*, 1963; Robinson, 2007).

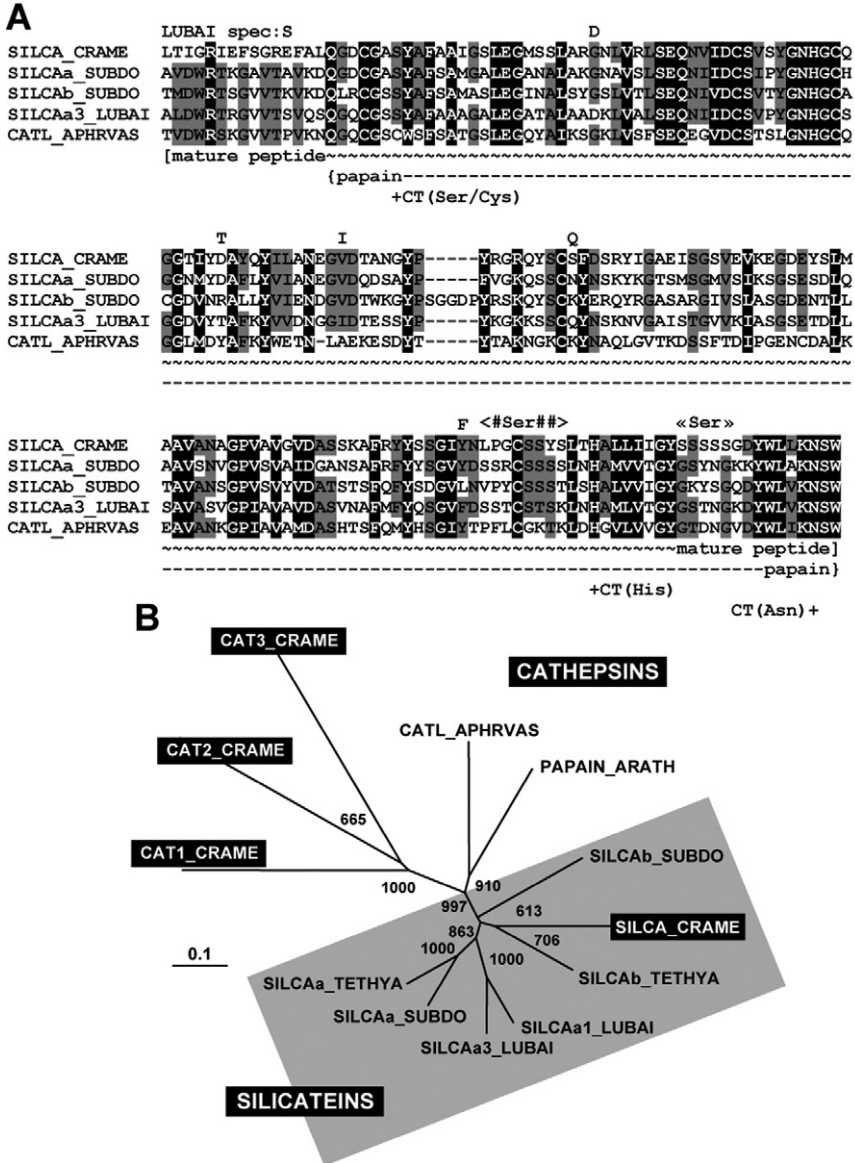


Figure 3.8 Hexactinellid silicatein sequence similarity to silicateins from demosponges and cathepsin (according to Müller *et al.*, 2008d). (A) The silicatein protein from *C. meyeri* was aligned with silicatein- α from *S. domuncula* (SILCAa.SUBDO) and silicatein- β from *S. domuncula* (SILCAb.SUBDO) and one isoform of silicatein- α from *L. baicalensis* (α -3) (SILCAa3LUBAI) as well as with the cathepsin L sequence from hexactinellid *A. vastus* (CATLAPHRVAS). Residues conserved (similar or related with respect to their physico-chemical properties) in all sequences are shown in white on

The model predictions reveal that the “conventional” Ser cluster is spatially separated from the “hexactinellid-specific” Ser cluster in *C. meyeri*. It is noticeable that, according to the model predictions, the “hexactinellid-specific” Ser cluster in *C. meyeri* spans the topologic position like the “conventional” Ser clusters in *S. domuncula* or in *L. baicalensis*. The position of the “hexactinellid-specific” Ser cluster (5 Ser residues) could be narrowed down to the outer loop of the exposed β -sheet. In detail, Ser169–171 are parts of the β -sheet, whereas Ser172–173 are placed in the loop, in comparison to the sequence from *S. domuncula*. Like for the other two demosponge models also the “conventional” Ser cluster in the *C. meyeri* model is in a loop, flanking the alpha helix.

Until now, no experimental data exist which could explain the potential role of the “hexactinellid-specific” Ser cluster in the silicatein molecule. In a recent report (Müller *et al.*, 2008b) we proposed, in line with previous suggestions in diatom systems (Hecky *et al.*, 1973; Lobel *et al.*, 1996a,b), that these Ser residues are involved in the interaction with silicic acid, via links between individual silicic acid molecules and the hydroxyl-containing side-chains of the Ser residue. Hence, it might be hypothesized that the “hexactinellid-specific” Ser cluster together with the “conventional” Ser cluster are involved in a strong stabilization of the interaction between silicatein and the polycondensated silica surfaces.

4. SYNTHESIS OF SPICULES IN DEMOSPONGIAE

4.1. Silicatein/silicase

In the initial phase to understand the mechanism of formation of giant basal spicules in hexactinellids, biochemical data existed only for spicule formation in demosponges. It was the group of Morse (Cha *et al.*, 1999; Shimizu *et al.*, 1998)

black, and those in at least four sequences in black on gray. The characteristic sites in the sequences are marked; the catalytic triad (CT) amino acids, Ser in silicateins and Cys in cathepsin, and His and Asn. The borders within the mature silicatein (mature peptide) and the peptidase-C1 papain family cysteine protease domain (papain) are given. The “conventional” serine cluster (<#Ser#>), and the “*C. meyeri*-specific” serine cluster (<<Ser>>) are marked. The aa conserved in all freshwater sequences, here shown in the *L. baicalensis* sequence, are listed above the alignment. (B) A radial phylogenetic tree was constructed after the alignment of these five proteins, together with the two *T. aurantium* silicateins- α and - β (SILCAa.TETYA and SILCAb.TETYA), a further silicatein- α from *L. baicalensis* (α -1) (SILCAa1LUBAI) as well as the three cathepsin sequences from *C. meyeri* (CAT1.CRAME; CAT2.CRAME and CAT3.CRAME) as well as the related papain-like cysteine peptidase XBCP3 from *Arabidopsis thaliana* (PAPAIN.ARATH, AAK71314). The radial tree was constructed after the alignment. The numbers at the nodes are an indication of the level of confidence for the branches as determined by bootstrap analysis (1000 bootstrap replicates).

who discovered that the organic filament in the central canal of the spicules is composed of a cathepsin L-related enzyme, which they termed silicatein. They cloned two of the proposed three isoforms of silicateins, the α - and β -form, from the demosponge *T. aurantium* (Cha *et al.*, 1999). Little later, these molecules were cloned also from other sponges, among them *S. domuncula* (Krasko *et al.*, 2000, 2002; Schröder *et al.*, 2004b). It could be clarified by our group that silicatein occurs in several isoforms in the axial filament (Müller *et al.*, 2007e). By application of the technique of two dimensional gel electrophoresis we demonstrated that the silicateins in the axial filament undergo stepwise phosphorylation. Five phospho-isoforms with pI values of 5.5, 4.8, 4.6, 4.5, and of 4.3 have been identified (Müller *et al.*, 2006a). The sizes of these phosphorylated proteins are around ~ 25 kDa, compatible with the predicted mature form of the silicatein. Interesting was the finding that under native conditions, in the absence of urea in the sample buffer, silicatein forms not only monomers but also dimers and trimers (Müller *et al.*, 2007a).

Surprising was also the finding that if a total tissue extract of *S. domuncula* was subjected to two-dimensional gel electrophoresis the predominant proteins were the silicateins, again present in five isoforms (Müller *et al.*, 2005, 2006a). The identification of the five isoforms was performed by Western blotting using polyclonal antibodies against silicatein. This finding suggests that silicatein is not only present within the spicules, but should exist to a considerable extent outside of the spicules. As an additional proof of the silicatein protein identified immunologically by Western blotting, electrospray ionization (ESI)-mass spectrometry (MS) was applied to confirm that those spots reflect silicatein (Müller *et al.*, 2006a).

In the course to further elucidate the metabolism of siliceous spicules in Demospongiae another enzyme, silicase, was identified from the marine sponge *S. domuncula*; silicase is able to depolymerize amorphous silica. The cDNA was isolated and the deduced polypeptide identified as an enzyme similar to carbonic anhydrases. Recombinant silicase displays besides a carbonic anhydrase activity the ability to dissolve amorphous silica under formation of free silicic acid (Eckert *et al.*, 2006; Schröder *et al.*, 2003b).

4.2. Morphology of spicules

From the demosponge species *S. domuncula* a special form of cell culture system, primmorphs, could be developed (Müller *et al.*, 1999) which turned out to be very suitable for a detailed understanding of spicule formation on cellular and subcellular level (Müller *et al.*, 2006a). Primmorphs, a special type of 3D-cell aggregates containing proliferating and differentiating cells, allow during incubation in medium supplemented with silicic acid the study of the differentiation of the sponge stem cells, the archaeocytes, to the spicule-forming sclerocytes (Müller *et al.*, 1999, 2004; Müller, 2006). In addition, the species *S. domuncula* represents a straightforward model since

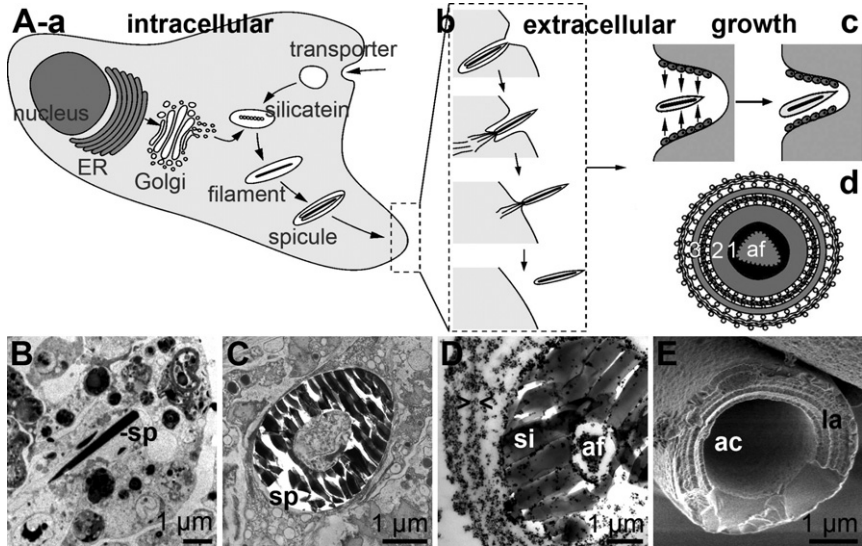


Figure 3.9 Formation of spicules in demosponges. (A) Schematic outline of spicule formation in the *S. domuncula*. (A-a) The initial steps of spicule synthesis occur intracellularly where the silicatein is processed to the mature form. During this processing silicatein undergoes phosphorylation. Very likely with the help of other proteins the silicatein molecules assemble to a rod, the axial filament. Around this filament the first deposition of silica proceeds. (A-b) The primordial, immature spicule is released from the cell, perhaps facilitated by cytoskeletal filaments. (A-c) Process of appositional growth of the spicule in the extracellular space (mesohyl). In the mesohyl, galectin molecules associate to strings (nets) that allow binding of silicatein molecules. Collagen fibers orient the silicatein–galectin strings concentrically around the growing spicules. (A-d) In the last step, biosilica deposition is mediated in two directions, originating both from the silicatein–galectin strings and from the surface of the spicule (centripetal and centrifugal). Finally, an additional further biosilica lamella is formed (3) which is layered onto the previous two lamellae (1 and 2). The initial biosilica layer is formed around the axial filament (af), existing in the axial canal of the spicule. (B–E) TEM/SEM analyses of the growth steps of spicules from *S. domuncula*. (B) An immature spicule (sp) growing within a sclerocyte. (C) A spicule (sp) in the phase of transport from the intracellular to the extracellular space. (D) Immunogold electron microscopy of cross sections through growing spicules in primmorphs; the immune complexes between the polyclonal anti-silicatein antibodies had been visualized with nanogold anti-rabbit IgG. Using this technique the concentric rings (><), formed from silicatein and galectin, around the silica layer (si) of the spicule can be visualized. The axial filament (af) likewise forms immuno-complexes with the antibodies. (E) Cross-cut through a spicule, showing the circular axial canal (ac) and the lamellated siliceous deposits (la) which surround the canal.

its skeleton is composed of only two types of megascleres, monactinal tylostyles and a smaller fraction of diactinal oxeas. The spicules reach up to 450 μm in length and diameters of 5–7 μm (Fig. 3.9E); they grow through apposition of lamellar silica layers. All spicules have an axial canal

of 0.3–1.6 μm in width in the center. To monitor the location and distribution of silicatein in and around the spicules, polyclonal antibodies (PoAb) were raised against purified filaments from spicules in rabbits (Müller *et al.*, 2005). Immunofluorescence studies with cryosections through tissue revealed that the surfaces of the spicules in the tissue and of isolated spicules are surrounded by sheets/cylinders composed of silicatein and galectin (Schröder *et al.*, 2006; Fig. 3.9D).

As outlined (Müller *et al.*, 2005, 2006a), the initial phase of spicule formation proceeds intracellularly in sclerocytes, where the spicules elongate up to 8 μm (Fig. 3.9B). As seen, these cells are loosely embedded in the mesohyl. Higher magnification reveals that these cells usually start to synthesize several spicules simultaneously; the lengths of the spicules observed reach values of 0.7–8 μm and diameters of up to 0.9 μm (Fig. 3.9B). Spicule formation starts within vesicles (Fig. 3.9B). After reaching a size of >5–8 μm , the sclerocytes contract and surround the spicule almost completely. Finally, the spicules are released into the extracellular space where they grow in length and in diameter by appositional growth (Fig. 3.9C and E). The maturation and the final form and size formation of the spicule occurs extracellularly.

Silicatein is present not only in the axial canal, but also in the extra-spicular and extra-cellular space (Müller *et al.*, 2005; Schröder *et al.*, 2006). It came surprising that also there the silicatein molecules are organized to larger entities. The immunogold electron microscopical analysis showed that the silicatein molecules are arranged along strings, which are organized in parallel to the surfaces of the spicules (Fig. 3.9D). A further progress in the elucidation of the morphogenesis of the spicules in the extracellular space came from the observation that there galectins which are the prevailing proteins in the extracellular space of demosponges (Diehl-Seifert *et al.*, 1985; Pfeifer *et al.*, 1993), associate with silicatein to form an organic cylinder within which the bio-silica lamella is formed (Schröder *et al.*, 2006). It is important for the understanding of the silicatein function that *S. domuncula* galectin is able to self-associate in the presence of Ca^{2+} (Diehl-Seifert *et al.*, 1985). Like galectin a second gene undergoes high expression if the cells are exposed to silicic acid; this is collagen (Krasko *et al.*, 2000). Like in the sponge *G. cydonium*, a model which had been initially used for the gene expression studies, also in *S. domuncula* it had been shown that galectin can form self-associated structures which have the ability to associate with collagen (Schröder *et al.*, 2006). The functional interaction between galectin and silicatein has been demonstrated by coimmunoprecipitation studies using antibodies raised against these proteins. The thin strings, formed by galectin/silicatein and collagen exist in close association with the silicate shell of the spicules on the outside of the spicules (Fig. 3.9D). It is self-explanatory that the antibodies also recognized the axial filament within the axial canal of the spicules (Fig. 3.9D). The immunogold electron microscopic images led to the assumption that collagen fibers mediate the

functional orientation of silicatein–galectin complexes by arranging them concentrically around the longer axis of a growing spicule. Then, silicatein mediates silica deposition onto the surface of the existing silica layer. Since the surface of a new siliceous spicule is also covered with silicatein, the appositional growth/thickening of a spicule proceeds from two directions (centrifugal and centripetal) (Müller *et al.*, 2005; Schröder *et al.*, 2006).

Based on the experimental data, the following schematic processes of silica deposition during spicule formation can be formulated (Fig. 3.9A; Müller *et al.*, 2007e).

4.2.1. The intracellular phase of spicule formation in the sclerocytes

Silica is taken up actively by a $\text{Na}^+/\text{HCO}_3^-[\text{Si}(\text{OH})_4]$ cotransporter (Schröder *et al.*, 2004a). In the first steps silicatein is synthesized as a pro-enzyme (signal peptide–propeptide–mature enzyme: 36.3 kDa) and processed via the 34.7 kDa form (propeptide–mature enzyme) to the 23/25 kDa mature enzyme. Very likely during the transport through the endoplasmic reticulum and the Golgi complex, silicatein undergoes phosphorylation and is transported into vesicles where silicatein forms rods, the axial filaments (Fig. 3.9A-a). After assembly to filaments the first layer(s) of silica is (are) formed. Finally, the spicules are released into the extracellular space where they grow in length and diameter by appositional growth. The immature spicules are extruded from the pinacocytes (Fig. 3.9A-b).

4.2.2. Extracellular phase (appositional growth)

Silicatein is present also in the extracellular space. As mentioned, the immunogold electron microscopic analysis showed that the silicatein molecules are arranged along strings, which are organized in parallel to the surfaces of the spicules. In the presence of Ca^{2+} , silicatein associates with galectin and allows the appositional growth of the spicules (Fig. 3.9A-c). Since the surface of a newly siliceous spicule is also covered with silicatein, the appositional growth/thickening of a spicule hence proceeds from two directions (centrifugal and centripetal).

4.2.3. Extracellular phase (shaping)

In the next step, the galectin-containing strings are organized by collagen fibers to net-like structures (Schröder *et al.*, 2006). It is very likely that collagen, which is released by the specialized cells the collencytes, provides the organized platform for the morphogenesis of the spicules (Fig. 3.9A-d). The longitudinal growth of the spicules can be explained by the assumption that at the tips of the spicules, the galectin/silicatein complexes are incorporated into deposited biosilica under formation and elongation of the axial canal.

5. SPICULE NETWORK IN SPONGES: A UNIQUE SKELETON

In living organisms four major groups of biominerals exist; (1) iron compounds, which are restricted primarily to Prokaryota, (2) calcium phosphates, found in Metazoa, (3) calcium carbonates, used by prokaryota, protozoa, plantae, fungi, and metazoa and (4) silica (opal) present in sponges and diatoms. The formation of the sponge skeleton is a multifaceted process. Even though these animals comprise the simplest body plan their biomineral structure formation is already highly complex and by far not completely understood (Müller, 2005). It could be demonstrated that, like in triploblasts, also the diploblastic Porifera skeleton formation has a pronounced effect on morphogenesis. For example, when animals grow under unfavorable conditions, that do not allow formation of the inorganic deposits (silica or calcium biominerals), growth of the specimens is extremely suppressed. Inhibition studies revealed that skeletogenesis of siliceous spicules is enzyme-mediated, more particularly, mediated by the Fe^{++} -dependent enzyme silicatein (Krasko *et al.*, 2000; Müller, 2005). The formation of siliceous spicules in sponges is surely genetically controlled and it can be considered that this process initiates the morphogenesis phase (Krasko *et al.*, 2000, 2002; Müller, 2006). Data demonstrated that at suitable concentrations, silicate induces genes, for example, those encoding collagen, silicatein, and myotrophin. A major step to elucidate the formation of the siliceous spicules on molecular level was the finding that the “axial organic filament” of siliceous spicules is in reality an enzyme, silicatein, which mediates the apposition of amorphous silica and hence the formation of spicules. First progress towards an elucidation of the axial filament formation was achieved (Murr and Morse, 2005; Müller *et al.*, 2007a) by the demonstration that the silicatein molecules from demosponges associate in a controlled way along fractals.

Collagen, also in sponges, is surely an important component in the morphogenesis of the spicules. It was already Schulze (1904) who described that comitalia of *Monorhaphis* are surrounded by a fibrous sheath. He even described the small holes (about 10 μm) in the net. By application of the SEM technique (Müller *et al.*, 2007d) the net could be identified as a collagen sheathing that is regularly interrupted by circular holes (7–10 μm). At higher magnification the uniformity in size and shape of the fibrils becomes clear. The diameter of the fibrils is ~ 25 nm and, with a periodicity of 65 nm, the fibrils can enlarge, forming large nodules of 40 nm (Müller *et al.*, 2007d). Since microtomography (MicroCT) analysis has become a standard technique for the visualization and quantification of the 3D structure of hard-structured materials below 10 μm it was advisable

to use this technique also for reconstructions of the other layer(s) of the giant basal spicule at the micrometer scale. Two dimensional (2D) cross sections have been recorded by MicroCT and the images obtained have been used for three dimensional (3D) reconstructions. 2500 separate 2D images have been superimposed from which the 3D reconstructions were computed from a 5-cm long part of a giant basal spicule, with a diameter of 2 mm. The reconstructions revealed that an organic envelope, made of collagen fibrils, surrounds the inorganic spicule. In addition, this rendering also disclosed that the surface of the silica material is not even but has a serrated relief structure. The protrusions are arranged in an organized pattern, an almost regular helical succession of small projections. While in younger parts of spicules, located closer towards the apex, the organic envelope is tightly attached to the spicule, at more basal regions within the 1–1.5-m long spicules this organic mantel surrounds them only very loosely (submitted for publication). Older parts of the spicules, which have lost the organic envelope, have smooth surfaces.

As mentioned, the surfaces of the spicules are covered by a collagen coat which leaves open holes of a diameter of around 10 μm . Interestingly, the surface of the giant basal spicules is spiked with “rectangular” protrusions of the same dimensions, which perfectly fit into the holes of the collagen net. The lamellae which underlie the protruding surface follow the carvings in a degressive manner. HR–SEM analysis discloses that the serrated relief structures, visualized by MicroCT, reflects those protrusions which are formed by the siliceous layers.

Taking these data into account it became possible to outline a process which could be the explanation of the spicule formation, including their morphogenesis. The appositional layering of the lamellae of the hexactinellid spicules has originally been described by Schulze (1904); Fig. 3.10A. He outlined that within each lamella, which he named “Siphone”, cellular structures exist, which had been termed “Spiculinlamellen” (Fig. 3.10A-a). His report influenced the successive scientists. This situation changed when the technique of HR–SEM was combined with those light microscopic approaches. Likewise, he realized already that the axial filaments vary in their diameters being wide at the beginning of the axial growth of the lamellae and becoming thin at the surface to the sub-terminal layer (Fig. 3.10A-b). A diagonal view of a broken giant basal spicule displays the sharp-edged layering of the lamellae; in none of those images a protruding organic layer or filaments could be identified between the lamellae (Fig. 3.10B). Coming back to Schulze (1904), he already pointed out the importance of the axial filament from the interior and the fibrous (collagen) sheet from the exterior for the development of the spicules. The outer surface of the giant basal spicules is surrounded by a fibrillar sheet layer which exposes the 10 μm protrusion, curving out from the lamellae. On the basis of these old and our recent data a solid scheme for the axial and

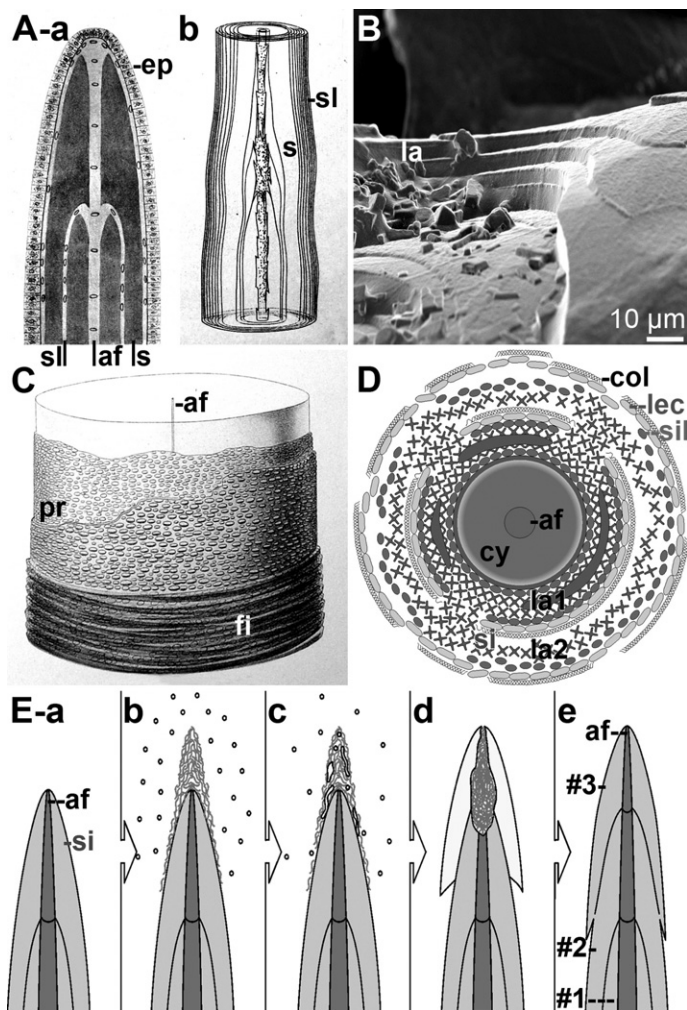


Figure 3.10 Growth of the giant basal spicules. (A-a) Original drawing of Schulze (1904) highlighting the lamellar organization of the spicule. He termed the lamellae “Siphone” (s) and the inter-lamellar space “Spiculin Lamellen” (sl). The surface of the cells are enveloped by an epithelial layer of cells (ep). The axial filament (af) forms the center. (A-b) The axial filament (af) changes its diameter during the process of lamellar formation of the “Siphone” (s). The diameter of the axial filaments is thicker at the surface of the newly forming lamella, and shrinks towards the surface of the sub-terminal layer. (B) Diagonal view of a broken giant basal spicule displaying sharp-edged layering (la) without any evidence for a protruding organic layer or filaments; SEM image. (C) Scheme explaining the growth of the spicules in the lateral direction (Schulze, 1904). It is highlighted that exterior of the spicules is surrounded by a solid fibrous (collagen) sheet (fi) which is followed to the interior by a separate layer which allows the exposure of the 10- μ m protrusions (pr). In the center is the axial filament (af). (D) Present-day schematic illustration of the formation of the giant basal spicules. The center of the spicule comprises the axial canal which is filled with the axial filament

diametral growth of the spicules can be given. The diametral growth proceeds lamellar-wise (Wang *et al.*, 2007). In analogy/homology to demosponges—also in the hexactinellids lamellar layers are formed in the spicules. The model implies (Fig. 3.10D) that the silicatein of the axial filament mediates the formation of the bio-silica cylinder. In further steps hollow cylinders, formed by two organic surfaces, surround the bio-silica cylinder. Both in centrifugal and centripetal direction an apposition of bio-silica occurs under formation of primary lamellae. With the progression to larger spicules the organic cylinder is proteolytically dissolved. In view of our recent results (Müller *et al.*, 2008e) the orientation of the organic cylinder and in consequence also of the form and shape of the spicules are directed by collagen. The existence of collagen in Hexactinellida has been proven by application of molecular biological tools and microscopic images. During longitudinal growth of the spicules, cone-like silica structures are formed via silicatein, which result in an apical–basal increase of the number of lamellae within the spicules (Fig. 3.10E). This mode of spicule growth is perfectly suitable for the formation of an increasing number of lamellae along the apical–basal axis of the spicules, and follows a suggestion that had already been proposed by Schulze (1904). The basal–apical growth pattern, increasing number of lamellae towards the basis, is facilitated by the fact that spicules from hexactinellids have an open tip. This structure allows the elongation of the axial filament within the axial canal. From studies with demosponges (*S. domuncula*) it is known that the first (few) silica lamella(e) of a spicule is/are formed intracellularly, while most of the silica material of the spicules is produced extracellularly by appositional layering of the lamellae (Müller *et al.*, 2005; Schröder *et al.*, 2006). In demosponges it remained unsolved by which mechanism an elongation of the axial filament can occur, since their tips are (always) closed.

(af; in red). Experimental evidence indicates that this filament is composed of silicatein. Around the axial filament at first the bio-silica cylinder (cy) is formed by silicatein and ortho-silicate (si). After completion of this core, the lamellae are synthesized within an organic hollow cylinder. The surfaces of the cylinder are composed of silicatein (sil; red ellipsoid balls) which are arranged both on the surface of the first silica cylinder and a proteinaceous tube/cage which is stabilized in its outer layer by lectin molecules (lec; yellow balls). The orientation of the tube/cage is governed by collagen (col; grey fibers). After completion of the first lamella (la1), a second lamella (la2), and the further ones are synthesized that give rise to the giant spicules. It can be assumed that with time the organic templates which mediate the bio-silica polymerization/condensation are disintegrated by proteases. (E) Schematic outline of the longitudinal, axial growth of the spicules by cone-like structural units (in green). The cone #3 is layered onto the underlying cones #2 and #1, made of ortho-silicate (si). The axial canal, which harbors the axial filament (af), that is composed of silicatein, represents a continuous structure that traverses the individual cones. It is proposed that silicatein is deposited from the extra-spicular space onto the spicules, forms there the lamellae, and then intrudes the axial filament. Finally, a complete stacking of cones is formed which build the multilamellar giant spicules.

The determination of the spicule morphology remains one of the most enigmatic processes in sponges. It is well known that the sizes and forms of the spicules are under species-specific control. However, only little information is presently known to define the control systems, governing the growth and form processes. Surely, the final basis will be a tuned gene regulatory network. The regulation on protein level will be based on the elongation of the axial filament and the interaction of the cells with the bio-silica structures, formed by silicatein. Hence, we might anticipate two directions of interacting control systems; firstly, the control of the formation of the axial filament from the silicatein molecules. Using two demosponge species it could be demonstrated that silicatein, especially if it is extracted from the spicules under mild, nonharsh conditions, readily forms dimers through noncovalent linkages. Subsequently, filaments/aggregates are formed from monomeric silicatein, suggesting a reassembly through fractal-like structures (Murr and Morse, 2005; Müller *et al.*, 2007a). Recently, our group succeeded to demonstrate that also in *Monorhaphis* the silicatein monomers assemble to larger aggregates in a controlled manner (submitted). If the enzyme is released from the bio-silica matrix in the presence of glycerol and subsequently transferred into the glycerol-free medium, (almost) immediately filamentous structures, which reach a size of around 1 μm , are formed (Fig. 3.11C and D). Addition of ortho-silicate to the reaction assay during this filament formation does not considerably change the size of the protein aggregates but allows silicates to precipitate on their surface (Fig. 3.11E). The silica nature of these clusters had been confirmed by energy dispersive X-ray spectroscopy.

The extracellular control of the spicule formation, especially with respect to their morphology is assumed to be under cellular control. The adjacent cells secrete both silicatein and galectin but also collagen forming the different proteinaceous layers within the axial cylinder and the lamellar zone (Fig. 3.11A). The surfaces of the growing spicules are covered by a sheet with open holes, allowing the underneath silica lamellae to invade. The activity and the specificity of the cells, surrounding the spicules, are controlled by the bio-silica through a backcoupling mechanism. At first the axial filament is formed (Fig. 3.11B-a). Subsequently, the first bio-silica layer is formed (Fig. 3.11B-b); this inorganic matrix display morphogenetic activity (Krasko *et al.*, 2000, 2002). This role of bio-silica could be demonstrated in the *S. domuncula* model with which a selective gene expression of collagen and silicatein could be established. In turn, and in analogy to *S. domuncula*, we assume that bio-silica of the lamellae attracts the cells loosely attached within the mesohyl of the sponge to, or close to, the surface of the spicule and causes their differentiation to sclerocytes (bio-silica-forming cells) and collencytes (collagen-forming cells), as reported (Schröder *et al.*, 2004b; Fig. 3.11B-c–B-d).

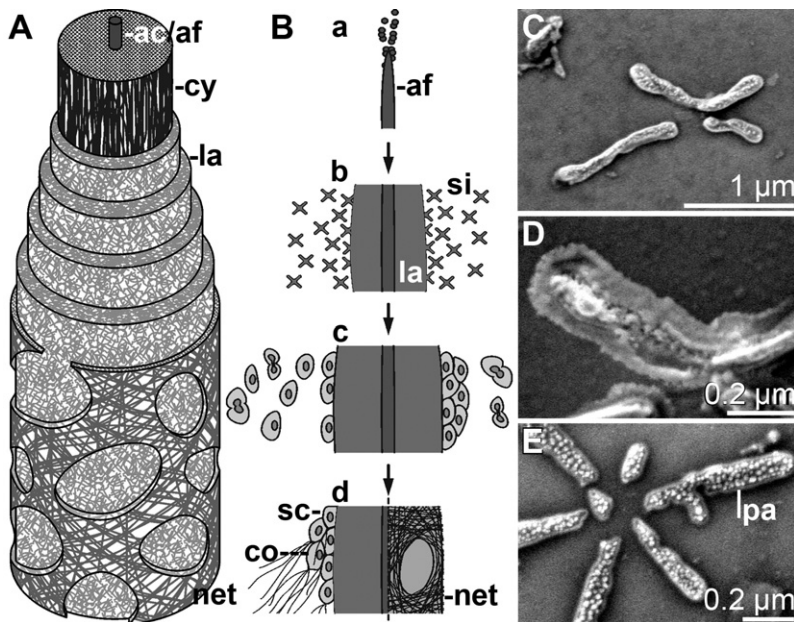


Figure 3.11 Towards the elucidation of the control mechanisms involved in the morphogenesis of the giant basal spicules. (A) Scheme summarizing the three morphological zones of the spicules. The silica structures (with their organic components); the axial canal (ac) (axial filament, af), the axial cylinder (cy) (axial barrel), and the lamellar zone (la) (lamellar coating). The surface of the spicule is surrounded by a net (net) which leaves open 10 μm holes, allowing the bio-silica lamellae to be squeezed in. (B) Backcoupling mechanism explaining the interaction between the bio-silica formation caused by the silicatein in the axial filament (af) and the morphogenetic activity, displayed by the product of silicatein, the bio-silica. (B-a) At first the axial filament (af) is formed which mediates the formation of bio-silica (B-b). (B-c) Bio-silica of the formed lamellae (l) attracts the cells to associate with the growing spicule and (2) causes a morphogenetic effect on the stem cells to differentiate into the sclerocytes (bio-silica-forming cells; sc) and the collencytes (collagen-forming cells; co). These two cell types allow the composition of the fibrous net (net) around the spicules. (C) Self-assembly of silicatein; SEM analysis. The lamellae of *Monorhaphis* had been extracted and the resulting 25/27-kDa protein was analyzed for the ability to organize into a filamentous structures; SEM analysis. After transfer of the silicatein from the glycerol-containing buffer to a glycerol-free medium, filamentous structures are rapidly formed. If exposed to ortho-silicate the smooth surfaces of the structures changed their smoothness under formation of rod-like/cuboid particles (pa) on their surfaces. (D) Higher magnification of (C). (E) Generation of rod-like/cuboid particles (pa) on the surface of the filamentous structures formed from the 24–27 kDa protein after incubation with ortho-silicate.

This mechanistic view offers a rational view how the different morphologies of the spicules could be formed and allow experimental testing in the future.

6. IMPORTANCE OF THE FINDINGS FOR NANOBIOTECHNOLOGICAL APPLICATIONS

Biogenic silica (biosilica), consisting of glassy amorphous silica, is formed in several aquatic and terrestrial organisms; however, only the sponges synthesize poly-silicate enzymatically. Since the discovery of silicatein, biosilicification has become an inspiration for the development of novel fabrication procedures in nanobiotechnology. Technical production of silica commonly requires high temperatures and pressures, and extremes of pH. Living organisms are, however, able to form silica under ambient conditions, at low temperature and pressure and near-neutral pH. Moreover, they produce their silica skeletons with high fidelity. Understanding the mechanism(s) of biosilica formation as well as identification of the components involved in this process and the constituents of biosilica is therefore of high importance for the technological/industrial application of the unique synthetic abilities of silica-forming plants and animals. Silica is, in principle, a mechanical fragile material. However, siliceous organisms use silica as a composite material.

Several different classes of biomolecules have been found to be associated with or embedded in the biosilica, including enzyme proteins, glycoproteins, and polyamines. In previous reviews (Müller *et al.*, 2007e; Schröder *et al.*, 2007, 2008; Wang and Wang, 2006), we reported about the present stage of knowledge on the structure, biochemical composition, and mechanisms of formation of biosilica, mainly in sponges, diatoms, and higher plants. We focused our attention particularly on biosilicification in sponges because of the enormous (nano)biotechnological potential of the sponge enzymes involved in this process. The enzymes involved in silica metabolism, in particular silicatein and silicase (protected by patents; silicatein [Müller and Schröder, 2007a] and silicase [Müller and Schröder, 2007b]), have attracted increasing attention because of their potential applications in the field of nanobiotechnology and biomedicine. Silica-based materials are used in many high-tech products including microelectronics, optoelectronics, and catalysts. Biocatalysis of silica formation from water-soluble precursors, in particular silicatein-mediated biosilica production, occurs under mild physiological conditions and is advantageous compared to technical (chemical) production methods that require high temperatures, pressures, or extremes of pH. The outstanding feature of the giant basal spicules from *Monorhaphis* will surely be seen in their role as a model of nature for the innovative development of further nano-composite materials of unique optical and mechanical properties, for example, by using them for the development of fundamentally new integrated optical elements based on peculiar waveguide properties, single-way waveguides with increased mode field diameter, and unique frequency and dispersion characteristics.

7. CONCLUDING REMARKS

Until 15 years ago the Porifera (sponges) had been an enigmatic animal taxon whose evolutionary origin, its phylogenetic position, and its genetic toolkit had been largely unknown. There had been one protein, the cell adhesion molecule galectin, which clarified those questions almost suddenly (Pfeifer *et al.*, 1993). Cloning and functional studies of that molecule solved the question on the evolutionary origin of the multicellular animals by the demonstration that all metazoan phyla originate from one common ancestor, the hypothetical Urmetazoa (Müller, 2001). After having established the monophyly of animals, and underscored the relevance of that phylum (Porifera) for the elucidation of the deep phylogeny of animals (Müller, 1998), it could be resolved that among the three classes included into the phylum Porifera, the evolutionary oldest class is represented by the Hexactinellida (Kruse *et al.*, 1998). This insight came as a surprise, since the members of that class are, based on their body plan, comprised with the most sophisticated body plan among the sponges. This enlightenment had been supported and flanked by the realization that the sponges comprise (almost all) basic functional circuits, known also from higher metazoan phyla. Regardless of that progress one main issue remained mysterious, the genetic basis for the construction of the highly complex skeleton built of spicules. Focusing on the siliceous sponges, major progress had been made in the understanding of the formation and the development of the spicules in the last few years. Furthermore, with the availability of the giant basal spicules from *Monorhaphis* substantial advancement in the insight of the construction of the siliceous spicules had been achieved and outlined in this review. The compiled results bridge, in an exemplary way, the gap usually existing between the cell's macro- and microscopic phenotype and the processes proceeding during the assembly of nano-bio-components, whose formation is genetically determined.

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THE BIOLOGY OF CAVEOLAE: ACHIEVEMENTS AND PERSPECTIVES

Marie-Odile Parat

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Abstract

Caveolae are specialized plasma membrane subdomains visualized more than 50 years ago as cave-like invaginations at the cell surface. They are rich in cholesterol, glycosphingolipids, and lipid-anchored proteins. Their signaling and trafficking capabilities influence multiple cellular processes, and are believed to require caveolin-1, a major protein component of caveolae in most

cell types. Today the structure and functions of caveolae are still the objects of intense research. Caveolin-1 is not anymore the only protein known to be required for caveolae formation, and functions for caveolin-1 outside of caveolae are being unveiled. Studying the phenotype of mice lacking caveolae has largely confirmed the roles attributed to this organelle and its defining protein. The consequences of mutation or ablation of caveolins in human disease are emerging. Recent evidence further suggests that caveolae and caveolin can be targeted for therapeutic purposes.

Key Words: Caveolin, Molecular dissection, Post translational modification, Cavin, Endocytosis, Endothelial cells, Nitric oxide synthase. © 2009 Elsevier Inc.

1. INTRODUCTION

Caveolae are specialized subdomains of the plasma membrane found in most cell types, and particularly abundant in highly differentiated cells such as endothelial cells, adipocytes, or muscle cells. They were first described as invaginations of the plasma membrane over 50 years ago (Palade, 1953; Yamada, 1955), but the molecular aspects of their formation and functions are still being unraveled. Insight into their function has come from the study of mice lacking caveolae, whose phenotype continues to undergo detailed examination. This review focuses on the structure, functions, and therapeutic perspectives offered by caveolae, with an emphasis on recent developments.

2. STRUCTURE OF CAVEOLAE

Electron microscopy allows the visualization of caveolae as ~70 nm omega-shaped invaginations of the plasma membrane or as circularized single or clustered vesicles underneath the plasma membrane (Fig. 4.1). Caveolae are specialized membrane microdomains enriched in sphingolipids, cholesterol, and the caveolin proteins. In physiological conditions, their shape or the presence of caveolin sets caveolae apart from the rest of cholesterol-rich membrane subdomains termed lipid rafts. It has been estimated that 144 caveolin molecules are present per invagination (Pelkmans and Zerial, 2005) in the form of homo and hetero-oligomers. Recent evidence has unveiled that caveolins are not the only proteins essential for the formation and function of caveolae (Hill *et al.*, 2008; Liu and Pilch, 2007).

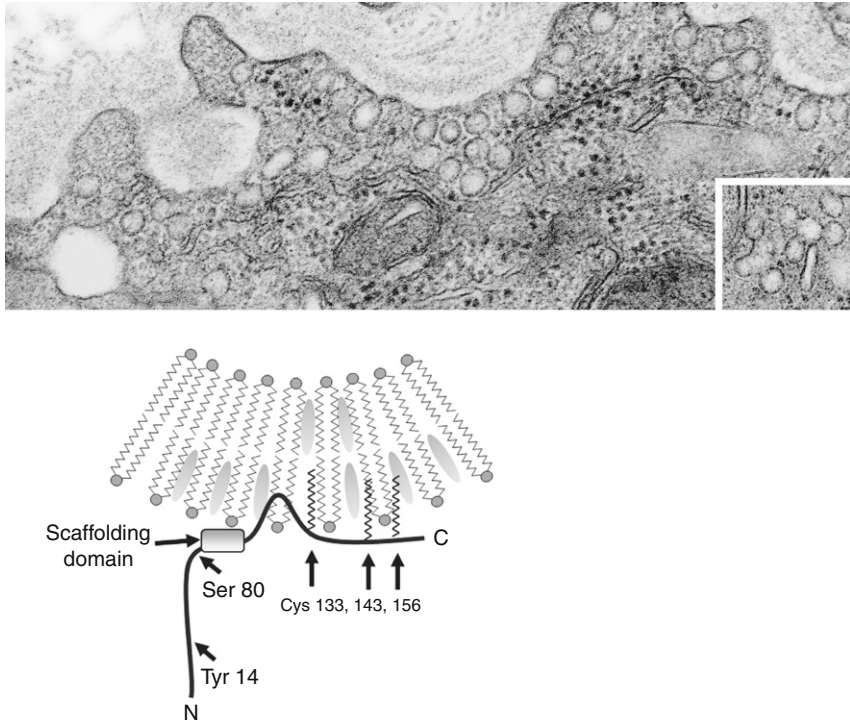


Figure 4.1 Caveolae structure and caveolin-1 schematic representation. Upper panel: electron micrograph of an endothelial cell of vasa vasorum in a rat aorta. Caveolae appear as 70 nm invaginations of the plasma membrane, as circularized vesicles, or fused to one another (inset). Lower panel: schematic of caveolin-1 domains and functionally important posttranslational modification sites.

2.1. Structural proteins

2.1.1. Caveolin-1

Caveolin-1 is the major member of the caveolin family (which also comprises caveolin-2 and the muscle-specific caveolin-3) and is expressed in most cell types. There is a link between caveolin-1 expression and caveolae formation, as caveolin-1-null cells have no caveolae (Drab *et al.*, 2001) and expression of caveolin-1 in cells devoid of caveolin-1 results in *de novo* caveolae formation (Fra *et al.*, 1995). However, caveolin-1 overexpression in cells already rich in caveolin-1 and caveolae has no further effect on caveolae number (Bauer *et al.*, 2005). Caveolin-1 has an unusual topology, with its C-terminus and N-terminus both facing the cytosol, connected by a membrane-embedded hydrophobic domain (Fig. 4.1). The structure of caveolin-1 has been studied in great detail.

2.1.1.1. Caveolin-1 α and β Two isoforms of caveolin-1 are seen on polyacrylamide gels after electrophoresis: a slower migrating isoform named caveolin-1 α and a faster migrating isoform named caveolin-1 β . Because (i) the first amino acid of caveolin-1 β is the highly conserved methionine 32 of caveolin-1 α and (ii) the sequence surrounding the AUG codon of methionine 32 of caveolin-1 α matched the Kozak nucleotide consensus for translation initiation, it was initially suggested that both isoforms are generated from a single mRNA transcript using the AUG that encodes methionine 32 as an alternate translation initiation site (Scherer *et al.*, 1995). However, later on the existence of two mRNA isoforms was reported using an RNAase protection assay (Ko *et al.*, 1998). This was later confirmed by a group which detected two mouse caveolin-1 mRNA variants. The full length variant generated mostly caveolin-1 α while the 5'-variant, lacking the first exon, generated exclusively caveolin-1 β (Kogo and Fujimoto, 2000). The authors confirmed that both α and β caveolin-1 isoforms are produced when a caveolin-1 cDNA construct, having no 5'-untranslated region (UTR), is transfected into mammalian cells (Kogo and Fujimoto, 2000). They further showed that *in vivo* expression of the full length and 5'-variant mRNAs correlated with expression of the α and β isoforms, respectively (Kogo *et al.*, 2004).

The functional specificity of each caveolin-1 isoform is poorly understood. It has been suggested that since their production is regulated independently at the transcriptional level with some level of cell type specificity, they may play unique physiological functions (Kogo *et al.*, 2004). One study proposed that caveolin-1 α and β have a different potential for caveolae formation, with caveolin-1 β hardly able to generate caveolae in the absence of caveolin-1 α (Fujimoto *et al.*, 2000). In contrast, baculovirus-based expression in sf21 insect cells indicated that either isoforms equally formed high molecular weight oligomers and stimulated the formation of intracellular vesicles of the size and buoyant density of caveolae (Li *et al.*, 1996b). In a recent study, targeted depletion of either caveolin-1 isoforms using antisense morpholino oligomers in developing zebrafish resulted in a substantial loss of caveolae (Fang *et al.*, 2006). Rescue by injection of translation-competent cRNAs further confirmed the isoform specificity, that is only the caveolin-1 α cRNA rescued the phenotype of the caveolin-1 α morphant while only caveolin-1 β cRNA rescued the phenotype of the caveolin-1 β morphant (Fang *et al.*, 2006). These results show nonredundant roles for caveolin-1 α and β in early zebrafish development. Further functional specificity of each isoform in development may include differential interaction with bone morphogenetic protein receptors (BMP) signaling (Nohe *et al.*, 2004, 2005).

Because caveolin-1 β is a naturally occurring, N-terminally truncated form of caveolin-1 α , the functions specifically attributed to the first 31 aminoacids of caveolin-1 α are anticipated to be lost in caveolin-1 β . This has

been demonstrated in few studies. For example, specific subcellular localization in transmigrating cells requiring tyrosine 14 of caveolin-1 α is lost in caveolin-1 β (Parat *et al.*, 2003). However, a rescue experiment in zebrafish morphants showed that caveolin-1 α cRNA with a mutation affecting tyrosine 14 could not rescue the phenotype of the caveolin-1 β morphant, suggesting that tyrosine 14 does not by itself define the functional difference between caveolin-1 α and β (Fang *et al.*, 2006).

2.1.1.2. Caveolin-1 molecular dissection—structure/activity of caveolin

2.1.1.2.1. Caveolin oligomerization Caveolin-1 ability to oligomerize has been documented by several groups (Monier *et al.*, 1995; Sargiacomo *et al.*, 1995). Caveolin-3 was also shown early on to possess oligomerization capabilities (Tang *et al.*, 1996). A GST-fusion peptide comprising amino acids 1–101 of caveolin-1 was shown to self oligomerize *in vitro*. This oligomerization capability was further mapped to aminoacids 61–101 (Sargiacomo *et al.*, 1995). More recently, a peptide made of aminoacids 82–101 was shown to form large, stable oligomers *in vitro* (Levin *et al.*, 2007). Caveolin-1 and caveolin-2 have been shown to form hetero-oligomers, possibly by interaction of their membrane-spanning domains (Das *et al.*, 1999). Finally, adjacent caveolin oligomers have been proposed to network with one another through caveolin-1 C-terminus–C-terminus and C-terminus–N-terminus interactions (Song *et al.*, 1997). Oligomers have been hypothesized to constitute the filamentous coat of caveolae (Fernandez *et al.*, 2002), to organize caveolae membrane, and to serve as a scaffold assembling caveolin-1-interacting molecules in caveolae (Sargiacomo *et al.*, 1995).

2.1.1.2.2. Caveolin-1 posttranslational modifications

2.1.1.2.2.1. Palmitoylation Caveolin-1 is palmitoylated on three cysteines located in the C-terminus of the protein (Dietzen *et al.*, 1995). Palmitoylation is known to target plasma membrane or caveolae proteins that would otherwise be cytoplasmic, except, ironically, in the case of caveolin: the defining protein of caveolae does not require palmitoylation for caveolae incorporation. This was demonstrated by biochemical isolation of caveolae after transfection of palmitoylation-deficient caveolin mutants first into cells that expressed endogenous caveolin-1 (Dietzen *et al.*, 1995) and later into cells devoid of endogenous caveolin-1 (Uittenbogaard and Smart, 2000). Another unusual aspect of caveolin-1 palmitoylation is that while for most proteins palmitoylation is a reversible modification, in the case of caveolin-1 it is an essentially irreversible, posttranslational modification (Parat and Fox, 2001). Palmitoylation has been shown to stabilize caveolin-1 oligomerization (Monier *et al.*, 1996), and to control the ability of caveolin-1 to bind to some of its protein binding partners and

to cholesterol (Di Vizio *et al.*, 2008; Lee *et al.*, 2001; Uittenbogaard and Smart, 2000; Uittenbogaard *et al.*, 2002).

2.1.1.2.2.2. Phosphorylation

2.1.1.2.2.2.1. *Tyrosine phosphorylation* Caveolin-1 was initially isolated and characterized as a substrate undergoing tyrosine phosphorylation by pp60src, the transforming protein of Rous Sarcoma Virus (Glenney and Soppet, 1992; Glenney and Zokas, 1989). It was later shown that cellular Abl and Src family kinases can phosphorylate tyrosine 14 of caveolin-1 (Lee *et al.*, 2000, 2001; Li *et al.*, 1996a; Sanguinetti and Mastick, 2003).

The significance of caveolin-1 tyrosine 14 phosphorylation has only partially been clarified. It occurs in response to growth factor and hormonal stimulation (Labrecque *et al.*, 2003; Lee *et al.*, 2000; Mastick *et al.*, 1995) or to cellular stress including oxidative stress (Parat *et al.*, 2002; Volonte *et al.*, 2001). Hyperosmotic shock induces the phosphorylation of caveolin-1 on tyrosine 14, and this induction is potentiated by cytoskeleton disruption (Sanguinetti *et al.*, 2003; Volonte *et al.*, 2001). Integrin activation also results in caveolin-1 tyrosine 14 phosphorylation (Radel and Rizzo, 2005; Wary *et al.*, 1999). Tyrosine 14 phosphorylation further plays a major role in caveolae endocytosis (Sverdllov *et al.*, 2007).

Experimental data point to a role of caveolin-1 Tyr¹⁴ in cell adhesion and migration processes. Phosphotyrosine-dependent protein interaction screens have shown that tyrosine 14 is required for caveolin-1 binding to Src-homology 2 (SH2)-containing proteins that regulate cell motility such as Grb7 (Lee *et al.*, 2000) or Csk (Cao *et al.*, 2002). Caveolin-1 tyrosine 14 is necessary for caveolin-1 anterior polarization in transmigrating cells (Parat *et al.*, 2003), integrin-regulated caveolae internalization (del Pozo *et al.*, 2005), growth factor-stimulated caveolae dynamics (Orlichenko *et al.*, 2006), the organization of plasma membrane into highly ordered domains at focal adhesions (Gaus *et al.*, 2006), and regulation of focal adhesion kinase exchange between focal adhesions and cytosolic pools (Goetz *et al.*, 2008). Tyrosine 14 also seems to be required to rescue the altered migration of caveolin-1-null cells in a scratch wound assay (Grande-Garcia *et al.*, 2007).

2.1.1.2.2.2.2. *Serine phosphorylation* Caveolin-1 serine phosphorylation has been suggested at serine 80 and possibly other serine residues (Fielding *et al.*, 2004; Garver *et al.*, 1999; Sargiacomo *et al.*, 1994; Schlegel *et al.*, 2001; Vainonen *et al.*, 2004). Caveolin-1 phosphorylation at serine 80 was proposed to regulate binding to the endoplasmic reticulum (ER) membrane and entry into the secretory pathway (Schlegel *et al.*, 2001). Mutation of serine 80 to alanine also appeared to increase the sterol binding ability of caveolin-1 (Fielding *et al.*, 2004). Phosphomimetic mutation of serine 80 to glutamic acid was shown to impair caveolae organization and dynamics (Kirkham *et al.*, 2008; Shigematsu *et al.*, 2003). Phosphorylation of serine 37 of caveolin-1- α and corresponding serine 6 of caveolin-1- β was also evidenced (Vainonen *et al.*, 2004). Much less is known about caveolin-1

serine phosphorylation compared to tyrosine phosphorylation, especially as far as the biological significance of the modification is concerned.

2.1.2. Caveolin-2

Since it is nonessential for caveolae formation and depends on caveolin-1 for stable expression and plasma membrane localization, caveolin-2 was considered an accessory protein for caveolin-1 and received far less attention than caveolin-1. Caveolin-2 is coexpressed with caveolin-1 in most cell types and interacts with caveolin-1 to form hetero-oligomers (Das *et al.*, 1999; Scherer *et al.*, 1996, 1997). Caveolin-2 may also interact with caveolin-3 in uncommon cell types where both isoforms are expressed (Capozza *et al.*, 2005; Rybin *et al.*, 2003; Segal *et al.*, 1999). In the absence of caveolin-1, caveolin-2 is retained in the Golgi complex and undergoes proteasomal degradation (Drab *et al.*, 2001; Mora *et al.*, 1999; Parolini *et al.*, 1999; Razani *et al.*, 2001). This raises the possibility that some phenotypic changes induced by lack of caveolin-1 may be due to concomitant reduction of caveolin-2 expression (Razani *et al.*, 2002b).

Caveolin-2 does not seem to be required for caveolae formation (Razani *et al.*, 2002b), however coexpression of caveolin-1 and -2 leads to the formation of more uniform (Li *et al.*, 1998) deeper (Fujimoto *et al.*, 2000), and more abundant (Lahtinen *et al.*, 2003) caveolae compared with expression of caveolin-1 alone, leading to the conclusion that caveolin-1 is sufficient for caveolae biogenesis but that caveolin-2 plays a modulatory role in this process. This function seems to be dependent on phosphorylation of serines 23 and 36 of caveolin-2 (Sowa *et al.*, 2003). Phosphorylation of caveolin-2 on each serine seems to occur in separate subcellular domains (Sowa *et al.*, 2008). Caveolin-2 can also undergo Src-dependant phosphorylation at tyrosine 19 (Lee *et al.*, 2002b) which seems to be involved in caveolae endocytosis (Kiss *et al.*, 2004). Tyrosine 27 was shown to be another Src phosphorylation substrate (Wang *et al.*, 2004). In contrast with caveolin-1, caveolin-2 does not undergo palmitoylation. The existence of a shorter isoform of caveolin-2, encoded by a splice variant mRNA and lacking the C-terminal domain of the protein, has been documented (Kogo *et al.*, 2002).

2.1.3. Caveolin-3

Caveolin-3 was identified as a muscle specific isoform of caveolin (Tang *et al.*, 1996; Way and Parton, 1995) and is structurally, functionally, and evolutionarily close to caveolin-1 (Kirkham *et al.*, 2008; Tang *et al.*, 1996). In particular, caveolin-3 is capable of generating caveolae upon transient expression in caveolin-null cells (Capozza *et al.*, 2005). Caveolin-3 interacts with several other proteins including nitric oxide synthase (NOS) (Feron *et al.*, 1996, 1998). Caveolin-3 localizes at the sarcolemma where it complexes with dystrophin and dystrophin-associated glycoproteins, playing a

key role in muscle structure and function as shown by the phenotype resulting from lack of caveolin-3 expression, overexpression of caveolin-3, or dominant negative mutations of caveolin-3 (Aravamudan *et al.*, 2003; Galbiati *et al.*, 2001a; Hagiwara *et al.*, 2000; Woodman *et al.*, 2004b). Caveolin-3 further interacts with the type I myostatin receptor, and inhibits the signaling of myostatin, a muscle-specific TGF- β superfamily member controlling muscle volume (Ohsawa *et al.*, 2006). Caveolin-3 may also play a role in maintaining the contractile phenotype of smooth muscle cells (Segal *et al.*, 1999).

2.1.4. Cavin

Cavin, also termed polymerase I and transcript release factor or PTRF, is a soluble protein that was first characterized as a cellular factor able to induce dissociation of paused ternary transcription complexes by promoting the release of both nascent transcripts and Pol I from the template (Jansa *et al.*, 1998). This factor was later found to be identical to a protein associated with the cytosolic surface of caveolae as determined by biochemical and imaging techniques, and was proposed as a caveolae marker (Aboulaich *et al.*, 2004; Vinten *et al.*, 2001, 2005; Voldstedlund *et al.*, 2001; McMahon *et al.*, 2006).

A critical role for cavin in caveolae formation has been recently unveiled by two independent groups (Hill *et al.*, 2008; Liu and Pilch, 2007). Cavin is recruited to plasma membrane caveolae domains by caveolin, and expression of full length cavin seems necessary for caveolae formation in the presence of caveolin-1 (Hill *et al.*, 2008). At present it is unclear whether the two proteins interact directly or rather through their lipid environment, a possibility raised by the observations that cavin is a phosphatidyl-serine-binding protein (Hill *et al.*, 2008), and that its association with caveolin-1 is cholesterol-dependant (Hill *et al.*, 2008; Liu and Pilch, 2007). Using various caveolin mutants and caveolins from a wide range of species, Hill *et al.* have shown that the ability of caveolin-1 to recruit cavin was linked to its capability to generate plasma membrane caveolae (Hill *et al.*, 2008).

Cavin seems to be abundant at the cytosolic face of caveolae (Vinten *et al.*, 2001, 2005). It has been estimated that cavin is roughly as abundant as caveolin-1 in caveolae (Hill *et al.*, 2008). Furthermore, cavin expression parallels caveolin expression in various tissues and cell lines (Hill *et al.*, 2008; Voldstedlund *et al.*, 2001). It is suggested that when the level of expression of cavin decreases, caveolin-1 diffuses in the plasma membrane and becomes internalized into the endolysosomal system where it is degraded (Hill *et al.*, 2008), explaining why downregulation of cavin results in lower expression of caveolin-1 (Hill *et al.*, 2008; Liu and Pilch, 2007). The characterization of the PTRF gene-disrupted mouse will further define the function of this protein, and help differentiate between the roles of caveolin-1 and those attributed to caveolae.

2.1.5. Flotillins: noncaveolar imitators of caveolins

The reggie/flotillin proteins consist of two prohibitin homology domain-containing family members (reggie-1/flotillin-2 and reggie-2/flotillin-1) which present similarities with caveolins, including their membrane topology (Bauer and Pelkmans, 2006) and fatty acid modification (Morrow *et al.*, 2002; Neumann-Giesen *et al.*, 2004), their oligomerization ability (Neumann-Giesen *et al.*, 2004; Salzer and Prohaska, 2001) and their enrichment in detergent-resistant membranes (Bickel *et al.*, 1997). Flotillins further share functional resemblance with caveolins, in that they generate microdomains that can be either flat or invaginated (Frick *et al.*, 2007), and they serve signaling (Baumann *et al.*, 2000; Neumann-Giesen *et al.*, 2007) and trafficking (Glebov *et al.*, 2006) functions. Flotillins have been proposed to be enriched in caveolae (Bickel *et al.*, 1997) and to interact with caveolin-1 (Volonte *et al.*, 1999). However, subsequent studies have determined that flotillins are associated with noncaveolar lipid rafts (Fernow *et al.*, 2007; Neumann-Giesen *et al.*, 2004; Rajendran *et al.*, 2007; Stuermer *et al.*, 2001).

2.2. Lipid composition

2.2.1. Cholesterol

Cholesterol plays a primordial role in the biogenesis, integrity, and functions of caveolae. Caveolae are enriched in cholesterol (Fujimoto *et al.*, 1997; Montesano, 1979; Simionescu *et al.*, 1983), and caveolin-1 binds to cholesterol (Murata *et al.*, 1995; Thiele *et al.*, 2000) possibly through a cholesterol recognition amino-acid consensus sequence shown in other cholesterol-interacting proteins (Li and Papadopoulos, 1998) and which in caveolin-1 overlaps with the scaffolding domain (Epand *et al.*, 2005). Serine 80 was documented to influence caveolin-1 cholesterol binding. Furthermore, even though Tyrosine 14 is not part of this cholesterol-binding domain, evidence indicates that tyrosine phosphorylation is inversely related to cholesterol binding (suggesting a tertiary structure interaction) (Fielding *et al.*, 2004). Cholesterol stabilizes caveolae oligomers (Monier *et al.*, 1996) and depletion or oxidation of cholesterol results in the loss of caveolae architecture (Rothberg *et al.*, 1992; Smart *et al.*, 1994). A less drastic alteration of caveolae cholesterol, namely the substitution of about 90% of cellular cholesterol with its biosynthetic precursor demosterol which contains one additional double bond between carbons 24 and 25, leads to heterogeneous caveolae size and variable number of caveolin-1 molecules per caveola (Jansen *et al.*, 2008). Interestingly, the substitution also resulted in increased caveolin-1 tyrosine 14 phosphorylation (Jansen *et al.*, 2008). Taken together, these data emphasize a complex relationship between caveolin-1 and cholesterol.

2.2.2. Sphingolipids

Sphingolipids complement cholesterol in the distinctive lipid organization of caveolae lipids and are found mostly in the exoplasmic leaflet of the bilayer. While the rest of the plasma membrane is mainly composed of phospholipids, cholesterol and sphingolipids can assemble laterally to form a liquid ordered phase—a property shared by membrane microdomains called rafts (Harder and Simons, 1997; Smart *et al.*, 1999).

2.3. The caveolar coat

Structural studies have revealed a striped or striated coat decorating the cytoplasmic surface of caveolae (Peters *et al.*, 1985; Rothberg *et al.*, 1992). This coat appeared to be made of filaments arranged in circular or spiral arrays in rapid-freeze, deep-etch images of the inner plasma membrane surface (Rothberg *et al.*, 1992). Recent images using ultrathin plastic sections (Parton *et al.*, 2006) or three dimensional reconstructions from electron microscope tomography (Richter *et al.*, 2008) revealed a spiked caveolar coat. The exact molecular composition of the coat is unknown. Caveolin-1 was proposed to be a component of the coat after immunogold detection (Rothberg *et al.*, 1992). Later studies proposed that caveolin-1 oligomers constituted the caveolar coat (Monier *et al.*, 1995). Experimental data indeed suggested that caveolin N termini, assembled into oligomers, could form structures resembling the caveolar coat filaments (Fernandez *et al.*, 2002). This is in contrast with one study suggesting that caveolin-1 only accumulates at the neck of caveolae (Thorn *et al.*, 2003). Physical models also suggest that the striations may be due to competing attractive (between caveolin molecules) and repulsive (membrane mediated) interactions at the surface of caveolar invaginations (Evans *et al.*, 2003). The recent discovery of a role for PTRF in the formation of caveolae (Hill *et al.*, 2008; Liu and Pilch, 2007) opens the possibility that PTRF may participate in the structure of the caveolar coat. Recent evidence also suggests that the serine/threonine kinase ARAF1 may participate in either assembly or maintenance of the caveolar coat (Pelkmans and Zerial, 2005).

2.4. Diaphragms

Endothelial cells can exhibit a particularly high number of caveolae and caveolae play an essential role in the function of the endothelium. In the cells of certain continuous endothelia, caveolae are equipped with an additional structure named stomatal diaphragm. These structures are seen by transmission electron microscopy as thin protein barriers anchored in the neck of caveolae (Stan, 2005, 2007). Stomatal diaphragms can also be observed associated with transendothelial channels and vesiculo–vacuolar

organelles, and fenestral diaphragms with endothelial fenestrae. A major component of these diaphragms has been identified as plasmalemmal vesicle protein PV-1, a glycoprotein capable of dimerization (Stan *et al.*, 1999a,b, 2004). PV-1 dimers are proposed to form radially oriented fibrils and PV-1 C-termini to form the central density of the diaphragms (Stan, 2007). This structure was recently confirmed by tomography of individual caveolae (Richter *et al.*, 2008). The function of stomatal diaphragms is unknown.

2.5. Biogenesis of caveolae

While there is a clear link between the expression of caveolin-1 (or -3) and the formation of caveolae (Drab *et al.*, 2001; Fra *et al.*, 1995; Galbiati *et al.*, 2001a), the mechanism by which caveolin might induce the curvature of the plasma membrane and participate in the special lipid composition of these microdomains is still the object of intense research. The current view is that after caveolin-1 synthesis in the ER, caveolae biogenesis begins along the secretory pathway with the assembly of caveolin-1 oligomers, in a stoichiometric complex with cholesterol (Chadda and Mayor, 2008). Oligomerization seems associated with the acquisition of detergent insolubility (Pol *et al.*, 2005). Cholesterol is essential to the oligomerization, and oligomerization is critical for the transit through the Golgi apparatus to the plasma membrane (Chadda and Mayor, 2008). Using live cell microscopy of green fluorescent protein (GFP)-caveolin-1- and red fluorescent protein (RFP)-caveolin-1 transfected cells, it has been documented that caveolae form in the Golgi apparatus and are delivered to the plasma membrane as stable units (Tagawa *et al.*, 2005).

The effect of caveolin-1 posttranslational modifications on caveolae biogenesis has been dissected out recently (Kirkham *et al.*, 2008). Palmitoylation did not modulate the ability of caveolin-1 to form caveolae, as shown by mutation of the three palmitoylatable cysteins of the C terminus of caveolin-1. Similarly, tyrosine 14 was apparently dispensable because truncated mutants that do not have tyrosine 14, including the naturally occurring caveolin-1- β , were able to form caveolae (Kirkham *et al.*, 2008). However, another study showed that EGF-regulated formation of caveolae was reduced by mutation of tyrosine 14, indicating that signaling events leading to caveolae formation may rely on caveolin-1 tyrosine 14 (Orlichenko *et al.*, 2006). Interestingly, mutation of serine 80 to alanine did not alter caveolae formation, but a phosphomimetic mutation of serine 80 did prevent caveolae formation, most likely through perturbation of caveolin-1 trafficking (Kirkham *et al.*, 2008). Analysis of the domains in caveolins that are important for caveolae formation using chimeric caveolins and caveolin mutants suggested a model in which caveolin may cause membrane deformation through a combination of amphipathic helix

insertion, interaction with cholesterol, and oligomerization, all of which are mediated by different regions of the protein (Kirkham *et al.*, 2008; Parton *et al.*, 2006). How additional proteins necessary for caveolae formation such as PTRF-cavin (Hill *et al.*, 2008) participate in the process of caveolae biogenesis, still needs to be elucidated.



3. FUNCTIONS OF CAVEOLAE

3.1. Endocytosis

Caveolae have been proposed in early literature to participate in the transport of macromolecules across endothelial cells through a process called transcytosis and involving caveolae internalization at the luminal surface of the endothelium, shuttling through the cell and release at the abluminal surface of the endothelium (Stan, 2002). Caveolae trafficking is even today the object of intensive study, and for most cell types the extent to which caveolae participate in constitutive endocytic processes is not entirely clear but their internalization under the appropriate stimulation has been demonstrated.

3.1.1. Mechanism of caveolae endocytosis

Caveolae contain the molecular machinery for vesicular transport and can undergo GTP-dependent, dynamin-mediated scission from the plasma membrane (Henley *et al.*, 1998; Oh *et al.*, 1998; Schnitzer *et al.*, 1995, 1996). Microinjected antibodies to dynamin II (Henley *et al.*, 1998) or expression of a dominant negative dynamin II lacking normal GTPase activity (van der Bliek *et al.*, 1993) therefore inhibit not only the scission of clathrin-coated vesicles but also GTP-induced fission, budding, and internalization of caveolae (Oh *et al.*, 1998; Yao *et al.*, 2005).

In addition to dynamin recruitment, internalization of caveolae requires local actin polymerization (Parton *et al.*, 1994; Pelkmans *et al.*, 2002), the presence of cholesterol (Orlandi and Fishman, 1998; Pelkmans *et al.*, 2002) and GM1 ganglioside at the cell surface (Choudhury *et al.*, 2006), and the involvement of kinases (Parton *et al.*, 1994; Pelkmans *et al.*, 2002, 2005). The best characterized of tyrosine kinases playing a role in caveolae endocytosis is Src, which can phosphorylate caveolin-1, caveolin-2, and dynamin during the internalization process (Sverdlov *et al.*, 2007). Small interfering RNA-induced silencing of Src caused caveolar structures to accumulate at the cell surface and reduced strongly the dynamics of caveolae (Pelkmans and Zerial, 2005). After endocytosis, the structure of caveolae remains stable even as they fuse with intracellular compartments (Tagawa *et al.*, 2005). In an original publication, it was suggested that caveolin-1 is actually a negative regulator of caveolae endocytosis, stabilizing the plasma membrane

association of caveolae, slowing down their internalization and thus allowing their visualization by electron microscopy (Le *et al.*, 2002).

3.1.2. Endocytosis in the absence of stimulation

Caveolae are quite static in the absence of endocytosis stimulus. This has been shown using photobleaching of GFP-caveolin-1-transfected cells (Thomsen *et al.*, 2002; Fig. 4.2), by total internal reflection fluorescence microscopy (TIR-FM) (Tagawa *et al.*, 2005), and by the lack of fusion between GFP-tagged and RFP-tagged caveolae (Tagawa *et al.*, 2005). These data are not in favor of a rapid and efficient endocytosis and recycling of caveolae (Hommelgaard *et al.*, 2005). Mobility of surface caveolin-1 can however be activated upon endocytosis stimulation (Tagawa *et al.*, 2005).

This picture was later refined when, using a combination of TIR-FM and epifluorescence microscopy, Pelkmans and Zerial showed that caveolae undergo continuous short range cycles of fusion and internalization during

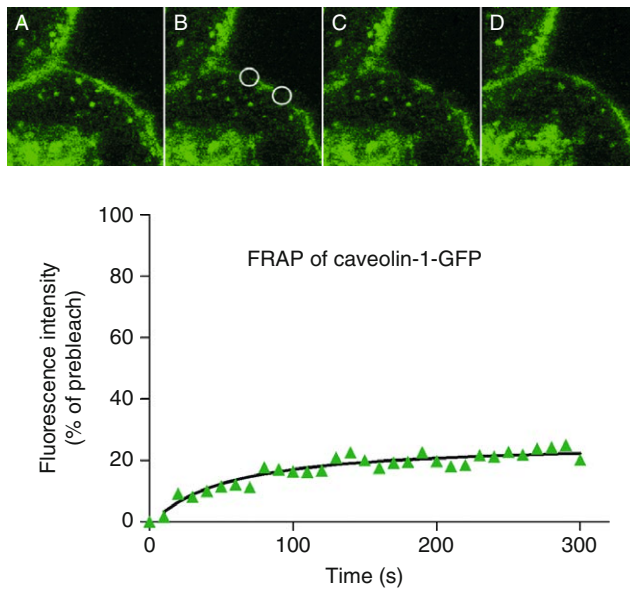


Figure 4.2 Low mobility of plasma membrane caveolin-1 in absence of endocytosis stimulation. MCF7 cells, which express minimal amounts of endogenous caveolin-1, were transfected with GFP-tagged caveolin-1. The mobility of the fluorescent protein was studied using FRAP analysis. (A) Fluorescence picture before bleaching. (B) Two bleach regions were selected at the cell periphery (white circles). (C–D) Fluorescence picture of the same area immediately after bleaching (C) and after 100 s (D). Lower panel: curve fit of mean fluorescence intensity at the bleach regions obtained from 26 measurements. The bleaching magnitude was 40%. The mobile fraction, calculated by comparing the fluorescence after full recovery with that before bleaching and just after bleaching, was $26.3 \pm 1.4\%$.

which they are restricted within a small cytoplasmic volume beneath the cell surface and thus do not travel long distances (Pelkmans and Zerial, 2005). Long range trafficking between the plasma membrane and intracellular pools can then occur following activation of internalization.

3.1.3. Endocytosis upon cell detachment

Cell detachment triggers internalization of caveolae. Upon loss of cell adhesion to the extracellular matrix, the ganglioside GM1, cholesterol, Glycophosphatidylinositol (GPI)-anchored proteins, and caveolin-1 are internalized in a reversible fashion. This process is inhibited by the expression of dominant negative dynamin II mutant. Furthermore, caveolin-1 harboring a tyrosine at position 14 is required for detachment-induced internalization of caveolae, as shown by experiments using caveolin-1-null cells reconstituted with wild type or Tyrosine 14 mutant caveolin-1 (del Pozo *et al.*, 2004, 2005; Echarri *et al.*, 2007; Salanueva *et al.*, 2007).

3.1.4. Other stimuli of caveolae endocytosis

Most of what is known about caveolae endocytosis comes from experiments performed with labeled albumin internalization in endothelial cells, simian virus 40 (SV40) entry into cells, and internalization of the ganglioside GM1-binding cholera toxin. Transcytosis of plasma macromolecules across endothelial cells has been shown to involve caveolae. In particular, electron microscopy revealed labeled serum albumin endocytosed through caveolae and traversing endothelial cells to be released at the abluminal side (Ghitescu and Bendayan, 1992; Schnitzer *et al.*, 1994). Binding of albumin to its caveolae-localized receptor Gp60 was shown to induce a signaling cascade comprising G-protein-coupled Src phosphorylation, Src-mediated dynamin phosphorylation, and caveolin-1 phosphorylation (Minshall *et al.*, 2000, 2002; Shajahan *et al.*, 2004a,b; Tiruppathi *et al.*, 1997). In the absence of caveolae, a compensatory transport mode has been proposed to take place through the opening of a paracellular permeability pathway (Miyawaki-Shimizu *et al.*, 2006; Predescu *et al.*, 2004; Schubert *et al.*, 2002) or through increased large pore volume flow in capillaries (Rosengren *et al.*, 2006).

For nonendothelial cells, abundant information about caveolae endocytosis has been gathered from experiments using SV40. Early electron microscopy experiments showed the virus in plasma membrane invaginations that were later shown to be caveolae (Anderson *et al.*, 1996; Stang *et al.*, 1997). After being trapped in caveolae, SV40 activates the signaling events characteristic of caveolae endocytosis (local tyrosine phosphorylation, dynamin-2 recruitment, actin tail formation, internalization, and release of caveolae in the cytosol (Pelkmans and Helenius, 2003; Pelkmans *et al.*, 2002). Endocytosed caveolae can then transfer to an

intermediate organelle, the caveosome, from where the virus traffics to the ER (Pelkmans *et al.*, 2001).

Similarly, labeled cholera toxin was shown to accumulate in caveolae (Montesano *et al.*, 1982; Parton, 1994). This toxin is made of an active A subunit and a binding B subunit which binds to GM1. Between internalization and the ER, where processing of the toxin releases the active subunit A peptide in the cytosol leading to toxicity, cholera toxin is seen in endosomes and caveosomes (Nichols, 2002; Parton and Richards, 2003; Parton and Simons, 2007; Tran *et al.*, 1987).

The picture became a little complicated with the demonstration that SV40 and cholera toxin could also enter cells devoid of caveolin-1 and caveolae, by cholesterol- and tyrosine kinase-dependent, but caveolin-1-, dynamin II-, and clathrin-independent pathway(s) (Damm *et al.*, 2005; Kirkham *et al.*, 2005). These internalization routes are referred to as non-caveolar lipid raft endocytosis, and occur in cells devoid of caveolin-1 and caveolae, but also in cells that have abundant caveolae. Caveolar and noncaveolar raft endocytosis pathways seem to share components of the same machinery (Echarri *et al.*, 2007; Kirkham and Parton, 2005).

3.2. Signaling

3.2.1. Caveolin-1 as a signaling regulator

Caveolin-1 capability to bind and regulate the activity of signaling proteins has been the object of a plethora of studies. The interaction has been mapped to aminoacids 61–101 of caveolin-1, a sequence which comprises the oligomerization domain (aminoacids 81–101), and termed the scaffolding domain. The partner sequence on the bound signaling protein was shown to be $\varphi X\varphi XXX\varphi$ or $\varphi XXXX\varphi X\varphi$ where φ represents an aromatic aminoacid, and the original hypothesis was that interaction between caveolin-1 scaffolding domain and a signaling protein would generally inhibit the signaling activity of the bound protein (Couet *et al.*, 1997). Not all caveolin-1 binding proteins contain one such sequence and the interaction does not always inhibit the signaling protein (Liu *et al.*, 2002b). Furthermore, interactions between signaling proteins and caveolin have been claimed based on cholesterol depletion, cofractionation, apparent colocalization using immunofluorescence techniques, and/or coimmunoprecipitation, and therefore need cautious interpretation (Parton and Simons, 2007). There is nonetheless solid evidence that the scaffolding domain of caveolin-1 can regulate signaling of known caveolin-1 interacting partners (Bucci *et al.*, 2000; Gratton *et al.*, 2003; Lin *et al.*, 2007). Recent evidence further points to a new, indirect mechanism by which caveolin-1 may regulate cell signaling, namely the modulation of palmitoylation of signaling proteins. This posttranslational modification is known to regulate the conformation, protein–protein and protein–lipid interaction, trafficking and subcellular

targeting of proteins, all of which are important determinants of the activity of signaling proteins. The palmitoylation profile of cells devoid of caveolin-1 was shown to be different from their wild type counterparts (Baran *et al.*, 2007).

3.2.2. Compartmentalization of signaling molecules in caveolae

Caveolae provide functional platforms where multiple sets of signaling molecules are preorganized and sequestered (White and Anderson, 2005). This was evidenced by cell fractionation, interaction with caveolin, and immunolocalization experiments. Organization of these signaling platforms provide spatial and temporal compartmentalization, as the signaling machinery is carried to different locations in cells by caveolae relocation. A combination of caveolae compartmentalization of a signaling protein and its regulation by direct interaction with caveolin-1 scaffolding domain sometimes results in exquisite regulation of that protein, as was demonstrated for endothelial nitric oxide synthase (eNOS): although the compartmentalization in caveolae of eNOS and other players of the signaling leading to endothelial nitric oxide production optimizes the process leading to eNOS activation, direct interaction with caveolin-1 maintains eNOS in an inactive state in the absence of stimulation (Sbaa *et al.*, 2005). Accordingly, caveolin-1-null mice have increased basal production of endothelial nitric oxide (Drab *et al.*, 2001), but reduced VEGF-induced nitric oxide production due to mislocalization of the VEGF receptor and loss of coupling to eNOS (Sonveaux *et al.*, 2004).

3.2.3. Role of caveolin-1 outside caveolae

In addition to caveolar caveolin-1, caveolin-1 has been identified on intracellular membranes, but also in an intracellular soluble form, and extracellularly as a secreted protein. Most importantly, caveolin-1 seems to play a role in these noncaveolar locations.

3.2.3.1. Intracellular caveolin Caveolin-1 has been visualized in intracellular membranes, and plays a role in membrane traffic (Liu *et al.*, 2002b). Caveolins are found associated with Golgi structures (Conrad *et al.*, 1995; Kurzchalia *et al.*, 1994; Luetterforst *et al.*, 1999; Smart *et al.*, 1994), the ER (Schlegel *et al.*, 2001; Smart *et al.*, 1996), caveosomes (Pelkmans *et al.*, 2001), lipid droplets (Liu *et al.*, 2004), and in certain cells mitochondria (Li *et al.*, 2001). The occurrence and function of cellular caveolin-1 outside of caveolae has recently been reviewed (Head and Insel, 2007). Functional evidence was collected from two cell types that do not show caveolae, but in which caveolin-1 is reportedly expressed and functionally active, namely neurons and lymphocytes (Head and Insel, 2007).

An additional pool of caveolin in the cell consists of cytoplasmic caveolin-1: Caveolin-1 interacts with chaperones (heat shock protein 56,

cyclophilin 40, cyclophilin A) to form a soluble complex with cholesterol (Uittenbogaard *et al.*, 1998) involved in the transport of newly synthesized cholesterol to caveolae (Smart *et al.*, 1996). Palmitoylation of cysteines 143 and 156 of the C-terminus of caveolin-1 is required for formation of this complex and for cholesterol transport (Uittenbogaard and Smart, 2000). Another such assembly was later identified, in which caveolin-1 was bound to cholesteryl ester in a complex made of cyclophilin 40, cyclophilin A, and annexin II (Uittenbogaard *et al.*, 2002). This complex transports cholesteryl ester from caveolae to endomembranes and requires cysteine 133 in the C-terminus of caveolin-1 (Uittenbogaard *et al.*, 2002). Of note, the fact that caveolin-1 can be found in lipoprotein complexes opens the possibility of the existence of secreted caveolin-1 (Liu *et al.*, 2002b).

3.2.3.2. Secreted caveolin-1 The existence of secreted caveolin-1 has emerged in the past few years. Caveolin-1 was identified in the lumen of serous, but not mucous, secretory vesicles of exocrine cells (Liu *et al.*, 1999), where it was not associated with the vesicles membrane but rather with a lipoprotein particle. In addition, both monomeric and oligomeric forms of caveolin-1 were detected in the conditioned media of cultured exocrine AR42J cells ectopically expressing caveolin-1. Targeting of caveolin-1 to the secretory pathway was later confirmed in other secretory cells including serous cells of the salivary gland and pituitary somatotrope endocrine cells (Li *et al.*, 2001). Phosphorylation on serine 80 has been proposed to mediate caveolin-1 entry into the secretory pathway (Schlegel *et al.*, 2001).

In addition, an impressive collection of data now links caveolin-1 secretion to prostate cancer cells. Increased expression of caveolin-1 was identified in metastatic prostate cancer cells (Yang *et al.*, 1998) and shown to mediate resistance to androgen deprivation (Nasu *et al.*, 1998). Secretion of caveolin-1 by prostate cancer cells was later documented (Tahir *et al.*, 2001), caveolin-1 was detected in the serum HDL₃ fraction of prostate cancer patients (Tahir *et al.*, 2001), and circulating caveolin-1 proposed as a biomarker and a prognostic marker for prostate cancer (Tahir *et al.*, 2003, 2006). What is most intriguing is that caveolin-1 secreted by prostate cancer cells seems to be biologically active, increasing the viability and growth of caveolin-1-negative prostate cancer cells *in vitro* and in tumor bearing mice (Bartz *et al.*, 2008; Tahir *et al.*, 2001). This effect can be antagonized by anticaveolin-1 antibody added to the cell culture conditioned media or administered intraperitoneally to mice (Tahir *et al.*, 2001). During perineural invasion of prostatic carcinoma, caveolin-1 secreted by perineural cells protects the prostate cancer cells from apoptosis as elegantly demonstrated *in vitro* and *in vivo* (Ayala *et al.*, 2006). Further evidence suggests that extracellular caveolin-1 can be taken up by endothelial cells in a scaffolding domain-dependent fashion, and induce proangiogenic behavior *in vitro* (Tahir *et al.*, 2008). In prostate cancer specimens, endothelial cell

caveolin-1 positivity and increased microvessel density were associated with tumor cell caveolin-1 expression (Yang *et al.*, 2007). The existence of extracellular, biologically active caveolin-1 opens up an entirely new area of research on caveolin in biology and physiology.

3.2.4. Caveolae internalization and signaling

Cells are known to make use of endocytosis to control the presence of receptors at the cell surface, thereby regulating cell signaling, receptor turnover, and the magnitude or duration of signaling events (Le and Wrana, 2005). It is, therefore, not surprising that in addition to their role in compartmentalizing signaling proteins in specific domains of the plasma membrane, caveolae also play a role in cell signaling through their endocytic capabilities. For example, caveolae internalization elicited by loss of contact with the extracellular matrix has been shown to cause loss of the small GTP-binding protein Rac-1 from the plasma membrane and to suppress Rac-1 activation. These data show that by regulating internalization of caveolae, integrins can control signaling pathways that rely on plasma membrane localization of signal transduction (del Pozo *et al.*, 2004; Echarri and del Pozo, 2006). Using the same system of cell-detachment-induced caveolae internalization, the authors further showed that the Ras-Erk pathway and the PI(3)K-Akt pathway were also downregulated upon cell detachment, but only in cells harboring caveolae. In contrast, the reduced activation of focal adhesion kinase (FAK) induced by loss of contact was unaffected by the presence or absence of caveolae (del Pozo *et al.*, 2005). Another example of signaling regulation by caveolae internalization is the nitric oxide production induced in endothelial cells upon gp60 activation by albumin, as detailed below (Maniatis *et al.*, 2006).

3.2.5. The example of eNOS

The regulation of eNOS activation by caveolae and caveolin-1 encompasses multiple aspects of the role of these organelles in signaling (Fig. 4.3). Endothelial NOS (Garcia-Cardena *et al.*, 1996b; Shaul *et al.*, 1996), but also downstream targets of nitric oxide signaling cascade (Zabel *et al.*, 2002) and recycling enzymes responsible for regeneration of the substrate for eNOS (Flam *et al.*, 2001; Solomonson *et al.*, 2003) are concentrated in caveolae. In caveolae, eNOS interacts with the scaffolding domain of caveolin-1, which maintains it in an inactive state (Feron *et al.*, 1996; Garcia-Cardena *et al.*, 1996a). This tonic inhibition is lost in caveolin-1-null endothelial cells, resulting in enhanced basal production of nitric oxide (Drab *et al.*, 2001). Conversely, inhibition of eNOS can be achieved by the scaffolding domain of caveolin-1 fused to a motif facilitating intracellular penetration of the peptide (Bucci *et al.*, 2000). This peptide, named cav-tratin, inhibits the consequences of endothelial nitric oxide production

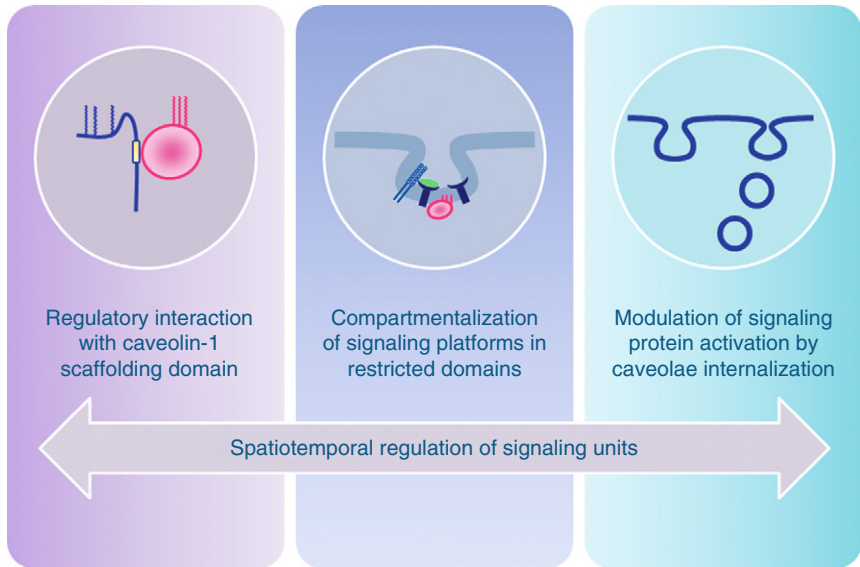


Figure 4.3 Various levels of signaling regulation by caveolin-1 and caveolae. Caveolin-1 interacts *via* its scaffolding domain with signaling proteins, which are generally maintained in an inactive state by the interaction. Caveolae compartmentalize signaling platforms in restricted domains of the plasma membrane, facilitating signaling cascades. Signaling protein regulation can further occur through caveolae internalization. Caveolae and caveolin-1 can combine these three mechanisms to organize spatiotemporal regulation of signaling units in cells.

in vivo (Bucci *et al.*, 2000; Gratton *et al.*, 2003) and corrects some nitric oxide-mediated aspects of caveolin-1 deficiency (Lin *et al.*, 2007).

Another feature of the regulation of eNOS activity is that while interaction with caveolin-1 results in tonic inhibition of eNOS, compartmentalization in caveolae (and therefore caveolin-1 expression) is required for some signaling cascades resulting in eNOS activation. This paradox has been reviewed in detail (Sbaa *et al.*, 2005). The activity of eNOS is further regulated by caveolae endocytosis. Cells have been shown to modulate nitric oxide production through caveolae internalization, leading to either receptor sequestration and inaccessibility to further stimulation (Dessy *et al.*, 2000), or to eNOS internalization associated with increased nitric oxide production (Chatterjee *et al.*, 2003). Dynamin seems to be involved both in caveolae fission and in direct interaction with eNOS resulting in increased eNOS activity. Recent experiments have shown that exposure of endothelial cells to albumin leads to the phosphorylation of eNOS on serine 1179 (a hallmark of eNOS activation), phosphorylation of caveolin-1 on tyrosine 14, dissociation of eNOS from caveolin-1, and nitric oxide production. These effects were prevented by the dynamin mutant that inhibits caveolae endocytosis (Maniatis *et al.*, 2006).

Lastly, the interaction of eNOS with caveolin-1 and caveolae exemplifies the spatiotemporal regulation of signaling offered by these organelles and their defining protein: caveolin-1 and caveolae accumulate in the trailing edge of endothelial cells migrating on a flat surface (Isshiki *et al.*, 2002; Parat *et al.*, 2003) while eNOS accumulates at the edge of the lamellipodium (Bulotta *et al.*, 2006). Through staining with antiphospho-eNOS antibody (pS1177), we further showed that the pool of eNOS which accumulates at the front of the cells and segregates away from caveolin-1 is activated (Fig. 4.4). In contrast, in transmigrating endothelial cells, caveolin-1 accumulates in the front extension of endothelial cells, away from caveolae (Parat *et al.*, 2003) and so does eNOS (Fig. 4.4). In this model, the pool of eNOS which colocalizes with caveolin-1 is inactive—there is virtually no colocalization between caveolin-1 and phospho-eNOS in the front extension of transmigrating endothelial cells, consistent with a role for caveolin-1 in maintaining eNOS in an inactive state.

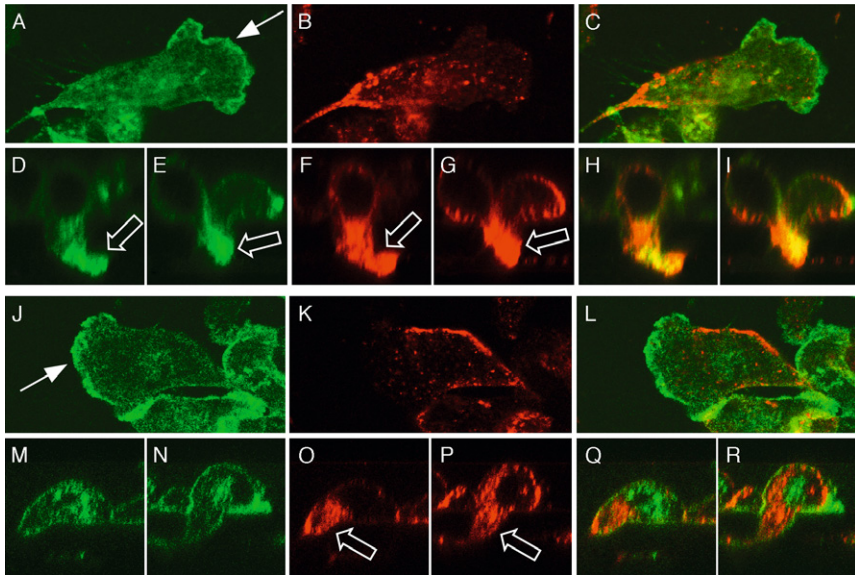


Figure 4.4 Spatiotemporal regulation of eNOS activation by caveolin-1 in migrating endothelial cells. Bovine aortic endothelial cells migrating in a two dimensional (on a flat surface) (A–C and J–L) or in a three-dimensional model (traversing the pores of a filter) (D–I and M–R) were fixed, stained, and labeled using rabbit antihuman caveolin-1 as primary antibody followed by biotinylated goat antirabbit antibody and Texas red-avidin (B, F, G, K, O, P), and mouse monoclonal anti-eNOS antibody (A, D, E) or mouse monoclonal antiphospho-eNOS (pS1177) antibody (J, M, N) followed by Alexa 488 goat antimouse IgG. The overlay of eNOS and cellular caveolin-1 (C, H, I) or phospho-eNOS and caveolin-1 (L, Q, R) are shown in the merged image. Accumulation at the edge of the lamellipodium is indicated by white arrows. Localization in the extension sent by cells through the filter pores is indicated by open arrows.

3.3. Mechanosensing

When endothelial cells are explanted from the *in vivo* environment where they are exposed to flow conditions varying with the cardiac cycle, and grown in culture, the abundance of caveolae decreases dramatically. In contrast, when cultured cells are subjected to chronic shear stress, the number of caveolae increases at the luminal surface of the plasma membrane (Boyd *et al.*, 2003). Caveolae further compartmentalize several signaling molecules involved in the response of endothelial cells to flow/shear stress. Consequently, caveolae have long been suspected to convert mechanical stimuli into chemical signaling.

Shear stress has been shown to elicit the relocation of caveolin-1 and caveolae in endothelial cells *in vitro* (Isshiki *et al.*, 2002), and increases in flow to activate eNOS at the luminal side of endothelial cells *in vivo* with apparent dissociation from caveolin (Rizzo *et al.*, 1998). In smooth muscle cells, caveolin-1 facilitates mechanosensitive signaling (Sedding *et al.*, 2005) and stretch-induced activation of extracellular signal-regulated kinase (ERK) was shown to be modulated by caveolae, in an actin cytoskeleton-dependent fashion (Kawabe *et al.*, 2004). An elegant study where caveolin-1 was selectively re-expressed in the endothelium of caveolin-1-null mice, demonstrated that flow-induced vascular remodeling depended on the expression of endothelial caveolin-1 and/or the presence of caveolae. The study further showed that caveolin-1/caveolae were required for optimal *ex vivo* flow-induced dilation of pressurized arteries, that the loss of caveolae impaired the flow dependant coupling to eNOS activation, and the authors concluded that both rapid and long term blood vessel mechanotransduction depends on endothelial caveolin-1 and caveolae (Yu *et al.*, 2006).

3.4. Cell adhesion and migration

Multiple functions for caveolin-1 and caveolae in the regulation of cell migration have been unveiled. The picture emerging from numerous studies is that of a complex modulatory role and varies with the cell type considered, the *in vivo*, *ex vivo*, or *in vivo* context; and, for *in vitro* studies, with the migration model and migratory conditions used. The phenotype of caveolin-1-null mice is not suggestive of major dysregulation of cell migration, so changes may be subtle or compensated for. For example, polarization of mouse embryo fibroblasts, persistence of directional migration, and wound closure were shown to be defective, but velocity higher in caveolin-1-null cells in an *in vitro* scratch wound assay (Grande-Garcia *et al.*, 2007). On the other hand, cell spreading on fibronectin was reported to be increased in caveolin-1-null or downregulated cells (Bailey and Liu, 2008) while exogenous caveolin-1 expression in cancer cells with low levels of caveolin-1 was shown to stabilize focal adhesion and lead to increased

motility (Goetz *et al.*, 2008). There are multiple mechanisms through which caveolin-1 and/or caveolae are expected to regulate cell migration, such as caveolae compartmentalization of migration-relevant signaling, caveolae position at the interface of cytoskeleton and extracellular matrix, possibly cholesterol transport (Navarro *et al.*, 2004), the connection of caveolin-1 with focal adhesions (Gaus *et al.*, 2006; Goetz *et al.*, 2008; Hill *et al.*, 2007;), and caveolae interaction with vimentin intermediate filaments (Berg *et al.*, 2008; Santilman *et al.*, 2007). The distribution of caveolin-1 and caveolae is asymmetric in migrating cells (Isshiki *et al.*, 2002; Lentini *et al.*, 2008; Parat *et al.*, 2003; Santilman *et al.*, 2006, 2007), further supporting a role for the protein or the organelle in cell migration (Grande-Garcia and del Pozo, 2008). *In vivo*, aspects of the phenotype of caveolin-1-deficient mice related to dysfunctional cell migration include delayed wound healing after punch biopsy (Grande-Garcia *et al.*, 2007) and decreased neovascularization in some (Sonveaux *et al.*, 2004; Woodman *et al.*, 2003), but not all (DeWever *et al.*, 2007; Lin *et al.*, 2007), angiogenesis models.

3.5. Lipid regulation

3.5.1. Regulation of cellular cholesterol

The relationship between caveolin-1 and cholesterol is complex as it appears that they can regulate each other's levels and trafficking in the cell (Frank *et al.*, 2002, 2006; Hailstones *et al.*, 1998; Ikonen and Parton, 2000; Pol *et al.*, 2005). Specifically, caveolin-1 expression has been documented to regulate cholesterol efflux (Fielding and Fielding, 1995; Frank *et al.*, 2001; Fu *et al.*, 2004) and caveolin-1 was shown to transport cholesterol through the cytoplasm in a chaperone complex. The complex consists of caveolin-1, cholesterol, and chaperones that differ depending on whether the complex traffics to or from plasma membrane caveolae (Smart *et al.*, 1996; Uittenbogaard and Smart, 2000; Uittenbogaard *et al.*, 1998, 2002;).

3.5.2. Lipid droplets

Lipid droplets (also termed lipid bodies or adiposomes) are complex, mobile, and metabolically active organelles serving a lipid storage function. They are made of a neutral lipid core surrounded by a monolayer of phospholipids and associated proteins. Caveolin-1 and caveolin-2 have been shown to associate with lipid droplets especially upon lipid loading of the cells (Liu *et al.*, 2004; Pol *et al.*, 2004). Furthermore, caveolin-1 was proposed to move bidirectionally between the plasma membrane and the surface of lipid droplets (Pol *et al.*, 2005). That caveolin may regulate lipid droplets is suggested by the fact that a dominant negative mutant of caveolin was localized to the surface of lipid droplets and caused an increase in neutral lipid accumulation (Pol *et al.*, 2001) while altering lipid droplet mobility and lipid redistribution and catabolism (Pol *et al.*, 2004).

3.6. Caveolin gene disruption and caveolae function

The generation of caveolin-1-gene disrupted mice has several remarkable features. First, four separate groups independently generated a caveolin-1 knockout mouse (Cao *et al.*, 2003; Drab *et al.*, 2001; Razani *et al.*, 2001; Zhao *et al.*, 2002) which are dissimilar with respect to the exon of the *CAV1* gene that was targeted, and the strain background in which the mice have been developed or to which they have been backcrossed. Second, certain aspects of the caveolin-1-null phenotype are discrepant between the independently generated gene-disrupted mice; for example, liver regeneration capabilities (Fernandez *et al.*, 2006; Mayoral *et al.*, 2007) or syngeneic B16 melanoma tumor angiogenesis (DeWever *et al.*, 2007; Woodman *et al.*, 2003). Last, even though an increasing number of functions were attributed to caveolae based on *in vitro* experiments, and the loss of caveolin-1 resulted in ablation of caveolae, the mice were shown to be viable, fertile, and exhibited relatively few phenotypical alterations in the absence of challenge. However, 7 years later, research publications keep refining our understanding of the altered responses exhibited by caveolin-1-null mice to various stresses/challenges.

The phenotype of caveolin-1 gene-disrupted mice seems more complex than initially thought and reflects the main functions that are attributed to caveolin-1 and caveolae. Although they are viable and fertile, *Cav1*^{-/-} mice show diverse abnormalities resulting in a reduced life span (Park *et al.*, 2003; Yang *et al.*, 2008). They exhibit pulmonary fibrosis and endothelial cell proliferation in the lungs, aberrant nitric oxide and calcium signaling in the cardiovascular system, dilated cardiomyopathy and pulmonary hypertension, nitric oxide-dependent microvascular hyper-permeability, increased plasma volume, altered blood vessel remodeling, and a reduced ability to perform physical work (Albinsson *et al.*, 2007; Cohen *et al.*, 2003a; Drab *et al.*, 2001; Hassan *et al.*, 2004; Park *et al.*, 2003; Razani *et al.*, 2001; Rosengren *et al.*, 2006; Schubert *et al.*, 2002; Wunderlich *et al.*, 2006; Yu *et al.*, 2006; Zhao *et al.*, 2002). Dysfunctional endothelial cells have been implicated as major players in the cardiovascular phenotype of caveolin-1-null mice, as some characteristics of the cardiovascular and pulmonary phenotype can be reversed by NOS inhibition, or by endothelial-specific reexpression of caveolin-1 (Albinsson *et al.*, 2007; Murata *et al.*, 2007; Schubert *et al.*, 2002; Wunderlich *et al.*, 2008; Yu *et al.*, 2006). In agreement with altered endothelial cell function, angiogenesis was impaired in caveolin-1 null mice challenged with a bFGF-loaded matrigel plug, or subcutaneous injection of melanoma (Woodman *et al.*, 2003). In a model of adaptive angiogenesis after artery resection, these mice also displayed impaired angiogenesis (Sonveaux *et al.*, 2004), in part due to a defect in endothelial progenitor cell mobilization (Sbaa *et al.*, 2006). In contrast, more recently, it was shown that tumor vessels were more abundant,

but less mature and more permeable, when tumors were implanted in caveolin-1-null mice compared to wild type mice (DeWever *et al.*, 2007; Lin *et al.*, 2007).

Other characteristics of caveolin-1-null mice include abnormal insulin signaling (Cohen *et al.*, 2003b; Razani *et al.*, 2002a), impaired utilization of circulating fatty acids (Fernandez *et al.*, 2006), dyslipidemia (Frank *et al.*, 2008; Razani *et al.*, 2002a) but, upon crossing with the apolipoprotein E-deficient mice, apparent resistance to atherosclerosis with downregulation of proatherogenic CD36 and VCAM-1 (Frank *et al.*, 2003). Recently, neurological abnormalities (Gioiosa *et al.*, 2008; Trushina *et al.*, 2006) and altered immune responses (Li *et al.*, 2005; Medina *et al.*, 2006) have also been unveiled. Caveolin-1 gene-disrupted males further show urogenital alterations including reduced renal calcium reabsorption and urolithiasis (Cao *et al.*, 2003), enlarged seminal vesicles (Le Lay and Kurzchalia, 2005), and hypertrophy of the bladder (Woodman *et al.*, 2004a). Finally, although these mice do not develop spontaneous tumors, they exhibit abnormal growth and differentiation of specific cell types (Lee *et al.*, 2002a; Yang *et al.*, 2008) and increased sensitivity to oncogene- or carcinogen-induced tumor formation (Capozza *et al.*, 2003; Williams *et al.*, 2003).

It is interesting to note that caveolin-2 gene disrupted mice had unaltered caveolae formation but exhibited a lung phenotype close to that of caveolin-1-null mice and similar exercise intolerance (Razani *et al.*, 2002b). Ultrastructural skeletal muscle alterations have also been recently described in both caveolin-1- and caveolin-2-null mice (Schubert *et al.*, 2007). Since caveolin-2 expression is dramatically reduced in the absence of caveolin-1, these observations open to a possibility that part of the phenotype of caveolin-1-null mice could be due to the loss of caveolin-2 rather than to the loss of caveolin-1 or caveolae.

Consistent with the role of caveolin-3 in skeletal and cardiac muscle, the phenotype of caveolin-3 gene-disrupted mice includes mild myopathic alterations in skeletal muscle (Galbiati *et al.*, 2001a; Hagiwara *et al.*, 2000), cardiomyopathy (Woodman *et al.*, 2002), and disorganization of the T-tubule system (Galbiati *et al.*, 2001a). Caveolin-1 and -3 double knock-out mice were also evaluated, and their phenotype was similar to the added phenotypes of caveolin-1-null mice and caveolin-3-null mice except for more pronounced cardiomyopathy (Park *et al.*, 2002).

3.7. Caveolin genetic analysis in humans

While genetic analysis of caveolin-3 in humans has revealed multiple muscle diseases associated with mutations in caveolin-3 (Aboumoussa *et al.*, 2008; Galbiati *et al.*, 2001b; Woodman *et al.*, 2004b), genetic analysis of caveolin-1 in humans is only emerging. A polymorphism in intron 2 of *CAV1*

(22375–22375 del AC) was suggested to be a quantitative trait locus of blood pressure, glycemia, and triglyceridemia and seems to be associated with protection against metabolic syndrome in hypertensive patients (Grilo *et al.*, 2006). In another study evaluating the same polymorphism, the prevalence of hypertension was not different, but systolic blood pressure higher for males with the ID or DD genotype compared to those with the II genotype (Yamada *et al.*, 2007). Most recently, a novel polymorphic purine complex in the 5′-UTR of the human *CAV1* gene has been identified, and alterations of this region were associated with predisposition to sporadic Alzheimer disease (Heshmati *et al.*, 2008).

Furthermore, patients with atypical lipodystrophy and hypertriglyceridemia who had no mutation in any known lipodystrophy gene were screened for potential mutations in the coding regions of *CAV1*. One patient and her father were found to have a heterozygous frameshift mutation I134fsdelA–X137 predicting premature termination with loss of the carboxy-terminal end of the protein. Another patient had a –88delC mutation in the 5′-UTR of *CAV1* with a potential effect on the reading frame (which was not demonstrated) (Cao *et al.*, 2008). Lastly, a homozygous nonsense mutation introducing a premature stop codon at position 38 was found in a patient with lipodystrophy, insulin resistance, and dyslipidemia. In that study, the lack of detectable caveolin-1 was shown by Western blot and immunofluorescence analyses and was associated, expectedly, with a lack of caveolin-2 expression (Kim *et al.*, 2008). More genetic analyses of caveolin-1 in humans are expected in the next few years and will shed light on the physiological roles of this protein, potentially suggesting new therapeutic approaches.



4. CAVEOLIN-1 AND CAVEOLAE AS THERAPEUTIC TARGETS

4.1. Cardiovascular diseases

The multiple roles of caveolae in the physiology of endothelial cells, and the cardiovascular phenotype of caveolin-1-null animals have prompted active research targeting caveolae and their defining protein in cardiovascular disease. The cholesterol-lowering drugs hydroxy-methylglutaryl-Coenzyme A reductase inhibitors (statins) have been reported to modify caveolin-1 expression. Their effect seems to depend on the statin itself, the cell type evaluated for caveolin-1 expression, and the preexisting condition studied. For example, atorvastatin was shown to decrease caveolin-1 expression, and thus promote eNOS activation, in cultured endothelial cells (Feron *et al.*, 2001). In another study, apoE^{-/-} mice were shown to have increased caveolin-1 expression in cardiac and aortic tissue, which was

corrected by rosuvastatin (Pelat *et al.*, 2003) while no effect of this statin was observed on the cardiac and aortic tissues of control mice. Similarly, hyperproliferating smooth muscle cells from patients with idiopathic pulmonary arterial hypertension (IPAH) had increased caveolin-1 expression, and treatment with lovastatin *in vitro* decreased caveolae number and the proliferation of these cells (Patel *et al.*, 2007). In contrast, in a rat model of IPAH, simvastatin restored caveolin-1 expression which was reduced in the endothelial cells of the pulmonary lesions (Taraseviciene-Stewart *et al.*, 2006). It, therefore, seems that statins are able to normalize the expression of caveolin-1 which is dysregulated in the various cell types involved in cardiovascular diseases, and these drugs are getting increased attention as caveolae-targeting agents in that context (Frank *et al.*, 2007).

As mentioned earlier, peptides derived from the scaffolding domain of caveolin-1 disrupt the binding of eNOS to caveolin and dose-dependently inhibit NOS activity *in vitro*. The scaffolding domain peptide has been successfully delivered intracellularly *in vitro*, *ex vivo*, and *in vivo* to prevent eNOS activation and thereby counter microvascular hyperpermeability. In addition to the correction of endothelial caveolin-1 deficiency (Lin *et al.*, 2007), potential therapeutic applications for the caveolin-1 scaffolding domain peptide include inflammation (Bucci *et al.*, 2000; Hatakeyama *et al.*, 2006; Song *et al.*, 2007), atherosclerosis (Rodriguez-Feo *et al.*, 2008), and tumor angiogenesis (Gratton *et al.*, 2003). In contrast, caveolin-1 scaffolding domain peptide was documented to prevent the development of pulmonary hypertension and medial hypertrophy in a model of monocrotaline-induced pulmonary hypertension (Jasmin *et al.*, 2006) and to protect isolated hearts in a model of ischemia, reperfusion, and polymorphonuclear neutrophil infiltration (Young *et al.*, 2001).

Finally, an attempt of siRNA-induced downregulation of caveolin-1 *in vivo* by injection of siRNAs in cationic liposomes demonstrated the feasibility of such an approach, and showed that transient downregulation of caveolin-1 in the endothelium could reversibly induce caveolae disappearance, augment nitric oxide production, and increase lung vascular permeability to albumin (Miyawaki-Shimizu *et al.*, 2006). In summary, proof of principle exists that caveolin-1 modulation can be achieved through a pharmacological or molecular approach and may prove useful in conditions that involve eNOS hyperactivation.

4.2. Cancer

Caveolin-1 can act both as a tumor promoter and as a tumor suppressor, and its expression has been reported to be increased or decreased in cancer cells. Caveolin-1 acts on cell proliferation, adhesion, epithelial to mesenchymal transition, migration, extracellular matrix remodeling, angiogenesis, inflammation, immunity, and apoptosis (Burgermeister *et al.*, 2008).

The consensus that seems to emerge in the literature is that caveolin-1 is a stage-specific and tissue-specific tumor modulator *in vivo* (Burgermeister *et al.*, 2008; Quest *et al.*, 2008; Shatz and Liscovitch, 2008).

4.2.1. Targeting caveolin-1 of cancer cells

Multiple studies have shown that modifying the expression of caveolin-1 in cancer cells *in vitro* before using them *in vivo* in a mouse tumor model could increase or decrease tumor take and/or metastasis (Cantiani *et al.*, 2007; Tirado *et al.*, 2006). Depending on whether tumor cells exhibit increased or decreased caveolin-1 expression, targeted downregulation *in vivo* using antisense or siRNA or overexpression of caveolin-1 can therefore be proposed as a therapeutic approach (van Golen, 2006), or as an adjuvant to cancer therapy. For example, *in vitro* data suggest that caveolin-1 downregulation could sensitize cancer cells to ionizing radiation (Cordes *et al.*, 2007), and *in vitro* and *in vivo* data show that stable transfection with antisense caveolin-1 cDNA vector restores sensitivity to androgen deprivation in prostate cancer cells (Nasu *et al.*, 1998).

In multiple myeloma cells, the proteasome inhibitor bortezomib has been shown to reduce caveolin-1 expression, and to prevent VEGF-induced caveolin-1 phosphorylation and cell migration (Podar *et al.*, 2004). Caveolin-1 was therefore identified as a molecular target of this drug in multiple myeloma. In the case of prostate cancer cells given a growth advantage by secreted caveolin-1, anticaveolin-1 antibodies have been trialed as an anticancer therapy in mice: as expected, intraperitoneal injections were shown to suppress orthotopic growth and metastasis of caveolin-1-secreting prostate cancer (Tahir *et al.*, 2001).

4.2.2. Taking advantage of caveolin-1 overexpression by some cancer cells

On the basis of overexpression of caveolin-1 in aggressive prostate cancer cells and in tumor-associated endothelial cells, the Thompson group developed a combined adenoviral gene therapy using (i) the caveolin-1 promoter upstream of the Herpes simplex virus thymidine kinase (*HSVtk*) gene and (ii) gancyclovir, which is turned into a toxic product by the enzyme. In addition to *in vitro* cytotoxicity, the authors showed decreased tumor wet weight, increased apoptosis, increased necrosis, and decreased microvessel density with this association in an orthotopic mouse prostate cancer model (Pramudji *et al.*, 2001).

4.2.3. Using caveolae in tumor vasculature to target tumor cells

Targeting the tumor vasculature is a highly promising approach for cancer treatment. Doing so with antibodies to proteins expressed exclusively in the caveolae of tumor endothelia has been the object of active research (Carver and Schnitzer, 2003). Such an approach would in theory offer the

combined advantages of tumor-specific endothelial expression of the target antigen (Oh *et al.*, 2004) and caveolae-mediated transcytosis of the antibody and its therapeutic cargo from the blood to the subendothelial space (Oh *et al.*, 2007).

4.2.4. Endothelial cell caveolin-1 as an antiangiogenic target

Caveolin-1 plays a key role in endothelial cell migration and angiogenesis, and it has been shown that both increasing and decreasing caveolin-1 expression can alter neovascularization. Increasing caveolin-1 expression in the endothelial cells of tumor vasculature via *in vivo* transfection was shown to reduce tumor growth, due to reduction in tumor blood flow by inhibition of endothelial nitric oxide production, and to decreased tumor angiogenesis (Brouet *et al.*, 2005). Endothelial-specific transgenesis of the caveolin-1 gene in mice impaired angiogenesis elicited by ischemia or an adenovirus encoding VEGF (Bauer *et al.*, 2005). On the other hand, *in vitro* experiments showed that siRNA- adenoviral- or antisense-mediated down-regulation of caveolin-1 reduced endothelial cell transmigration to serum (Beardsley *et al.*, 2004), capillary like tube formation (Griffoni *et al.*, 2000; Liu *et al.*, 2002a), and angiogenesis in the chorioallantoic membrane assay (Griffoni *et al.*, 2000). Furthermore, caveolin overexpression was shown to increase, and its downregulation to decrease, the adhesion of lymphocytes to tumor endothelium, an important factor in immune response to cancer (Bouzin *et al.*, 2007). Targeting the expression of caveolin-1 in tumor vasculature endothelial cells therefore represents a potentially valid means of targeting cancer, or of improving existing cancer treatments.

5. CONCLUDING REMARKS

As more in-depth knowledge about caveolins and caveolae structure and functions is generated, new questions arise that will need to be answered. Exciting new developments include the discovery of cavin as another protein than caveolin-1 necessary for caveolae formation and function, which brings new expectations regarding the concerted mechanism by which the two proteins help generating caveolae. Another perplexing feature of caveolin is its versatile location in membrane bilayers, lipid monolayers, but also soluble lipoprotein-like complexes, with unknown mechanisms leading to its secretion. Furthermore, the existence of extracellular caveolin-1 that is functionally active *in vivo* poses the question of how secreted caveolin-1 can act on a neighbouring or a distant cell, and new findings in this area may have implications beyond prostate cancer. Finally, the next years will probably see more reports of caveolin mutations or ablation in human disease, which will enhance our knowledge of caveolae

physiological roles and reinforce the potential of caveolae and caveolin therapeutic targeting.

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PHARMACOLOGY OF CILIATED PROTOZOA—DRUG (IN)SENSITIVITY AND EXPERIMENTAL DRUG (AB)USE

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Abstract

Most data on the effects of drugs as inhibitors, modulators, or stimulators have been collected with higher eukaryotic, mainly mammalian cells. Although in cell biological experiments with lower eukaryotes, including ciliates, the same drugs have frequently been applied, many results remained questionable for several reasons. Most drugs had to be used in unusually high concentrations. Moreover, drug effects have rarely been verified at the biochemical or molecular level. Data steadily emerging from genomics of ciliates, mainly *Paramecium tetraurelia* and *Tetrahymena thermophila*, show that drug-binding sites have only occasionally been conserved during evolution. They may vary or be totally absent in ciliate orthologs or specifically in certain paralogs. We here try to evaluate data available so far on the pharmacology of ciliates. In the future, domain analysis and drug screenings may detect compounds specifically effective in specific ciliated protozoa, including pathogenic forms, and, thus, yield an important basis not only for cell biology but also for ecotoxicology.

Key Words: Ciliates, Drug effects, *Paramecium*, Pharmacology, Protozoa, *Tetrahymena*. © 2009 Elsevier Inc.

1. INTRODUCTION

Many chemical compounds, natural or artificial, are known to modulate cellular functions as stimulators, inhibitors, or blockers. The pharmacological effects of such “drugs” may be specific for certain processes, but frequently they entail a variety of side effects. While most effects have been established mainly with mammalian cells, use of such drugs has frequently been expanded to species down to the protozoan level. Over the years, such attempts have resulted in many failures and errors. This is also true of ciliated protozoa, where most data have been collected from work with *Paramecium tetraurelia* and *Tetrahymena thermophila*. Their recent genomic analysis (Aury *et al.*, 2006; Eisen *et al.*, 2006) now allows us to look into molecular details required for binding and activity of drugs whose importance as tools in cell biology is undisputable. See genome databases <http://www.genoscope.cns.fr/paramecium> and <http://paramecium.cgm.cnrs-gif.fr> for *P. tetraurelia* and <http://www.ciliate.org/> for *T. thermophila*, respectively. For *Tetrahymena*, the protein data can be retrieved from another database, <http://www.tigr.org/tdb/e2k1/ttg/>.

Recent analyses, mainly with *Paramecium*, have shown that sensitivity to an “established” drug may vary from “orthodox” to totally ineffective even for one type of molecule when different paralogs are compared within one ciliate species (Sehring *et al.*, 2007). Some of the discrepancies between the effects of drugs on higher and lower eukaryotic systems can now be

explained by sequence data. Such data also shed some doubts on many previous analyses when unusually high concentrations had to be applied, so one may expect unspecific side effects.

While most drugs were established with mammalian cells and probed with protozoa only later on, there are a few examples where the sequence of developments has been to the opposite. An example is artemisinin, a putative inhibitor of the endoplasmic reticulum/sarcoplasmic reticulum (ER/SR) Ca^{2+} -ATPase (SERCA-pump) in *Plasmodium* (Uhlemann *et al.*, 2005). It was used as a decoction from the *Artemisia* plant (family Asteraceae) already over 2200 years ago in Chinese medicine, but its pharmacology is being elucidated only now. *Plasmodium* is a member of the phylum Apicomplexa and, thus, closely related to the phylum Ciliophora (ciliates), both belonging to the group of “Alveolata.”

Therefore, the motivation to deal with the pharmacology of ciliates is severalfold. First, drugs are important research tools. Second, the rationale of their function is widely amenable to molecular analysis. Third, one may consider the potential application of data from easy-to-analyze ciliates to their pathogenic relatives, including other ciliates (such as *Ichthyophthirius*) and other members of the phylum Apicomplexa (*Plasmodium*, *Toxoplasma*, etc.) although considerable scepticism is advised in any such data transfer without careful tests.

One reason of the widely diverging effects of drugs at lower evolutionary levels may be that, in nature, a target (or a target bearing cell, e.g., a ciliated protozoan) will hardly ever meet any of the relevant ligands. This is in evident contrast to “higher” systems. A striking example is the unpleasant toxic effect of the plant *Veratrum* (Liliaceae, *V. album* in Europe) whose Na^{+} -channel agonist veratridine “teaches” cows to avoid it carefully, as one can observe every year in alpine pastures. In fact, most channel activators, inhibitors, or blockers (such as agatoxins or conotoxins) are active only within a range of animal phyla or they are produced only by small numbers of species for chemical warfare against specific species. Nevertheless, some target molecules in ciliates interestingly may have “orthodox” drug-binding sites, while these may be absent from some paralogs. This is the case, for example, with the capacity of *Paramecium* filamentous actin (F-actin) to bind the bicyclic heptapeptide phalloidin from the mushroom *Amanita phalloides* (Sehring *et al.*, 2007). In contrast, phalloidin is considered a reliable diagnostic tool for mammalian F-actin (Wieland and Faulstich, 1978). The question arises why any *Paramecium* actin form should ever be able to bind phalloidin. Differential localization of drug-sensitive and -insensitive paralogs in *Paramecium* suggests different molecular properties required at different sites of the cell body—a hypothesis to be followed up in the future. The same may be found in future experiments with the more recently

discovered membrane-permeable (in mammalian cells) bicyclic pentapeptide jasplakinolide (Bubb *et al.*, 2000).

Cell biology of ciliates (and of most other protozoa) still suffers from our considerable ignorance of specific inhibitors, modulators, or stimulators for experimental purposes. An example is the ongoing debate on the identity of some intracellular Ca^{2+} -release channels. For instance, ryanodine—an alkaloid isolated from the South American plant *Ryania* (family Flacourtiaceae), the classical inhibitor of Ca^{2+} -release channels—has no recognizable effect in *Paramecium* (Länge *et al.*, 1995). In contrast, the situation is fairly clear, of course, with endogenous modulators produced by the cells themselves, such as inositol 1,4,5-trisphosphate (InsP_3). Even then, the response to exogenous artificial modulators may vary between higher and lower eukaryotic systems.

In this review, we try to evaluate published data critically, to develop a rationale, wherever possible, on a molecular basis and to show up gaps to be filled by future research. A substantial body of literature could not be included, not in the least for the sake of brevity. We encourage more systematic analysis of the pharmacological studies with ciliated protozoa in the future.

2. EARLY SUCCESS STORY—CALMODULIN AND ITS EFFECT ON ION TRANSPORT

Before any pharmacology of ciliates could emerge, they were a subject of intense electrophysiological analysis (Eckert, 1972; Eckert and Brehm, 1979; Machemer, 1988). *Paramecium* mutants were detected that showed aberrant swimming behavior and simultaneously defects in different plasmalemmal cation channels (Oertel *et al.*, 1977; Saimi and Kung, 2002). In parallel, drugs originally established for mammalian cells have been tested. Subsequently attempts have been made to complement lacking channel functions by injecting wild-type cytosol and to identify the defective component. With *Paramecium*, in an even more advanced stage, complementation cloning has been developed (Haynes *et al.*, 1996; Meyer and Cohen, 1999) which finally was extended to an indexed genomic library (Keller and Cohen, 2000). This culminated in function repair by transfection with the respective wild-type genes (Haga *et al.*, 1982; Haynes *et al.*, 1996; Kerboeuf *et al.*, 1993) and in homology-dependent inactivation of genes which, thus, could be identified in more extensive studies (Keller and Cohen, 2000; Meyer and Cohen, 1999).

From studies along these lines, calmodulin (CaM) has been detected as a positive modulator of different plasmalemmal cation channels in *Paramecium* (Kung *et al.*, 1992; Saimi and Kung, 2002). This includes voltage-dependent Ca^{2+} -channels in the ciliary membrane (Preston and Saimi, 1990; Saimi and

Kung, 2002) as well as some nonciliary (“somatic”) cation channels, such as anteriorly concentrated depolarizing Na^+ -channels (Saimi and Ling, 1990) and posteriorly enriched hyperpolarizing K^+ -channels (Preston *et al.*, 1990). Remarkably, voltage-dependent Ca^{2+} -channels are inactivated by the very same Ca^{2+} they conducted into the cilia (Brehm and Eckert, 1978). Also remarkably, the underlying Ca^{2+} /CaM-regulated channels have been detected in neurons only two decades later (Levitan, 1999; Peterson *et al.*, 1999).

On this background, established “anti-CaM” drugs have been explored by electrophysiology with *Paramecium* cells. The drug designated “W7” (N-[6-aminohexyl]-5-chloro-1-naphthalene-sulfonamide hydrochloride) has, thus, proved an efficient CaM inhibitor in *Paramecium* (Hennessey and Kung, 1984) as was the case with R24571 (later on termed calmidazolium) (Ehrlich *et al.*, 1988). In contrast, the related compound, W5, served as an inactive control, just as with mammalian cells. Several other drugs, for example, trifluoperazine, may be considered with more caution, as they exert a variety of effects even in higher eukaryotes. Nevertheless, in *Paramecium* trifluoperazine produced similar electrophysiological effects as W7 (Ehrlich *et al.*, 1988; Erxleben and Plattner, 1994).

In the 1970s, *Paramecium* became a favorite object for some electrophysiologists, assuming that they would be more easy to analyze, not in the least for the availability of useful mutants. In retrospect, this assumption may appear naïve, as alone the number of K^+ -channels in a *Paramecium* cell by far surmounts that in any other organism including man (Haynes *et al.*, 2003). Even though many genes have been duplicated fairly recently and retained with only small changes (Aury *et al.*, 2006) so that the “effective” number may be about half, this renders pharmacology with *Paramecium* particularly intriguing.

Among the plethora of many CaM-dependent enzymes in ciliates, the effect of “anti-CaM” drugs has not yet been explored. This is true, for example, for the plasma membrane Ca^{2+} -ATPase/pump (PMCA) and for the protein phosphatase 2B (PP2B, calcineurin). The PMCA and the PP2B catalytic subunit (SU) are both endowed with a CaM-binding domain (Carafoli, 2005; Rusnak and Mertz, 2000). W7, trifluoperazine, fluphenazine, and calmidazolium have been shown to inhibit a type of soluble Ca^{2+} -dependent ATPase isolated from *Paramecium* (Levin *et al.*, 1989).

While it has been assumed for a long time that *Paramecium* possesses only one CaM gene, ongoing exploration of its genome has revealed 14 (R. Kissmehl, unpublished results). Therefore, one now has to consider the large number of CaM target molecules in conjunction with the potentially widely varying reaction of different protein isoforms to drugs, as detected with some other *Paramecium* proteins (see subsequent sections). One now would have to ask, therefore, whether “anti-CaM” drugs would affect different isoforms in the same way.

In sum, “anti-CaM” drugs currently look exceptional in their specificity in ciliates. However, considering the wide variation of CaM-binding sites, as far as analyzed (I. M. Sehring, unpublished results), it may be premature to assume identical drug effects for all CaM paralogs.



3. CATION CHANNELS, PUMPS, AND EXCHANGERS

3.1. Plasmalemmal cation channels

3.1.1. Inhibitors/blockers of plasmalemmal cation channels

After the special aspect of CaM-activated channels, specific ion channel activities will be discussed. Tetraethylammonium (TEA^+) is exceptional as an inhibitor as it is capable of inhibiting K^+ -channels in mammalian cells and in *Paramecium* (Eckert and Brehm, 1979). This is not the case with the “standard” Na^+ -channel blocker, tetrodotoxin, as this toxin is inefficient with *Paramecium*. Similarly, the plethora of inhibitors of the different families of voltage-gated Ca^{2+} -channels are inefficient with ciliates. This is true for verapamil (or its derivative, D600 = methoxyverapamil) in *Paramecium* (Eckert and Brehm, 1979) as well as for agatoxins, conotoxins, diltiazem, and nifedipine, according to trials with the marine ciliate *Euplotes vannus* (Krüppel and Wissing, 1996) though in these cells a Ca^{2+} -dependent K^+ -outward current was inhibited by D600 (Krüppel *et al.*, 1991). Nevertheless, beyond any electrophysiological backing, a variety of such channel inhibitors have been applied to *Paramecium* cells and to other ciliates until quite recently and many times before, with the intention to pinpoint alleged interactions with specific cell surface receptors and signal transduction mechanisms.

Also by electrophysiology, amiloride has been described as an inhibitor of hyperpolarization-sensitive Ca^{2+} -influx channels (Preston, 1990; Preston *et al.*, 1992). Backed by electrophysiology, amiloride may be a useful drug, though in mammalian cells it also inhibits a Na^+/H^+ -antiporter (Bhartur *et al.*, 1999; Li *et al.*, 1991) and, specifically with hepatocytes, plasmalemmal $\text{Na}^+/\text{Mg}^{2+}$ - and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -antiporters (Cefaratti *et al.*, 2000). In *Paramecium*, experiments with amiloride have suggested that activation of hyperpolarization-sensitive Ca^{2+} -influx channels may be the primary step in trichocyst exocytosis in response to stimulation by aminoethyl-dextran (AED) (Kerboeuf and Cohen, 1996). While this assumption was theoretically appealing, using other methodologies we could not find such effects, neither with AED nor with veratridine (see below) stimulation of exocytosis of trichocysts, the dense core-secretory vesicles of *Paramecium* (Erxleben and Plattner, 1994). Moreover, in our whole cell-patch clamp analyses with *Paramecium*, we have demonstrated that neither de- nor hyperpolarization can induce trichocyst exocytosis.

Collectively, with the exception of TEA^+ , blockers of plasmalemmal cation channels available appear ineffective with ciliates.

3.1.2. Activators of plasmalemmal cation channels

Note that CaM-activated channels in the cell membrane of *Paramecium* have been discussed separately in [Section 2](#).

Aconitin (from the ranunculacean plant, *Aconitum*), batrachotoxin (from the skin secretion of poisonous dart frogs), and veratridine (from the liliacean, *Veratrum*) are all alkaloids that maintain plasmalemmal Na⁺-influx channels of mammalian cells in an open state ([Ameri, 1998](#); [Barnes and Hille, 1988](#); [Bloomquist, 1996](#)). No such effects, but rather widely different ones are known from ciliates, at least for veratridine in *Paramecium*.

In *Paramecium*, veratridine causes a rise in intracellular 3',5'-cyclic guanosine monophosphate (cGMP), probably due to a Ca²⁺-influx ([Schultz and Klumpp, 1993](#); [Schultz and Schade, 1989](#)) since the respective guanylate kinase is activated by Ca²⁺ ([Schultz and Klumpp, 1993](#)). Another target of Ca²⁺ are trichocyst exocytosis sites ([Plattner and Klauke, 2001](#)) that are also activated by veratridine with EC₅₀ ~ 1 mM, paralleled by cGMP formation ([Knoll et al., 1992](#)). Maximal stimulation by veratridine occurs in presence of extracellular calcium, [Ca²⁺]_e between ~10⁻⁴ and 10⁻³ M ([Blanchard et al., 1999](#); [Plattner et al., 1994](#)). This effect cannot be attributed to a membrane-permeabilizing effect since isolated cortical Ca²⁺-stores (alveolar sacs) retain ⁴⁵Ca²⁺ even when exposed to 2.5 mM veratridine and since the secretagogue effect of veratridine *in vivo* is highest at intermediate [Ca²⁺]_e values. Remarkably, no exocytosis was induced by either batrachotoxin or aconitin.

Originally, it had been assumed that in *Paramecium* veratridine would activate voltage-dependent Ca²⁺-influx channels ([Schultz and Klumpp, 1993](#); [Schultz et al., 1986](#)), just like in a depolarization response. This is known to result in ciliary reversal (backward swimming) and cGMP formation ([Schultz and Schade, 1989](#); [Schultz et al., 1986](#)). In fact, we observed by fluorochrome analysis that intracellular calcium, [Ca²⁺]_i, increases in response to the secretagogue effect of veratridine ([Plattner et al., 1994](#)), but we also registered spillover of Ca²⁺ from somatic domains into cilia. This is an alternative explanation to that mentioned above. In fact, the same phenomena occurred when we analyzed *pawn* mutants (devoid of ciliary voltage-dependent Ca²⁺-influx channels; [Beisson et al., 1980](#)). *Pawn* cells respond by ciliary beat reversal also to AED ([Plattner et al., 1984, 1985](#)) and not only to veratridine ([Plattner et al., 1994](#)). Both are known not to involve depolarization signals ([Erxleben and Plattner, 1994](#)). All our data, therefore, make voltage-dependent Ca²⁺-channels unlikely targets of veratridine.

In retrospect, veratridine is unique in activating, in the somatic (non-ciliary) cell membrane, a Ca²⁺-conductance whose molecular basis, however, has not been identified as yet. Any other Na⁺-channel agonist had no effect comparable to that of veratridine ([Plattner et al., 1994](#)).

3.2. Intracellular cation channels and pumps

3.2.1. Intracellular Ca^{2+} -release channels

Their nature and subcellular localization is of paramount importance, just like that of plasmalemmal channels, considering the multitude of strictly localized cellular functions regulated by local Ca^{2+} signals (Berridge, 2006). Ca^{2+} -release channel analysis has posed considerable problems with protozoa, be it the InsP_3 receptor or the ryanodine receptor-type Ca^{2+} -release channels though these channels are fairly well characterized in higher eukaryotes (Fleischer, 2008; Foskett *et al.*, 2007; Mikoshiba, 2007; Pointing, 2000). For any other activators of Ca^{2+} -stores, such as nicotinic acid adenine dinucleotide phosphate (NAADP^+) and perhaps also for cyclic adenosinediphosphoribose (cADPR) (Guse, 2002; Lee, 2004) receptors are not known in any system at this time, although the latter is considered by most authors as an activator of ryanodine receptors. InsP_3 (derived from phosphatidylinositol-4,5-bisphosphate, PIInsP_2), cADPR and NAADP^+ (both derived from NADH), together with sphingosine-1-phosphate (Young and Nahorski, 2002), are the metabolic products currently known to activate Ca^{2+} -release from internal stores in higher eukaryotes. Clearly, receptors for metabolic activators should be more easily amenable to specific activation and identification. Although some inhibitors of these pathways have been found, receptors for the latter two activators (or even for the latter three, but in particular for NAADP^+), are currently under debate even in mammalian systems.

Both ryanodine and InsP_3 receptors have different pharmacological properties (Taylor and Traynor, 1995), but the different modulators are known each to exert many side effects (Taylor and Broad, 1998). Also, simultaneous occurrence in the same membrane continuum (smooth muscle SR) has recently been documented (McCarron and Olson, 2008). Thus, identification and localization of InsP_3 or ryanodine receptor orthologs in low eukaryotes is currently a challenge.

A step forward in the unraveling of Ca^{2+} -channels in protozoa was the identification of the *IplA* gene in *Dictyostelium* (Traynor *et al.*, 2000) which possesses regions related to InsP_3 receptor sequences, but so far no evidence for InsP_3 interaction exists. Despite the availability of rising sequence data due to various genome projects, no such sequences were identified in full in any other protozoa. Only recently full sequencing and cloning of an InsP_3 receptor and proof of its function as such has been achieved in *Paramecium* (Ladenburger *et al.*, 2006). This receptor is more aberrant than observed among metazoans and the relationship of the region corresponding to the well-characterized ligand-binding domain of mammalian InsP_3 receptors (Bosanac *et al.*, 2002; Yoshikawa *et al.*, 1996) seems to be restricted to amino acids which are essential for InsP_3 interaction (Fig. 5.1). Although a detailed biochemical analysis is still lacking, the



Figure 5.1 InsP₃-binding domain of the *P. tetraurelia* InsP₃ receptor. This figure is compiled on the basis of the work by [Ladenburger et al. \(2006, 2009\)](#). Alignment of the putative InsP₃-binding domain of the *Paramecium* PflnsP₃RN1 and PflnsP₃RN2 (accession No. CAI39149; CAI39148) in comparison to known ligand-binding domains from mouse (*Mm*) InsP₃R type 1 (accession No. P11881) and rat (*Rn*) InsP₃R type 3 (accession No. NP_037270). Sequences are shown in single-letter code and are numbered on the left side. Residues that are identical are highlighted in black, similar residues are shaded. The highlighted blue amino acids are involved in InsP₃ binding, those in red (arrow heads 3,6 and 8) are essential for InsP₃ binding ([Yoshikawa et al., 1996](#)). Note that in *Paramecium*, the amino acids essential for InsP₃ binding are preserved as identical (black background) or “similar” (gray background) amino acids, while some of the less essential ones (blue arrow heads 1,2,4,5,7,9 and 10) deviate.

InsP₃-binding domain homologously expressed in *P. tetraurelia* binds [³H] Ins 1,4,5-trisphosphate (Ladenburger *et al.*, 2006).

In mammalian cells, InsP₃ formation and its effects can be inhibited by Li⁺ (Halcher and Sherman, 1980) which, however, can inhibit several widely different metabolic pathways (Williams *et al.*, 2004). In *Paramecium* and *Tetrahymena* cells, Li⁺ can affect surface pattern formation (Beisson and Ruiz, 1992; Jerka-Dziadosz and Frankel, 1995), chemoresponses (Wright *et al.*, 1992), and the activity of contractile vacuoles (Ladenburger *et al.*, 2006). Direct effects on targeting of InsP₃ receptors by Li⁺ has been demonstrated not only in human cells (Seelan *et al.*, 2004), but also in *Paramecium* where InsP₃ receptors are redistributed in the presence of Li⁺ (Ladenburger *et al.*, 2006).

Based on these novel findings, one now may probe InsP₃ receptor inhibitors. Neomycin, an aminoglycoside antibiotic, has frequently been used for this purpose (Phillippe, 1994), even in *Paramecium* (Robinette *et al.*, 2008), but this compound may exert a variety of effects (Lu *et al.*, 1998). For instance, it can inhibit also ryanodine receptor-type Ca²⁺-channels at a high-affinity Ca²⁺-binding site (Laver *et al.*, 2007), Ca²⁺-influx channels in hepatocytes (Huges *et al.*, 1988), and Ca²⁺-currents in *Paramecium* (Gustin and Hennessey, 1988). Therefore, confidence in that drug as an InsP₃ receptor inhibitor must be very low (for further aspects related to InsP₃, see Section 6.3.1). With ciliates, there is no experience with any other inhibitors or activators of InsP₃ receptors, such as xestospongin or adenophostin, as they are established for higher eukaryotic systems. Caution is advised particularly since, for instance in *Dictyostelium*, such drugs affect key players of Ca²⁺ dynamics other than putative InsP₃ receptors (Malchow *et al.*, 2008).

Ryanodine is a standard activator of Ca²⁺-release channels in the SR of muscle and in the ER of many other mammalian cells. In contrast, ryanodine has no effect in *Paramecium* whose alveolar sacs, the flat subplasmalemmal Ca²⁺-stores closely attached to the cell membrane, strikingly resemble the SR in several respects (Länge *et al.*, 1995; Plattner and Klauke, 2001). These cortical Ca²⁺-stores are mobilized during stimulated trichocyst exocytosis, causing a secondary Ca²⁺-influx (store-operated Ca²⁺-influx, SOC) (Hardt and Plattner, 2000) required for full activation of trichocyst exocytosis (Klauke *et al.*, 2000). The fact that this can be activated also with caffeine (Klauke and Plattner, 1998), another nonphysiologic activator of ryanodine receptors frequently used (Sawynok and Yaksh, 1993), pointed to the presence of ryanodine receptor-like Ca²⁺-release channels in these stores. Unfortunately, disturbingly high concentrations of tens of millimolar have to be applied not only with *Paramecium* but also with mammalian cells (Cheek and Barry, 1993; Verkhatsky and Shmigol, 1996). Further support came from experiments with 4-chloro-*m*-cresol (4CmC; Klauke *et al.*, 2000), a membrane-permeable activator of mutated ryanodine receptors

in men (Fessenden *et al.*, 2003, 2006; Herrmann-Frank *et al.*, 1996; Tegazzin *et al.*, 1996). In *Paramecium*, release of Ca^{2+} from alveolar sacs can be stimulated by 4CmC even in lower concentrations than used, for example, to activate ryanodine receptor-type Ca^{2+} -release channels in lymphoma cells (McCarthy *et al.*, 2003).

Only very recently, we could find in *Paramecium* a candidate gene for Ca^{2+} -release channels compatible with these observations and localize its translation product to the alveolar sacs, that is, to their outer side facing the cell membrane (Ladenburger *et al.*, 2009). Surprisingly, this type of channel combines signature elements of InsP_3 as well as of ryanodine receptors. For the latter, two conserved regions could be identified, one corresponding to the pore segment and the other one overlapping with the domain responsible for 4CmC activation, as described for the mammalian channels (Fessenden *et al.*, 2003, 2006). This goes along with observations during gene-silencing experiments where knockdown of this receptor type causes impaired Ca^{2+} -release when stimulated with 4CmC. In agreement with our *in vivo* observations, activation of these channels by ryanodine seems unlikely, as the conserved glutamine residue, Gln⁴⁸⁶³, in the predicted carboxy-terminal membrane-spanning sequence, which mediates ryanodine interaction in mammalian receptors (Wang *et al.*, 2003), is absent (Ladenburger *et al.*, 2008).

Any other types of inhibitors of these Ca^{2+} -release channels, as they are frequently used with mammalian systems, appear problematic to use with protozoan cells at this time, as the molecular background information is still too poor. This became evident in a recent genomic screening not only of *Paramecium* (Ladenburger *et al.*, 2008) but also of Apicomplexa (Nagamune and Sibley, 2006). For *Toxoplasma*, a mixed type, InsP_3 receptor-/ryanodine receptor-type Ca^{2+} -release channel is postulated to govern some secretory processes accompanying host cell invasion (Lovett *et al.*, 2002), but no molecular identification has been achieved so far. Any pharmacology along these lines would be of paramount interest.

Taken together, we conclude that 4-chloro-*m*-cresol is the only activator of (non- InsP_3 receptor-type) Ca^{2+} -release channels effective in ciliates at low concentrations, while ryanodine is inefficient (as far as we could realize up to now). Caffeine is also efficient, but requires disturbingly high concentrations, just as with mammalian cells.

3.2.2. Inhibitors of Ca^{2+} -pumps

The main types of Ca^{2+} -ATPases (Ca^{2+} -pumps) are the plasma membrane Ca^{2+} -ATPase (PMCA) and the SR/ER-type Ca^{2+} -ATPase (SERCA) in the main internal Ca^{2+} -stores, SR, and ER (Carafoli, 2005). They both belong to the P-type ATPases, meaning the formation of a phosphorylated intermediate during the pumping cycle. Only the PMCA contains a CaM-binding regulatory domain at its carboxy-terminus. The PMCA also occurs

in ciliates, but no specific inhibition studies have been performed, neither in *Paramecium* (Wright and VanHouten, 1990) nor in *Tetrahymena* (Wang and Takeyasu, 1997), where it has been identified at a molecular level.

Two genes encoding SERCAs have been found in *Paramecium*, both closely resembling each other (Hauser *et al.*, 1998). Their way through the cell has been followed by expression as a green fluorescent protein (GFP)-fusion protein, revealing passage through the ER and final delivery to alveolar sacs, so that little remains in the ER (Hauser *et al.*, 2000).

The SERCA in *P. tetraurelia* proved insensitive to thapsigargin (Kissmehl *et al.*, 1998), the standard inhibitor in mammalian cells (Inesi and Sagara, 1994; Thastrup *et al.*, 1990). Thapsigargin is a sesquiterpene lactone isolated from the mediterranean plant *Thapsia garganica* (family Umbelliferae, now called Apiaceae). With higher eukaryotes it is routinely applied to empty the ER as the most prominent Ca^{2+} -store and, thus, to induce a store-operated Ca^{2+} -influx (Takemura *et al.*, 1989). Therefore, thapsigargin is generally an important diagnostic tool.

Sequence analysis shows the absence of the putative thapsigargin-binding domain in the *P. tetraurelia* SERCA, PtSERCA (Hauser *et al.*, 1998) (Fig. 5.2). From the two other established, widely different SERCA inhibitors, that is, tertiary butyl(benzo)hydroquinone (briefly designated as *t*-butylhydroquinone) and cyclopiazonic acid (Inesi and Sagara, 1994; Papp *et al.*, 1993; Witcome *et al.*, 1992), we tried out the first one by application in the usual concentrations to isolated alveolar sacs, but reduction of $^{45}\text{Ca}^{2+}$ -sequestration thus achieved was small (Länge *et al.*, 1995). Only suspiciously higher concentrations induced trichocyst discharge (Plattner and Klauke, 2001). Also in *Paramecium* cells, the chemoresponse to exogenous GTP was inhibited by SERCA inhibitors in the following sequence: *tert*-butylhydroquinone > cyclopiazonic acid > thapsigargin (lowest sensitivity) (Wassenberg *et al.*, 1997). Since this reaction remains unaffected by previous emptying of cortical Ca^{2+} -stores (Sehring and Plattner, 2004) another store may provide the endogenous Ca^{2+} -component required for this reaction. With isolated “pellicle” fractions (cell membranes with adhering alveolar sacs) thapsigargin was reported to reduce Ca^{2+} -uptake at submicromolar concentrations (Wright *et al.*, 1993). This Ca^{2+} -uptake has been attributed to a pump in the cell membrane. If so, this pump would have unorthodox toxin sensitivity and these membranes would have to be resealed during isolation. To reassure sequestration, rather than adsorption, release by adding a Ca^{2+} -ionophore would be a standard test. This would definitely prove previous sequestration into closed compartments (Länge *et al.*, 1995). Unfortunately in *Tetrahymena*, the molecular identification of the SERCA-type pump, just as any other P-type ATPase, has also not yet led to pharmacological investigation up to now (Wang and Takeyasu, 1997).

In apicomplexans, SERCA activity is inhibited by adequately low concentrations of thapsigargin (Nagamune *et al.*, 2007) and by artemisinin

A Phosphorylation site

P. tetraurelia	306	KVAVALAVAAIPEGLPAVITTTCLALGARRMAKOKAIV--RKLPKVQTLG-CTTIICSDKTGTGLTTNEMC
Rabbit SERCA2B	297	KIAVALAVAAIPEGLPAVITTTCLALGTRRRMAKNAIV--RSLPSVETLG-CTSVICSDKTGTGLTTNQMS
D. melanogaster	297	KIAVAVAVAAIPEGLPAVITTTCLALGTRRRMAKNAIV--RSLPSVETLG-CTSVICSDKTGTGLTTNQMS
A. franciscana	300	KIAVALAVAAIPEGLPAVITTTCLALGTRRRMAKNAIV--RSLPSVETLG-CTSVICSDKTGTGLTTNQMS
P. falciparum	314	KISVALAVAAIPEGLPAVITTTCLALGTRRRMAKNAIVFTRKLQSVETLG-CTTVICSDKTGTGLTTNQMT
L. mexicana	313	KVAVALAVAAIPEGLPAVITTTCLALGARRMAKNAIV--RDLPSVETLGRCT-VICSDKTGTGLTTNMQS
T. brucei	303	KVAVALAVAAIPEGLPAVITTTCLALGTRRRMAKNAIV--RDLPSVETLGRCT-VICSDKTGTGLTTNMQS
S. mansoni	298	KIAVALAVAAIPEGLPAVITTTCLALGTRRRMAKNAIV--RSLPSVETLG-CTTVICSDKTGTGLTTNQMT
T. vaginalis	295	KIAISLAVAAIPEGLPAVITTTCLALGTRRRMAKNAIV--TK-LPAVETLG-CTSVICSDKTGTGLTTNQMT
A. thaliana	329	EIAVALAVAAIPEGLPAVITTTCLALGTRRRMAKNAIV--RKLPSVETLG-CTTVICSDKTGTGLTTNQMA

B FITC-binding site /T₁ tryptic site

P. tetraurelia	484	-NEFAK-RA-TLEFTRDRKSMSV-----LASSQNEKGNVLF-IKGAPDYLLKST
Rabbit SERCA2B	480	-----K-KEFTLEFSRDRKSMSVYCT-----PNKPSRTSMS-K-----MF-VKGAPEGVIDRCT
D. melanogaster	480	-----K-KEFTLEFSRDRKSMSVYCT-----PLKASRLGTGPK-----LF-VKGAPEGVLERCT
A. franciscana	484	-----K-KEFTLEFSRDRKSMSVYCV-----PLKAGLLSNGPK-----MF-VKGAPEGVLDRCT
P. falciparum	708	-KQI-KIIEFT--FTREKRLMSV-----IVEN-----KKKEIILY-CKGAPENI IKNCK
L. mexicana	495	-----L-KKATLEFTRSRKSMSVCCST-----RHS-----LF-VKGAPEEILKRCCT
T. brucei	478	-----K-KNATLEFTRSRKSMSVHVSTVTGSP-----ASSTNN-----LF-VKGAPEEVLRR-S
S. mansoni	485	-----E-RKFTLEFSRDRKSMSVYVI-----P-QSQIS-GSKEK-----LF-VKGAPEEILDRCT
T. vaginalis	473	YKEYPKVR--THEFTRARKSMS--C---IVGK-----NTL-VKGAPEEVLAKCD
A. thaliana	500	-SELEQ-RIATLEFDRDRKSMSGV-----MVDSSSGNK-----LLLKGAPEEVLKST

C Membrane spanning domain III (thapsigargin binding)

P. tetraurelia	257	DPLSKRLDEFQDKLAKYVT---YICIIQVW--MNIGNFSDPAY
Rabbit SERCA2B	247	TPLQOKLDEFGEQLSKVIS---LICIAVWI--INIGHFNDFVH
D. melanogaster	247	TPLQOKLDEFGEQLSKVIS---VICVAVWA--INIGHFNDFAH
A. franciscana	250	TPLQOKLDEFGEQLSKVIS---VICVAVWA--INIGHFNDFAH
P. falciparum	263	TPLQIKIDLFQOQLSKIIF---VICVTVWIFTNFKHFSDFIH
L. mexicana	256	TPLQKLDEFQVLLSGVIG---YICLFVFW--ANL-----L
T. brucei	246	TPLQVKLDEFQVLLSGVIG---YICLVVFA--VNL-----V
S. mansoni	248	TPLQOKIDFQQLSKVIT---FICIAVWC--INIGHFNDFVH
T. vaginalis	246	TPLQRLDNF---SKYISVGILFICVITW-FA-NISKFE-VG
A. thaliana	272	TPLKKKLNEFGEVLTMIIG---LICALVWL--INVKYF-----L

Figure 5.2 Continued

(Eckstein-Ludwig *et al.*, 2003; Nagamune and Sibley, 2007; Nagamune *et al.*, 2007; Uhlemann *et al.*, 2005). Here, the putative thapsigargin-binding sites resemble more those in mammals than in their sister phylum, the Ciliophora (Fig. 5.2). Any effect of artemisinin on ciliates remains to be analyzed.

Surprisingly, caffeine was found to inhibit SERCA activity in *Paramecium* (Länge *et al.*, 1996) by inhibiting the formation of the energized phosphointermediate (Kissmehl *et al.*, 1998). However, this cannot explain the Ca^{2+} -mobilization effect of caffeine (see Section 3.2.1) since Ca^{2+} -reuptake in alveolar sacs is very slow, with a half-life of ~ 60 min, according to analyses with widely different methods (Mohamed *et al.*, 2003). In contrast, Ca^{2+} -mobilization by caffeine is very fast and, thus, leads to rapid trichocyst exocytosis (Klaue and Plattner, 1998).

To sum up, the classical SERCA inhibitor, thapsigargin, is inefficient in *Paramecium*, while *t*-butyl-hydroquinone inhibited some stimulated responses. As a side effect, caffeine inhibits the SERCA pump by inhibiting formation of the phosphointermediate. No drug comparable in efficiency to artemisinins in *Plasmodium* is known so far from ciliates.

3.2.3. Inhibitors of H^+ -ATPase

Such inhibitors are important for analyzing a variety of functional aspects of organelle acidification also in *Paramecium* (Wassmer *et al.*, 2008), for example, phagosome formation (Ishida *et al.*, 1997). Moreover, an H^+ -gradient generated by an H^+ -ATPase can be used by the cell indirectly for secondary active transport processes.

In *Paramecium*, osmoregulation occurs via an H^+ -ATPase in the decorated spongione of the contractile vacuole complex (Allen, 2000; Fok *et al.*, 1995). This may also apply to other cells endowed with such an organelle. The electrochemical H^+ -gradient built up by this H^+ -pump (Stock *et al.*, 2002b) can be exploited for the extrusion of water by a chemiosmotic process and of an excess of ions, including Ca^{2+} , by a secondary active process (Stock *et al.*, 2002a). Application of the H^+ -ATPase inhibitor concanamycin B compromises cell viability by osmotic swelling (Grønlien *et al.*, 2002b). Remarkably, the other inhibitor, bafilomycin, does not work

Figure 5.2 Characteristics of the *P. tetraurelia* SERCA-type Ca^{2+} -ATPase. From Hauser *et al.* (1998). (A) Considerable interspecies conservation of the active center with the aspartate residue (“D”) undergoing phosphorylation during each pumping cycle. (B) Considerable variability in a region frequently used for biochemical/biophysical analyses, with a lysine residue (“K”) that binds FITC, while the framed area was used to produce SERCA-specific antipeptide antibodies for localization to alveolar sacs (see Section 3.2.2). The considerable interspecies variability of the thapsigargin-binding domain (C) explains the insensitivity of *Pt*SERCA in contrast to, for example, mammalian cells.

in *Paramecium* (Fok *et al.*, 1995). Here, the H^+ -ATPase is an important component also in re-establishing Ca^{2+} -homeostasis after massive Ca^{2+} -signaling accompanying synchronous exocytosis stimulation (I. M. Sehring and H. Plattner, in preparation). Along these lines we found that, in a first step, centrin, assembled in the cell cortex as a major component of the “infraciliary lattice,” serves as a highly efficient Ca^{2+} -binding protein (Sehring *et al.*, 2008), that is, as an immobile Ca^{2+} -buffer. It can down-regulate Ca^{2+} after massive exocytosis stimulation within ~ 20 s. Second, Ca^{2+} is gradually released from cortical centrin. Third, a highly efficient extrusion mechanism operating faster than any of the known Ca^{2+} -ATPases/pumps is provided by the contractile vacuole complex (Ladenburger *et al.*, 2006) which can extrude Ca^{2+} steadily released from the low-affinity Ca^{2+} -binding sites of centrin contained in the infraciliary lattice. Concomitantly, concanamycin B inhibits downregulation of the Ca^{2+} -signal (I. M. Sehring and H. Plattner, in preparation).

In sum, concanamycin B blocks the H^+ -ATPase in *Paramecium*, in contrast to the other inhibitor widely used, bafilomycin. Considering the numerous isoforms of subunits that combine to form a functional H^+ -ATPase molecule, many different holoenzyme isoforms can exist in a *Paramecium* cell (Wassmer *et al.*, 2005, 2006). Any consequences on drug sensitivity are not known.

3.3. Exchangers/antiporters and ionophores

3.3.1. Inhibitors of exchangers/antiporters and cotransport systems

Among the multitude of cation antiporters in plant and animal cells, in the latter a plasmalemmal Na^+/Ca^{2+} -antiporter is of particular importance. It is required, for instance, for rapid extrusion of Ca^{2+} from cardiac myocytes (Choi and Eisner, 1999). (Na^+ -homeostasis can be rapidly re-established by the plasmalemmal Na^+/K^+ -ATPase/pump). A SERCA-type Ca^{2+} -pump is enriched in the extensively large longitudinal SR which pumps Ca^{2+} back into the SR for reuse, that is, for release from the much smaller terminal cisternae in response to cell membrane depolarization and Ca^{2+} -influx (Ca^{2+} -induced Ca^{2+} -release, CICR) (Mackrill, 1999). The terminal cisternae are connected to the longitudinal SR and the next contraction cycle can, thus, be initiated by the next wave of $[Ca^{2+}]_i$ increase, based on the supportive function of the Na^+/Ca^{2+} -antiporter in the cell membrane.

The cortical Ca^{2+} -stores in ciliates, the alveolar sacs, closely resemble the SR of muscle cells, particularly that of skeletal muscle, in several respects (Länge *et al.*, 1995). This includes the occurrence of a store-operated Ca^{2+} -influx (SOC, initiated by emptying stores, causing Ca^{2+} -influx from outside in a secondary step), rather than a CICR (with a primary Ca^{2+} -influx causing a secondary Ca^{2+} -release from stores). A CICR occurs, for

example, in cardiac myocytes (Mackrill, 1999). However, some experiments using amiloride as an inhibitor of hyperpolarization-sensitive Ca^{2+} -influx channels (Preston, 1990; Preston *et al.*, 1992) have suggested that a CICR-type mechanism would also occur in *Paramecium* during exocytosis stimulation (Kerboeuf and Cohen, 1996). Subsequently the SOC, rather than the CICR model, has obtained support by direct measurement of Ca^{2+} -release from alveolar sacs upon exocytosis stimulation even at low extracellular Ca^{2+} -concentration, $[\text{Ca}^{2+}]_e$, when no influx can occur (Hardt and Plattner, 2000). Similar results were obtained (Mohamed *et al.*, 2002) with double mutants devoid of any stimulated Ca^{2+} -influx (Kerboeuf and Cohen, 1990). Along these lines, in a study dedicated to Na^+/H^+ -exchange in *Tetrahymena* no inhibitory effect of amiloride could be established (Kramhøft and Jessen, 1992). This is opposite to the effects with mammalian cells (see Section 3.1.1). In consequence of these data and considering the multiple effects of amiloride (see Section 3.1.1), one may envisage some other effects of amiloride in *Paramecium*.

For downregulation of $[\text{Ca}^{2+}]_i$, in *Paramecium* the Ca^{2+} -pumping efficiencies of the PMCA- and that of the SERCA-type pump in alveolar sacs appear too sluggish (Mohamed *et al.*, 2003; Plattner and Klauke, 2001; Wright and VanHouten, 1990). A $\text{Na}^+/\text{Ca}^{2+}$ -antiporter system has been inferred from experiments with *Euplotes*, based on results achieved with the inhibitor, Bepridil (Burlando *et al.*, 1999). However, we rather detected another mechanism for Ca^{2+} -downregulation which operates in the absence of Na^+ . It involves Ca^{2+} -binding to cortical centrin (Sehring *et al.*, 2008) followed by steady dissociation and extrusion mainly via the contractile vacuole system (see Section 3.2.3).

As noticed in Section 3.1.1, amiloride inhibits different cation antiporters in mammals (Cefaratti *et al.*, 2000) and, thus is always problematic to use. Generally there are no really specific inhibitors available for most antiporter systems (Blaustein and Lederer, 1999).

Cotransport for the internalization of sugars and Na^+ , as known from mammals, also exists in ciliates. The uptake of inositol by *Tetrahymena* is inhibited by typical inhibitors such as phlorizin and cytochalasin B (in concentration well above those required for F-actin depolymerization in mammalian cells) (Kersting and Ryals, 2004).

Our most important conclusion is as follows. While a variety of $\text{Me}^+/\text{Me}^{2+}$ -antiporters show up in the *Paramecium* database we still know neither their localization nor their pharmacology. The situation may be better with cotransporters.

3.3.2. Ionophores

These form transmembrane ion channels frequently of high ion specificity, in some cases with a specific exchanger function.

Ca^{2+} -ionophores are routinely used to mimic the effect of a Ca^{2+} -signal that normally would occur during cell stimulation. Concomitantly, the Ca^{2+} -ionophores A23187 or ionomycin can induce exocytosis of trichocysts in *Paramecium* (Plattner, 1974) and, as shown later, of mucocysts in *Tetrahymena* cells (Melia *et al.*, 1998; Wissig and Satir, 1980) when Ca^{2+} is added to the medium.

Monensin, an ionophore isolated from a *Streptomyces* species, acts as a Na^+/H^+ -antiporter frequently used to release protons from acidic compartments. This allows one to monitor effects of organelle acidification on trafficking (Mollenhauer *et al.*, 1990). Along these lines monensin could be safely applied to *Paramecium* to study processing of secretory protein precursors to mature products (Adoutte *et al.*, 1984; Gautier *et al.*, 1994) and phagosome processing depending on acidification (Fok and Ueno, 1987).

Any ionophore effect is due to its intrinsic pore-forming structure and, therefore, should be usable in widely different systems. Therefore, ionophores are generally sound tools to analyze the relevance of certain cations for organelle function. They are spontaneously inserted into membranes and their modus operandi does not remarkably depend on the cell type analyzed.



4. FUNCTIONS OF SPECIFIC COMPONENTS

4.1. Lysosomal acid phosphatase

To serve as a lysosomal key enzyme, acid phosphatase has to be discriminated from any other phosphatases, notably alkaline phosphatase. With higher eukaryotes, L(+)-tartrate serves as an inhibitor of the former, levamisole (or similar compounds) of the latter. In *Paramecium*, lysosomal acid phosphatase is also L(+)-tartrate sensitive, while a soluble (cytosolic) alkaline phosphatase is sensitive to tetramisole (Fok, 1983). This corresponds to effects observed with mammalian cells. For protease inhibitors, see Section 7.2.2.

4.2. Peroxisomal catalase

The key enzyme, catalase, is normally inhibited by 3-amino-1,2,4-triazole. This is frequently exploited when peroxisomes have to be identified at the electron microscope level by an osmophilic reaction product formed from 3,3'-diaminobenzidine. That this also works with the ciliates, *Tetrahymena* and *Paramecium*, has been shown simultaneously by Fok and Allen (1975) and Stelly *et al.* (1975).

4.3. Mitochondrial activity

Before oxidative phosphorylation/cell respiration can conserve energy as ATP, protons are tunneled from the perimitochondrial space back into the mitochondrial matrix, the F-type ATPase/ATP synthase molecule serving as an H^+ -canal. As also generally known, this is preceded by the electron transfer via the cytochrome chain that can be inhibited by cyanide due to the presence of a heme group in cytochromes. However, in plants a cyanide-insensitive form of cell respiration has been known for some time already, until this was also detected in *Paramecium* (Doussi re *et al.*, 1979). This pathway can be inhibited by salicylhydroxamic acid (SHAM) in plants (Lambers *et al.*, 1983; McDonald *et al.*, 2002) and in *Paramecium* (Doussi re *et al.*, 1979).

It follows that SHAM has to be combined with cyanide for inhibition of oxidative phosphorylation/cell respiration in *Paramecium* to any substantial (95%) degree (Ogura *et al.*, 1985). Antimycin A, another uncoupler of oxidative phosphorylation, is ineffective with *Paramecium* (Doussi re *et al.*, 1979).

4.4. *Clostridium* neurotoxins

Bacterial neurotoxins from *Clostridium* species encompass tetanus toxins (TeTX) and botulinum toxins (BoNT) causing in man extreme contraction (tetanus) or relaxation (botulism). The pathogenic effects are due to interference with neurotransmission in different neuromuscular junctions by blocking exocytotic transmitter release (TeNT: Humeau *et al.*, 2000; BoNT: Marvaud *et al.*, 2002). This is achieved by the metalloendoprotease activity of the toxin light chains which are reported to specifically cleave SNARE proteins (Montecucco and Schiavo, 1995; Montecucco *et al.*, 2004; Schiavo *et al.*, 2000). (SNAREs = soluble N-ethylmaleimide [NEM]-sensitive factor [NSF] attachment protein receptors.) SNAREs first mediate membrane-to-membrane docking and finally fusion (Jahn and Scheller, 2006; Jahn *et al.*, 2003). Both membrane docking and fusion can be inhibited by the *Clostridium* neurotoxins. Unfortunately, NSF as a thiol reagent can react in the intact cell with many molecules (Whiteheart *et al.*, 2001), so its use *in vivo* has been restricted to the context of SNARE functional localization studies with carefully permeabilized *Paramecium* cells (Kissmehl *et al.*, 2002).

A number of isoforms of each type of SNAREs have been identified also in *Paramecium* (Kissmehl *et al.*, 2007; Schilde *et al.*, 2006, 2008). In higher eukaryotes, each of the *Clostridium* toxins recognizes only a specific sequence motif at which it cuts a SNARE protein (Marvaud *et al.*, 2002; Montecucco *et al.*, 2004), and, thus, this property has been used as a diagnostic of SNAREs. The heavy chain is required only for toxin uptake

into cells and finally into their cytosol, but this can be circumvented by injecting light chains (as it has been done with *Paramecium* cells; D. Vetter and H. Plattner, unpublished results).

A specific cleavage activity has been shown with BoNT-A in *Paramecium* cells where it can prevent the redocking of trichocysts which previously had been detached from the surface (D. Vetter and H. Plattner, unpublished results). This in turn has been achieved by high concentrations of cytochalasin B (Pape and Plattner, 1990), that is, when this drug is known to also inhibit plasmalemmal transport systems in higher eukaryotes (see Section 3.3.1). This analysis was facilitated by using a temperature-sensitive mutant cell line, *nd9*. In these cells, under nonpermissive culture temperature conditions, trichocysts are attached only at the alveolar sacs (“docking site I”), rather than forming a functional “docking site II” (required for SNARE-mediated exocytosis) (Pape and Plattner, 1990). This sequence of docking steps is important as SNARE complexes, once assembled, may be insensitive to *Clostridium* neurotoxins (Hayashi *et al.*, 1994; Wellhöner, 1982). In fact, in microinjection series applying different light chain *Clostridium* toxins, only BoNT-A was able to prevent trichocyst redocking (D. Vetter and H. Plattner, unpublished results), suggesting involvement of SNAP25 or of a similar protein in this process. Although later analyses revealed the presence of a SNAP25-like protein in *Paramecium* (Schilde *et al.*, 2008) we also observed that this protein is not cleaved by BoNT-A or -E. The failure of BoNT-E is due to the absence of essential amino acids in the binding and cleavage sites. BoNT-A is inactive probably because some of the amino acids relevant for toxin binding are not contained in the sequence serving as a substrate, even though the cleavage sites may be preserved (Fig. 5.3). Unexpectedly, a mutated (inactive) form of BoNT-A was seen to yield a higher molecular weight complex with another, yet unidentified protein (Schilde *et al.*, 2008).

Therefore, *Clostridium* neurotoxins, some of the most important diagnostics for the identification of SNAREs, do not work in the ciliate analyzed, *Paramecium*.

5. CYTOSKELETON

5.1. Drugs directed against microtubules

Ciliates contain several populations of microtubules, with α - and β -tubulins from different genes (Dupuis-Williams *et al.*, 1996), located at defined sites (Adoutte *et al.*, 1991; Cohen and Beisson, 1988; Fleury *et al.*, 1995). This includes the cell cortex, where they may be involved in surface pattern formation (Aufderheide *et al.*, 1980). Beyond cilia, some “somatic” microtubules emanate from ciliary basal bodies (Plattner *et al.*, 1982). Some of

		BoNT/A α-exosite							BoNT/E B-region		BoNT/E cleavage site				BoNT/A cleavage site				cleaved by BoNT	
		-7	-6	-5	-4	-3	-2	-1	0	+1	+2	+3	+4	+5	+6	+7	+8	E	A	
SNAPc																				
<i>Pt</i> SNAP	118	DDIY	DEMIN	QTNQ	MKYQ	QLLT	NQATT	NCNT	QLDR	INQK	ADK	CTVC	MSKQ	NEKTK	KKTI			174	-	-
<i>Hs</i> SNAP23	153	EENLT	QVGS	TLGN	LKDM	ALNIG	NEIDA	QNPO	TKRIT	DKAD	TNRDR	IDIAN	ARAK	KLI				209	-	-
<i>Hs</i> SNAP25	147	DENLE	QVSG	LIGN	LRHM	ALDVG	NEID	QNRQ	IDRI	EKADS	SNKTR	IDIAN	QRAIT	KML				203	+	+
<i>Rn</i> SNAP23	152	EENLT	QVGS	TLGN	LKMA	LDVG	NEIDA	QNQO	TKQK	ITEK	ADTN	KNR	IDIAN	TRAK	KLI			208	+	+
<i>Hs</i> SNAP29	203	DSNL	DELS	MGLGR	LKDIAL	GMQTE	IEEQ	DDIL	DLRL	ITTK	VDKLD	VNTK	STER	KVRQ	L			258	?	-
<i>Hm</i> SNAP25	154	EQNM	GEVSN	NI	SNLR	MAVD	MGSE	IDSN	RQVD	RINN	KMTS	SNQL	RISD	ANKR	ASKLL			210	?	+
<i>Dm</i> SNAP24	154	DENL	GOVNS	MLGN	LRMA	LDVG	SELEN	QNKQ	VDRI	NAKG	DANN	IRMD	GVN	KRAN	NLL			210	-	-
<i>Dm</i> SNAP25	155	EENMG	QVNT	MIGN	LRMA	LDVG	SELEN	QNRQ	IDRI	NRKGS	NEAR	IAVAN	QRAH	CLL				211	-	-
<i>At</i> SNAP29	193	DEALT	DL	SALL	GEL	LKMA	VDG	GTATER	CTNEL	DHLQ	DNADEL	NYR	VKQ	SNQ	RARYLL			249	?	?
<i>At</i> SNAP30	205	DDGL	SDL	SDTL	LGDL	LKMA	VDG	MGSE	IDK	NKAL	DHLG	DDVD	ELNS	RVQ	GANQ	RARHLL		261	?	?
<i>At</i> SNAP33	242	DDGL	SDL	SDTL	LGEL	LKMA	VDG	MGSE	ITEK	KNKGL	DHLH	DDVD	ELN	FRV	QQS	NQGR	RLL	298	+	+
<i>Sc</i> Sec9p	595	DRNL	DOI	QVSN	RLK	KMAL	TTG	KELD	SCQK	RLNN	ITE	STD	DL	DLN	LHM	NTNR	LACTR	651	-	-
<i>Sc</i> Spo20p	337	YGNL	EQIK	AVSG	DLK	IMAH	AFGR	FEAC	NTRM	FDI	ENN	VQAD	NA	LQAK	RYR	LEKVI		393	-	-

Figure 5.3 Potential binding and cleavage sites for BoNT-A and BoNT-E identified based on the alignment of the Qc-SNARE motif of *Paramecium tetraurelia* SNAP25 (*Pt*SNAP; gi|124391995) with the Qc-SNARE motifs of other SNAP25 homologues. Data compiled on the basis of [Schilde et al. \(2008\)](#). *Homo sapiens* synaptosomal-associated protein 23, SNAP23 (*Hs*SNAP23; gi|1374813), SNAP25 (*Hs*SNAP25; gi|14714976), SNAP29 (*Hs*SNAP29; gi|6685982), *Rattus norvegicus* SNAP23 (*Rn*SNAP23; gi|117558349), *Hirudo medicinalis* SNAP25 homologue (*Hm*SNAP25; gi|1923252), *Drosophila melanogaster* synaptosomal-associated protein 24 SNAP24 (*Dm*SNAP24; gi|8163739), SNAP25 (*Dm*SNAP25; gi|548941), *Arabidopsis thaliana* synaptosomal-associated protein SNAP25-like SNAP29 (*At*SNAP29; gi|15241436), SNAP30 (*At*SNAP30; gi|15222976), and SNAP33 (*At*SNAP33; gi|15240163), *Saccharomyces cerevisiae* t-SNARE component Sec9 (*Sc*Sec9p; gi|730733) and SNAP25 homologue Spo20p (*Sc*Spo20p; gi|6323659). Conserved amino acids of the heptad repeats of the SNARE motif are shown white on black background, residues critical for binding of BoNTs white on gray background and other conserved residues black on gray. Amino acid positions of the respective proteins are indicated on both sides. Boxed are the binding sites for BoNT-A and BoNT-E, respectively, that are necessary for substrate binding as well as their cleavage sites ([Breidenbach and Brunger, 2004](#); [Chen and Barbieri, 2007](#)). The cleavage sites for BoNT-A and BoNT-E are indicated by arrows below. Note that in the *Paramecium* SNAP, most of the amino acids critical for cleavage by BoNT-E are conserved, in contrast to BoNT-A. However, the amino acids critical for toxin binding are not conserved for both these toxins. As a result, *Pt*SNAP is not cleaved.

these run parallel to the cell surface, while some others emanate in vertical direction and reach deep into the cell interior. Some of these help in positioning the nuclei (Adoutte *et al.*, 1991; Cohen and Beisson, 1988), while some others, in *Paramecium* and *Tetrahymena*, serve saltatory docking of mitochondria and trichocysts (Aufderheide, 1977, 1980; Glas-Albrecht *et al.*, 1991; Plattner *et al.*, 1982).

In mammalian cells, a variety of posttranslational modifications of tubulins occur (MacRae, 1997) and the selective positioning and function of posttranslationally modified tubulins has been documented (Banerjee, 2002). In different domains of *Paramecium* and *Tetrahymena* cells, different posttranslational modifications of tubulin have been detected, such as acetylation (cilia), glutamylation, polyglycylation, etc. (*Paramecium*: Adoutte *et al.*, 1991; *Tetrahymena*: Gaertig, 2000; Libusová and Dráber, 2006; Penque *et al.*, 1991; Xia *et al.*, 2000). This variability may be one reason why drug sensitivity of different microtubule subpopulation within individual mammalian cell types may vary considerably, as known since a long time already (Saxton *et al.*, 1984). This is also true for nonciliary microtubules in *Paramecium* whose nocodazole sensitivity decreases with tubulin acetylation (Torres and Delgado, 1989). In general, such microtubules, as they occur in cilia, have very low drug sensitivity.

Nocodazole is routinely used as a microtubule depolymerizing agent with *Tetrahymena* (Grønlien *et al.*, 2002a; Kaczanowski *et al.*, 1985) and *Paramecium* (Cohen and Beisson, 1988). Another standard drug, colchicine, remains totally inefficient even in millimolar (!) concentrations toward cell division in *Paramecium* (Pape *et al.*, 1991) and *Tetrahymena* (Kuzmich and Zimmermann, 1972). This can explain why 20 mM colchicine had to be applied (together with amiloride to reduce ion fluxes; see Section 3.3.1) to avoid reciliation after ethanol-induced deciliation (Schultz *et al.*, 1997). At such extraordinary concentrations colchicine also inhibits some Ca^{2+} -influx channels (Schultz *et al.*, 1997).

A systematic analysis of “antitubulin” drugs on the basis of cell division inhibition in *Paramecium* cells has resulted in the following sensitivity sequence: nocodazole, trifluralin (mainly used with plants; Lignowski and Scott, 1972), parbendazole > the *Vinca* toxins vinblastine > vincristine $\gg$ colchicine (Pape *et al.*, 1991). The first group is efficient at usual, low concentrations without any recognizable side effects. The last group, including colchicine (a standard drug with higher eukaryotic cells), is inefficient even at concentrations exceeding by orders of magnitude those used, for example, with mammalian cells. Only the microtubule population involved in acidosome-mediated phagosome acidification has been reported to be more sensitive to colchicine (Fok *et al.*, 1985). Furthermore, its less toxic derivative, colcemid (*N*-deacetyl-*N*-methylcolchicine), can block in *Tetrahymena* cell division (Kuzmich and Zimmermann, 1972) and

regeneration of cilia after experimental deciliation (Rosenbaum and Carlson, 1969) at much lower concentration.

Vinblastine in tens of micromolar concentration also disrupts a microtubule population relevant for cell division in *Stentor* (Diener *et al.*, 1983). The microtubule stabilizing agent, taxol, obtained from the *Taxus* (yew) tree is also efficient in *Paramecium* (Pape *et al.*, 1991). Later on, a taxol derivative was used to label microtubule subpopulations in a variety of ciliates (Arregui *et al.*, 2002). Interestingly both stereoisomers of tubulazole are active in *Paramecium* (Pape *et al.*, 1991), just like in *Plasmodium* (Dieckmann-Schuppert and Franklin, 1989), whereas in mammalian cells only the *cis*-form is active.

To establish a feedback between exposure to “antitubulin” drugs and transcription of the α -tubulin gene in *Tetrahymena*, again almost 10 mM colchicine, but only 10^3 times lower concentrations of oryzalin had to be applied, while taxol remained without any effect (Stargell *et al.*, 1992).

Considering the spectrum of posttranslational modifications of tubulins in ciliates, it remains open to what extent the different drug sensitivities indicated above would apply to the variety of other microtubule subpopulations. In fact, a wide variation of nocodazole sensitivity has been reported for some *Paramecium* mutants, from hypersensitive (Jerka-Dziadosz *et al.*, 1998) to resistant (Torres *et al.*, 1991).

Taken together, data currently available indicate that the sensitivity of ciliate microtubules to established drugs differs widely from that in higher eukaryotes. Nocodazole and possibly some drugs effective in plants may be exceptional. Molecular modeling with the published sequences of ciliate tubulins and identification of drug-binding sites would be a rewarding task.

5.2. Drugs directed against actin

Both monomeric (globular) and polymeric (filamentous) forms of actin, G- and F-actin, respectively, can be stabilized by specific drugs routinely used in cell biology of higher eukaryotes. Among the first type are cytochalasin B or D (binding to the barbed end of actin filaments) as well as latrunculin A. The second type is represented by the bicyclic peptides phalloidin and jaspilakinolide. Side effects have been reported for cytochalasin B (Cooper, 1987), more than for cytochalasin D. The drugs shift the G-/F-actin equilibrium unilaterally, implying that F-actin can be removed by cytochalasins or latrunculin, but stabilized by phalloidin or jaspilakinolide (Allingham *et al.*, 2006; Cooper, 1987). The drug-target interaction is mediated by up to six specific amino acids serving as specific binding sites for the respective type of depolymerizing, and by three amino acids for repolymerizing drugs (phalloidin: Belmont *et al.*, 1999; Drubin *et al.*, 1993; jaspilakinolide: Bubb *et al.*, 1994; latrunculin A: Ayscough *et al.*, 1997). As shown in Fig. 5.4, only a subset of *Paramecium* actin isoforms possess these binding sites in full or in part (Sehring *et al.*, 2007). When an antibody

against a conserved *Paramecium* actin domain (that may recognize mainly subfamily 1 members; [Sehring et al., 2007](#)) was used for immunogold electron microscope analysis, this labeled, among other details, some cortical filaments as well as the surface of phagocytic vacuoles ([Kissmehl et al., 2004](#)). How does that compare with drug effects?

Already early experiments with *Paramecium*, using cytochalasin B, have cast serious doubts on its capability to “dissolve” microfilaments, while a variety of overt artifacts had been observed ([Sibley et al., 1977](#)). Later on, cytochalasins (mainly type D) have been applied again in studies particularly with

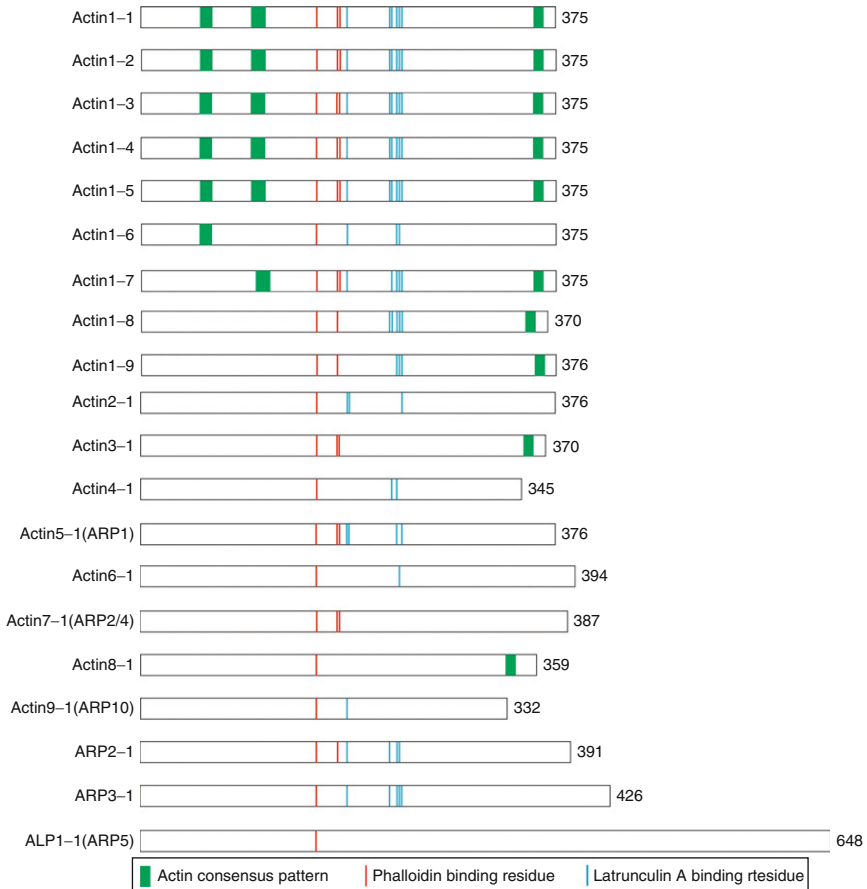


Figure 5.4 Characteristics of *Paramecium* actin. Not only some actin isoforms in *P. tetraurelia* may miss the actin consensus pattern (green), but also some are devoid of the binding motifs for F-actin stabilizing (red, phalloidin) and F-actin destabilizing drugs (blue, latrunculin A, valid also for cytochalasins). From [Sehring et al. \(2007\)](#). Green (1st two and last bar/all broad bars), red (3rd to 5th bar), blue (6th to 11th bar).

Tetrahymena (Grønlien *et al.*, 2002a) and *Paramecium* (Allen and Fok, 1985; Cohen *et al.*, 1984; Kersken *et al.*, 1986a,b; Zackroff and Hufnagel, 1998), for example, to inhibit phagocytosis—a clearly actin-based process. Different steps, that is, pinching off, acidosome and lysosome fusion and retrieval of “ghosts” from spent phagosomes from the cytoproct, had different cytochalasin B sensitivity (Allen and Fok, 1983; Fok *et al.*, 1985, 1987). Concentrations of the different cytochalasins and of their derivatives required to impair phagosome formation vary considerably, though they are consistently above those required in “higher” cell systems (Zackroff and Hufnagel, 1998). Interestingly, when *Tetrahymena* cells are exposed to such drugs over several cell cycles phagocytosis may become drug insensitive (Zackroff and Hufnagel, 2002). One may consider substitution by less drug-sensitive isoforms as they occur in *Paramecium* (Sehring *et al.*, 2007), but this has not been elucidated.

Surprisingly docking of trichocysts to the cell membrane can also be inhibited by cytochalasin B (Beisson and Rossignol, 1975) which, in contrast to cytochalasin D, can also undock trichocysts under appropriate conditions (Pape and Plattner, 1990); see also “*Clostridium* toxins” (Section 4.4). This is surprising since actin is unlikely to be present directly at docking sites proper although trichocyst tips are surrounded by some actin (Kissmehl *et al.*, 2004). Since side effects have been published for cytochalasin B (see Section 3.3.1) but hardly for cytochalasin D, this may point out an effect which is unrelated to actin, altogether. Only in the last few years studies on the role of actin in secretory organelle docking and in removing empty “ghosts” after exocytosis have been very much intensified with mammalian cells (Giner *et al.*, 2007). No similar studies are available from ciliates.

Many effects of actin-binding drugs would be expected considering the manyfold involvement of F-actin in cellular processes (Soldati and Schliwa, 2006; see also “background” references in Sehring *et al.*, 2007) and its wide distribution inside, for example, a *Paramecium* cell (Kissmehl *et al.*, 2004; Sehring *et al.*, 2006). This ranges from cilia, basal bodies, trichocyst tips, cytopharynx, to different stages of phagocytic vacuole processing and, finally, the cytoproct. Widely different isoforms are encoded by 25 genes (Sehring *et al.*, 2007) and many of them are differentially localized (Sehring *et al.*, 2006). Their sensitivity to “standard” drugs must widely vary, considering the absence of some or all binding sites for drugs that normally would favor de- or repolymerization (Sehring *et al.*, 2007). For instance, the isoform forming the cleavage furrow, PtAct4, has no phalloidin-binding sites (Sehring *et al.*, 2007), as documented in Fig. 5.4, thus explaining its insensitivity previously recognized (Cohen *et al.*, 1984). Vice versa, high concentrations of cytochalasin B had also to be used, for example, in *Euplotes aediculatus*, to inhibit conjugant separation (Geyer and Kloetzel, 1987).

Fluorescently labeled phalloidin was microinjected into *Paramecium* cells to pinpoint F-actin in the cell cortex and around phagocytic vacuoles, including nascent ones (Kersken *et al.*, 1986a,b). After longer times, cortical

label disappeared, while transcellular filament bundles not previously seen emerged (Figs. 5.5 and 5.6). This may be a good way to manipulate intracellular F-actin for any further functional analyses.

Our summarizing view is as follows. Cytochalasin D as well as phalloidin (and probably the somewhat smaller jasplakinolide molecule) may act specifically as “antiactin” drugs also in the ciliates analyzed so far. However, the effects achieved with “antiactin” drugs may encompass only a small fraction of a large spectrum of actin forms and of their functions, as suggested by the manifold, sometimes pleiotropic effects of gene-silencing experiments (Sehring *et al.*, 2006). Furthermore, the high divergence of actins from different ciliate groups (Kim *et al.*, 2004) may require even more scrutinized work. So far, no such extensive analyses have been made with any ciliate other than *Paramecium*. Some of the results previously reported in the ciliate literature depended on the application of unusually high drug concentrations, so that their relevance may require re-examination.

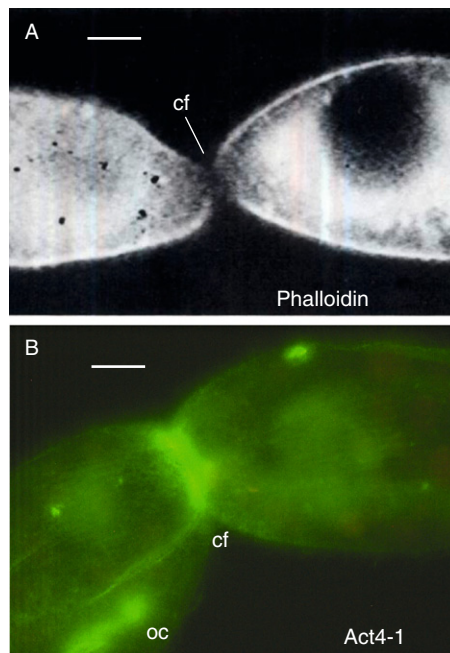


Figure 5.5 Labeling of the cleavage furrow in *P. tetraurelia* cells. Dividing *Paramecium* cells never showed any labeling of the cleavage furrow (cf) with injected fluorescently labeled phalloidin (A), but a cleavage furrow can be visualized with antibodies against *PtAct4-1* that is specifically incorporated into the cleavage furrow (B). Also note cortical labeling in (A) and antibody labeling of the oral cavity (oc) in (B). Bars = 10 μ m. (A) is from Kersken *et al.* (1986a) and (B) is an unpublished micrograph from the series published by Sehring *et al.* (2006).

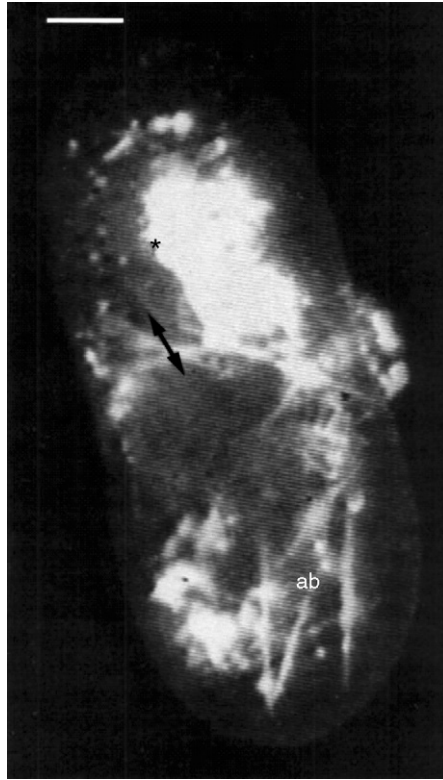


Figure 5.6 Microinjected fluorescently labeled phalloidin over longer time periods causes rearrangement of F-actin in a *P. tetraurelia* cell. Note removal of cortical staining (to be compared with Fig. 5.5A) and occurrence of transcellular fluorescently labeled filament bundles. Asterisk marks site with an (unlabeled) contractile vacuole, “ab” labels newly formed actin bundles and the double arrow the position of the oral apparatus. Bar = 10 μm . From Kersken *et al.* (1986b).

6. SIGNALING, INTRACELLULAR TRAFFICKING, AND SIGNAL TRANSDUCTION

6.1. Protein kinases and phosphatases

Protein phosphorylation and dephosphorylation are important processes during signal transduction. The plethora of protein kinases includes Ser/Thr- and Tyr-kinases. While phosphorylation of Ser/Thr residues is very common in protists, Tyr-phosphorylation is still highly questionable. Any attempts to block (receptor) Tyr-phosphorylation, for example, by genistein in the ciliates *Paramecium* and *Tetrahymena* (Robinette *et al.*, 2008) face the

absence of any genes encoding Tyr-kinases in these organisms according to recent progress in genomics. This also compromises results achieved with antibodies against Tyr-phosphorylation sites in *Tetrahymena* (Christensen *et al.*, 2003). Any prognostication of Tyr-phosphorylation sites relying on mammalian databases imply similar structural details for substrate–enzyme interaction that, in most cases, have not been verified. Moreover, real proof of Tyr-phosphorylation would require additional methods, for example, mass spectroscopy. Identification of useful drugs would require binding studies. At this time, it looks as if Tyr-phosphorylation would first appear in evolution in those protists that show close relationship to multicellular organisms. Among them are choanoflagellates (King *et al.*, 2008; Li *et al.*, 2008) and *Dictyostelium* (Williams *et al.*, 2005) that both also possess cell adhesion molecules or precursors thereof. In most recent work, Manning *et al.* (2008) found a plethora of Tyr-kinases in choanoflagellates, but neither in *Dictyostelium* nor in *Tetrahymena*. However, in the latter two genera, they found conventional Tyr-specific phosphatases and phospho-Tyr-binding sites.

Generally there is considerable uncertainty about the efficiency of protein kinase inhibitors in ciliates. Staurosporine (a “broad band,” i.e., non-specific protein kinase inhibitor; Rüegg and Burgess, 1989) and genistein have been used to study the effect on phagocytosis in the ciliate *Uronema* (Hartz *et al.*, 2008). In *Paramecium primaurelia*, synthesis of the glycosylphosphatidylinositol (GPI) anchor of variant surface antigens has been inhibited by low concentrations of two protein kinase inhibitors: (1-[5-isoquinoline-sulfonyl]-2-methylpiperazine, H-7) and staurosporine (Azzouz *et al.*, 2001). H-7 also inhibited a cGMP-dependent protein kinase (Miglietta and Nelson, 1988). These biochemical experiments with *Paramecium* indicate that such inhibitors may be potentially useful, although probably without high specificity.

Among protein phosphatases (PP), types 1, 2A, and 2C in mammalian cells are inhibited by okadaic acid, microcystin, and some other toxins (Rusnak and Mertz, 2000), at characteristic concentrations, while 2B (calcineurin) is not (Cohen *et al.*, 1990). The sequence of sensitivity is PP2A > PP1 > PP2C $\gg$ 2B (practically insensitive). The inhibitory concentrations required are, thus, one of the criteria for classification of these phosphatases. PP2B is usually sensitive specifically to the immunosuppressant cyclosporin A. In *Paramecium*, PP1, PP2A, and PP2C (Klump *et al.*, 1990b) as well as PP2B had been identified by biochemical methods (Kissmehl *et al.*, 1997) and by molecular biology (Fraga *et al.*, 2008). In contrast to mammalian orthologs, PP2A-like activity in *Paramecium* is practically insensitive to okadaic acid (Klump *et al.*, 1990a). The effect of cyclosporine A and rapamycin on PP2B of ciliates remains to be elucidated. This would be even more important as calcineurin governs a variety of cellular functions (Aramburu *et al.*, 2004), probably also in *Paramecium* where it is localized to distinct sites (Momayezi *et al.*, 2000).

Our conclusion is as follows. The examples discussed above clearly show once again how much background information is needed for a useful application of allegedly specific drugs in ciliates. The reverse sensitivity of PP1 and 2A in *Paramecium* to standard inhibitors (Klumpp *et al.*, 1990a) caution one to simply transfer data from higher eukaryotes to ciliates. While Ser/Thr-kinases and phosphatases are well established, tyrosyl phosphorylation in ciliates remains elusive. Any further pharmacology of protein kinases and phosphatases in ciliates has to be complemented by molecular biology and biochemistry.

6.2. Monomeric G-proteins

A very high number of sequences encoding monomeric G-proteins show up in the *Paramecium* database. Among them, ADP-ribosylation factors (Arf) are of particular interest because of their key role in vesicle trafficking (Hurtado-Lorenzo *et al.*, 2006). While Arf has not yet been characterized in ciliates, modulators of small G-proteins (GTPases) such as guanine nucleotide exchange factors (GEF) have been annotated (Mouratou *et al.*, 2005). They contain only partially conserved sequences, so that this may also be expected for Arf. Knowledge of the Golgi-relevant Arf1 ortholog would be particularly interesting as the Arf1-binding site in the mammalian ortholog is blocked by the toxin brefeldin A—a cyclopentanecrotonic acid derivative isolated from *Penicillium brefeldianum* (Donaldson *et al.*, 1992). This is commonly used to manipulate the Golgi apparatus in higher eukaryotes (Klausner *et al.*, 1992). The reversible dissociation of Golgi stacks by brefeldin A-mediated inhibition of ER → Golgi vesicle transport would be an important diagnostic. While brefeldin A can be used to monitor Golgi → apicoplast trafficking (DeRocher *et al.*, 2005), however, even in plants disturbingly high concentrations had to be used (Moreau *et al.*, 2007). This is even more so with *Paramecium* (Kissmehl *et al.*, 2007) where we unfortunately have also been unable in several attempts to pinpoint any of the otherwise typical Golgi-specific antigens as GFP-fusion proteins. Encouraged by Golgi-specific sequences retrieved from the *P. tetraurelia* genomic database we made attempts with the adaptor protein component AP1μ1 and with GRASP, but no labeling has been achieved with different constructs (C. Schilde and H. Plattner, unpublished results). This and the low sensitivity to brefeldin A underscores the importance of further attempts to look for pharmacological tools for any further analysis of Golgi dynamics in the future.

In a combination of overexpression as GFP-fusion proteins combined with fluorescence microscopy and anti-GFP antibody labeling at the EM level, we could localize to the *Paramecium* Golgi apparatus essentially two components of the trafficking machinery, namely Syx5 and Sec22 (Kissmehl *et al.*, 2007). Both were sensitive to brefeldin A, though at “unorthodox,”

high concentrations. Therefore, it appeared advisable to support these data by electron microscopic immunoanalysis. We, thus, could assign *PtSyx5* and *PtSec22* to Golgi structures of low brefeldin A sensitivity (Kissmehl *et al.*, 2007).

It should be noted that the general assumption of selective inhibition of trimeric, but not of monomeric G-proteins, by AlF_4^- (Barritt and Gregory, 1997) is difficult to maintain as this compound also inhibits other processes, for example, some mediated by monomeric G-proteins of the Arf-type (Le Stunff *et al.*, 2000) and P-type ATPases such as the SERCA-type Ca^{2+} -ATPases (Inesi *et al.*, 2006; Missiaen *et al.*, 1988). With the exception of Arf, pharmacology of this important aspect of vesicle trafficking is poor in general and in particular with ciliates.

6.3. Trimeric G-proteins, PI_nSP_2 turnover, nucleotide cyclases, and phosphodiesterases

Most eukaryotic cells, from *Dictyostelium* on (Williams *et al.*, 2005), can dynamically assemble trimeric G-proteins from an α -, β -, and γ -SU. This complex, when coupled to receptors with seven-transmembrane domains, can mediate signal transfer from the cell surface to the interior. As also generally known, this can encompass activation, for example, of an adenylylate cyclase or a phospholipase C (PL-C). The latter mediates turnover of phosphatidylinositol-4,5-bisphosphate (PI_nSP_2), as generally known. As a consequence, protein kinase A (PK-A) can be activated, or the cleavage products from PI_nSP_2 , diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($InsP_3$) can activate protein kinase C (PK-C) and $InsP_3$ receptor-type Ca^{2+} -release channels, respectively. With higher eukaryotes, based on this textbook knowledge, pharmacology of these different aspects is frequently exploited in cell biology. A full cycle has been ascribed, for instance, to *Tetrahymena*, based on drug effects (Hassenzahl *et al.*, 2001)—but how solid is the information behind?

6.3.1. Trimeric G-proteins

First of all, the substrate of PL-C, PI_nSP_2 , does occur in *Paramecium* (Kaneshiro, 1987) and *Tetrahymena* (Leondaritis *et al.*, 2005; Wang *et al.*, 2001). However, evidence for the existence of trimeric G-proteins in ciliates (*Paramecium*) is circumstantial. It is based on the following observations: First, the occurrence of genomic sequences compatible with a β -SU (Forney and Rodkey, 1992) and second, the binding of commercial (heterologous) ABs against α - and β -SU and the ADP-ribosylation by pertussis toxin of the α -SU, with molecular weights of ≥ 41 and ≤ 36 kDa, respectively (De Ondarza *et al.*, 2003). Occurrence of trimeric G-proteins in *Paramecium* has also been implied from experiments on GPI anchor biosynthesis, based on the experience with higher eukaryotes, which in

Paramecium, however, contains ceramide (Kaneshiro *et al.*, 1997). This assumption for GPI synthesis in *Paramecium* was supported by additional hints, such as the inhibitory effect of the nonhydrolyzable GTP analogue, GTP- γ -S, and the modulatory effect of cholera and pertussis toxin (Azzouz *et al.*, 2001). Furthermore, injected GTP- γ -S activates, while GTP- β -S inhibits some Ca^{2+} -currents in the euryhaline species, *Paramecium calkinsi* (Bernal and Ehrlich, 1993) and in *P. multimicronucleatum* (Nakaoka *et al.*, 1997) as if trimeric G-proteins would modulate those Ca^{2+} -channels. Similarly some signatures compatible with the occurrence of G-proteins and protein kinase C are found in the membrane-bound form of the pheromone Er-1 of *Euplotes raikovi* (Ortenzi *et al.*, 2000). Also fragmentary hints have been collected with *Stentor coeruleus* (Marino *et al.*, 2001).

In an analysis of a putative GABA_B-type (metabotropic) receptor that in mammals is coupled to trimeric G-proteins, and its endocytosis in *Paramecium*, a variety of inhibitors have been applied (Ramoino *et al.*, 2003, 2006). This included the GABA_B receptor agonist baclofen (4-amino-3-[4-chlorophenyl]butanoic acid), and inhibitors of endocytosis via clathrin- and caveolin-specific endosomes. Also in this case it would now be possible and, therefore, mandatory to document the occurrence of sequences assignable to the GABA_B receptor and to caveolin in the *Paramecium* genome and/or proteome.

It follows from all this fragmentary knowledge about the aspects addressed in this subchapter that this is one of the most important fields to be settled in the future to achieve a firm basis of signal transduction in ciliates.

6.3.2. Adenylate cyclase

In studies on phagocytosis with the ciliate *Uronema*, the antagonist for the α -SU, NF023, has been applied (Hartz *et al.*, 2008), although no pharmacological or molecular background is known from that system. As to adenylate cyclase, the mammalian β -adrenergic activator isoproterenol has been applied to *Paramecium* in conjunction with the (non-cAMP specific) adenylate cyclase activator forskolin and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (Wiejak *et al.*, 2007). (Note that contradictory effects of isoproterenol alone on digestive vacuole processing have been published with *Tetrahymena* (Fok and Shockley, 1985).) In *Paramecium*, however, the classical activators and inhibitors may be problematic to use without detailed biochemical analyses and molecular biology, considering the following results achieved *in vitro*. In fact, there is some contradiction in the literature. When the purified enzyme was analyzed neither fluoride or forskolin (Kudo *et al.*, 1985; Schultz *et al.*, 1987) nor cholera toxin, pertussis toxin, or GTP analogs exerted any effect (Schultz *et al.*, 1992). In contrast, forskolin at remarkably low concentrations was able to efficiently modulate cAMP levels in *Euplotes* during pheromone signal transduction (Apone

et al., 2003). Here, the signal transduction pathway could also be interrupted by the membrane-permeable derivative *N*-2'-*O*-dibutyryl-cyclic AMP which is more resistant to phosphodiesterase. Also *in vivo*, formation of the GPI anchor of variant surface antigens in *P. primaurelia* was stimulated by both, forskolin and another cAMP derivative, Br-cAMP (Azzouz *et al.*, 2001), but the level of interference remains uncertain. Again *in vivo*, the forward swimming response of *Paramecium* to different drugs affecting cyclic nucleotide effects showed wide variation: While 3-isobutyl-1-methylxanthine and monobutyryl-cAMP (in contrast to dibutyryl-cAMP) caused accelerated swimming, the cyclase activator forskolin had no effect (Bonini *et al.*, 1986). 3-Isobutyl-1-methylxanthine affected the cAMP-dependent chemoresponse to different stimuli (Van Houten, 1990).

In mammalian cells, isoquinoline-type inhibitors of protein kinases, H7 and H8, are reported to inhibit more or less selectively protein kinase C and A, respectively (Hidaka *et al.*, 1984). Both inhibited hyperpolarization-induced accelerated forward swimming in *Paramecium* (Yang *et al.*, 1997). This is known to involve cAMP formation (Schultz and Klumpp, 1993) within a subsecond time scale (Yang *et al.*, 1997), and, concomitantly, protein kinase A (Hamasaki *et al.*, 1989).

This brief overview confronts us with numerous positive results achieved with drugs related to cAMP turnover *in vivo* that for unknown reasons are in contrast to the inefficiency of such drugs with the isolated enzymes.

6.3.3. Phospholipase C and protein kinase C

The *Paramecium* and *Tetrahymena* databases contain no stringent evidence with sufficient statistical significance, neither for a PL-C nor for a PK-C. However, in *Paramecium* an InsP₃ receptor-type Ca²⁺-release channel with typical features could be cloned, including a channel domain and an InsP₃-binding domain containing the amino acid residues crucial for InsP₃ binding (Ladenburger *et al.*, 2006); see Fig. 5.1. This does not stringently imply occurrence of a PL-C pathway since, for instance in *Dictyostelium*, a PL-C-independent pathway of InsP₃ formation has been established (Van Dijken *et al.*, 1995). Also the effects of Li⁺ on chemotactic response in *Paramecium* were said not to be directly attributable to phosphoinositide metabolism according to the responses to other inhibitors (Wright *et al.*, 1992). In fact, the early steps of this signal transduction along the pathways under consideration remain largely open in ciliates. What we have additionally found in our work on InsP₃ receptors in *Paramecium* is the spontaneous occurrence of small, short Ca²⁺-puffs along the elements of the contractile vacuole complex, that is, where we can produce a significant Ca²⁺-response by uncaging of caged InsP₃ and where we have localized InsP₃ receptors by the electron microscopic immunogold technique (Ladenburger *et al.*, 2006). This may imply that search of dramatic effects in response to defined cell stimulation

may lead one to an irrelevant track, unless such a phenomenon could be detected in the future. It remains to be stated that with ciliates, no experience exists with established InsP_3 receptor inhibitors, such as xestospongins, as used with higher eukaryotes.

As to PK-C, circumstantial evidence of its occurrence in *Paramecium* has been obtained using the stimulating effect of a phorbol ester and the inhibitory effect of peptides interfering with a PK-C substrate, MARCKS (myristoylated alanine-rich C-kinase substrate) *in vivo* (Hinrichsen and Blackshear, 1993). Similarly Wijek *et al.* (2001) have described effects of a phorbol ester and of the PK-C inhibitor GF 109203X on endocytosis in *Paramecium* when exposed to an agonist of mammalian β -adrenergic activator.

Altogether we recognize considerable gaps in the biochemical and pharmacological aspects discussed in the present context. Considering the availability of powerful tools from molecular biology, we should no more feel comfortable with circumstantial evidence only.

6.3.4. Ca^{2+} /polycation-sensing receptor

In higher eukaryotic systems trimeric G-proteins are also coupling the extracellular “ Ca^{2+} /polyvalent cation-sensing receptor” to a PL-C-based signal transduction pathway in mammalian cells (Breitwieser *et al.*, 2004; Brown, 2000). These receptors were originally defined as a sensor for changes in $[\text{Ca}^{2+}]_e$ to which some mammalian cells may become exposed, allowing for a rapid response by intracellular signal transduction (Hofer, 2005). They turned out to mediate a rapid response also to extracellular polycations in a variety of cells (Quinn *et al.*, 1997; Shorte and Schofield, 1996). Both these aspects would be important also for ciliate cell biology. First, these cells may “see” widely varying $[\text{Ca}^{2+}]_e$. Second, the polycation AED is meanwhile a generally accepted and frequently applied secretagogue (Plattner *et al.*, 1984, 1985) that perfectly mimics natural stimuli for exocytosis in *Paramecium* (Plattner and Klauke, 2001). Later on, the highly positively charged protein, lysozyme, has been introduced as a secretagogue in *Paramecium* (Hennessey *et al.*, 1995); simultaneously it has been prized as a chemorepellent. From our previous work, it appears clear that the secretagogue effect operates in a similar or even identical way as AED (Plattner *et al.*, 1984, 1985), while the chemorepellent effect may be due to a spillover of Ca^{2+} into cilia, also as with AED in *Paramecium* (Husser *et al.*, 2004; Plattner *et al.*, 1984).

As in mammalian cells (Breitwieser *et al.*, 2004), the Ca^{2+} /polyvalent cation-sensing receptor in *Paramecium* clearly follows the rule saying that the stimulation effect increases with the number/density of amination residues (Plattner *et al.*, 1985). Remarkably, mere addition of extracellular Ca^{2+} to the medium causes an immediate increase of $[\text{Ca}^{2+}]_i$, but no exocytosis ensues (Erxleben *et al.*, 1997). Whether the signal transduction underlying

AED stimulation follows the PL-C-mediated pathway is not clear, particularly since some other pathways also seem to emerge for the Ca^{2+} /polycation-sensing receptor in higher eukaryotic systems (Brown, 2000). Direct activation of a variety of plasmalemmal cation-conducting channels has been discussed for mammalian systems (Williams, 1997) and envisaged as a possibility also for *Paramecium* (Klauke *et al.*, 2000).

6.4. Overall section summary

In sum, clearly no stringent molecular data have been provided so far for trimeric G-proteins in ciliates. Thus, any pharmacological approaches must be considered as merely tentative. In general terms, all receptor studies with ciliates would require substantiation by profound analyses on a molecular level. There are good modulators available for cAMP-dependent responses in ciliates, although it remains unexplained why results achieved *in vitro* differ from those *in vivo*. Even more problematic is the pharmacology of PL-C and PK-C in ciliates. Also to be settled are important details of the signal transduction pathway during stimulation by exogenous polycations.

7. OTHER FACTORS AND TOOLS

7.1. Glycosylphosphatidylinositol anchor

GPI-anchored “variant surface antigens” cover the entire surface of ciliates. They have been analyzed in great detail in *Paramecium* (Azzouz *et al.*, 2001) and to some extent in *Ichthyophthirius* (Clark *et al.*, 2001). In the latter, a fish skin parasite, variant surface antigens are of particular interest with regard to host cell infection. In *Paramecium*, their function remains unsettled, though their production rate may surmount that in other cell types. Here, ceramide (a sphingosine base covalently bound to an acyl chain in a way resembling a peptide bond) serves to fix the GPI anchor in the cell membrane (Azzouz *et al.*, 1995, 2001). Inhibitors of enzymes required for ceramide synthesis have been established in pathogenic flagellates (Denny *et al.*, 2006), but not yet probed with ciliates. Van Houten and her group have manipulated the expression of GPI-anchored variant surface proteins in *Paramecium* and also shown the effects on chemotactic responses (Paquette *et al.*, 2001). Among the inhibitors and activators they used some affecting trimeric G-proteins (see Section 6.3.1) and protein kinases (see Section 6.1).

GPI-anchored surface proteins should be releasable from the *Paramecium* cell surface by heterologous GPI-specific phospholipase C (Azzouz *et al.*, 1995). Though an endogenous enzyme (Deregnaucourt, 1992), probably constitutively produced (Paquette *et al.*, 2001), does exist and putative genes for this enzyme have been found in the database, its characterization and

subcellular localization still awaits elucidation. *In vitro*, the enzyme-mediated release of GPI-anchored proteins could be inhibited by *p*-chloromercuri-phenylsulfonic acid (Deregnaucourt *et al.*, 1988) and 2-nitro-4-carboxy-phenyl-*N,N*-diphenylcarbamate (Paquette *et al.*, 2001). A partial sequence of a GPI-specific phospholipase C gene has been reported from *Euplotes crassus* (Klobutcher *et al.*, 1991), but this has not been followed up. Remarkably this type of enzymes from bacterial sources, when applied to *Tetrahymena*, has no effect, as observed early on (Ko and Thompson, 1992) and many times later on with different ciliates.

In sum, there are compounds available from the literature that can inhibit the cleavage of the GPI anchor also in ciliates. For inhibition of the synthesis of phosphatidylinositol-containing proteins in *Tetrahymena* (Pak *et al.*, 1991), see also Sections 7.2.1 and 7.3.2.

7.2. Protein synthesis and degradation

7.2.1. Inhibitors of protein synthesis

With ciliates, few systematic analyses have been performed with inhibitors of protein synthesis. Early on, cycloheximide has been shown to inhibit protein synthesis at rather low concentrations in *Tetrahymena* (Wang and Hooper, 1978) where it also inhibits regeneration of cilia after deciliation (Rosenbaum and Carlson, 1969). To inhibit formation of new secretory organelles, cycloheximide has been eventually applied in conjunction with ³H and ³⁵S amino acid labeling and polyacrylamide gel electrophoresis/autoradiography in *Paramecium* and *Tetrahymena*, respectively (Pape and Plattner, 1990; Turkewitz *et al.*, 1991). In experiments with *Paramecium*, inhibition of trichocyst biogenesis during redocking (after previous detachment from the cell membrane) has also been achieved with some other protein synthesis inhibitors, puromycin and actinomycin D, and electrophoresis was eventually combined with electron microscopic autoradiography (Pape and Plattner, 1990). Similarly mucocyst reformation has been inhibited by cycloheximide or actinomycin D (Sauer and Kelly, 1995). Biosynthesis of phosphatidylinositol-containing proteins in *Tetrahymena* was also inhibited by cycloheximide (Pak *et al.*, 1991). Also with *Tetrahymena*, cycloheximide resistance of modified cells has been exploited to assess phenotypic assortment (Bowman *et al.*, 2005; Doerder *et al.*, 1992) and actinomycin D to inhibit protein synthesis in the course of α -tubulin expression rundown experiments (Stargell *et al.*, 1992). The differential sensitivity of *Chlorella* endosymbionts and their hosts, *Paramecium bursaria*, to cycloheximide and puromycin, respectively, was taken advantage of to produce algae-free paramecia (Ayala and Weis, 1987).

All these data support the usefulness of cycloheximide, possibly also actinomycin D and other compounds, as inhibitors of protein synthesis in ciliates.

7.2.2. Protease inhibitors

These may be considered from two points of view, first, as inhibitors of artificial protein degradation during cell fractionation and, second, as tools for elucidating *in vivo* functions.

Protease inhibitors are indispensable for preserving proteins during isolation and subsequent processing by biochemical methods. Ciliates seem to be particularly sensitive to endogenous proteases or these are of rather low sensitivity to some of the inhibitors generally used, for instance, phenylmethylsulfonylfluoride (PMFS) (Straus *et al.*, 1992). Cocktails of increasing complexity have been designed (although the necessity of all of the components included has not been specified in detail). Early on, pepstatin, chymostatin, leupeptin, aprotinin, benzamidine, and *o*-phenanthroline have been applied with *Tetrahymena* (Turkewitz *et al.*, 1991). A systematic analysis with *T. thermophila* have qualified antipain, chymostatin, leupeptin, as well as CPI I and CPI II (calpain inhibitor I and II) as most powerful inhibitors of artificial proteolysis (Straus *et al.*, 1992). Subsequently, Sauer and Kelly (1995) have reduced the number of inhibitors to leupeptin, chymostatin, and antipain. With *Paramecium*, in the absence of such systematic studies and to be on the safe side, we usually combine antipain, aprotinin, chymostatin, E64, leupeptin, Pefabloc SC, and pepstatin A, sometimes complemented by TAME (*N*-*p*-tosyl-L-arginine methylester) and Na-(*p*-toluene sulfonyl)-L-arginine, occasionally also by PMFS. For details, see recent publications (e.g., Ladenburger *et al.*, 2006 for *Paramecium*; Melia *et al.*, 1998 for *Tetrahymena*).

In more specific terms, addressing cathepsin L (a cysteine protease), in *Paramecium* the most useful inhibitors are cystatin, *N*^α-tosyl-lysylchloromethane (Suzuki *et al.*, 1998; Völkel *et al.*, 1996) and E64, complemented by the protease inhibitor leupeptin (Suzuki *et al.*, 1998). E64 was also useful for affinity-based isolation of cathepsin L from *Tetrahymena* (Herrmann *et al.*, 2006). Posttranslational processing of secretory products from *Tetrahymena* mucocysts by endopeptidase cleavage has been inhibited by *N*-tosyl-L-phenylalanyl-chloromethyl ketone (Collins and Wilhelm, 1981).

Altogether there is a collection of useful protease inhibitors available for ciliates.

7.3. Miscellaneous

7.3.1. Lithium and lanthanum

The potential use of Li⁺ for downregulating InsP₃ receptors in *Paramecium* and its potential pitfalls has been discussed by Ladenburger *et al.* (2006); see Sections 3.2.1 and 6.3.3. La³⁺ as a diagnostic tool to specify SOC in *Paramecium* in conjunction with fluorochrome analyses has been used by Klauke *et al.* (2000). Of course, the pharmacological use of Li⁺ and La³⁺ and

of some other cations depends on the context of analysis and of support by some other independent criteria. See also [Section 6.3.3](#) for Li^+ -effects.

7.3.2. Glycosylation

N-glycosylation of proteins is an important posttranslational modification with implications concerning folding control and targeting. This has been also shown with *Toxoplasma gondii* where the standard inhibitor, tunicamycin, is effective at the usual low concentrations ([Luk *et al.*, 2007](#)). Indications for ciliates are scarce. In *Tetrahymena*, the synthesis of phosphatidylinositol-containing proteins was inhibitable by tunicamycin ([Pak *et al.*, 1991](#)) which may affect their glycan moiety.

7.3.3. Fatty acid synthesis

Fatty acid synthesis is inhibited, as shown in *Paramecium*, by $\leq 10 \mu\text{M}$ cerulenin ([Rhoads *et al.*, 1987](#)), the standard inhibitor that binds to the β -ketoacyl carrier protein synthase contained in the heteromultimeric fatty acid synthase.

7.3.4. Various pharmacological tools applied to ciliates

Surprisingly *Paramecium* and *Tetrahymena* react by chemotactic reactions to a variety of drugs that otherwise are known from mammalian pharmacology, such as capsaicin, etc. ([Rodgers *et al.*, 2008](#)). Any more pharmacological details are unknown.

Many other drugs from the standard repertoire of mammalian cell biology have been applied to ciliates. Examples are inhibitors of antiporters such as amiloride and some others, such as doxorubicin (adriamycin) or EIPA (5-[N-ethyl-N-isopropyl]amiloride), some less specific “anti-CaM” drugs such as wortmannin or the antipsychotic (“neuroleptic”) drugs, phenothiazines chlorpromazine and trifluoperazine, as well as the local anesthetic dibucaine. (Note that the last three can release trichocyst, probably by somehow mobilizing Ca^{2+} —whatever the mechanism may be.) Only rarely have the effects of these drugs been followed up in detail. Consider the following examples. Trifluoperazine was able to inhibit in *Paramecium* plasmalemmal CaM-activated influx channels, just like any of the more specific “anti-CaM” drugs ([Erxleben and Plattner, 1994](#)). HPLC analysis after wortmannin application in *Tetrahymena* revealed its capacity to inhibit replenishing phosphatidylinositol-3-phosphate and phosphatidylinositol-3,5-phosphate pools ([Leondaritis *et al.*, 2005](#)). Heparin, ruthenium red, AlF_4^- , and fluoride are some other chemicals with widely different effects in other eukaryotes. Finally, one has to consider the pleiotropic effects of the frequently used aminoglycoside drug, neomycin (see [Section 3.2.1](#)).

Compounds forming pores in membranes by complexing 3-hydroxy-sterols, such as cholesterol, work equally well in ciliates as in mammalian

cells, as the principle is the same. This holds true of filipin (Olbricht, 1984), digitonin, and saponin. We routinely used the latter two, recently preferably saponin, for cell permeabilization. In particular saponin at $\sim 0.01\%$ allows for so careful permeabilization of *Paramecium* cells that supravital effects can be studied (Kissmehl *et al.*, 2002).

In retrospect, a variety of side effects have been published for most drugs over the years, while they were prized to be specific for certain cellular functions at the beginning. This may be even more so with lower eukaryotes, such as ciliates. Many of the data collected with ciliates over the years, unpublished or published (Lumpert, 1990), frequently appear rather problematic.

8. CONCLUDING REMARKS

The pharmacology of higher eukaryotic cells, notably mammalian cells, has accumulated considerable knowledge and experimental potential, be it on a merely pragmatic or on a defined molecular basis. In contrast, pharmacology of lower eukaryotes lags far behind. Most drugs established for higher eukaryotic systems are not applicable to lower eukaryotes because of lacking effects or requirement of unusually high concentrations with likely side effects. This also holds true of ciliated protozoa, *Paramecium* and *Tetrahymena* being the most favored model organisms. Their cell biological investigation is, thus, missing very important tools. The great number of drugs applied to ciliates has only occasionally been tested for their molecular effects. Many compounds have been used only on a pragmatic basis and more or less tacitly accepted by the scientific community when some effects had been achieved with “reasonable” concentrations (comparable to mammalian cells).

In retrospect, such trials (many of which have not been included here) may have appeared justified in the past, in the absence of molecular biology as a tool that became available only in quite recent years. However, nowadays any further use of pharmacological modulators must be backed by molecular biology and/or biochemistry. In retrospect, this postulate emerges from the large number of failures in ciliate pharmacology with allegedly established drugs, particularly when confronted with facts emerging from molecular biology (e.g., absence of binding sites, etc.).

With ciliates, only a small selection of drugs have reliable “orthodox” effects (Table 5.1). Examples are CaM inhibitors, as found by electrophysiological analysis of Ca^{2+} /CaM-activated plasmalemmal Me^{+} - or Me^{2+} -influx channels. Other examples are some (only some!) drugs activating Ca^{2+} -release channels in intracellular Ca^{2+} -stores or some (again only some!) drugs inhibiting the Ca^{2+} -ATPase (SERCA-type pump) in such

stores. However, even in these cases, “standard” activators (ryanodine) or inhibitors (thapsigargin) are frequently ineffective. An impressive warning is the observation that the effect of artemisinin as a SERCA inhibitor in *Plasmodium* depends on a single amino acid (Uhlemann *et al.*, 2005). Widely different, orthodox or aberrant, types of reaction are also seen with tubulin- and actin-directed drugs. Considerable differences may occur between paralogs even within one species, for example, with actins in *Paramecium*. Most ion channel blockers (with the exception, e.g., of TEA⁺) and many other compounds that are highly efficient in higher eukaryotes proved ineffective in ciliates. Ongoing analysis of the *P. tetraurelia* and *T. thermophila* genomes reveals some clues to this discrepancy. This may include aberrant sequences in the drug interaction domains, as shown for the SERCA inhibitor thapsigargin, for the actin-directed drugs phalloidin, jaspalakinolide, and latrunculin A, as well as for *Clostridium* toxins.

Such analyses become increasingly important, not only to establish specific drugs as tools in the cell biology of ciliates (and their relatives, the parasitic Apicomplexa), but also to elucidate the pharmacological/toxicological background of some ecological aspects as well as some evolutionary aspects of (in)sensitivity to drugs and consequently to avoid their experimental abuse in ciliates. Unfortunately, it appears that drug sensitivity in the different members of the phylum Alveolata may widely diverge. Moreover, some drugs may have widely different effects in different protists. *Dictyostelium* is a striking example in so far as, for example, xestospongin and W7 inhibit targets quite different from those described above for mammals or ciliates (Malchow *et al.*, 2008). Also in contrast to ciliates, *Dictyostelium* appears to be the only member of the protozoan kingdom for which existence of receptors with seven transmembrane domains (Prabhu and Eichinger, 2006), usually coupled to trimeric G-proteins, and tyrosine phosphorylation have been documented on a molecular level (Williams *et al.*, 2005).

From another point of view, it remains a challenging question why certain proteins contain “orthodox”-binding sites for some drugs (e.g., for phalloidin in some of the *Paramecium* actin isoforms) which they never “see” in nature. Why are such signatures retained up to the mammalian level, while the drugs are inefficient with some of the isoforms in ciliates—even within one species? Are such drug-binding sites functionally important in a way yet to be determined (for instance, polymerization properties and/or capacity to interact with some other proteins)? Or, are they transmitted during evolution as an irrelevant feature allowing for later acquirement of a selective property? In other words, is the capacity to bind certain drugs part of a selection mechanism during evolution?

In retrospect, pharmacology of ciliates lags far behind that in higher eukaryotes and, thus, requires more systematic work in the future. Looking into the future, steadily accumulating data from molecular biology may

Table 5.1 Selection of some drugs that may potentially prove useful for experiments with live ciliates^a

Drug	Pharmacological effect	Useful conc. ^b	References
W7	Anti-CaM effect	~10–100 μ M	Erxleben and Plattner (1994) and Hennessey and Kung (1984)
Calmidazolium	Anti-CaM effect	≥ 1 μ M	Ehrlich <i>et al.</i> (1988)
TEA ⁺	K ⁺ -channel inhibitor	~1 mM	Eckert and Brehm (1979) and Erxleben and Plattner (1994)
Veratridine ^c	See Section 3.1.1 for ciliates	1 mM	Blanchard <i>et al.</i> (1999)
4-Cl- <i>m</i> -cresol	Ryanodine substitute	500 μ M	Klauke <i>et al.</i> (2000)
Concanamycin B	H ⁺ -ATPase inhibitor	30 nM	Grønlien <i>et al.</i> (2002b)
A23187/ionomycin	Ca ²⁺ -ionophore	70 μ M	Plattner (1974)
Monensin	Na ⁺ /H ⁺ exchanger	20 μ M	Gautier <i>et al.</i> (1994)
L(+)-tartrate	Acid phosphatase inhibitor	10 mM	Fok (1983)
3-Amino-1,2,4-triazole	Catalase inhibitor	1–20 mM	Fok and Allen (1975) and Stelly <i>et al.</i> (1975)
Salicylhydroxamic acid	Inhibitor of cyanide-insensitive respiration	1 mM	Doussière <i>et al.</i> (1979)
Nocodazole	Microtubule destabilizer	>15 μ M	Pape <i>et al.</i> (1991)
Parbendazole	Microtubule destabilizer	>1.2 μ M	Pape <i>et al.</i> (1991)
Taxol	Microtubule stabilizer	≥ 2 μ M	Pape <i>et al.</i> (1991)
Cytochalasin B, D	F-actin destabilizer	450 μ M	Cohen <i>et al.</i> (1984)
		40 μ M	Zackroff and Hufnagel (1998)
Phalloidin	F-actin stabilizer	≥ 20 μ g/ml ^d	Kersken <i>et al.</i> (1986b)
Cycloheximide	Protein synthesis inhibitor	0.7–9 mM	Pape and Plattner (1990) and Turkewitz <i>et al.</i> (1991)

(continued)

Table 5.1 (continued)

Drug	Pharmacological effect	Useful conc. ^b	References
Protease inhibitors	See Section 7.2.2	See References	Melia et al. (1998) and Ladenburger et al. (2006)
Okadaic acid	Protein phosphatase inhibitor ^e	See Section 6.1	Klumpp et al. (1990a)
Aminoethyl-dextran ^f	Activator of exocytosis, SOC	$\geq 1 \mu\text{M}$	Plattner et al. (1985)
Cerulenin	Fatty acid synthesis inhibitor	$9 \mu\text{M}$	Rhoads et al. (1987)
Saponin	Membrane permeabilization ^g	$\sim 0.01\%$	Routine laboratory use

^a This list is not complete and actual usefulness of a certain drug has to be explored with the actual ciliate cell type and under the conditions of culturing, for example, ions contained in the medium and other variables. Also note that the table does not discriminate between drug effects on different cell functions (e.g., effects of “antitubulin” drugs on cell division or intracellular transport). Before use the original literature should be consulted, particularly on concentrations to be applied.

^b Pilot values, for rough orientation only (see also footnote a); consult original literature.

^c Veratridine in *Paramecium* activates somatic Ca^{2+} -influx channels, while its “orthodox” effect (with mammalian cells) is to keep Na^+ -channels in an open state. Its effect greatly depends on $[\text{Ca}^{2+}]_e$.

^d Equivalent to $25 \mu\text{M}$, injected into *Paramecium* cells.

^e Okadaic acid has reverse effects on PP1 and PP2A in *Paramecium* as compared to mammalian cells ([Klumpp et al., 1990a](#)).

^f AED effect depends on degree of amination (number of NH_2 -groups per dextran carrier).

^g Formation of small pores in membranes by complexation 3-hydroxy-sterols, for example, cholesterol. Under carefully selected conditions, cells can survive about 30 min ([Kissmehl et al., 2002](#)).

yield important clues to a reliable pharmacology of ciliates. A rational basis of drug use will also provide an invaluable tool for ongoing use of ciliates as powerful model systems in cell biology.

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CELL AND MOLECULAR BIOLOGY OF NUCLEAR ACTIN

Wilma A. Hofmann

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Abstract

Actin is a highly conserved protein and one of the major components of the cytoplasm and the nucleus in eukaryotic cells. In the nucleus, actin is involved in a variety of nuclear processes that include transcription and transcription regulation, RNA processing and export, intranuclear movement, and structure maintenance. Recent advances in the field of nuclear actin have established that functions of actin in the nucleus are versatile, complex, and interconnected. It also has become increasingly evident that the cytoplasmic and nuclear pools of actin are functionally linked. However, while the biological significance of nuclear actin has become clear, we are only beginning to understand the mechanisms that lie behind the regulation of nuclear actin. This review provides

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an overview of our current understanding of the functions of actin in the nucleus.

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1. INTRODUCTION

Actin is a 43-kDa protein that was originally identified and purified by [Straub \(1942\)](#) in Albert Szent-Gyorgyi's lab in Hungary. It was initially purified as a major component of the contractile proteins from skeletal muscle and is still best known as filamentous protein that is involved in muscle contraction. Actin is a highly conserved, widely distributed protein that is present in eukaryotic and prokaryotic cells alike.

In the cytoplasm actin exists in two forms, G-actin and F-actin. Muscle cell actin is organized into relatively stable thin filaments. In the cytoplasm of nonmuscle cells, actin monomers also readily polymerize in an ATP-dependent process to form microfilaments. However, in contrast to thin filaments in muscle cells that are very stable, microfilaments are highly dynamic in nature. That is, they can form rapidly, rearrange, and reform. Considering the multiple forms of actin in combination with the fact that actin is also one of the most abundant proteins in eukaryotic cells, comprising 1–15% of the total proteins, it is not surprising that the functions of actin are numerous. As a component of the cytoskeleton, actin is involved in cell motility and maintenance of the cell shape; in endocytosis, exocytosis, and secretion; in cell division; mRNA transport and translation; in signal transduction, and synaptic transmission.

The forms and functions of actin in the cytoplasm are regulated by a vast array of actin-binding proteins (ABPs). To date, 60–100 types of ABPs have been identified ([dos Remedios et al., 2003](#)). ABPs can be roughly divided into two groups. One regulates assembly and disassembly of actin filaments as well as the length, stability, and form of the actin filaments. They also regulate interactions between actin filaments and other components of the cytoskeleton. The other group consists of the superfamily of molecular motors called myosins that bind to actin and use the energy from ATP hydrolysis to generate force for the unidirectional movement along actin filaments.

The presence of actin in cell nuclei appears to have been first reported by Ohnishi and colleagues who purified actin from nuclei of calf thymus cells ([Ohnishi et al., 1963, 1964](#)). The early work on nuclear actin was met with extensive criticism and the very presence of actin in the nucleus was questioned for many years, which was partly due to the fact that thymocyte

nuclei are particularly difficult to purify. Another main problem was the detection of actin in the nucleus by immunofluorescence. Most of the known functions of actin in the cytoplasm involve polymerization into filaments, which can be stained by fluorescent phalloidin. However, under normal conditions, nuclei are not stained by phalloidin. Therefore, actin detected in isolated nuclei or subnuclear fractions was thought to represent a cytoplasmic contaminant due to the high amount of actin protein in the cytoplasm.

Finally, however, [Clark and Merriam \(1977\)](#) and [Clark and Rosenbaum \(1979\)](#) demonstrated the presence of actin in *Xenopus* oocyte nuclei beyond any doubt. Removal of the nuclear envelope of manually isolated oocyte nuclei and minimal cytoplasmic contamination revealed the presence of actin in the remaining nuclear gel. [Bertram et al. \(1977\)](#) reported changes in actin concentrations in nuclei of mouse fibroblasts depending on the proliferative state of cells, while the amount of cytoplasmic contamination stayed the same. [Pederson \(1977\)](#) used a refined method to isolate chromatin from *Dictyostelium discoideum* in which the cytoplasmic contamination of the purified chromatin fraction was estimated to be less than 0.3%. He reported that the concentration of actin in this chromatin fraction exceeded the cytoplasmic concentration by several orders of magnitude ([Pederson, 1977](#)). Subsequently, actin was also detected in nuclei of mammalian cells by immunoelectron microscopic studies. [Nakayasu and Ueda \(1985a\)](#) and [Miller et al. \(1991\)](#) showed that cell-permeable crosslinkers like potassium chromate or *cis*-platinum can crosslink actin to DNA. Now, almost half a century later it is clear that actin is a constitutive component of somatic cell and oocyte nuclei. A selected list of organisms and cell types in which actin has first been identified is shown in [Table 6.1](#).

The amount of actin in nuclei seems to be consistent among species as well as cell types. Actin has been estimated to comprise 4–11% of the nuclear nonhistone proteins in *Physarum polycephalum* ([Jockusch et al., 1974](#)), HeLa cells ([Peterson and McConkey, 1976](#)) rat liver cells ([Douvas et al., 1975](#)), and amphibian oocytes ([Clark and Merriam, 1977](#); [De Robertis et al., 1978](#); [Gounon and Karsenti, 1981](#); [Lestourgeon et al., 1975](#)). In various amphibian oocytes this translates into an amount of 0.1–0.22 μg and a concentration of 2.4–3.2 mg/ml ([Clark and Rosenbaum, 1979](#); [Merriam and Hill, 1976](#); [Scheer et al., 1984](#)).

In the last decade enormous progress has been made in identifying the functions of actin in the nucleus. Considering the amount of nuclear actin and extrapolating from the diversity of functions of cytoplasmic actin, it is not surprising that actin in the nucleus has been implicated in a variety of nuclear processes. While the biological functions of nuclear actin have become clearer, the mechanisms of how actin works in the nucleus are still not understood, however.

Table 6.1 Selected list of species and cell types in which nuclear actin has been identified

Species and/or Cell type	References
Protists	
<i>Physarum polycephalum</i>	Jockusch <i>et al.</i> (1971, 1974)
<i>Amoeba proteus</i>	Goldstein <i>et al.</i> (1977b)
<i>Dictyostelium discoideum</i>	Fukui (1978); Pederson (1977)
<i>Tetrahymena pyriformis</i>	Katsumaru and Fukui (1982)
<i>Acanthamoeba castellanii</i>	Kumar <i>et al.</i> (1984)
<i>Acetabularia</i>	Soyer-Gobillard <i>et al.</i> (1996)
<i>Dinoflagellate</i>	Soyer-Gobillard <i>et al.</i> (1996)
Plants	
<i>Sauromatum guttatum</i>	Skubatz <i>et al.</i> (2000)
<i>Allium cepa</i>	Cruz <i>et al.</i> (2008)
Insects	
<i>Spodoptera frugiperda</i>	Volkman (1988)
<i>Drosophila melanogaster</i>	Sauman and Berry (1994)
Amphibians	
<i>Xenopus laevis</i>	Merriam and Hill (1976)
<i>Pleurodeles waltlii</i>	Gounon and Karsenti (1981)
<i>Rana temporaria</i>	Parfenov <i>et al.</i> (1995)
Birds	
duck erythroblasts	Maundrell and Scherrer (1979)
chicken liver	Crowley and Brasch (1987)
Mammalian cell types	
Thymus cells	Ohnishi <i>et al.</i> (1963, 1964)
Liver cells	Douvas <i>et al.</i> (1975)
Epithelial cells	Lestourgeon <i>et al.</i> (1975)
Myoblasts	Paulin <i>et al.</i> (1976)
Kidney cells	Pagoulatos and Yaniv (1978)
Ovary cells	Brunel and Lelay (1979)
Lymphocytes	Nakayasu and Ueda (1983)
Neural cells	Amankwah and De Boni (1994); Milankov and De Boni (1993); Sahlas <i>et al.</i> (1993)
Oocytes	Funaki <i>et al.</i> (1995)

This review will focus on the one hand on the emerging biological functions of nuclear actin. On the other it will highlight some of the challenges that we face when trying to understand the mechanisms behind the biological functions of nuclear actin.

2. BIOLOGICAL FUNCTIONS OF NUCLEAR ACTIN

The nuclear processes in eukaryotic cells involve the preservation of the genetic information, that is, DNA replication and repair. On the other hand, extra- and intracellular signals are transmitted into the nucleus where they regulate the transcriptional activity of genes. Nuclear processes that are involved in effective gene regulation include intranuclear movement of chromosome regions, as well as remodeling and modification of these regions. Transcription itself requires binding of transcription factors to regulatory regions of DNA, formation of preinitiation complexes, and recruitment of RNA polymerase complexes before RNA is synthesized as transcription proceeds. The resulting RNA finally has to be processed and exported out of the nucleus. Within 10 years after the identification of actin in the nucleus, nuclear actin had been implicated in most of these processes.

It is obvious that all events that lead to the regulation of gene expression are highly interconnected, which makes it difficult to correlate an observed effect with a specific process. An additional complication arises from the close proximity in which these events occur inside the nucleus. However, during the last decade advances in methodology have allowed us to isolate many of these processes and analyze them separately, which in turn has led to a better understanding of the involvement of actin.

2.1. Structure of the nucleus

Actin functions in the assembly and stabilization of the nuclear envelope. The nuclear lamina is associated with the inner nuclear membrane and the peripheral chromatin. In most cells it is mainly composed of A- and B-type lamins as well as lamin-associated proteins (Stewart *et al.*, 2007). Clubb and Locke (1998) described the existence of a layer of actin inside the nucleus along the nuclear envelope that colocalized with lamin, suggesting a lamin-actin perinuclear shell. Actin was also shown to interact with the C-terminal domain of lamin A (Sasseville and Langelier, 1998) as well as with the lamina-associated protein emerin (Fairley *et al.*, 1999; Lattanzi *et al.*, 2003). It has been suggested that a nuclear protein complex consisting of emerin, actin, and lamin exists at the nuclear envelope that stabilizes the nuclear membrane against mechanical stress (Holaska and Wilson, 2007; Holaska *et al.*, 2004). In addition, the importance of actin in nuclear envelope assembly was demonstrated in a cell-free *Xenopus* egg extract in which actin accumulates at the inner nuclear membrane after the lamina forms (Zhang *et al.*, 1996). Later it was found that an interaction between actin and the 4.1R protein is necessary for nuclear envelope assembly (Krauss *et al.*, 2002, 2003).

Actin has also been proposed to play an important role in maintaining the internal nuclear structure through association with a nuclear fraction referred to as a “nuclear matrix”. The nuclear matrix fraction is defined as a chromatin-free, insoluble framework after extractions of nuclei with detergents, digestion with nucleases, and solubilization with salt buffers. It has been suggested that such a matrix exists *in vivo* playing an important role in maintaining spatial order within the nucleus. Proposed functions of the nuclear matrix are connected to DNA replication and/or repair, gene transcription, RNA splicing, and transport (Berezney, 2002; Berezney *et al.*, 1996; Hancock, 2000). Actin was shown to be associated with this isolated fractions in various cell types (Bachs *et al.*, 1990; Capco *et al.*, 1982; Fey *et al.*, 1984; Mattern *et al.*, 1996, 1997; Nakayasu and Ueda, 1984, 1985a,b, 1986; Okorokov *et al.*, 2002; Schroder *et al.*, 1987; Valkov *et al.*, 1989; Verheijen *et al.*, 1986). Even though the nucleus can be partitioned into functional compartments and it is appealing to think of an underlying “nuclear skeleton” that organizes these compartments, it should be noted that the very existence of the nuclear matrix is controversial and is being discussed extensively (Pederson, 1998, 2000).

However, a number of recent studies seem to confirm an intranuclear filament network in the *Xenopus* oocyte model, with actin as an integral component. Kiseleva *et al.* (2004) analyzed *Xenopus* oocyte nuclei by field emission scanning electron microscopy and saw filaments containing actin and protein 4.1. It was suggested that this network of filaments provide chromatin free channels through which RNPs or proteins could diffuse inside the nucleus (Kiseleva *et al.*, 2004). Bohnsack *et al.* (2006) analyzed sections of *Xenopus* oocyte nuclei after cryofixation by confocal microscopy and identified bundles of actin filaments. It was suggested that these meshwork of intranuclear actin filaments might stabilize the mechanical integrity of *Xenopus* oocyte nuclei (Bohnsack *et al.*, 2006; Gall, 2006).

In summary, the body of existing data suggests that actin contributes to a variety of protein associations whose arrangement with the nuclear outline and in the nuclear interior positions them to function as organizers of the nuclear structure. However, the existing data come mainly from the *Xenopus* oocyte model. Further studies are needed to elucidate the functions of actin in association with the nuclear envelope and other nuclear structures in the oocyte model system as well as in somatic cells.

2.2. Chromatin remodeling

The structure of chromatin and epigenetic marking of histones is of extreme importance in regulating gene transcription. A group of multiprotein complexes is involved in transcription regulation by modifying histones or altering the chromatin structure. These complexes can be divided into two groups, chromatin-remodeling and chromatin-modifying complexes. The chromatin-remodeling complexes are ATP-dependent complexes that

use the energy of ATP hydrolysis to remodel chromatin by locally disrupting or altering the association of histones with DNA. The chromatin-modifying complexes, on the other hand, are the histone acetyltransferase (HAT) and histone deacetylase complexes (HDAC). They regulate the transcriptional activity of genes by altering the level of acetylation of nucleosomal histones associated with the genes. Actin and actin-related proteins (Arps) can be found in complexes with a wide variety of chromatin remodeling and modifying complexes.

Arps constitute a group of proteins that share 17–60% homology with conventional actin and are considered to be an evolutionarily conserved class of eukaryotic proteins. Even though all Arps share sequence homology, members of the Arp family are diverse among themselves. A common structural feature shared by Arps and conventional actins is the so-called actin fold that contains the ATP/ADP-binding site. However, only Arp1 and Arp4 have been shown to bind and hydrolyze ATP. Moreover, several studies have demonstrated substantial differences in the surface structure of individual Arps, suggesting that Arps are functionally distinct (Frankel and Mooseker, 1996).

Actin and Arps were first identified as integral components of the mammalian SWI/SNF chromatin-remodeling complex (Zhao *et al.*, 1998). To date actin and Arps have been found in a wide variety of chromatin-remodeling and modifying complexes as stoichiometric components. Szerlong *et al.* (2008) recently identified a specific domain, the helicase-SANT-associated (HAS) domain, in a number of chromatin altering complexes that is necessary for the binding of actin and Arps. Alterations in this domain resulted in a loss of actin and Arps in these complexes and in a loss of function, confirming the importance of actin and Arps as components of chromatin remodeling and modifying complexes. While there is accumulating information about functions of Arps in chromatin-remodeling and modifying complexes (Blessing *et al.*, 2004; Chen and Shen, 2007; Farrants, 2008; Meagher *et al.*, 2005; Olave *et al.*, 2002) not much is known about the function of actin itself in chromatin remodeling. However, it was shown that actin is required for the ATPase activity of the Brg 1 subunit of the SWI/SNF complex and involved in the association of this complex with nuclear structures (Zhao *et al.*, 1998). Figure 6.1 shows the functions that have been suggested for actin and Arps as components of chromatin altering complexes. The implication of actin in both the structure and biochemistry of SWI/SNF complexes in combination with the recent identification of a common actin and Arp binding domain in chromatin altering complexes makes it imperative to study further its functional contribution to chromatin remodeling.

2.3. Intranuclear chromosome movement

In a eukaryotic cell, the genomic DNA can be morphologically and functionally divided into heterochromatin and euchromatin. Our current understanding is that heterochromatin is a densely packed region of the

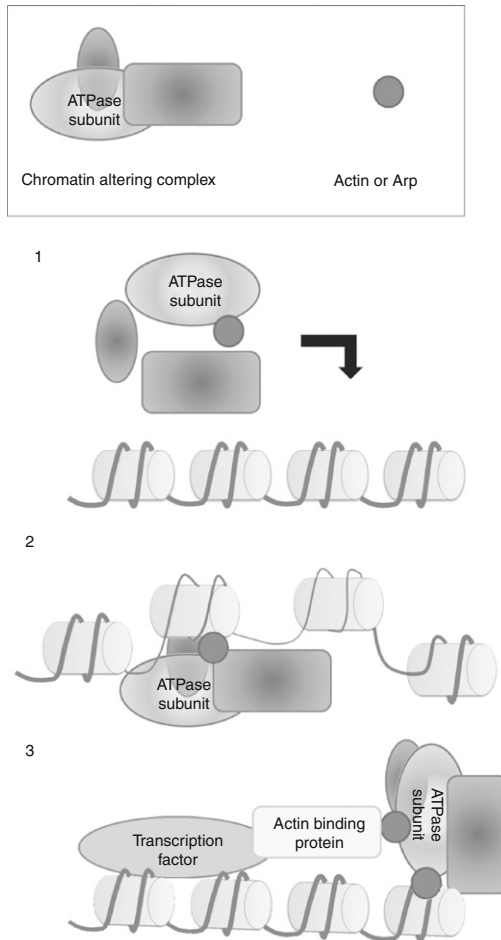


Figure 6.1 Potential functions for actin and actin-related proteins in chromatin remodeling. (1) Assembly and structural integrity of the chromatin remodeling complexes. Deletion studies on yeast chromatin remodeling and modifying complexes suggest that actin and certain Arps are important for assembly and structural integrity of these complexes. Deletion of Arp8 in the INO80 complex, for example, led to the absence of not only Arp8 but also actin and Arp4 (Shen *et al.*, 2003). Arp7 and Arp9 have been shown to be important for the structural integrity of the yeast SWI/NSF and RSC chromatin remodeling complexes (Cairns *et al.*, 1998; Peterson *et al.*, 1998) and Arp4 is required for the structural integrity of the NuA4 chromatin modifying complex (Galarneau *et al.*, 2000). (2) Association of chromatin altering complexes with the chromatin. *In vitro* studies have shown that several Arps are able to bind directly to core histones or nucleosomes (Galarneau *et al.*, 2000; Harata *et al.*, 1999; Shen *et al.*, 2003). These findings suggest that Arps could have a function in targeting and/or anchoring of these complexes to the chromatin. (3) Recruitment through interaction with actin-binding proteins. As described in Section 3.3 an increasing number of actin-binding proteins are being identified as transcription factors and transcriptional coregulators. These actin-binding proteins could act as transcriptional regulators by facilitating the recruitment of chromatin altering complexes through their interaction with the actin present in these complexes.

nucleus, often close to the nuclear periphery, that constitutes a transcriptionally repressive environment. Euchromatin on the other hand constitutes spread out chromosomes within the nuclear interior that are easily accessible to transcription factors and represents that fraction that is actively transcribed. However, this chromatin organization is dynamic and changes in chromosome rearrangement occur during mitosis, meiosis, and sometimes when gene translocations take place upon transcription activation or inhibition.

Numerous studies have shown an association of actin with various states of chromatin (Milankov and De Boni, 1993; Sauman and Berry, 1994). It was suggested that actin plays a role in mitotic and meiotic chromosome condensation and segregation in various organisms and cell types (Goldstein *et al.*, 1977a; Jockusch *et al.*, 1971; Sanger, 1975). Actin was also implicated in chromatin rearrangement during apoptosis (Widlak *et al.*, 2002) where actin was found to localize in the center of fragmented nuclei with the suggestion for a role in nuclear fragmentation (Neradil *et al.*, 2005). Recent studies have now demonstrated that actin and the actin-binding protein nuclear myosin I (NMI) are involved in the translocation of activated genes (Fig. 6.2). Chuang *et al.* (2006) used a transgenic gene activation assay in which the VP16 activator domain was introduced into an inactive chromosome region that usually locates at the periphery of the nucleus. The authors showed that, upon activation, this domain translocates from the periphery towards the interior of the nucleus in an active and directed process that is independent of the transcriptional activity. Moreover, and most relevant to the present review, it was demonstrated that this curvilinear movement is facilitated through an actin-myosin interaction (Chuang *et al.*, 2006).

An involvement of actin in intranuclear chromosome movement was also confirmed by Dundr *et al.* (2007). In this study, the authors analyzed the association of snRNA genes with Cajal bodies that occurs when these genes are actively transcribed. Monitoring fluorescent proteins that were tagged to inducible U2 snRNAs genes as well as to the Cajal body proteins demonstrated that inactive U2 snRNA genes do not associate with the Cajal bodies. Upon activation however, the chromosome region containing the U2 snRNA genes moved towards the Cajal bodies. In agreement with Chuang *et al.* (2006), the authors found that this movement is also directed and actin dependent.

Myosins are a large family of actin based molecular motors. All myosins have a defined domain organization with an N-terminal head that contains the ATP- and actin-binding sites (motor domain), a neck region with IQ motifs that bind to calmodulin or light chains, and a highly variable tail domain which is important for cargo binding. They can roughly be divided into two subclasses, the conventional myosin II and the unconventional myosins. While the myosin II associate with their tails to form filaments, the unconventional myosins do not form filaments. Myosins are by definition

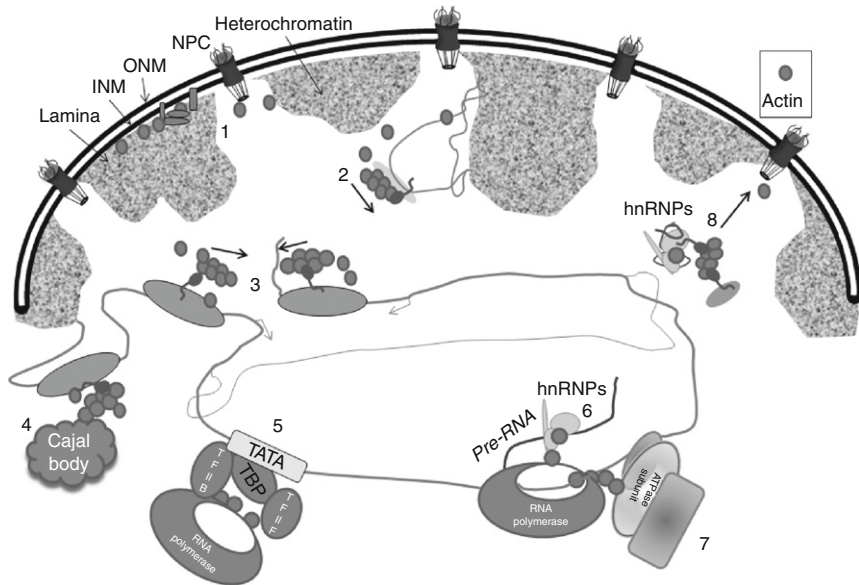


Figure 6.2 Nuclear functions of actin. (1) Assembly, stabilization, and connection of the nuclear envelope through interaction of actin with emerin, lamin A, and protein 4.1 (Section 2.1) 2–4 Actin facilitated intranuclear chromosome movement (Section 2.2). (2) Movement of chromosomes away from heterochromatin after activation of transcription. (3) Chromosomal reorganization during transcription activation. (4) Movement of chromosome regions towards Cajal bodies. (5) Assembly of transcription initiation complexes (Section 2.4.1). (6–7) Regulation of transcription elongation through recruitment of chromatin modifying complexes and RNA packaging proteins (Section 2.4.2). (8) Nuclear export of proteins and RNA (Section 2.5). ONM: outer nuclear membrane; INM: inner nuclear membrane; NPC: nuclear pore complex.

motorprotein which means that they use the hydrolysis of ATP to perform mechanical work. Most importantly, the ATPase activity of myosin depends on the binding to actin.

There are several open questions in regard to the actin-myosin facilitated movement of chromosome regions. One of them is how both are recruited to the sites that are being moved. While Chuang *et al.* (2006) showed that the movement is independent of active, ongoing transcription, the extent to which chromatin remodeling of these sites plays a role in determining the subsequent movement is not clear. Both, actin (Section 2.2.) and NMI (Cavellan *et al.*, 2006; Percipalle *et al.*, 2006) are components of chromatin remodeling complexes (Fig. 6.1). Therefore, it is possible that both are recruited through their association with these complexes. However, actin as a component of the SWI/SNF complex also seems to have a role during the process of remodeling by itself (Zhao *et al.*, 1998). It remains to be established if and how the functions of actin during chromatin remodeling and

chromosome movement are connected. Another interesting question concerns the directionality of the movement, specifically how it is determined in which direction the chromosome region moves. While gene translocation towards the interior of the nucleus seems to be a common mechanism, other genes have been found to translocate towards the outer regions of the nucleus (Bartova *et al.*, 2002).

2.4. Actin in transcription

Eukaryotic cell nuclei contain three distinct classes of RNA polymerases that are termed RNA polymerase I, II, and III. Each polymerase synthesizes specific RNAs. RNA polymerase I is located in a specific nuclear compartment, the nucleolus, where it is responsible for ribosomal RNA (rRNA) synthesis (except 5S rRNA). RNA polymerase II is located in the nucleoplasm and responsible for the transcription of all protein-coding genes into messenger RNA (mRNA) and for transcription of most small nuclear RNAs (snRNA) and most microRNA precursors. RNA polymerase III is also localized in the nucleoplasm, transcribing 5S rRNA, transfer RNA (tRNA), U6 snRNA, and signal recognition particle RNA among other RNAs.

Actin was first mentioned in connection with the transcriptional process in 1979 when Smith *et al.* (1979) identified actin as a protein that copurified with RNA polymerase II from the slime mold *P. polycephalum*. Subsequently, actin was discovered in transcriptionally active extracts from human HeLa cells and from calf thymus (Egly *et al.*, 1984). This study indicated that actin might act as a coactivator for transcription. The first clear evidence for a direct role of actin in transcription was provided by Scheer *et al.* (1984). The authors showed that microinjecting antibodies directed against actin, as well as ABPs like fragmin, into the nuclei of amphibian oocytes, led to a retraction of chromosome loops, sites at which active transcription takes place (Scheer *et al.*, 1984). A close association of actin with the polymerase was also observed in insect cell nuclei and it was suggested that actin is responsible for the recruitment of the RNA polymerase to the DNA (Sauman and Berry, 1994). Experiments in one-cell mouse embryos showed an association of actin with the newly formed transcription sites upon activation of transcription (Nguyen *et al.*, 1998), a finding that was also observed in human lymphocytes after activation of transcription (Kysela *et al.*, 2005). As a number of recent studies have elucidated the role of actin in transcription it has become obvious that actin is involved in the process of transcription in more than one way (Fig. 6.2).

2.4.1. Basal transcription

2.4.1.1. RNA polymerase I Actin has been shown to localize not only in the nucleoplasm but also in the nucleolus in both mammalian oocytes and somatic cells (Funaki *et al.*, 1995; Nowak *et al.*, 1997; Soyer-Gobillard

et al., 1996). Kysela *et al.* (2005) used electron microscopy to show that most of the nucleolar actin localizes to fibrillar centers in resting lymphocytes. During activation of these cells, the total amount of actin in the fibrillar centers decreased while the amount of actin in the granular compartment increased, indicating an recruitment of actin from possible storage sites to sites of active RNA polymerase I transcription. A colocalization with actively transcribing ribosomal genes was shown by Fomproix and Percipalle (2004) and Philimonenko *et al.* (2004) and the direct involvement of actin in transcription by RNA polymerase I was demonstrated by Philimonenko *et al.* (2004) and Ye *et al.* (2008). Actin physically associates with the RNA polymerase I core enzyme independent of ongoing transcription (Philimonenko *et al.*, 2004) as well as with the promoter of coding regions of rDNA genes (Philimonenko *et al.*, 2004; Ye *et al.*, 2008). Furthermore, microinjection experiments showed that antibodies directed against actin inhibit RNA polymerase I transcription in *in vivo* as well as *in vitro* transcription assays.

2.4.1.2. RNA polymerase II *In vivo* microinjection experiments showed that antibodies directed against actin inhibit transcription of RNA polymerase II (Hofmann *et al.*, 2004). Several studies demonstrated an association of actin with the mammalian (Hofmann *et al.*, 2004; Kukalev *et al.*, 2005; Smith *et al.*, 1979) as well as yeast (Mitsuzawa *et al.*, 2003) RNA polymerase II core complex. In fact, even highly purified RNA polymerase II contains trace amounts of actin, suggesting that actin might be a subunit of the RNA polymerase II (Hofmann *et al.*, 2004). Mitsuzawa *et al.* (2003) identified specifically an association of actin with the Rbp7 subunit of phosphorylated yeast RNA polymerase II while Kukalev *et al.* (2005) showed an association of actin with the phosphorylated C-terminal domain of the large subunit. The functional significance of the association of actin with RNA polymerase II was demonstrated in a minimal *in vitro* transcription system that only consists of a DNA template and purified transcription factors (Hofmann *et al.*, 2004). Most importantly this system analyses specifically the basal process of transcription independently of other transcription-related processes like chromatin remodeling. In this system antibodies against actin inhibited RNA production while addition of actin stimulated transcription up to 8-fold. It has also been demonstrated that actin is necessary for specifically the start of transcription by RNA polymerase II because preinitiation complexes cannot assemble at the promoter in the absence of actin (Hofmann *et al.*, 2004). Besides a role in transcription initiation there is also conclusive evidence that actin plays a role in transcription elongation through a complex interaction with RNA-processing factors and chromatin remodeling factors (Section 2.4.2.).

2.4.1.3. RNA polymerase III Hu *et al.* (2003) have shown that actin tightly associates with purified RNA polymerase III. Moreover, *in vivo* experiments demonstrated that actin is present at the promoter region of the actively transcribing U6 snRNA gene, an association that is lost when transcription is inhibited. Moreover, it was shown that after inhibition of transcription actin partly dissociated from the RNA polymerase III complex, which resulted in an inactive form of RNA polymerase III. Furthermore, in an *in vitro* assay, adding exogenous actin to this inactive RNA polymerase III activated transcription, demonstrating a crucial role for actin in transcription by RNA polymerase III (Hu *et al.*, 2004). This study also showed that actin specifically binds to three RNA polymerase III subunits RPC3, RPABC2, and RPABC3. Interestingly, the last two subunits are common to all three RNA polymerases and therefore could represent universal sites for RNA polymerase-actin interactions. However, studies on yeast RNA polymerase II have identified the Rpb7 subunit as an interaction site for actin and this subunit is not shared among the RNA polymerases (Mitsuzawa *et al.*, 2003). Further studies are clearly needed to analyze the interaction of actin with the various subunits of the three RNA polymerases in detail.

2.4.1.4. Viral transcription Support for a direct function of actin in the transcriptional process also comes from studies on virus replication. Studies with human parainfluenza virus (De *et al.*, 1991, 1993) and respiratory syncytial virus have shown that recruitment of host cell actin is required for and stimulates transcription by the viral RNA polymerases (Burke *et al.*, 1998; Huang *et al.*, 1993; Mazumder and Barik, 1994).

2.4.2. Transcription regulation and RNA processing

Besides a role for actin in basal transcription there is also emerging evidence, at least for RNA polymerase II transcription, that actin is involved in the regulation of transcription through an association with the RNA product as well as with chromatin remodeling factors (Fig. 6.2).

Actin by itself does not bind to RNA but an association of actin with heterogeneous nuclear ribonucleoprotein particles (hnRNPs) and small nuclear ribonucleoprotein particles (snRNPs) has been reported consistently. Pagoulatos and Yaniv (1978) showed that actin is associated with hnRNPs in monkey kidney cells after infection with the SV40 virus. Subsequently, actin was identified as a component of hnRNPs in a variety of mammalian cells (Brunel and Lelay, 1979), avian erythroblasts (Maundrell and Scherrer, 1979), and *Xenopus* oocytes (Gounon and Karsenti, 1981). Actin was found in complex with nuclear DNA Helicase II/Helicase A, a protein associated with hnRNPs (Zhang *et al.*, 2002) and identified as a component of snRNPs that form active splicing complexes or spliceosomes. Confocal microscopy showed a dynamic spatial relationship between actin and snRNPs which suggests that they may functionally interact in response to

changes in transcriptional activity (Sahlas *et al.*, 1993). In addition, treatment with actin targeting drugs induced a selective release of precursor RNA (Schroder *et al.*, 1987).

A series of studies has now shed some light on the functionality of the association of actin with hnRNPs. Percipalle *et al.* (2002) reported the association of actin specifically with some hnRNP A/B-type proteins in mammalian cells. Subsequently, it was demonstrated that an interaction of actin and the hnRNP protein hrp65–2 in the *Chironomus tentans* experimental system (Percipalle *et al.*, 2003) and an actin–hnRNP U protein interaction in the mammalian system (Kukalev *et al.*, 2005) are required for transcription of RNA polymerase II. Finally, Sjolinder *et al.* (2005) and Obrdlik *et al.* (2008) showed that the assembly of actin into the hnRNP complex that assembles along the growing pre-mRNA recruits a HAT to the transcribing gene. It was suggested that the presence of actin in the hnRNP might act as a platform for binding of chromatin remodeling complexes, which in turn is required for ongoing transcription (Sjolinder *et al.*, 2005). The importance of an actin–RNP–RNA polymerase II association in transcription elongation was also supported by recent studies that show an association of two actin-regulating proteins, N-WASP and the Arp2/3 complex, with the RNA polymerase II and components of the splicing complex (Wu *et al.*, 2006; Yoo *et al.*, 2007).

2.4.3. Actin-myosin complexes in transcription

Two myosins, namely NMI and myosin VI, have been implicated in the transcriptional process. NMI is the first nucleus-specific myosin that was identified and is a unique isoform of myosin Ic (Nowak *et al.*, 1997; Pestic-Dragovich *et al.*, 2000). NMI, like other myosin I molecules, consists of a single heavy chain and a globular head. It has a short tail and is unable to self-associate into filaments. In addition, NMI contains a unique N-terminal extension that is not found in any other myosin and is required for the localization of NMI to the nucleus (Pestic-Dragovich *et al.*, 2000). *In vivo* and *in vitro* studies have demonstrated that NMI is critically involved in transcription by RNA polymerases I and II (Fomproix and Percipalle, 2004; Hofmann *et al.*, 2006b; Kysela *et al.*, 2005; Nowak *et al.*, 1997; Pestic-Dragovich *et al.*, 2000; Philimonenko *et al.*, 2004; Ye *et al.*, 2008). Interestingly, there seem to be differences in the involvement of actin and NMI in transcription by RNA polymerases I and II. In case of transcription by RNA polymerase II actin is necessary for the formation of the preinitiation complexes (Hofmann *et al.*, 2004). The involvement of NMI however, comes at a later stage during transcription activation (Hofmann *et al.*, 2006b). For RNA polymerase I transcription it was shown that actin associates with RNA polymerase I independent of ongoing transcription. However, the association of NMI with the RNA polymerase I is dependent on ongoing transcription (Philimonenko *et al.*, 2004). Nonetheless, an actin–NMI association is important during transcription elongation (Ye *et al.*, 2008). In addition to NMI,

myosin VI has also been shown to be involved in transcription by RNA polymerase II (Vreugde *et al.*, 2006). Myosin VI is a very interesting myosin. In the cytoplasm, myosins move along actin filaments in a certain direction and myosin VI moves in a direction that is opposite to all other myosins. Myosin VI has been shown to associate with RNA polymerase II as well as with actively transcribing genes and a role for myosin VI in transcription in an *in vitro* assay was demonstrated (Vreugde *et al.*, 2006). The involvement of these two molecular motors in the transcriptional process is highly intriguing and constitutes a particular active domain of this overall field (de Lanerolle *et al.*, 2005; Grummt, 2006; Hofmann *et al.*, 2006a; Percipalle and Farrants, 2006).

In summary, we have so far at least three different established functions of actin during transcription, at least for RNA polymerase II. *In vitro* experiments on naked DNA templates have shown that actin recruits RNA polymerase II to the preinitiation complex and therefore plays a role during transcription initiation. Secondly, an interaction of actin with NMI, and possibly with myosin VI is involved during activation of transcription and, if we extrapolate from data obtained on RNA polymerase I transcription, during transcription elongation. Another role for actin appears to involve the recruitment of chromatin remodeling complexes during transcription elongation that is also crucial for ongoing transcription elongation. Finally, an association of actin with the forming hnRNPs has been established that is also necessary for ongoing transcription. The challenge lies now in establishing how actin does all this.

2.5. Nuclear export and intranuclear transport

That proteins and RNAs can move along curvilinear tracks inside the nucleus has been known for a long time. Tracks of mRNA have been observed in the nucleoplasm by *in situ* hybridization both on light and electron microscopy (Huang and Spector, 1991; Lawrence *et al.*, 1989). Meier and Blobel (1992) analyzed the movement of Nopp 140, a nucleolar phosphoprotein that shuttles between nucleolus and cytoplasm and suggested that it moves between the nucleolus and the cytoplasm along a curvilinear track through the nucleoplasm. Similarly, a single track was observed on electron microscope level for the HIV-1 Nef protein within the nucleus (Murti *et al.*, 1993). Even though it was not fully analyzed at this time, the authors already suggested that these tracks could be facilitated through actin (Meier and Blobel, 1992). Parfenov *et al.* (1995) analyzed two stages of *Rana temporaria* oocytes by electron microscopy and found differences in the length of actin filaments. Stages that are active in rRNA transcription contained distinct intranuclear tracks composed of actin bundles that reached from nucleoli to the envelope. Stages that are not transcriptionally active showed short stretches of actin bundles, mainly in association with nucleoli but none extending to the envelope. A possible

interpretation was that the changes in the actin filaments are related to the synthetic state of the oocytes because during highly active synthesis of rRNA the actin filaments could provide a track for fast export.

A functional involvement of actin in the Rev-dependent nuclear export of HIV-1 pre-mRNA in HeLa cells was demonstrated by [Kimura et al. \(2000\)](#). As mentioned above, associations of actin with snRNPs and hnRNPs had been reported since 1979 and therefore a role for actin in RNA processing and export had been suggested ([Reddy and Busch, 1983](#)) (see also [Section 2.4.2.](#)). In an elegant study with *C. tentans* polytene chromosomes, [Percipalle et al. \(2001\)](#) showed that actin becomes associated with the hnRNP protein hrp36 from the time hrp36 binds to the growing Balbiani ring mRNA. The authors then demonstrated by electron microscopy that actin accompanies these hnRNPs from the site of transcription through the nuclear pore complex into the cytoplasm ([Fig. 6.2](#)). This study indeed suggests that actin could have a function in the nuclear export of RNA-protein complexes. In support of a role of actin in nuclear export of RNP complexes [Cisterna et al. \(2006\)](#) demonstrated an involvement of an actin-myosin complex in the export of the small ribosomal subunit after it is assembled in the nucleolus. That actin-dependent export of RNAs and proteins from the nucleus can occur independently of other nuclear processes was shown by microinjection studies in *Xenopus* oocytes. [Hofmann et al. \(2001\)](#) showed that the actin-dependent export of retroviral RNAs occurs independently of the transcriptional process. This study also showed that not only retroviral RNAs but also host proteins are exported from *Xenopus* oocyte nuclei as well as cultured monkey cells in an actin-dependent manner. An active actin-dependent intranuclear translocation was also suggested for the movement of a certain form of nuclear speckles that form upon transcriptional inactivation ([Wang et al., 2006](#)).

Finally, viruses also seem to recruit nuclear actin to facilitate movement inside then nucleus. [Forest et al. \(2005\)](#) showed that herpes simplex virus 1 (HSV-1) capsids are transported inside the nucleus in a temperature- and energy-dependent manner along a curvilinear track. The authors demonstrated that this intranuclear transport is actin- and probably myosin-dependent. This study was also supported by [Feierbach et al. \(2006\)](#) who further demonstrated that herpes virus induces nuclear actin filament formation and that actin is required for assembly and subsequent export of the viral capsids, perhaps via an actin-myosin V complex.

2.6. Prokaryotic actin: A long stretch?

Obviously, prokaryotes do not have a nucleus. So what is the link between prokaryotic actins and eukaryotic nuclear actin? Actin is one of the most conserved proteins and actin homologues in eubacteria include the proteins MreB, ParM, and MamK. In Archaea, the actin homolog Ta0583 was identified in *Thermoplasma acidophilum* ([Roeben et al., 2006](#)). While the

sequence similarity between prokaryotic and eukaryotic actin is rather low, the structural homology is amazingly high (Roeben *et al.*, 2006). Moreover, this structural homology seems to translate into functional similarity. In prokaryotes and eukaryotes alike, actin polymerizes into filaments which are important for maintaining cell shape and polarity (Shih and Rothfield, 2006). Most interestingly, from the viewpoint of eukaryotic nuclear actin, this functional similarity also includes an interaction with the prokaryotic RNA polymerase and a role in chromosome movement.

Most prokaryotic cells have a single chromosome that, while not being surrounded by a nuclear membrane, often occupies a specific region of the cell that is called a nucleoid (Thanbichler *et al.*, 2005). MreB has been shown to play a central role in chromosome partitioning, at the stage of the separation of the origin regions. Depletion of MreB in *Bacillus subtilis* or in *Caulobacter crescentus*, or the overproduction of a dominant negative MreB allele in *Escherichia coli* severely affected the proper DNA localization and segregation (Gitai *et al.*, 2005; Kruse *et al.*, 2003; Soufo and Graumann, 2003). Most interestingly, not only was an association of MreB with DNA demonstrated but also an association of MreB with the bacterial RNA polymerase. A large-scale analysis of protein complexes in *E. coli* showed an interaction with MreB and the RNA polymerase (Butland *et al.*, 2005). In another study, Kruse *et al.* (2006) demonstrated a specific interaction between MreB and RNA polymerase by using *in vivo* and *in vitro* assays. In addition to showing a physical association between RNA polymerase and MreB, the authors also demonstrated that depletion or inactivation of MreB leads to defects in chromosome separation similar to the defects observed when the RNA polymerase is inactivated. The implication here is that the RNA polymerase is associated with the bacterial cytoskeleton and moves along the bacterial actin filaments causing thereby chromosomal segregation.

Recent developments in the field of bacterial actin establish that actin is indeed a highly conserved protein with conserved functions. These include on one hand functional homology of cytoskeletal actin that involve roles in intracellular motility and maintenance of the cell structure. On the other hand, this homology also extends to a central role of actin in the transcriptional process and in the translocation of chromosomes. Actin has now been shown to associate with bacterial, viral, and eukaryotic RNA polymerases and this association is crucial for transcription to take place in all three systems.

3. MECHANISMS OF ACTIN FUNCTION IN THE NUCLEUS

3.1. Nucleocytoplasmic translocation of actin

The eukaryotic nucleus is separated from the cytoplasm by the nuclear envelope which leads to spatial and temporal separation of nuclear and cytoplasmic processes. Transfer of proteins and protein–RNA complexes

occurs through the nuclear pore complexes and is mediated by import and export receptors.

The entry and exit of actin into and from the nucleus is not fully understood but seems to be complex and highly regulated. So far, we only have very limited information about the nuclear import or export of actin. Early work indicated that actin shuttles constantly between the nucleus and the cytoplasm. Microinjection experiments in *Amoeba proteus* and *Xenopus* oocytes have shown that upon injection of actin into the cytoplasm part of it enters the nucleus. Conversely a transfer of labeled nuclei into unlabelled cytoplasm in *A. proteus* showed that part of the nuclear actin leaves the nucleus while exclusively nuclear proteins are retained (De Robertis *et al.*, 1978; Goldstein *et al.*, 1977b). Treatment with DMSO (Fukui, 1978; Fukui and Katsumaru, 1979; Sanger *et al.*, 1980b) or heat shock (Iida *et al.*, 1986) leads to the disappearance of cytoplasmic actin stress fibers and the reversible appearance of nuclear actin rods (see also Section 3.2.). Sanger *et al.* (1980b) followed labeled actin during the formation of these rods and saw a translocation of actin from the cytoplasm to the nucleus during the formation and a reverse translocation after disappearance of the rods. One hypothesis is that the loss of cytoplasmic filaments leads to an increase in soluble actin that then enters the nucleus and forms the intranuclear actin rods. In support of this theory, DMSO treatment of isolated nuclei failed to induce the nuclear actin bundles (Fukui and Katsumaru, 1980) presumably, because no additional actin could be imported from the cytoplasm. However, Pendleton *et al.* (2003) showed that while ATP depletion leads to the appearance of intranuclear actin filaments, it also leads to an increase of cytoplasmic stress fibers. Therefore it seems that disassembly of cytoplasmic actin filaments might cooccur but does not seem to be a prerequisite for the import of actin into the nucleus.

Bertram *et al.* (1977) showed an increase of actin in the nucleus from 5% to 9% in proliferating cells depending on the growth phase and changes in nuclear actin have also been observed after inhibition of transcription (Brasch, 1990; Crowley and Brasch, 1987), in connection to senescence (Kwak *et al.*, 2004), during mitosis (Meijerman *et al.*, 1999), and during cellular transformation (Rao *et al.*, 1997). Thus, at the same time as bidirectional nucleocytoplasmic translocation of actin has been demonstrated to accompany various changes in the cell, the cause as well as the effect of this translocation, remains unknown.

3.1.1. Nuclear import

Actin itself does not contain a known nuclear localization signal (NLS) and the conditions under which actin enters the nucleus are not quite clear. Circumstantial evidence suggests that actin could enter the nucleus in complex with small actin binding proteins that contain NLSs (Fig. 6.3).

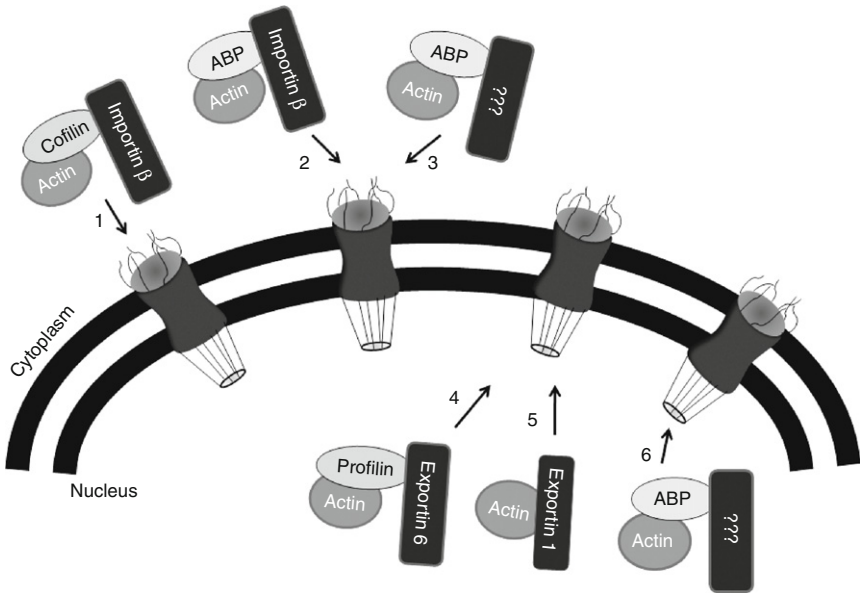


Figure 6.3 Nucleocytoplasmic translocation of actin. (1–3) Nuclear Import of Actin. Actin itself does not contain a nuclear localization signal (NLS) and no actin-specific import receptor has been identified so far. However, actin is thought to enter the nucleus in complex with actin-binding proteins. One candidate is the small actin binding protein (ABP) cofilin that does contain a classical NLS and enters the nucleus through the import receptor importin β (1). As described in more detail in [Section 3.3](#), a number of other actin-binding proteins have been shown to enter the nucleus either through the import receptor importin β (2) or through not yet identified import pathways (3). It is feasible that actin might enter the nucleus in complex with these ABPs. (4–6) Nuclear Export of Actin. Actin contains 2 functional leucine rich nuclear export signals that facilitate export through the export receptor exportin 1 (4). It was also shown that actin can exit the nucleus in complex with the actin binding protein profilin through the export receptor exportin 6 (5). Similar to nuclear import it is also possible that actin might leave the nucleus in complex with other ABPs through not yet identified export pathways (6).

In contrast to actin, cofilin, for example, possesses a functional SV40-type NLS that facilitates nuclear import of proteins through the import receptor importin- β ([Abe et al., 1990](#); [Iida et al., 1992](#); [Matsuzaki et al., 1988](#)). It was shown that cofilin translocates to the nucleus upon DMSO treatment and heat shock where it colocalizes with the newly formed intranuclear actin rods ([Abe et al., 1993](#); [Nishida et al., 1987](#); [Yonezawa et al., 1988](#)). [Pendleton et al. \(2003\)](#) showed that latrunculin B treatment in mast cells induced intranuclear filament formation, which could be inhibited by antibodies to cofilin. However, it is unclear if the translocation of cofilin into the nucleus is associated with the nuclear import of actin or with the formation of the nuclear filaments by itself. It is also not clear if cofilin

then is generally involved in the nuclear import of actin or specifically associated with the nuclear import of actin under conditions that lead to nuclear actin rod formation.

3.1.2. Nuclear export of actin

Actin is exported from the nucleus by at least two different export pathways (Fig. 6.3). Actin possesses two classical leucine-rich nuclear export signals that are present in all isoforms and that are functional and necessary for the export of actin via the export receptor exportin 1 (Wada *et al.*, 1998). These authors demonstrated that treatment of cells with leptomycin B, an inhibitor of exportin 1, leads to an accumulation of actin in the nucleus. In addition, after LMB treatment, paracrystal-like actin structures were found in the nucleus similar to those induced by DMSO or heat stress (see Section 3.2.) (Wada *et al.*, 1998).

Recently, an additional export pathway has been identified. The export receptor exportin 6 seems to be responsible for the nuclear export of actin that is bound in a complex with the small actin-binding protein profilin (Stuven *et al.*, 2003). However, exportin 6 is not expressed in *Xenopus* oocytes (Bohnsack *et al.*, 2006). These authors microinjected labeled actin into *Xenopus* oocyte nuclei and did not observe export of this actin. This is somewhat surprising since dynamic exchange of actin between the nucleus and cytoplasm has been demonstrated in *Xenopus* oocytes (Clark and Merriam, 1977; Paine, 1984). Nuclear translocation of actin has been observed in a variety of cell types in higher and lower eukaryotes. In addition, even though *Xenopus* oocytes contain a high amount of total actin due to their size, the overall concentration of actin is comparable to the concentration of actin in somatic cells. However, the actin used in these experiments was translated *in vitro*. Due to the complex three dimensional folding pathways it is inherently difficult to obtain properly folded and active actin by *in vitro* translation (Tian *et al.*, 1995). It is possible therefore that the observed lack of export was due to improper folding which might lead to masking of potential export factor binding sites. While characterization of the functional export pathways provided the necessary first-order explanation of the molecular biology of nucleocytoplasmic partitioning of actin, resolving the apparent discrepancy between the nucleocytoplasmic exchange of actin that was seen and its export that went undetected in the important *Xenopus* oocyte model may prove particularly informative.

3.2. The form of actin in the nucleus

As discussed here, an increasing number of nuclear processes have been shown to involve actin. The question now is how actin is involved. Most of the known functions of actin in the cytoplasm involve dynamics of actin polymerization. Thus, when we think about actin and how actin

works we ultimately envision actin filaments (Pollard *et al.*, 2000, 2001). It is not clear however what form actin has in the nucleus. Traditionally the drug phalloidin is used to visualize actin filaments by immunofluorescence microscopy. But under physiological conditions there is very little evidence for filamentous actin in the nucleus based on a lack of nuclear phalloidin staining.

As was alluded to earlier, distinctive actin bundles (also called actin rods or paracrystals) can be induced in the nucleus in a variety of cell types from different organism under a number of conditions. Table 6.2 shows a list of conditions that lead to the formation of these actin rods. What exactly induces the formation of these bundles and if they are formed from actin that is already in the nucleus or through increased nuclear import of actin is not known. Sanger *et al.* (1980a) showed that in untreated conditions actin staining in the nucleus is diffuse. Within 30 min of exposure to medium containing DMSO, cells begin to round up and lose their cytoplasmic actin bundles. At the same time, bundles of actin filaments appear in the nuclei of the cells. One to two hours after removal of DMSO the cells revert to their flattened shape, lose the nuclear actin bundles and regain the cytoplasmic bundles. As discussed in Section 3.1.1, one hypothesis is that the disassembly of the cytoplasmic actin leads to an influx of actin into the nucleus. Sanger *et al.* (1980b) used microinjection of fluorescently labeled actin and demonstrated that this actin easily incorporated into the cytoplasmic actin filaments. After treatment with DMSO translocation of this fluorescently labeled actin into the nucleus as well as incorporation into the intranuclear rods was observed. After removal of DMSO the fluorescently labeled actin left the nucleus and incorporated into the cytoplasmic filaments again. On the other hand, as mentioned above, Pendleton *et al.* (2003) showed that nuclear actin rods also formed after ATP depletion, which lead to an increase in cytoplasmic stress fibers. However, it could be possible that actin monomers that are freed during rearrangement of the cytoplasmic actin filaments might be imported into the nucleus.

On the other hand differential reactions of certain cell types to cellular stress have been observed. While myoblasts form intranuclear actin rods upon treatment with DMSO as well as after heat shock, differentiated myotubes only form intranuclear actin rods after treatment with DMSO but not after heat shock (Abe *et al.*, 1993). Meijerman *et al.* (1997, 1999) have observed redistributions of intranuclear G-actin after heat shock though no formation of intranuclear actin rods and no changes in the total nuclear actin concentration.

An unresolved issue is whether the actin rods that are formed under various conditions are identical in structure. In some cases these actin rods could be decorated with cofilin or heavy meromyosin but could not be stained by phalloidin. This could be explained by studies that show that binding of cofilin and phalloidin to actin is competitive. Hence, the binding

Table 6.2 Conditions that lead to intranuclear actin rod formation

Condition	Species and/or cell type	References
5–15% DMSO	<i>Dictyostelium discoideum</i>	Fukui, 1978
	<i>Amoeba proteus</i>	Fukui and Katsumaru, 1979
	<i>Tetrahymena pyriformis</i>	Katsumaru and Fukui, 1982
	HeLa (mammalian epithelial cells)	Fukui and Katsumaru, 1979
	PtK2 (rat kangaroo cells)	Osborn and Weber, 1980b; Sanger <i>et al.</i> , 1980a
	WI38 (human fibroblasts)	Sanger <i>et al.</i> , 1980b
Heat Shock	mammalian myoblasts and myotubes	Abe <i>et al.</i> , 1993; Ono <i>et al.</i> , 1993
	mammalian myoblasts	Abe <i>et al.</i> , 1993; Ono <i>et al.</i> , 1993
	HeLa, CHO (hamster ovary cells), fibroblasts	Iida and Yahara, 1986; Welch and Suhan, 1985
Cytochalasins	mammalian fibroblasts	Yahara <i>et al.</i> , 1982
	mammalian one-day embryo	Nguyen <i>et al.</i> , 1998
	<i>Zea mays</i> epidermal cells	Braun <i>et al.</i> , 1999
Latrunculin	<i>Zea mays</i> root hair cells	Jiang <i>et al.</i> , 1997
	mammalian mast cells	Pendleton <i>et al.</i> , 2003
	ATP depletion	Pendleton <i>et al.</i> , 2003
A23187 + Mg ²⁺ (ionophore)	mammalian mast cells	Osborn and Weber, 1980b
	PtK2	Osborn and Weber, 1980a
Trifluoperazine	HeLa	Osborn and Weber, 1984
Forskolin	CHO	Osborn and Weber, 1984
Virus infection: AcMNPV (baculovirus)	<i>Spodoptera frugiperda</i>	Charlton and Volkman, 1991; Volkman, 1988
	PRV (Pseudorabies virus) HSV (Herpes simplex virus)	Feierbach <i>et al.</i> , 2006

Table 6.2 (continued)

Condition	Species and/or cell type	References
Mutations in α -skeletal muscle actin (V163L; V163M; R186G; H40Y; D286G)	leads to myopathies in patients; also tested in: mammalian myoblast, myotubes, and fibroblasts	Bathe <i>et al.</i> , 2007; Costa <i>et al.</i> , 2004; Ilkovski <i>et al.</i> , 2004; Sparrow <i>et al.</i> , 2003; Domazetovska <i>et al.</i> , 2007; Hutchinson <i>et al.</i> , 2006

site for phalloidin would already be occupied by cofilin (Yonezawa *et al.*, 1988). In other cases phalloidin staining of these actin structures was observed. Whether these differences really reflect distinctive actin structures or if the differences in staining are related to fixation methods that do or do not remove ABPs, thereby freeing the phalloidin binding sites, remains to be ascertained. What is also not clear is the physiological relevance of these nuclear actin rods. Intranuclear rod formation that appears after inhibition of nuclear export of actin is accompanied by a decreased viability of the cells (Wada *et al.*, 1998). In addition, mutations in α -actin with concurrent rod formation lead to severe myopathies that correlate with a decrease in the mitotic index (Domazetovska *et al.*, 2007), though a clear cause-and-effect connection has not been established yet.

Under most physiological conditions however, actin filaments are not seen in the nucleus *in situ* and phalloidin does not recognize nuclear structures. It is still heavily debated if this is because the nucleus does not contain actin filaments at all under nontreated conditions, or if the binding sites for phalloidin on nuclear actin are occupied by other proteins. Another possibility is that actin forms nucleus-specific structures that are not common to the actin structures known from the cytoplasm. In support of the latter possibility, a number of actin antibodies have been developed that stain specifically the nucleus. Gonsior *et al.* (1999) developed the monoclonal 2G2 antibody against actin that recognized actin aggregates in the nucleus. Based on the X-ray diffraction studies, the 2G2 recognizes an epitope that is buried in the F-actin structure but recognizable when actin is complexed with profilin (Schoenenberger *et al.*, 2005). Another antibody (1C7) that was raised against an epitope that is exposed when actin forms the so-called lower dimer (LD) also does not recognize cytoplasmic actin filaments but seems to be nucleus-specific (Schoenenberger *et al.*, 2005). Interestingly, these antibodies differ in their staining pattern which gave rise to the suggestion that a variety of unconventional and perhaps nucleus-specific actin forms are present in the nucleus (Jockusch *et al.*, 2006). McDonald *et al.* (2006) measured the dynamics of nuclear actin in cells transfected with tagged β -actin by FRAP analysis.

The authors showed that the nucleus contained fast- as well as slow-recovering populations of nuclear actin and suggested that the slow species represented polymerized actin that seems to have distinctive dynamics that differed from the actin dynamics in the cytoplasm.

In summary, large and perhaps ordered aggregates of actin are known to form in the nucleus under conditions that appear to increase the free actin concentration in the cell by disassembling the cytoplasmic polymers. Under normal conditions nuclear actin appears to exist in an equilibrium between monomers and higher order structures or heterotypic complexes with other proteins.

3.3. Actin-regulating proteins in the nucleus

To understand the various forms that actin can take, it is imperative to understand the principles that lie behind the intrinsic propensity of actin to form filaments. Actin is a protein with a complex three-dimensional structure. Atomic structure determination of the actin molecule shows that it is folded into two large domains each consisting of two sub-domains (Otterbein *et al.*, 2001). Between the two domains lies a deep cleft, the so-called ATP-binding pocket, as well as the binding site for a divalent cation. *In vitro*, monomeric actin has a low ATPase activity but polymerization into filaments occurs with concomitant hydrolysis of ATP. The tendency to form filaments depends on the critical concentration (C_c) of actin. The C_c is defined as the concentration of actin monomers in equilibrium with actin filaments. Therefore at monomer concentrations below the C_c actin will stay monomeric. At concentrations above C_c actin monomers will spontaneously polymerize into filaments until the critical concentration is reached. The C_c of actin depends on a number of factors including temperature, salt concentration and which specific cation is used (Gordon *et al.*, 1977).

The concentration of actin *in vivo*, in the cytoplasm as well as in the nucleus, is well above the critical concentration for polymerization. However, in the cytoplasm this polymerization process is regulated by a large number of ABPs that regulate the form of actin by nucleating, crosslinking, bundling, or severing actin filaments, by inducing filament branching or by sequestering actin monomers and thereby regulating actin polymerization.

A considerable number of these cytoplasmic ABPs have also been found in the nucleus. Interestingly, in many cases the nuclear functions of these ABPs are not necessarily related to actin dynamics but rather to regulation of transcription, DNA repair, and mRNA splicing through interaction with specific nuclear factors. Some of the cytoplasmic ABPs and their proposed nuclear functions are described below.

The gelsolins consist of a class of proteins that are found from lower eukaryotes to mammals. Members of the gelsolin family share 3–6 repeats of a conserved domain and are responsible for capping and/or severing actin

filaments in the cytoplasm (Burtnick *et al.*, 2001; Way and Weeds, 1988). Several members of the gelsolin family, namely gelsolin (Salazar *et al.*, 1999), CapG (Onoda *et al.*, 1993), supervillin (Wulfschlegel *et al.*, 1999), and FliI, (Davy *et al.*, 2000) have been identified in the nucleus. A series of studies have independently identified CapG, gelsolin, supervillin, and FliI as transcriptional coactivators or repressors for several nuclear hormone receptors. CapG was shown to modulate transcriptional activity in a reporter assay (De Corte *et al.*, 2004) and supervillin (Ting *et al.*, 2002) and gelsolin (Nishimura *et al.*, 2003) were shown to interact with the androgen receptor and enhance the transcriptional activity of the androgen receptor as well as other nuclear hormone receptors. Another study showed that FliI can associate directly with the estrogen receptor and the thyroid hormone receptor as well as with other coactivators of nuclear hormone receptors thereby enhancing their transactivation (Lee *et al.*, 2004). Tropomodulin is another F-actin capping protein that has been shown to localize to the nucleus where it seems to play a role in differentiation related transcription regulation (Kong and Kedes, 2004).

A number of actin filament cross linking and bundling proteins have also been found in the nucleus. These include α -actinin that has been shown to translocate to the nucleus (Honda *et al.*, 1998) where it associates with a group of histone deacetylases that regulates a number of transcription factors (Chakraborty *et al.*, 2006). Several spectrins have been identified in the nucleus and have been implicated in DNA repair, chromatin remodeling, and transcription regulation through interaction with nuclear complexes that are associated with these functions (Sridharan *et al.*, 2003 *et al.*, 2006). In addition, an association with PML bodies and the nuclear matrix has been reported (Tse *et al.*, 2001). Filamin A has been shown to associate with transcription regulators (Berry *et al.*, 2005) and myopodin (Weins *et al.*, 2001) with the nuclear matrix (Fuentes-Mera *et al.*, 2006). Members of the nesprins have been shown to play an important role in nuclear envelope assembly (Libotte *et al.*, 2005; Zhang *et al.*, 2005, 2007). Actin polymerizing proteins that include members of the WASP family (N-WASP) as well as the Arp2/3 complex have been implicated in transcription regulation (Wu *et al.*, 2006; Yoo *et al.*, 2007) through interaction with the splicing factor containing complex PSF–NonO.

G-actin binding proteins with nuclear functions include thymosin β 4 that has been shown to interact with ATP-dependent DNA helicase II and to regulate specific gene expression (Bednarek *et al.*, 2008). In addition, profilin has been implicated in a number of nuclear processes (Jockusch *et al.*, 2007) that include associations with components of snRNPs and therefore in slicing of pre-RNAs (Skare *et al.*, 2003). In addition, profilin has been identified as a receptor that targets actin for nuclear export (Stuven *et al.*, 2003).

In summary, a large number of proteins that regulate actin dynamics in the cytoplasm are also present in the nucleus. However, the mere existence of these proteins does not imply that they regulate the form of actin in the nucleus. Many of these ABPs have been shown to interact with specific transcription factors or nuclear factors that are involved in RNA splicing or DNA repair. Alternatively, through their association with specific transcription or splicing factors they could act as a platform to recruit actin containing transcription complexes or chromatin remodeling complexes to specific sites (Fig. 6.1).

3.4. Methods for identifying the form of nuclear actin

Studies using nucleus specific antibodies (Gonsior *et al.*, 1999; Schoenenberger *et al.*, 2005) as well as the analysis of nuclear actin dynamics in live cells (McDonald *et al.*, 2006) are steps towards the identification of actin forms in the nucleus. However, attempts to correlate the forms of actin to specific nuclear function have so far been relatively unsuccessful. The application of methods traditionally used to analyze the form of actin in the cytoplasm to the nucleus has yielded confusing and often contradictory results. Therefore, this section will adopt a critical perspective on the methods used to correlate nuclear actin form to function, which might explain the unsatisfactory results achieved so far.

3.4.1. Determination and visualization of G-and F-actin in the nucleus

Two common methods to determine the amount of G-actin in an extract are the DNase I inhibition assay and the “F-actin sedimentation” assay. While both assays can be used successfully to determine the concentration of the monomeric and soluble G-actin, the results do not give information on whether the remaining actin is in a filamentous form. The sedimentation assay is based on centrifugation at 100,000 *g* (Phillips *et al.*, 1980) for the purpose of separating F-actin and G-actin. However, several considerations have to be taken into account. Firstly, short oligomeric actin in addition to G-actin may remain in the supernatant. Secondly, the pellet fraction does not only contain filamentous actin but also actin that is associated with large complexes. Considering that nuclear actin is associated tightly with large nuclear complexes such as the chromatin remodeling complexes, the various RNA polymerase complexes, hnRNP complexes, etc., this actin, even though it is not polymerized, will still be in the pelleted fraction.

The DNase I inhibition assay is based on the fact that soluble monomeric actin forms a stable 1:1 complex with DNase I. After complex formation, the DNase I enzyme loses its activity (Blikstad *et al.*, 1978; Fox *et al.*, 1981). This assay can determine the fraction of the total actin that is unbound. However, it may not necessarily be expected that DNase I

might recognize monomeric actin that is associated with nuclear complexes. It was shown for example that DNase I binds to the same actin-binding site as thymosin $\beta 4$ (Ballweber *et al.*, 1994) and for most of the nuclear proteins that bind to actin, the binding site has not been established.

An important consideration for the determination of the form of actin in an extract is the method of preparation. Conditions used in the extract preparation can significantly affect the polymerization state of actin. A common procedure to prepare nuclei or nuclear extract is the use of sonication to either lyse the cells or reduce the viscosity due to the nuclear chromatin. However sonication has been shown to be a potent actin polymerization inducer. *In vitro* experiments demonstrated that sonic vibrations for very short periods of time (2–10s) lead to rapid polymerization of actin within a few minutes (Maruyama, 1981). Therefore, a nuclear extract that has been sonicated might contain artificially produced actin filaments. Other issues are changes in salt concentration and which cations are used to prepare the extract. As mentioned above these conditions can significantly affect actin. De Robertis *et al.* (1978) suggested that the gel-like structure that is observed after the nuclear envelope is removed from *Xenopus* oocyte nuclei might be an effect of the divalent cations that are used in the preparation method, which would significantly induce actin polymerization.

Care should also be taken in evaluating data obtained by microscopy. Fluorescently labeled DNase I and vitamin-D binding protein, while binding to soluble uncomplexed actin, most likely will not detect actin that, while monomeric, is assembled into large nuclear complexes. It is also known that the methods of fixation for immunofluorescence microscopy and for electron microscopy induce changes in the actin conformation. OsO_4 and dehydration in acetone or ethanol, commonly used fixation methods, alter the actin structure and can lead to disorder and aggregation of filaments (Maupin-Szamier and Pollard, 1978; Small, 1981). Similarly, critical-point drying preparations for cryoelectron microscopy have been shown to lead to actin filament aggregation (Koestler *et al.*, 2008; Resch *et al.*, 2002). This continuing debate over actin visualization methods in the cytoplasm may benefit future studies of nuclear actin.

3.4.2. Drugs and mutations used to correlate nuclear actin form with function

3.4.2.1. Cytochalasin D Cytochalasin D is a fungal metabolite that induces actin depolymerization. However, the actions of cytochalasin D are complex and not entirely understood. On one hand cytochalasin D binds to G-actin and inhibits the addition of this G-actin to actin filaments. Existing F-actin fibers then depolymerize as the effective concentration of free G-actin becomes limiting. However, effects of cytochalasin D also involve a capping of the actin filament. Moreover, in some cell types,

binding of cytochalasin D to G-actin also results in proteolytic degradation of monomeric actin (Sampath and Pollard, 1991).

3.4.2.2. Latrunculin A and B The toxins latrunculin A and B form 1:1 complexes with G-actin. Thereby, on one hand they sequester actin monomers and on the other hand lead to actin depolymerization due to a decrease in free G-actin concentration (Coue *et al.*, 1987; Spector *et al.*, 1989). However, the effects of latrunculin also include a change in the ATP binding pocket that inhibits nucleotide exchange of actin. In addition, binding of latrunculin to actin inhibits binding of thymosin β 4, and therefore might be expected to also affect binding of other proteins to actin (Yarmola *et al.*, 2000). Furthermore, it has been shown that latrunculin A can affect the expression of actin and possibly of other ABPs through a feedback mechanism (Bershadsky *et al.*, 1995).

3.4.2.3. Swinholide A Swinholide A is isolated from the marine sponge *Theonella swinhoei*. It is known to bind two actin monomers. It also has the potential to cap the barbed ends of actin filaments and to sever actin filaments by intercalating between two actin protomers within the filament. Formerly it was thought to stabilize physiologically relevant actin dimers, however recent solution of its cocrystal structure with actin showed that the two actin monomers are unrelated and do not belong to the same dimer (Klenchin *et al.*, 2005). In addition, the site of swinholide binding to actin is an important site of association with actin binding proteins.

3.4.2.4. Phalloidin Phalloidin is isolated from the mushroom *Amanita phalloides*. It is an F-actin stabilizing peptide (Low and Wieland, 1974) that changes the critical concentration on both ends of an actin filament and thereby prevents the dissociation of the monomers from the filaments (Coluccio and Tilney, 1983).

3.4.2.5. Jasplakinolide Jasplakinolide is derived from the Indo-Pacific marine sponge *Jaspis johnstoni*. The actions of jasplakinolide are different *in vivo* and *in vitro*. *In vitro* jasplakinolide stabilizes actin filaments (Bubb *et al.*, 1994). *In vivo*, however, the effects of jasplakinolide are complex. Jasplakinolide sequesters actin monomers and also releases actin from other monomer sequestering proteins. This leads to a dissociation of the existing actin filaments. Jasplakinolide then, by induction of increased nucleation, causes formation of disorganized actin filaments (Bubb *et al.*, 2000).

3.4.2.6. BDM 2,3-Butanedione monoxime (BDM), also known as diacetyl monoxime (DAM) has been used in many studies as an inhibitor of the ATPase activity of myosins to identify the functions of nuclear myosins. However, while effectively inhibiting the ATPase activity of muscle myosin

II, it was shown that BDM has no effect on the ATPase activity of nonmuscle myosins. Among the myosins that were specifically tested were: *Acanthamoeba* myosin-IC, chicken myosin-V, and porcine myosin-VI (Ostap, 2002). Moreover, it was shown that BDM is an especially unsuitable drug to use because it affects a large number of proteins. Among the observed effects of BDM was an effect on actin dynamics. Treatment of cells with BDM was shown to inhibit the incorporation of new actin monomers into filaments and to lead to a relocation of the actin regulatory proteins Arp3, Vasp, and WAVE1 for example (Yarrow *et al.*, 2003).

3.4.2.7. Actin mutants A number of engineered actin gene constructs have been used to investigate domains within the protein that are involved in nuclear functions (Chuang *et al.*, 2006; Posern *et al.*, 2004; Vartiainen *et al.*, 2007; Ye *et al.*, 2008). Two types of mutations have been used to this end. The first type targets specific sites of the ATP-binding pocket whose mutation leads to deficiencies in ATP-binding, hydrolysis, or in release of the phosphate. The other type targets actin polymerization through inhibition of actin-actin interactions. The most prominent effects observed in the cytoplasm are the ability of these actin mutants to change the cytoskeletal structure through stabilization or destabilization of actin filaments. However, when interpreting the observed results one also has to take into account that these mutations alter the ability of actin to interact with ABPs. The conformation of the ATP-binding site and changes in this conformation upon ATP-hydrolysis and P_i release greatly affect ABP binding. Profilin, for example, has a higher affinity for ATP-actin than for ADP-actin (Vinson *et al.*, 1998). Cofilin (Blanchoin and Pollard, 1998) and gelsolin (Laham *et al.*, 1993), on the other hand, bind ADP-actin tighter than ATP-actin. That mutations therefore also change binding of ABPs was also demonstrated by (Posern *et al.*, 2002).

In summary, it may be questioned whether the observed effects on nuclear actin function by the above described methods can be wholly attributed to changes in nuclear actin dynamics. In evaluating the “results”, several factors have to be taken into consideration. Drugs as well as mutations in the actin protein may change the interaction of actin with nuclear ABPs. “Actin depolymerizing” drugs, for example, bind actin monomers and therefore induce a depolymerization of actin filaments by reducing the monomeric actin pool. However, an alternative interpretation may be that actin-depolymerizing drugs bind actin monomers and prevent binding of these monomers to potential binding partners.

In this regard, it should be noted that changes in the cytoskeleton induced by drugs may have strong effects on nuclear events. For example, altering the structure of actin leads to changes in the gene expression of actin as well as other ABPs (Lyubimova *et al.*, 1997; Schmitt-Ney and Habener, 2004). An excellent example for how changes in cytoplasmic actin affect

nuclear events is the work by Treisman and coworkers (Miralles *et al.*, 2003; Posem *et al.*, 2002, 2004; Sotiropoulos *et al.*, 1999; Vartiainen *et al.*, 2007). This group showed that the coactivator MAL binds to cytoplasmic G-actin. Upon depletion of the G-actin pool by induced actin polymerization, MAL is released from the bound actin. Subsequently MAL enters the nucleus and induces transcription of serum response factor regulated genes. In this case, increase in transcription through actin polymerization is not related to nuclear actin but to cytoplasmic actin. Finally, it should be noted that cytochalasins as well as latrunculin depolymerize cytoplasmic actin filaments. But, both of them, while depolymerizing cytoplasmic filaments actually induce actin filament formation in the nucleus (see Table 6.2).

Therefore, methods used to analyze actin dynamics in the cytoplasm may not be universally suitable to analyze actin dynamics in the nucleus. Results obtained from these studies are open to a number of interpretations and cannot easily be correlated with nuclear actin dynamics.

3.5. Actin isoforms in the nucleus

So far, not much attention has been paid to the different actin isoforms in regard to nuclear actin and its various functions. Eukaryotic cells usually express several actin isoforms. In mammals the actin family consists of six actin isoforms. There are four tissue-specific α actins: α -skeletal actin (α -SKA), α -cardiac actin (α -CAA), α -smooth muscle actin (α -SMA), and γ -smooth muscle actin (γ -SMA). In addition, two other actin isoforms are ubiquitously expressed. They are cytoplasmic β -actin (β -CYA) and cytoplasmic γ -actin (γ -CYA). The primary structure of the various actin isoforms is highly conserved and α -actins share >93% amino acid sequence identity with the nonmuscle β - and γ -isoforms. The β and γ cytoplasmic actins in mammalian cells differ by only four conservative amino acid substitutions in the N-terminus.

Early studies on nuclear actin showed that the same actin isoforms that are found in the cytoplasm are also present in the nucleus (Nakayasu and Ueda, 1983; Vandekerckhove *et al.*, 1981). These early studies on nuclear actin are probably the most reliable in regard to information about the isoforms because two-dimensional gel electrophoresis was used to identify the different actin isoforms by isoelectric focusing. A number of later studies has identified specifically β -actin as part of nuclear complexes by using mass spectrometry. However, it remains to be determined if this can be taken as a general finding. Most of these studies were done in cell types that do not contain α -actin to begin with. In addition, as mentioned above, the two cytoplasmic actin isoforms differ only in four amino acids at the very N-terminus. It is not known if in these studies N-terminal peptide sequencing was performed. Considering that β - as well as γ -actin was identified in the nucleus, a possibility that should not be discounted is that the actin

in these complexes could be either β - or γ -actin. Studies in muscle cells that contain the α -actin isoforms have shown that α -actins are also present in the nucleus. Actin is expressed as β - and γ -isoforms in myoblasts but these isoforms are downregulated during muscle differentiation while expression of α sarcomeric actin is induced (Lloyd and Gunning, 2002). α - and β -actin isoforms were identified both in the cytoplasm as well as the nucleus in myotubes (Abe *et al.*, 1993; Lattanzi *et al.*, 2003; Nagaoka *et al.*, 1995; Ono *et al.*, 1993). Thus, it appears that there is no distinction between the actin isoforms in terms of nuclear localization. Interestingly, a few studies have also reported nucleus specific isoforms (Bremer *et al.*, 1981; Kumar *et al.*, 1984; Nakayasu and Ueda, 1986) although no further information about these is available.

Increasing evidence shows that the actin isoforms (at least in the cytoplasm) are not only spatially segregated but also functionally distinct (Khaitlina, 2001, 2007; Rubenstein, 1990). How this functional segregation is achieved is not quite clear but recently a posttranslational modification of β - but not γ -actin was identified that contributes to the functional separation between the two cytoplasmic actin isoforms (Karakozova *et al.*, 2006; Kashina, 2006).

It has also been shown that various ABPs distinguish between actin isoforms. Ezrin associates specifically with β -actin filaments but not with α -actin filaments (Shuster and Herman, 1995), different isoforms of profilin associate differentially with specific actin isoforms (Segura and Lindberg, 1984), and betaCap73 binds specifically to β -actin (Shuster *et al.*, 1996). In addition, it appears that binding of phalloidin to various actin isoforms differs. In light of increasing evidence of a functional difference between the actin isoforms that at least appears to be partially mediated through differential binding of ABPs, it would be very interesting to analyze whether there is a functional difference in the actin isoforms in the nucleus.

4. CONCLUDING REMARKS

Actin has been implicated in a large number of nuclear processes and it has become apparent that the functions of actin in the nucleus are as diverse and numerous as we know them to be in the cytoplasm. Some of these are schematically depicted in Fig. 6.2. It also appears that the functions of actin in nuclear processes are highly conserved. However, while in some cases we have a clearer picture of the function and involvement of actin has been established, we do not know the “How.” Increased understanding of nuclear processes and the complexes that are involved has allowed us to analyze them separately *in vitro*. However, nuclear processes are highly interconnected in ways that are not fully understood. Transcription depends

on the remodeling and modification of the chromatin, as well as on the assembly of the transcription machinery. Transcription factors shuttle between the nucleus and the cytoplasm or must be recruited from intranuclear storage sites. Transcription activators transmit intra- and extracellular signals to the nucleus where specific genes are activated or repressed. Expression of these genes depends on the translocation and interaction of specific chromosome regions which in turn may depend again on the remodeling and modifications of these regions. As described above, actin is involved in most of these processes and perhaps in more than one way in some of them. The challenge lies now in finding the connection between the different functions of actin in the nucleus and how they are regulated. An appealing idea is that the many ABPs that have been described as transcription coactivators might recruit actin, which is known to be part of various functional complexes (RNA polymerase complexes, chromatin remodeling complexes, or RNA processing complexes), to the sites of transcription activation. Then binding of actin to different ABPs could facilitate, for example, the switch from a function in transcription initiation to transcription elongation.

Alternatively, it is conceivable that the various actin isoforms may have differential functions (and these may still be affected through interaction with different ABPs). Exploring further the interrelation between the cytoplasmic and the nuclear pool of actin may provide kinetic clues to unraveling the pathways of actin complex formation in the nucleus. Overall, from this point on, it is likely that the study of actin functions in the nucleus will increasingly go hand in hand with the study of the nuclear functions themselves.

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SMALL UBIQUITIN-LIKE MODIFIERS IN CELLULAR MALIGNANCY AND METASTASIS

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Abstract

Small ubiquitin-like modifiers (SUMOs) mediate a variety of cellular functions of protein targets mainly in the nucleus but in other cellular compartments as well, and thereby participate in maintaining cellular homeostasis. SUMO system plays important roles in transcriptional regulation, DNA damage responses, maintaining genome integrity, and signaling pathways. Thus, in some cases, loss of regulated control on SUMOylation/deSUMOylation processes causes a defect in maintaining homeostasis and hence gives a cue to cancer development and progression. Furthermore, recent studies have revealed that SUMO system is involved in cancer metastasis. In this review, we will summarize the

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possible role of SUMO system in cancer development, progression, and metastasis and discuss future directions.

Key Words: SUMO, SUMOylation, deSUMOylation, Ubc9, SENP, Cancer.

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1. INTRODUCTION

Posttranslational protein modifications can endow target proteins with a new function either by altering the conformation of modified proteins or by providing a new interface for protein-protein interactions. These protein modifications can be divided into two categories based on the associated modifiers. The first category involves modification via the attachment of nonproteinaceous molecules, such as those with phosphoryl, methyl, and acetyl groups in their structure. The second category involves modification via genetically encoded small proteins called ubiquitin and ubiquitin-like proteins (ubl) (Hunter, 2007). Ubiquitin has been known to be a modifier that marks target proteins to be delivered to the cellular demolishing machinery, namely, 26S proteasome (Ciechanover, 1998; Hershko and Ciechanover, 1998; Hochstrasser, 1996). However, recent studies revealed that monoubiquitination and certain types of polyubiquitination, which are dependent on the lysine residues on which branches are formed, do not function as degradation signals (Haglund and Dikic, 2005; Hicke, 2001). These ubiquitination types allow target proteins to interact with new binding partners, and hence play important roles in the formation of chromatin structure and propagation of signals from the cell surface to the nucleus (Haglund and Dikic, 2005; Hicke, 2001). Modification by ubls does not direct proteins to the proteolytic machinery (Kerscher *et al.*, 2006; Welchman *et al.*, 2005). Rather, the mechanism of action is more or less similar to that observed in monoubiquitination (Kerscher *et al.*, 2006; Welchman *et al.*, 2005).

Small ubiquitin-like modifiers (SUMOs) are the best studied members among ubls and primarily play a role in the nuclear compartment. Although some targets are found in the cytoplasm, plasma membrane, and endoplasmic reticulum, the majority of SUMO targets identified thus far are nuclear proteins (Geiss-Friedlander and Melchior, 2007; Hay, 2005). As their major function in the nucleus, SUMOylation is involved in diverse nuclear events such as nuclear-cytoplasmic trafficking, transcriptional regulation, maintenance of genome stability, and DNA repair (Geiss-Friedlander and Melchior, 2007; Hay, 2005). Recent studies have suggested the possible involvement of the SUMO system in pathological conditions such as cancer in addition to the regular function in normal cells (Dawlaty *et al.*, 2008; Kim *et al.*, 2006). SUMO targets include a number of oncogenic transcription

factors, the well-known tumor suppressor protein p53, components of chromatin-remodeling complexes, and proteins in the telomerase complex. Dysregulation of SUMOylation on those proteins, together with other oncogenic signals, is possibly involved in promoting cell growth and the loss of the tumor suppressing role of target proteins. SUMOs were originally identified and studied in depth in the yeast system. However, in this review, we will deal with SUMOs mainly in the context of the mammalian system. We will start with an introduction to SUMO proteins, and enzymes associated with SUMOylation and deSUMOylation processes. Thereafter, we will focus on the possible roles of the SUMO system in the processes of cancer initiation, progression, and metastasis by introducing published examples in detail.

2. SUMOS AND SUMO-MODIFYING ENZYMES

Similar to ubiquitin and other ubls, SUMOs can be attached to cellular target proteins with the aid of three types of enzymes: SUMO-activating enzyme (E1), SUMO-conjugating enzyme (E2), and SUMO ligases (E3). In most cases, except in a few SUMO target proteins such as RanGAP1 (Mahajan *et al.*, 1997; Matunis *et al.*, 1996), the detectable portion of the SUMOylated protein from the entire individual protein is extremely minor, suggesting dynamic regulation of SUMOylated proteins by deSUMOylating proteases. Thus far, it has been characterized four isoforms of SUMO proteins, a heterodimeric E1 enzyme, an E2, 10 E3s, and six deSUMOylating proteases.

2.1. SUMO proteins

The SUMO protein was initially identified in yeast *Saccharomyces cerevisiae* as a suppressor of mutations in the centromere protein MIF2 and designated Smt3p (Meluh and Koshland, 1995). Almost simultaneously, the mammalian homolog of the yeast protein was discovered and referred to using multiple names, such as SUMO-1 (small ubiquitin-like modifier 1), UBL1 (ubiquitin-like 1), PIC1 (promyelocytic leukemia protein (PML)-interacting protein 1), sentrin, and GMP1 (GTPase-activating protein (GAP)-modifying protein 1) (Boddy *et al.*, 1996; Mahajan *et al.*, 1997; Matunis *et al.*, 1996; Okura *et al.*, 1996; Shen *et al.*, 1996). Thereafter, three more isoforms of SUMO-1, namely, SUMO-2, -3, and -4, were identified in the mammalian system (Fig. 7.1) (Guo *et al.*, 2004; Lapenta *et al.*, 1997; Mannen *et al.*, 1996). Genes encoding each SUMO protein are located on independent chromosomes in humans (SUMO-1, 2q33; SUMO-2, 17q25.1; SUMO-3, 21q22.3; and SUMO-4, 6q25). While SUMO-1, SUMO-2, and SUMO-3 are expressed in most tissues, SUMO-4 mRNA is detected in a limited number of tissues, including the kidney, lymph node, and spleen,

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SUMO-1 MSDQEARPSTEDLDGDKKEGEYIKLKVIQGDSSEIHFKVMTHLKKLKESYCQRGVPNSLRFLEGQRIADNHTFKELGMEEEDVIEVQEQTGGSTV
SUMO-2 MADEKPKEGVKT----ENNDHINKVAGQGSVVQFKIKRTELSKLMKAYCERQLSMRQIRFREDGQPINETDTPAQLEMEDEDTIDVFQQTGGGVY
SUMO-3 MSEEKPKEGVKT----ENDHINKVAGQGSVVQFKIKRTELSKLMKAYCERQLSMRQIRFREDGQPINETDTPAQLEMEDEDTIDVFQQTGGGVYESSLAGHSF
SUMO-4 MANEKPEEVKT----ENNDHINKVAGQGSVVQFKIKRTELSKLMKAYCERQLSMRQIRFREDGQPINETDTPAQLEMEDEDTIDVFQQTGGGVY

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Figure 7.1 Comparison of the primary sequences of human SUMO-1 to SUMO-4. Based on SUMO-2/-3 sequences, identical residues are shown in red and conservative changes are in blue. Triangle represents the cleavage site for the maturation of SUMOs.

suggesting differential regulation by SUMO-4 compared to other SUMO proteins (Bohren *et al.*, 2004; Guo *et al.*, 2004). The products of SUMO genes are small proteins with sizes around 10 kDa and are homologous with ubiquitin in their 3D structure (Bayer *et al.*, 1998; Huang *et al.*, 2004). Similar to ubiquitin, all four SUMO isoforms are expressed as their precursors with the C-terminal extension of several amino acids; these precursors require processing to expose the C-terminal Gly-Gly residues that are essential for their conjugation to target proteins. Although SUMO-2 and SUMO-3 proteins are expressed from separate genes, matured forms are almost identical in their amino acid sequences (~97% identical) and only differ from each other with respect to three amino acids in their N-terminal extensions. Thus, SUMO-2 and SUMO-3 can be categorized into a subfamily distinct from that of SUMO-1 which shows only ~50% identity to SUMO-2/-3. The predicted amino acid sequences of the latest member of the family SUMO-4 are much closer to those of SUMO-2/-3 than that of SUMO-1 (~87% identity to SUMO-2). SUMO-4 can be conjugated to target proteins when the matured form is expressed ectopically (Guo *et al.*, 2004, 2005). However, a group reported that the maturation process of SUMO-4 to expose C-terminal Gly-Gly residues is inhibited by a unique proline residue located at position 90; hence, native SUMO-4 appears not to be able to form covalent isopeptide bonds with substrates (Owerbach *et al.*, 2005). Furthermore, endogenous SUMO-4 protein was not detectable upon Ubc9 affinity purification while SUMO-1 and SUMO-2/-3 were identified in HEK293 cells and human kidney tissues (Bohren *et al.*, 2007). SUMO-4 has been implicated as a candidate gene for the susceptibility to type 1 diabetes (Wang and She, 2008). However, it will be critical for the identification of endogenous SUMO-4 protein to support its biological importance. Further, it needs to be clarified whether SUMO-4 fulfills its biological functions through conjugation to cellular targets similar to other SUMO family members or through non-covalent interaction with its binding partners.

The 3D structures of SUMO proteins resemble the overall structure of ubiquitin, characterized by a tightly packed $\beta\beta\alpha\beta\beta\alpha\beta$ -fold (Bayer *et al.*, 1998; Huang *et al.*, 2004). SUMO-1 and SUMO-2 possess an identical folding but present significantly different charge distributions at a surface region near the C-terminus (Huang *et al.*, 2004). SUMO proteins possess long and highly flexible N-terminal extensions that are absent in ubiquitin. Unlike ubiquitin

that generates several distinct polyubiquitin chains through internal lysine residues, only SUMO-2/-3 form a single type of poly-SUMO chains through a lysine residue in the N-terminal extension (Tatham *et al.*, 2001). SUMO-1 cannot form polymeric chains due to the lack of lysine residues in the N-terminal region; however, SUMO-1 is found in poly-SUMO chains possibly at the end of the chain, thus acting on the chain termination signal (Matic *et al.*, 2008). The physiological importance and role of poly-SUMO chains have recently been revealed in the mammalian system. Poly-SUMO chains appeared to provide strong, repetitive recognition sites with multiple copies of SUMO interaction motifs (SIMs) on RNF4, an ubiquitin E3 ligase, which adds ubiquitin to poly-SUMOylated proteins (Lallemand-Breitenbach *et al.*, 2008; Tatham *et al.*, 2008; Weisshaar *et al.*, 2008). The hypothesis that SUMOylation may enhance proteasomal degradation of target proteins by the SUMOylation-mediated ubiquitination process was first proposed in the yeast system (Prudden *et al.*, 2007; Sun *et al.*, 2007; Uzunova *et al.*, 2007; Xie *et al.*, 2007). It has been established that in human acute promyelocytic leukemia (APL) cells, PML protein and its oncogenic fusion form with the retinoic acid receptor α (PML-RAR α) are SUMOylated and degraded by the ubiquitin-proteasome system upon arsenic treatment (Chen *et al.*, 1997; Lallemand-Breitenbach *et al.*, 2001; Muller *et al.*, 1998; Shao *et al.*, 1998; Zhu *et al.*, 1997). However, the mechanism how SUMOylation induces this protein degradation remains unelucidated. It is now clear that arsenic treatment induces poly-SUMOylation by SUMO-2/-3 on PML and PML-RAR α and that, in turn, the poly-SUMO chains recruit RNF4 through interaction with SIMs (Lallemand-Breitenbach *et al.*, 2008; Tatham *et al.*, 2008; Weisshaar *et al.*, 2008). Ultimately, RNF4 adds ubiquitin to PML and PML-RAR α and this leads to their degradation by 26S proteasome. In addition to PML and PML-RAR α , a clock-controlled gene product called BMAL1 is poly-SUMOylated by SUMO-2/-3 and subsequently subjected to ubiquitin-proteasome-mediated degradation (Lee *et al.*, 2008). In this case, it is not known whether RNF4 is the poly-SUMO-specific ubiquitin E3 ligase. Recent proteomic approaches identified a number of SUMO-2 target proteins that were simultaneously conjugated with ubiquitin (Schimmel *et al.*, 2008). Based on these findings, there appear to be a greater number of cellular proteins that are regulated by SUMO-directed, ubiquitin-dependent proteolysis.

In addition to the ability to perform polymeric chain formation, SUMO family members demonstrate functional heterogeneity. SUMO-1 and SUMO-2/-3 preferentially modify different cellular proteins under physiological conditions. For example, RanGAP1 is a good target for SUMO-1 but is poorly conjugated by SUMO-2/-3 (Saitoh and Hinchey, 2000). On the other hand, Topoisomerase II and centromere protein-E (Zhang *et al.*, 2008b) are preferentially modified by SUMO-2/-3 (Azuma *et al.*, 2003, 2005; Zhang *et al.*, 2008b). Some other proteins, such as PML, are substrates for both SUMO-1 and SUMO-2/-3 modification (Fu *et al.*, 2005;

Lallemand-Breitenbach *et al.*, 2008; Muller *et al.*, 1998). Proteomic analysis also supported the target specificity of SUMOs by revealing that cellular proteins are preferentially attached either by SUMO-1 or by SUMO-2, or by both SUMOs (Vertegaal *et al.*, 2006). It is not clear which factors determine differential target specificity since SUMOs utilize the same enzymes for conjugation processes. One possible explanation might be a spatiotemporal availability of SUMO proteins in certain physiological contexts.

2.2. SUMOylation processes and enzymes

The SUMOs are destined to finally attach to the cellular proteins by forming isopeptide bond between the ϵ -amino group of lysine residues on target proteins and the C-terminus of SUMOs. The conjugation processes are achieved by a sequential reaction of a series of enzymes that are analogous but distinct from those of ubiquitin and other ubls (Fig. 7.2). The matured form of SUMOs with exposed C-terminal Gly-Gly residues is activated by the activating enzyme E1, a heterodimeric protein consisting of SUMO-activating enzyme subunit 1 (SAE1, also called Uba2) and SAE2 (Aos1), in an ATP-dependent adenylation on its C-terminus (Desterro *et al.*, 1999; Johnson *et al.*, 1997; Okuma *et al.*, 1999). Activated SUMOs form thiolester bond between active site cysteine residue of E1 and the C-terminus of SUMOs. SUMO proteins are subsequently transferred to

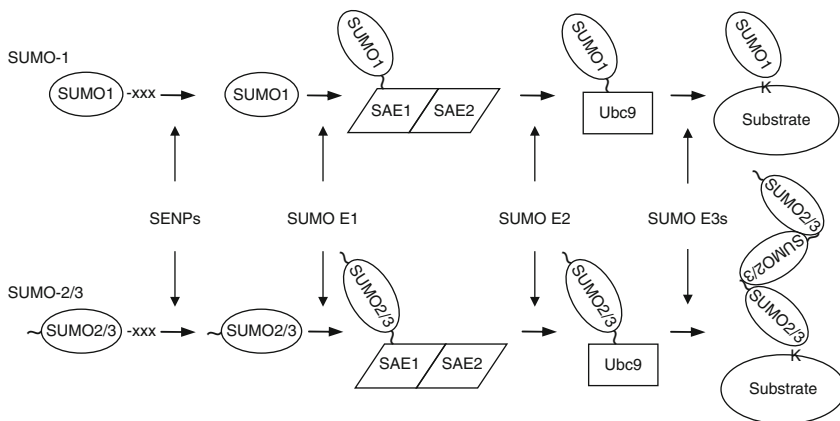


Figure 7.2 The SUMOylation pathway. The C-terminal extensions of SUMOs are processed by SENPs prior to conjugation. The matured SUMOs are activated by a heterodimeric E1 consisting of SAE1 and SAE2 in an ATP-dependent adenylation on its C-terminus. SUMO proteins are subsequently transferred to Ubc9, a unique SUMO E2. SUMO E3s guide the Ubc9-SUMO complex to specific targets so that Ubc9 can aid the complete formation of isopeptide bond by utilizing the energy stored in the thiolester bond. Only SUMO-2/-3 form a single type of poly-SUMO chains through a lysine residue in the N-terminal extension that is missing in SUMO-1.

Ubc9, a unique SUMO E2, by transthioylation reaction. At the end of the reaction, SUMO E3s guide the Ubc9-SUMO complex to specific targets so that Ubc9 can aid the complete formation of isopeptide bond by utilizing the energy stored in the thiolester bond.

2.2.1. SUMO E1

The only function of SUMO E1 is to initiate conjugation reaction, and thus this enzyme appears to not be a target for intensive regulation in cells. Although SUMOylation in other cellular compartments has been recently reported (Geiss-Friedlander and Melchior, 2007), SUMOylation mainly occurs in the nucleus; therefore, majority of both SAE1 and SAE2 localize to the nucleus (Azuma *et al.*, 2001). The protein levels of SAE2 increase during the S-phase and reduce in the G₂-phase, consistent with the pivotal role of SUMOylation in cell cycle progression (Azuma *et al.*, 2001).

SUMOylation is a key step for forming PML nuclear bodies in which many virally expressed proteins have accumulated, and the increase in the size of PML nuclear bodies is inversely correlated with the efficiency of viral infection (Everett, 2001; Regad and Chelbi-Alix, 2001). Thus, SUMOylation has been implicated to play an antiviral role. Certain viruses are known to prevent the activation of host defense systems as a counteraction to antiviral responses (Samuel, 2001). Interestingly, an adenoviral protein Gam1 interacts with SAE1/SAE2 and induces the degradation of SAE1 by recruiting Cullin-really interesting new gene (RING) ubiquitin E3 ligase with its suppressor of cytokine signaling (SOCS)-like domain (Boggio *et al.*, 2007). These findings may indirectly support the antiviral role of SUMOylation.

2.2.2. SUMO E2

The SUMO system comprises a single E2, Ubc9, in all eukaryotes (Johnson and Blobel, 1997; Lee *et al.*, 1998; Saitoh *et al.*, 1998; Schwarz *et al.*, 1998). Ubc9 has a primary structure similar to that of ubiquitin E2s but shows enzymatic specificity only to SUMOs (Desterro *et al.*, 1997; Johnson and Blobel, 1997; Schwarz *et al.*, 1998). Structural analyses revealed that the surface area of Ubc9 for SUMO binding is mainly positively charged and that the surface of SUMO has an overall negative charge (Bencsath *et al.*, 2002; Liu *et al.*, 1999; Tatham *et al.*, 2003). Thus, the charge distribution might facilitate interaction between SUMO and Ubc9 and in discriminating SUMO from other ubls. The most intriguing feature of Ubc9 among ubl-E2s is the recognition potential for a specific signature motif, that is, ψ KXE (ψ , bulky hydrophobic amino acid; K, lysine; X, any amino acid; E, glutamic acid), on the substrates (Rodriguez *et al.*, 2001; Sampson *et al.*, 2001). With this ability, Ubc9 can promote the SUMOylation of targets even without the aid of E3 ligases, at least *in vitro*. In addition to the basic signature motif, a more extended version of it with additional

phosphorylation site has been reported recently (Hietakangas *et al.*, 2003). Heat shock transcription factor 1 (HSF1) is a key inducer of stress-response proteins and has been found to be SUMOylated in a stress-inducible manner (Hietakangas *et al.*, 2003). The SUMOylation on HSF1 is entirely dependent on the phosphorylation of serine residues that reside five amino acids downstream of the SUMOylation site. In addition to HSF1, the phosphorylation-dependent SUMOylation motif has been reported in a number of cellular targets such as HSF4b, myocyte enhancer factor 2A (MEF2A), and GATA-1 (Hietakangas *et al.*, 2006). It will be interesting to examine the manner in which phosphorylation regulates interaction between the signature sequence and Ubc9. Although majority of SUMO targets accept SUMO on the ψ KXE signature motif, certain proteins, such as ubiquitin-conjugating enzyme E2-25K, are SUMOylated on the other site than the signature motif (Pichler *et al.*, 2005). The ψ KXE signature motif enhances Ubc9 interaction with majority of substrates but appears not to operate with respect to all targets.

In addition to mediating SUMO conjugation reactions, Ubc9 functions as SUMOylation target; further, autoSUMOylation of Ubc9 appears to modulate target discrimination without affecting thiolester formation or catalytic activity (Knipscheer *et al.*, 2008). The effects of autoSUMOylation on Ubc9 vary depending on the target proteins: no autoSUMOylation effects toward HDAC4, E2-25K, PML, or TDG, inhibition of Ubc9 function on RanGAP1, and strong activation of Ubc9 for the SUMOylation of Sp100 were observed. Detailed mechanistic and structural analyses suggested that SUMOylation on Ubc9 can create a new interface between SUMO on Ubc9 and SIM in a subset of substrates, in addition to the original interaction between Ubc9 and ψ KXE motif on substrates (Knipscheer *et al.*, 2008). In this manner, autoSUMOylation can enhance the efficiency of the SUMOylation reaction even without the aid of E3 ligases for certain substrates that contain SIM motif. If the SIM motif on the substrate is not positioned or oriented properly, autoSUMOylation does not enhance substrate SUMOylation as in the case of HDAC4, E2-25K, PML, or TDG. Thus, it is probable that autoSUMOylation of Ubc9 represents another level of control mechanisms to enlarge target specificity according to cellular context, since autoSUMOylated Ubc9 was found in a tissue-specific manner (Nacerddine *et al.*, 2005).

2.2.3. SUMO E3s

Although *in vitro* SUMOylation can be achieved without the aid of E3, the efficiency and target specificity of conjugation reactions by Ubc9 are enhanced with the support of E3 ligases (Hochstrasser, 2001). SUMO E3s accelerate conjugation reactions by bringing the substrate in close proximity to Ubc9 $\sim$ SUMO and also by increasing the catalytic activity of Ubc9. SUMO E3 ligases contain three subtypes of enzymes: classical type

harboring RING-like domain for E2 binding, nonclassical type without any homology domain or motif to known ubl E3s, and the dual-function type that acts with both ubiquitin and SUMOs (Table 7.1). The classical E3s for ubiquitin and ubls comprise conserved structural domains on which E2 interacts. It can be inferred from this viewpoint that the protein inhibitor of activated STAT (PIAS) family members and MMS21 (also called NSE2) belong to the classical type of E3s. They comprise the SP-RING domain for Ubc9 binding, which is similar to the RING domain of ubiquitin E3s

Table 7.1 SUMO E3 ligases and their targets in the mammalian systems

Name	Representative cellular targets
<i>Classical SP-RING type</i>	
PIAS1	p53 (Kahyo <i>et al.</i> , 2001; Schmidt and Muller, 2002), GRIP1 (Kotaja <i>et al.</i> , 2002b), AR (Nishida and Yasuda, 2002), PPAR γ (Ohshima <i>et al.</i> , 2004), ER α (Sentis <i>et al.</i> , 2005), PR (Daniel <i>et al.</i> , 2007; Jones <i>et al.</i> , 2006), and MBD1 (Lyst <i>et al.</i> , 2006)
PIASx	AR (Kotaja <i>et al.</i> , 2002a; Nishida and Yasuda, 2002), GRIP1 (Kotaja <i>et al.</i> , 2002b), STAT-1 (Rogers <i>et al.</i> , 2003), PPAR γ (Ohshima <i>et al.</i> , 2004), and SF-1 (Lee <i>et al.</i> , 2005)
PIAS3	IRF-1 (Nakagawa and Yokosawa, 2002), ER α (Sentis <i>et al.</i> , 2005), MBD1 (Lyst <i>et al.</i> , 2006), c-Myb (Sramko <i>et al.</i> , 2006), and Tel (Roukens <i>et al.</i> , 2008)
PIASy	LEF-1 (Sachdev <i>et al.</i> , 2001), C/EBP α (Subramanian <i>et al.</i> , 2003), c-Myb (Dahle <i>et al.</i> , 2003), GATA-1 (Collavin <i>et al.</i> , 2004), SF-1 (Lee <i>et al.</i> , 2005), NEMO (Mabb <i>et al.</i> , 2006), and YY1 (Deng <i>et al.</i> , 2007)
MMS21	hSMC6 and TRAX (Potts and Yu, 2005), and TRF1 and TRF2 (Potts and Yu, 2007)
<i>Nonclassical type</i>	
RanBP2	Topoisomerase II <i>in vivo</i> (Dawlaty <i>et al.</i> , 2008), HDAC4 (Kirsh <i>et al.</i> , 2002), Sp100 (Pichler <i>et al.</i> , 2002), and PML (Tatham <i>et al.</i> , 2005) <i>in vitro</i>
Pc2	CtBP (Kagey <i>et al.</i> , 2003), SIP1 (Long <i>et al.</i> , 2005), HIPK2 (Rosic <i>et al.</i> , 2006), and Dnmt3a (Li <i>et al.</i> , 2007)
HDAC4	MEF2 (Gregoire and Yang, 2005; Zhao <i>et al.</i> , 2005), LXR β (Ghisletti <i>et al.</i> , 2007), and HIC1 (Stankovic-Valentin <i>et al.</i> , 2007)
<i>Dual-function type</i>	
TOPORS	p53 (Weger <i>et al.</i> , 2005); several more putative targets, including Sin3A, from proteomic approach (Pungaliya <i>et al.</i> , 2007)
TRAF7	c-Myb (Morita <i>et al.</i> , 2005)

(Kotaja *et al.*, 2002a; Nishida and Yasuda, 2002; Potts and Yu, 2005; Sachdev *et al.*, 2001; Schmidt and Muller, 2002; Zhao and Blobel, 2005). The PIAS family of proteins comprises four members in the mammalian system, namely, PIAS1, PIAS3, PIASx, and PIASy; further, PIASx has two splicing variants: PIASx α and PIASx β (Shuai and Liu, 2003). A mutational analysis in the SP-RING domain of PIAS1 resulted in the loss of Ubc9 binding, confirming the role of the domain for the interaction with E2 (Kahyo *et al.*, 2001). PIAS family members function as transcriptional coregulators in cytokine signaling as well as in a variety of other cellular processes, including the p53 pathway, nuclear receptor signaling, and Wnt signaling (Schmidt and Muller, 2003). The role of PIAS proteins as transcriptional coregulators has been largely investigated independent of SUMO ligase activity (Shuai and Liu, 2005). However, a recent study revealed the link between SUMO ligase activity and the transcriptional coregulator function of PIAS1 protein (Liu *et al.*, 2007), suggesting the possibility that SUMO ligase activity of other PIAS proteins might be important for their transcriptional coregulator function. Consistent with their roles in transcription, a number of transcription factors, including p53, LEF-1, c-Jun, androgen receptor, STAT-1, and c-Myb, have been indicated to be PIAS substrates (Kahyo *et al.*, 2001; Nishida and Yasuda, 2002; Sachdev *et al.*, 2001; Schmidt and Muller, 2002; Sramko *et al.*, 2006; Ungureanu *et al.*, 2003). PIASy has been suggested to be essential to accurately direct Topoisomerase II to specific chromosomal regions that is crucial for sister-chromatid separation in mitosis (Diaz-Martinez *et al.*, 2006). However, this possibility was not supported by the results of the genetic studies performed on PIASy-knockout mice, since PIASy-deficient mice and cells did not demonstrate chromosome segregation abnormalities (Dawlaty *et al.*, 2008; Roth *et al.*, 2004; Wong *et al.*, 2004). Another SP-RING containing protein, that is, MMS21, was initially identified in a multiprotein complex that is required for growth and DNA repair in *S. cerevisiae* (Zhao and Blobel, 2005). Biochemical and genetic experiments confirmed the SUMO ligase activity of MMS21 and provide that this activity is required for the maintenance of nucleolar integrity, telomere clustering, silencing, and length regulation (Zhao and Blobel, 2005). MMS21 has SUMO ligase activity and participates in the DNA repair process in humans (Potts and Yu, 2005). Further, MMS21 is critical for the recruitment of telomeres to PML bodies, which is a hallmark of ATL cells, through the SUMOylation of multiple telomere-binding proteins, including TRF1 and TRF2 (Potts and Yu, 2007).

A polycomb protein Pc2, a histone deacetylase HDAC4, and a nucleoporin RanBP2 (also called Nup358) belong to nonclassical SUMO E3 ligases from the viewpoint that they do not contain any established, conserved domain of E3s (Kagey *et al.*, 2003; Pichler *et al.*, 2002). Pc2 is a member of polycomb group proteins that form large multimeric complexes

that are involved in the stable repression of gene expression (Wotton and Merrill, 2007). The number of substrates of Pc2 is relatively few compared to that of other SUMO E3s. The Pc2 substrates in the list comprise the transcriptional corepressor CtBP, Smad-interacting protein 1 (SIP1), homeodomain-interacting protein kinase 2 (HIPK2), and DNA methyltransferase 3a (Kagey *et al.*, 2003; Li *et al.*, 2007; Long *et al.*, 2005; Roscic *et al.*, 2006). HDAC4 is implicated in SUMOylation of MEF2 and liver X receptor (Ghisletti *et al.*, 2007; Zhao *et al.*, 2005). HDAC4 that contains an N-terminal Ubc9-interacting domain increases SUMOylation of MEF2 on the same lysine residue for acetylation, and thereby, represses transcriptional activity of MEF2 (Zhao *et al.*, 2005). In addition, LXR SUMOylation by HDAC4 is critical for the LXR-mediated transrepression of subsets of immune response genes (Ghisletti *et al.*, 2007). Thus, it appears that Pc2 and HDAC4 potentiate transcriptional repression by SUMOylation of target proteins. The mechanisms via which Pc2 and HDAC4 enhance substrate SUMOylation have not been studied well. Since these enzymes do not have significant sequence similarity to other known SUMO E3s, further structural studies will elucidate new insights on the E3 function of nonclassical E3s. The third protein RanBP2 has initially been observed as a binding partner of SUMOylated RanGAP1, which traps it at the cytoplasmic face of the nuclear pore complex (Mahajan *et al.*, 1997; Matunis *et al.*, 1998). Subsequently, the SUMO ligase activity of RanBP2 was identified; this activity is observed to reside in a ~30 kDa domain of RanBP2 that does not resemble any established ubl E3s but directly interacts with Ubc9 and strongly enhances SUMOylation of the SUMO-1 target Sp100 (Pichler *et al.*, 2002). Extensive structural analyses suggested that RanBP2 acts as an E3 by binding both SUMO and Ubc9 to enhance the catalytic property of Ubc9 and to position SUMO ~ Ubc9 in an optimal orientation (Pichler *et al.*, 2005; Reverter and Lima, 2005; Tatham *et al.*, 2005). RanBP2 has been implicated in nucleocytoplasmic transport as a part of the nuclear pore complex (Chook *et al.*, 1999). However, RanBP2 appears to not be an essential component for nucleocytoplasmic trafficking. Depletion of RanBP2 showed no significant effect on assembly of nuclear pore complex and nuclear protein import, whereas it caused some reduction in the nuclear export of nuclear export signal (NES)-containing proteins and mRNA (Bernad *et al.*, 2004; Hutten and Kehlenbach, 2006). In addition, hypomorphic mouse embryonic fibroblasts expressing approximately a quarter of RanBP2 protein did not show any overt defect in the nuclear export of mRNA and in the bidirectional nucleocytoplasmic transport of proteins (Dawlaty *et al.*, 2008). RanBP2 is suggested to play a role in mitosis (Arnaoutov and Dasso, 2005). During mitosis, RanBP2 is located on spindles and at kinetochores of chromosomes (Joseph *et al.*, 2002, 2004). In addition, depletion of RanBP2 in cells caused mitotic defects including mislocalization of several kinetochore components leading to aberrant

kinetochore structure, formation of multipolar spindles, and mitotic arrest (Joseph *et al.*, 2004; Salina *et al.*, 2003). Given that, RanBP2 appears to be more closely involved in mitosis than nucleocytoplasmic trafficking.

Lastly, Topoisomerase I-interacting protein (TOPORS) and tumor necrosis factor receptor-associated factor 7 (TRAF7) are dual-function E3 ligases that act with both ubiquitin and SUMOs (Morita *et al.*, 2005; Weger *et al.*, 2005). TOPORS, a binding partner of Topoisomerase I and p53, has an ubiquitin E3 ligase activity for p53 (Rajendra *et al.*, 2004) and was also suggested as a SUMO E3 for p53 (Weger *et al.*, 2005). Recent proteomic screening identified several potential TOPORS targets for SUMOylation, including Sin3A, which are involved in chromatin modification or transcriptional regulation (Pungaliya *et al.*, 2007). TOPORS has been implicated as a tumor suppressor in lung, colon, and brain tumors (Bredel *et al.*, 2005; Oyanagi *et al.*, 2004; Saleem *et al.*, 2004); however, the relationship between tumor suppressor function and SUMO ligase activity has not been studied. TRAF7, with its self-ubiquitination activity, can stimulate SUMOylation of proto-oncogene product c-Myb, and TRAF7-mediated SUMOylation sequesters c-Myb to the cytosol, probably to negatively regulate c-Myb function (Morita *et al.*, 2005). For these dual-function E3 ligases, more extensive studies on the mechanism and structure will be required to elucidate the manner in which these proteins recognize both substrates unlike other E3 ligases. Although new SUMO E3 ligases are being identified with time, the number of SUMO ligases identified thus far is relatively small compared to the huge number of ubiquitin E3 ligases. Furthermore, increasing numbers of SUMO targets residing in cellular compartments other than the nucleus are being identified; however, the associated E3 ligases have not been described. From this viewpoint, it can be inferred that many E3 ligases remains unidentified.

2.2.4. SUMOylation enhancer

In addition to SUMO E1, E2, and E3s, a protein called RSUME (RWD-containing SUMOylation enhancer) was identified as a new member of the SUMOylation pathway, which enhances gross cellular SUMOylation by SUMO-1, SUMO-2, and SUMO-3 (Carbia-Nagashima *et al.*, 2007). RSUME enhances overall cellular SUMO-1 conjugates without altering E1 levels in transfection and increased I κ B SUMOylation in the *in vitro* assays. The ability of RSUME to activate general SUMOylation is distinct from the action of E3 ligases that specify a narrow range of substrates. RSUME appears to physically interact with both SUMO-1 and Ubc9, increase noncovalent binding of SUMO-1 to Ubc9, and enhance Ubc9 thioester formation and SUMO polymerization. In biological systems, RSUME is suggested to be involved in NF- κ B signaling and hypoxic response by enhancing SUMOylation of I κ B and HIF-1 α , respectively. It will be interesting to have a knockout mouse model of RSUME to observe

the manner in which RSUME is involved in SUMOylation events and elucidate the biological effects of RSUME deficiency under physiological conditions.

2.3. Enzymes for deSUMOylation process and its physiological roles

SUMO-specific proteases (SENPs) are typical cysteine proteases that contain the His/Asp/Cys catalytic triad at its carboxyl (C)-terminus. Three major roles of SENPs can be proposed in biological systems. The first is to process C-terminal extension of SUMOs; this extension requires removal before the initiation of conjugation reaction. The second is removing SUMO proteins from their polymeric chains in the case of SUMO-2/-3. The last and the most important function of SENPs are to reverse SUMOylation by separating SUMO proteins from their conjugates depending on the cellular context. For precursor processing, SENPs hydrolyze the peptide bond between the C-terminal Gly-Gly residues of SUMO and the extra extensions. For deSUMOylation from cellular targets or from poly-SUMO chains, SENPs cleave isopeptide bond between the C-terminal carboxyl group of Gly in SUMOs and the ϵ -amino group of lysine residues in targets or in SUMOs. Thus, individual SENP shows a range of catalytic activities toward precursor processing and isopeptide bond cleavage as well as demonstrates different preferences to SUMO paralogs (e.g., SUMO-1, SUMO-2, and SUMO-3). These characteristics of SENPs are well summarized in another review (Drag and Salvesen, 2008). SUMOylation is an extremely dynamic process in cells and the extent and duration of SUMOylation are regulated at the level of conjugation by E1, E2, E3s, as well as deconjugation process by SENPs. Yeast *S. cerevisiae* has two deSUMOylating enzymes, Ulp1 and Ulp2; of these, Ulp1 is indispensable for cell growth and viability (Li and Hochstrasser, 1999, 2000). In humans, initial research to discover SENPs in the human genome revealed seven candidate proteins, that is, SENP1-SENTP7, which have homology to Ulp proteins at its C-terminal regions (Yeh *et al.*, 2000). SENP4 is likely to be a pseudogene, since no matching mRNA has been found. The homology comparison between Ulp proteins and human SENPs are well described elsewhere (Hay, 2007). Among these candidates, biological roles of at least five enzymes, including SENP1, SENP2, SENP3, SENP5, and SENP6 (SUSP1), have been elucidated (Table 7.2).

2.3.1. SENP1

SENP1 is the first identified and the most extensively characterized enzyme among human SENPs. SENP1 possesses endopeptidase activity for SUMO precursor processing as well as isopeptidase activity for deconjugation of

Table 7.2 SUMO-specific proteases in humans

Name	Cellular localization	Substrate specificity
SEN1	Nucleus (Bailey and O'Hare, 2004; Gong <i>et al.</i> , 2000) and nuclear-cytoplasmic shuttling (Kim <i>et al.</i> , 2005b; Li <i>et al.</i> , 2008)	Both precursor processing and isopeptidase activity (Gong <i>et al.</i> , 2000; Mikolajczyk <i>et al.</i> , 2007; Xu and Au, 2005) Specific to SUMO-1 <i>in vivo</i> from knockout studies (Yamaguchi <i>et al.</i> , 2005)
SEN2	Nuclear pore (Hang and Dasso, 2002; Zhang <i>et al.</i> , 2002), nucleus (Nishida <i>et al.</i> , 2000), and nuclear-cytoplasmic shuttling (Itahana <i>et al.</i> , 2006)	Preferential isopeptidase activity than precursor processing to all SUMO paralogs (Mikolajczyk <i>et al.</i> , 2007; Reverter and Lima, 2006; Zhang <i>et al.</i> , 2002)
SEN3	Nucleolus and nucleus (Nishida <i>et al.</i> , 2000)	Isopeptidase activity to SUMO-2/-3 (Gong and Yeh, 2006; Nishida <i>et al.</i> , 2000)
SEN5	Nucleolus and nucleus (Di <i>et al.</i> , 2006; Gong and Yeh, 2006) and cytoplasm (Zunino <i>et al.</i> , 2007)	Both precursor processing and isopeptidase activity to SUMO-2/-3 (Di <i>et al.</i> , 2006; Gong and Yeh, 2006; Mikolajczyk <i>et al.</i> , 2007)
SEN6	Nucleus (Choi <i>et al.</i> , 2006; Mukhopadhyay <i>et al.</i> , 2006)	Isopeptidase activity to SUMO-1 (Choi <i>et al.</i> , 2006) and more preferentially to SUMO-2/-3 (Mukhopadhyay <i>et al.</i> , 2006) Weak precursor processing activity (Kim <i>et al.</i> , 2000)
SEN7	Nucleus (Cheng <i>et al.</i> , 2006)	Isopeptidase activity preferentially to SUMO-2/-3 (Lima and Reverter, 2008)

SUMO from targets, toward SUMO-1, SUMO-2, and SUMO-3 *in vitro* (Gong *et al.*, 2000; Mikolajczyk *et al.*, 2007; Xu and Au, 2005). In the comparison of enzymatic activity among purified C-terminal catalytic domains of SENPs, SENP1 appeared to possess the strongest potential for both precursor processing and isopeptide bond cleavage (Mikolajczyk *et al.*, 2007). Although SENP1 cleaves peptide and isopeptide bond for all three SUMO paralogs *in vitro*, SENP1 is likely to be very specific to SUMO-1 *in vivo*, since the depletion of SENP1 in mice showed decreased SUMO-1

processing and accumulation of SUMO-1 conjugates with no effect on SUMO-2/-3 in both events (Yamaguchi *et al.*, 2005). SENP1 localizes in the nucleus or the cytoplasm, depending on the cellular contexts and cell types (Bailey and O'Hare, 2004; Gong *et al.*, 2000; Kim *et al.*, 2005b; Li *et al.*, 2008). SENP1 contains three nuclear localization signals (NLS) in the N-terminal region (NLS1) and C-terminal regions (NLS2 and NLS3), and also harbors an NES at its C-terminus (Bailey and O'Hare, 2004; Kim *et al.*, 2005b). NLS1 plays major a role in nuclear localization; however, NLS2 and NLS3 are necessary for the complete retention of SENP1 in the nucleus (Bailey and O'Hare, 2004; Kim *et al.*, 2005b). The C-terminal NES is critical for the cytoplasmic deposition of SENP1 (Kim *et al.*, 2005b). In the nucleus, SENP1 regulates a variety of cellular events, including transcription, by modulating the SUMOylation status of target proteins. For example, SENP1 enhances transcriptional activity of androgen receptors via deSUMOylation of HDAC1, which is responsible for decreased deacetylase activity (Cheng *et al.*, 2004). DeSUMOylation of the CRD1 domain, a strong transcriptional repression domain, in p300 by SENP1 enhances c-Jun transcriptional activity by relieving the *cis*-repression of p300 activity (Cheng *et al.*, 2005). Even though an increasing number of SENP1 targets have been found in the nucleus, cytoplasmic substrates are yet to be identified. Thus, it remains to be clarified whether cytoplasmic localization of SENP1 is an active process involving cytoplasmic targets or a passive phenomenon merely to sequester SENP1 to the exterior of the nucleus.

2.3.2. SENP2

SENP2 has a preference for deSUMOylation as compared to precursor processing. Initial research showed that SENP2 can cleave C-terminal extension of SUMO-1 and SUMO-3, and also the isopeptide bond between SUMO-1/SUMO-3 and associated substrates *in vitro* (Zhang *et al.*, 2002). More extensive biochemical assays and structural analyses of SENP2-substrates complexes revealed that SENP2 catalyzes deSUMOylation reaction more efficiently than precursor processing (Mikolajczyk *et al.*, 2007; Reverter and Lima, 2006). SENP2 was found in association with the nuclear face of nuclear pore complexes by binding to a nucleoporin, Nup153 (Hang and Dasso, 2002; Zhang *et al.*, 2002). Ectopic expression of mutant SENP2 lacking the interaction motif with Nup153 leads to nonspecific deSUMOylation in cells (Hang and Dasso, 2002), suggesting that proper subcellular localization of SENPs might be an important tool to control *in vivo* function of SENPs. Consistent with this idea, overexpression of SENP2 does not alter SUMOylation of RanGAP1, which resides in the cytoplasmic space of nuclear pores, while SENP2 cleaves the isopeptide bond between SUMO and RanGAP1 *in vitro* (Hang and Dasso, 2002). In addition to the primary localization to the nuclear pore complexes, SENP2 can shuttle between the nucleus and the cytoplasm with its NLS and NES

(Itahana *et al.*, 2006). The cytoplasmic fraction of SENP2 undergoes degradation attributable to the ubiquitin-proteasome system, indicating that it might be another regulatory mechanism of SENP2 (Itahana *et al.*, 2006). SENP2 is involved in transcriptional regulation and determining cellular location of certain proteins. SENP2-mediated deSUMOylation of several transcriptional regulators such as p300, CREB-binding protein (CBP), and Elk-1 reverts transcriptional repression by SUMOs (Girdwood *et al.*, 2003; Kuo *et al.*, 2005; Yang *et al.*, 2003).

DeSUMOylation of ZNF451, a PML body-associated transcriptional coregulator, redistributes it from the nuclear domain to nuclear speckles and the nucleoplasm (Karvonen *et al.*, 2008). While most SENP2 functions have been described in the nucleus, its non-nuclear role has also been reported. The voltage-gated potassium channel, Kv1.5, is a newly identified SUMO target; SENP2 serves as a deSUMOylating enzyme for Kv1.5 (Benson *et al.*, 2007). Reversible SUMOylation appears to control the electrophysiological properties of the voltage-sensitive channel.

2.3.3. SENP3

SENP3 has a distinct specificity to SUMO-2/-3 conjugates over SUMO-1 conjugates *in vitro* and *in vivo*; further, it binds preferentially to SUMO-2/-3 (Gong and Yeh, 2006; Nishida *et al.*, 2000). SUMO C-terminal processing property of SENP3 has not been characterized. SENP3 has been predicted to play a role in the nucleolus with its concentrated nucleolar distribution (Nishida *et al.*, 2000). In the nucleolus, SENP3 is associated with nucleophosmin (NPM1), a crucial factor for ribosome biogenesis and a target of SUMOylation, and catalyses deSUMOylation of NPM1-SUMO-2 conjugates (Haindl *et al.*, 2008). Depletion of SENP3 inhibits the conversion of the 32S rRNA species to the 28S form, which is the same defect as that observed in NPM1 depletion cases. The control of SUMOylation of NPM1 by SENP3 is essential for 28S rRNA maturation. Similar to other SENPs, SENP3 is also involved in transcriptional regulation. The transcriptional and myogenic activities of MEF2D is modulated by SUMO-2/-3 conjugation as well as deSUMOylation by SENP3 (Gregoire and Yang, 2005).

2.3.4. SENP5

SENP5 has similarity to SENP3 in terms of SUMO paralog specificity and subcellular localization. SENP5 possesses both precursor processing and isopeptidase activity toward SUMO-2/-3 in preference to SUMO-1 (Di *et al.*, 2006; Gong and Yeh, 2006; Mikolajczyk *et al.*, 2007). SENP5 localizes mainly to the nucleolus with its N-terminal stretch of amino acids (Di *et al.*, 2006; Gong and Yeh, 2006); however, it is also distributed in the nucleus (Gong and Yeh, 2006). A recent study revealed additional localization of SENP5 in the cytosol and cytosolic SENP5 separates SUMO-1 from its cellular conjugates (Zunino *et al.*, 2007). The cytosolic

fraction of SENP5 appeared to be involved in the regulation of mitochondrial morphology (Zunino *et al.*, 2007). A dynamin-related protein (DRP1), which is required for mitochondrial fission and is recruited from the cytosol to the mitochondrial outer membrane to coordinate membrane scission, has been identified as a SUMO-1-conjugated protein (Harder *et al.*, 2004). SUMOylation of DRP1 enhances DRP1-mediated mitochondrial fission by increasing the stability of DRP1. In contrast, SENP5 is found to be responsible for deSUMOylation of DRP1 and thus, silencing of SENP5 results in a fragmented and altered mitochondrial morphology. This is a new instant of cytoplasmic function of SENPs. The most intriguing function of SENP5 is regulation of cell division. Depletion of SENP5 by RNA interference (RNAi) caused inhibition of cell proliferation, aberrant nuclear morphology, and appearance of binucleated cells, which is consistent with mitotic and cytokinetic defects (Di *et al.*, 2006). Blocking of SUMOylation, as observed in cases of *Ubc9* deletion, also caused a huge defect in chromosome segregation (Nacerddine *et al.*, 2005). Thus, balanced control of SUMOylation status of certain target proteins by SUMOylation and deSUMOylation is critical for proper progression of cell division.

2.3.5. SENP6 (SUSP1)

SENP6 is one of the members of early identified human SENPs referred to as SUMO-specific protease 1 (SUSP1) (Kim *et al.*, 2000). In the initial studies on catalytic activity, SUSP1 showed precursor processing activity when the enzyme and the substrate SUMO-1- β -galactosidase were coexpressed in *Escherichia coli* and also in an *in vitro* cleavage assay with SUMO-1-PESTc as a substrate (Kim *et al.*, 2000). The deSUMOylating activity of SUSP1 has been elucidated in subsequent studies; this involves deconjugation of SUMO-1 from retinoid X receptor α (Choi *et al.*, 2006) and SUMO-2/-3 from a clock-controlled gene BMAL1 (Lee *et al.*, 2008). In terms of SUMO paralog specificity, SUSP1 preferentially separates SUMO-2/-3 from its conjugates over SUMO-1 (Mukhopadhyay *et al.*, 2006). SUSP1, together with SENP7, demonstrated much lower catalytic activity against both SUMO-linear fusion and isopeptide bond among SENP family members (Mikolajczyk *et al.*, 2007). SUSP1 is predominantly localized in the nucleoplasm with its N-terminal domain serving as a determinant for the localization (Choi *et al.*, 2006; Mukhopadhyay *et al.*, 2006), although it was initially reported in the cytoplasm (Kim *et al.*, 2000). The depletion of SUSP1 caused redistribution of SUMO-2/-3 to PML bodies and led to an increase in the number and size of PML bodies, indicating the role of SUSP1 in PML body maintenance (Mukhopadhyay *et al.*, 2006). SUSP1 is also involved in the regulation of protein stability by cleaving poly-SUMO-2/-3 chains from BMAL1, otherwise degraded by the ubiquitin-proteasome system (Lee *et al.*, 2008).

2.4. Knockout mouse models for SUMO system

The physiological roles of the SUMO system have been extensively studied in yeast with the implication of its pivotal roles in cell cycle progression. This SUMO system is much more complicated in the mammalian system than in yeast: single *SUMO* gene in yeast *S. cerevisiae* versus four isoforms in mammals and a much greater number of E3 ligases and deSUMOylating enzymes in the mammalian system. Recent progress in knockout studies revealed the physiological importance of individual members of the SUMO system in mammalian biology as well as redundancy among SUMO proteins or among subgroup of enzymes. The results of knockout studies are briefly summarized in Table 7.3.

2.4.1. SUMO-1

A link between SUMO and human diseases has been proposed by a study that *SUMO-1* was suggested as a candidate gene responsible for cleft lip phenotype (Alkuraya *et al.*, 2006). In a patient with cleft lip and palate, karyotype analysis identified a balanced reciprocal chromosomal translocation t(2;8)(q33.1;q24.3), which does not alter gross gene expression in general. Instead, the 2q breaking point interrupted the expression of *SUMO-1* gene, indicating that SUMO-1 disruption might be a cause of cleft lip. The *Sumo-1*-targeted mice generated by the gene-trap method exhibited similar cleft lip phenotype in heterozygotes (8.7% of total heterozygotes). A certain proportion of heterozygotes and a greater number of homozygous knockouts showed lethality in the embryonic stage or immediately after birth, suggesting the presence of developmental defects in case of Sumo-1 deficiency.

Inconsistent with the observations of Alkuraya *et al.* (2006), a recent conventional knockout study on *Sumo-1* gene from independent group reported neither the cleft lip phenotype nor any developmental defects (Zhang *et al.*, 2008a). Targeted disruption of the last three exons of the murine *Sumo-1* gene abolished SUMO-1 expression in mice as well as SUMO-1 conjugation on RanGAP1 in embryonic fibroblasts. However, Sumo-1-deficient mice are completely normal with no developmental defects or cleft lip in both heterozygotes and homozygous knockout mice. Rather, in this study, functional compensation of SUMO-1 by SUMO-2/-3 was proposed by demonstrating an increased modification of established SUMO-1 targets by SUMO-2/-3. Presently, it is difficult to evaluate the accuracy of the results concerning the developmental and cleft lip phenotype in SUMO-1-knockout mice. Since the thorough analysis of *SUMO-1* gene-trapped mice has not been published yet, the study will help us gain a clear understanding of this discrepancy.

Table 7.3 Phenotypes of knockout models for SUMO pathways

Gene name	Phenotypes	References
<i>SUMO-1^a</i>	Partial embryonic lethality and cleft lip phenotype Normal development; no cleft lip phenotype; functional compensation of SUMO-1 deficiency by SUMO-2/-3	Alkuraya et al. (2006) Zhang et al. (2008a)
<i>Ubc9</i>	Embryonic lethality; severe mitotic defects in chromosome condensation and segregation; abnormality in nuclear architecture, including nuclear envelope dysmorphism and disruption of nucleoli and PML nuclear bodies; defects in nucleocytoplasmic trafficking	Nacerddine et al. (2005)
<i>PIAS1</i>	Partial embryonic lethality; runts; significant defects in downregulating Jak/Stat and NF- κ B signaling; hyperinflammatory response	Liu et al. (2004, 2005)
<i>PIASx</i>	Normal development; minor reduction in the testis weight but fertile	Santti et al. (2005)
<i>PIASy</i>	Normal development; slight reduction in IFN- γ or Wnt signaling Defects in downregulating Jak/Stat and NF- κ B signaling in dendritic cells	Roth et al. (2004) and Wong et al. (2004) Tahk et al. (2007)
<i>PIAS1/PIASy^b</i>	Embryonic lethality	Tahk et al. (2007)
<i>RanBP2</i>	Embryonic lethality	Aslanukov et al. (2006)
<i>RanBP2</i> hypomorphic	Normal development; normal global SUMOylation by SUMO-1 and SUMO-2/-3 Chromosome segregation defects with the formation	Dawlaty et al. (2008)

(continued)

Table 7.3 (*continued*)

Gene name	Phenotypes	References
<i>SENP1</i>	of anaphase bridges; higher incidence of spontaneous and carcinogen-induced tumorigenesis Embryonic lethality; severe fetal anemia due to defects in erythropoietin production	Cheng <i>et al.</i> (2007)

^a The phenotypes of SUMO-1 knockout from two independent groups are inconsistent. See text.

^b *PLAS1/PLASy* represents double knockout of both genes.

2.4.2. SUMO E2 Ubc9

Ubc9 was originally identified with a phenotype involved in cell cycle regulation in *S. cerevisiae* ([Seufert *et al.*, 1995](#)). Ubc9-deficient cells were observed to be defective in cell cycle progression at G₂- or early M-phase, causing the accumulation of large budded cell with a single nucleus, short spindle, and replicated DNA ([Seufert *et al.*, 1995](#)). In other organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster*, depletion of Ubc9 also resulted in developmental defects in embryonic stages ([Epps and Tanda, 1998](#); [Jones *et al.*, 2002](#)). The deletion of *Ubc9* gene in mice was also detrimental to the developmental processes causing embryonic lethality at the early postimplantation stage, which is accompanied by the apoptotic cell death of the inner cell mass region ([Nacerddine *et al.*, 2005](#)). More detailed analyses of Ubc9-deficient blastocysts cultured *in vitro* revealed severe mitotic defects in chromosome condensation and segregation. Approximately 10% of Ubc9-deficient mitotic cells show anaphase bridges that are similar to the phenotype of *RanBP2*-knockout cells ([Dawlaty *et al.*, 2008](#); [Nacerddine *et al.*, 2005](#)). In addition, Ubc9 deficiency caused abnormality in nuclear architecture, including nuclear envelope dysmorphism and disruption of nucleoli and PML nuclear bodies ([Nacerddine *et al.*, 2005](#)). Ran-GAP1 also failed to accumulate at the nuclear pore complex causing mislocalization of Ran protein, suggesting a defect in nucleocytoplasmic trafficking. *Ubc9* deletion in mice, which causes complete blocking of SUMOylation, is excessively fatal to study its roles in live animals. Conditional gene deletion might also cause rapid regression of the *Ubc9*-deleted organs or tissues so that it renders the analysis of the effects of Ubc9 deficiency difficult. The strategy of generating hypomorphic alleles, which is adapted for *RanBP2* deletion in mice, might be an alternative strategy to avoid its lethal phenotype ([Dawlaty and van Deursen, 2006](#); [Dawlaty *et al.*, 2008](#)). Interestingly, the overexpression of the dominant negative form of

Ubc9 that impairs enzymatic activity in MCF-7 breast cancer cells is associated with an increased sensitivity of these cells to anticancer drugs (Mo *et al.*, 2005); further, MCF-7 cells expressing the dominant negative form of Ubc9 showed reduced growth ability in the xenograft mouse model (Mo *et al.*, 2004). In conclusion, Ubc9, in conjunction with SUMOylation, is a fundamental protein for cell survival in mammalian systems and might play a role in tumorigenesis.

2.4.3. SUMO E3 PIASs

Although an increasing number of PIAS substrates have been reported, knockout studies for PIAS1, PIASy, and PIASx exhibited trivial defects in the general SUMOylation ability of individual knockout mice (Liu *et al.*, 2004; Roth *et al.*, 2004; Santti *et al.*, 2005; Wong *et al.*, 2004). The gross phenotype of PIASy-knockout mice is mild, showing no developmental defects and slight reduction in IFN- γ or Wnt target gene expression in embryonic fibroblasts (Roth *et al.*, 2004; Wong *et al.*, 2004). PIASx-deficient mice are normal and fertile except for minor reduction in the testis weight in mutant males (Santti *et al.*, 2005). PIAS1-knockout mice demonstrated the most characteristic phenotype among PIAS-deleted animals that have been generated thus far. PIAS1-deficient mice were observed to be runts and were partially lethal in the perinatal stage (Liu *et al.*, 2004). PIAS1-knockout mice and cells showed significant defects in downregulating Jak/Stat and NF- κ B signaling upon cytokine stimulation, because PIAS1 selectively inhibits the recruitment of STAT-1 or NF- κ B to the endogenous gene promoters (Liu *et al.*, 2004, 2005). The recruitment of PIAS1 to the target promoters requires its phosphorylation on the 90th serine residue by I κ B kinase α (IKK α) (Liu *et al.*, 2007). Interestingly, IKK α -mediated phosphorylation of PIAS1 requires SUMO ligase activity of PIAS1, since RING domain-mutated PIAS1 failed to be phosphorylated on the 90th serine residue and thus was unable to repress both the TNF-induced and the IFN-induced genes (Liu *et al.*, 2007). Thus, it is obvious that SUMO ligase activity of PIAS1 and SUMOylation play pivotal roles in transcriptional regulation, although the exact mechanism warrants further investigation in detail. In contrast to its profound effects on cytokine signaling, single deletion of PIAS1 was not essential in regulating the physiological activity of p53. PIAS1 has been expected to play a role in regulating p53 function, since SUMOylation on p53 is enhanced by PIAS1 (Kahyo *et al.*, 2001; Schmidt and Muller, 2002). Unexpectedly, PIAS1 deficiency did not demonstrate any difference in γ -irradiation-induced, p53-dependent apoptotic cell death between wild-type and PIAS1-deficient thymocytes (Liu *et al.*, 2004). The mild phenotype of single *PIAS* gene deletion in mice is probable to be caused from the functional redundancy of PIAS family members. In accordance with this hypothesis, PIAS1/PIASy double-knockout mice are embryonic lethal prior to day 11.5, showing much more severe defects in

development than individual deletion (Tahk *et al.*, 2007). Furthermore, PIASy deficiency in dendritic cells in which PIAS1 expression is low resulted in the enhanced expression of NF- κ B and STAT-1-dependent genes (Tahk *et al.*, 2007), indicating the overlapping function of PIAS1 and PIASy. Taken together, the presence of minor reduction in gross SUMOylation in each of the PIAS knockouts and the absence of defects in p53-dependent signaling in PIAS1-null cells might be the result of redundancy among PIAS proteins.

2.4.4. SUMO E3 RanBP2

RanBP2-null mice are embryonic lethal (Aslanukov *et al.*, 2006; Dawlaty *et al.*, 2008). The hypomorphic mice expressing approximately a quarter of RanBP2 protein are developmentally normal and the global patterns of SUMOylation by SUMO-1 and SUMO-2/-3 were not affected in them (Dawlaty *et al.*, 2008), suggesting that RanBP2 promotes SUMOylation in a narrow range of target proteins. The most pronounced phenotype among the hypomorphic mice was of chromosome segregation defects with the formation of anaphase bridges. These mice were also susceptible to spontaneous as well as chemical-induced tumorigenesis. These phenomena will be discussed in the later part of the review in detail.

2.4.5. DeSUMOylating protease SENP1

SENP1 is critical for mouse development (Cheng *et al.*, 2007; Yamaguchi *et al.*, 2005). SENP1-deficient mice displayed embryonic lethality between embryonic day 12.5 and 14.5, and placental abnormalities were suggested as a cause of embryonic death (Yamaguchi *et al.*, 2005). In an independent knockout study, SENP1-deficient embryos showed severe fetal anemia with much fewer erythrocytes than their wild-type littermates; further, the erythroid cells in the fetal liver were more apoptotic in knockout embryos (Cheng *et al.*, 2007). Further analysis revealed the pivotal role of SENP1 in the regulation of developmental hypoxia via deSUMOylation of hypoxia-inducible factor 1 α (HIF-1 α) (Cheng *et al.*, 2007). The detailed mechanism of SENP1 function in hypoxia response will be described later on in the paper.

3. ROLES OF SUMOS IN CANCER DEVELOPMENT, PROGRESSION, AND METASTASIS

Cancer is a chronic genetic disease arising by the multistep accumulation of abnormal changes in the genome. The accumulation of acquired genetic changes allows normal cells to achieve the hallmarks of cancer, which are the capacity of signal-independent growth, insensitivity to

growth-inhibitory signals, evading apoptosis, immortality, angiogenic potential, and the ability of tissue invasion and metastasis (Hanahan and Weinberg, 2000). In addition to the cancerous cellular transformation, the microenvironments surrounding cancer cells such as inflammatory situation are well known to be major contributors of cancer development and progression (Karin, 2006b; Mantovani *et al.*, 2008). The SUMOylation system appears to be involved in many aspects of cancer, from initiation to metastasis, directly or indirectly. SUMOylation targets include a number of oncogenic transcription factors as well as the tumor suppressor p53 (Kim and Baek, 2006). The abolition of SUMOylation leads to genomic instability as proven in Ubc9 and RanBP2-knockout mice models (Dawlaty *et al.*, 2008; Nacerddine *et al.*, 2005); this provides a cue to initiate malignant cell transformation. In addition, SUMOylation regulates hypoxic response, inflammation status, and the expression of metastatic suppressor genes, which are closely related to the progression and spread of cancer (Cheng *et al.*, 2007; Kim *et al.*, 2006; Liu *et al.*, 2007). Here, we will summarize and discuss the possible link between the SUMO system and cancer (Fig. 7.3).

3.1. Possible involvement of SUMO in the initiation and progression of cancer

3.1.1. Regulation of nuclear hormone receptor function by SUMO

Nuclear hormone receptors, such as androgen receptors (AR) in the prostate cells, and estrogen receptors (ER) and progesterone receptors (PR) in the breast are well-known etiological factors of hormone-dependent

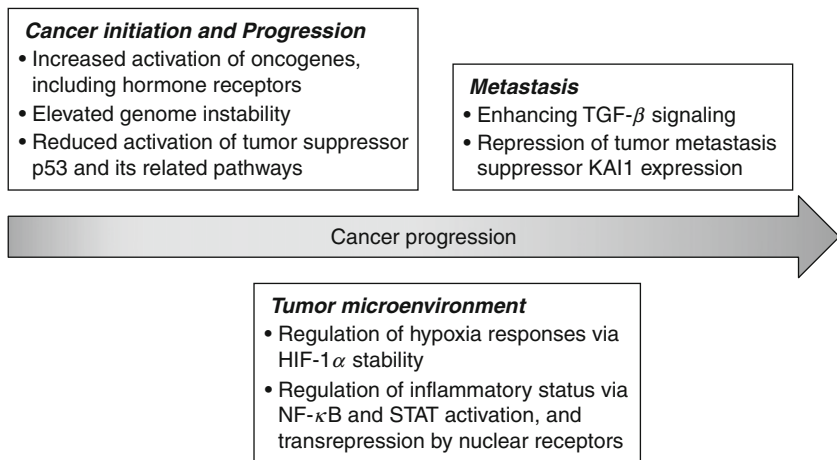


Figure 7.3 Contribution of misregulated SUMOylation to cancer initiation, progression, and metastasis.

malignancies. Their transcriptional abilities are under the control of the SUMO system by direct modification of nuclear receptors or by targeting their coregulators. AR is a critical transcription factor for the growth and survival of prostate cells in normal as well as malignant states (Balk and Knudsen, 2008). AR has long been identified as a SUMO target in which SUMOylation reduces hormone-induced transcriptional activity of AR (Poukka *et al.*, 2000). PIAS1 and PIASx α mediate SUMOylation of AR in a ligand-dependent manner, which repress transcriptional potential of AR (Kotaja *et al.*, 2002a; Nishida and Yasuda, 2002). In addition to AR, several of its coregulators, such as GRIP1, HDAC1, and pontin, have been implicated to be mediated by SUMOylation and deSUMOylation processes. SUMOylation of coactivator protein GRIP1 is necessary for colocalization with AR in the nucleus as well as for its coactivator function (Kotaja *et al.*, 2002b). DeSUMOylation of HDAC1 by SENP1 enhances AR-dependent transcription via reduction of deacetylase activity in the transcription complex (Cheng *et al.*, 2004). In accordance with this report, SENP1 is overexpressed in human prostate cancers specimens (Cheng *et al.*, 2006). Further, chronic androgen exposure of LNCaP prostate cancer cells caused an elevation of SENP1 gene expression (Bawa-Khalfe *et al.*, 2007). Forced reduction in SENP1 levels in prostate cancer cells abrogates androgen-dependent transcription and cell growth (Bawa-Khalfe *et al.*, 2007). Furthermore, SENP1 overexpression in the prostate of transgenic mice leads to the development of prostatic intraepithelial neoplasia at an early age (Cheng *et al.*, 2006). The SUMOylation of another AR coregulator protein, that is, pontin, which is a component of the chromatin-remodeling complex, has been implicated as a critical regulatory mechanism of AR function in relation to prostate cancer development. Pontin was purified in a complex with Ubc9 and SUMOylated in an AR agonist-enhanced manner (Kim *et al.*, 2007). The SUMO modification of pontin elevates its transcriptional activation function via its nuclear retention and increases its coactivator-binding ability; this leads to enhanced proliferation and growth of prostate cancer cells by activating a subset of AR target genes involved in tumor progression (Kim *et al.*, 2007).

ER and PR play essential roles in the physiology of the mammary gland as well as breast cancer development (Feigelson and Henderson, 1996; Lanari and Molinolo, 2002). The transcriptional activity of ER and PR has been shown to be regulated by the SUMO system; however, the causal relationship between ER or PR SUMOylation and breast cancer development has not been studied well compared to AR. Both ER α and PR are SUMOylated in a ligand-induced manner; however, the consequence of this SUMOylation event is contradictory to some extent (Daniel *et al.*, 2007; Sentis *et al.*, 2005). SUMOylation on ER α within the hinge region is critical for ER α -dependent transcriptional activation (Sentis *et al.*, 2005), while PR SUMOylation represses its transcriptional activity (Daniel *et al.*,

2007). The readouts of coregulator SUMOylation also differ between cases. SUMOylation of coactivator AIB1 attenuated its transactivation ability (Wu *et al.*, 2006). On the other hand, SUMOylation of SRC-1 increased PR-SRC-1 interaction as well as nuclear retention of SRC-1 without changing the stability of SRC-1 (Chauchereau *et al.*, 2003).

In nuclear hormone receptor-associated transcription machinery, SUMOylation and deSUMOylation processes appear to regulate the transcriptional events in complicated ways; further, the readouts of SUMOylation might be entirely context dependent. Thus, any cellular context that can alter the SUMOylation status in a direction to cause increased nuclear receptor activity, for example, chronic induction of SENP1 in relation to AR as shown above, might induce hyperproliferation of cells, and hence contribute to cancer development and progression together with other carcinogenic signals.

3.1.2. Maintenance of genome stability in mitosis by SUMO

As the major phenotypes of defective mutants for SUMOylation or deSUMOylation in yeast *S. cerevisiae* and *Schizosaccharomyces pombe* are closely related to chromosome segregation, it is clear that the SUMO system plays a pivotal role in maintaining genome stability during mitosis in eukaryotic cells (Watts, 2007). The role of SUMOs in mitosis in the yeast system is well described elsewhere (Dasso, 2008; Watts, 2007). In multicellular organisms, loss of genetic stability results in uncontrolled expression of certain genes involved in cell cycle regulation, and thereby, frequently contributes to cancer development. The results from *Ubc9*-knockout models demonstrating aberrant chromosome structures in mitosis provide substantial explanation on the importance of SUMOylation in the mammalian cell cycle as described before. Inhibition of SUMOylation by SENP2 overexpression leads to cell cycle arrest (Zhang *et al.*, 2008b). In addition, large numbers of SUMO targets identified by proteomic screening approaches, at least in yeasts, include chromatin and genome stability-related proteins (Hannich *et al.*, 2005; Wohlschlegel *et al.*, 2004). From these observations, one can expect that SUMOylation is closely related to the maintenance of genome stability. In the mammalian system, SUMO-1 and SUMO-2/-3 appear to perform distinct functions in mitosis by modifying individual substrates in different regions. SUMO-2/-3 localizes to centromeres and condensed chromosomes, whereas SUMO-1 localizes to the mitotic spindle and spindle midzones (Zhang *et al.*, 2008b). In particular, SUMO-2/-3 modification of the microtubule motor protein CENP-E is essential for cell cycle progression by targeting CENP-E to the kinetochore. SUMO ligases have also been suggested to be key molecules required for correct chromosome segregation. PIASy was found to be required for accurate chromosome segregation by directing Topoisomerase II to specific chromosome regions in human cells (Diaz-Martinez *et al.*, 2006). However, mouse models lacking

PIASy did not demonstrate any critical defects in mouse development (Roth *et al.*, 2004; Wong *et al.*, 2004) and chromosome segregation (Dawlaty *et al.*, 2008). One possible explanation is the redundancy among PIAS family members in the mouse system. The redundancy between PIAS1 and PIASy has been proven, at least in part, by showing that PIAS1/PIASy double-knockout mice demonstrated more severe phenotypes than each of the single-deficient mice in development as well as in the repression of target gene expression in immune response (Tahk *et al.*, 2007), although PIASy single knockdown was sufficient to observe chromosomal segregation defects in human cells. Thus, it will be interesting to analyze whether chromosomal segregation defects could be observed in PIAS1/PIASy double-knockout models.

The most dramatic defect in chromosome segregation was detected in RanBP2 hypomorphic mouse model (Dawlaty *et al.*, 2008). As described previous section, RanBP2 hypomorphic mice and embryonic fibroblasts expressing around a quarter of RanBP2 protein compared to wild-type exhibited severe aneuploidy caused by abnormal chromosomal segregation with formation of anaphase bridge. Mechanism studies revealed that RanBP2-mediated SUMOylation of Topoisomerase II α (Topo II α) is a critical step for the targeting of the protein to inner centromeres where Topo II α decatenates DNA for proper separation of sister chromatids in mitosis. Importantly, RanBP2 hypomorphic mice demonstrated much higher incidence of tumor formation in the skin, lung, liver, and many other organs and tissues compared to wild-type mice when a carcinogen, that is, 7,12-dimethylbenzanthracene (DMBA) was administered to the skin at early age. In addition, RanBP2 hypomorphic mice exhibited high frequency and short latency of spontaneous tumor occurrence than wild-type mice. Thus, it is obvious from this example that maintenance of chromosome stability by SUMOylation is critical to secure the expression of genetic information and malfunction of the SUMO system can cause cellular malignancy.

3.1.3. Regulation of tumor suppressor p53 and p53-related pathways by SUMO

As a critical protein for cell fate determination and tumor suppression, p53 is a target of extensive posttranslational modifications that regulate its stability and biological activity (Bode and Dong, 2004). The SUMOylation system participates in these complex regulatory repertoires for the stability of p53 as well as many aspects of p53-related pathways, such as DNA damage responses and cell senescence. The protein level of p53 should be maintained at low levels in normal cells via the ubiquitin-proteasome system, while stress conditions stabilize p53; this, in turn, induces cell growth arrest, senescence, and apoptosis (Brooks and Gu, 2006; Dai *et al.*, 2006). Although SUMOylation of p53 has long been reported, the biological effect of this

modification remains controversial whether it is positive or negative to p53 function (Gostissa *et al.*, 1999; Kwek *et al.*, 2001; Muller *et al.*, 2000; Rodriguez *et al.*, 1999; Schmidt and Muller, 2002). In addition to directly modifying p53, the SUMO system regulates p53 via the modification of MDM2, a major ubiquitin E3 ligase that maintains low p53 levels in normal cells (Chen and Chen, 2003; Xirodimas *et al.*, 2002). It has been established that MDM2 possesses self-ubiquitination potential to erase itself from the cells under stress that leads to p53 accumulation (Lavin and Gueven, 2006). In contrast, the stabilization of MDM2 in normal cells found to be achieved by SUMOylation that inhibits self-ubiquitination of MDM2 resulting in the accumulation of MDM2 and the degradation of p53 (Lee *et al.*, 2006). It is of considerable interest to elucidate the mechanism via which cells recognize stress and regulate SUMOylation status. In the mouse system, a deSUMOylating enzyme called SUSP4 is induced upon UV damage and removes SUMO from MDM2; this causes the degradation of MDM2 and the accumulation of p53 (Lee *et al.*, 2006), representing the critical role of the SUMOylation system in p53 regulation. It will be interesting to observe whether SENP2, the closest human homolog of SUSP4, plays a similar role in the p53 pathway or if other members of the SENP family participate in the pathway in humans. Interestingly, SUMO-1 levels in oral squamous cell carcinoma (SCC) tissues were much higher than that in normal oral epithelium. MDM2 was predominantly present in the SUMOylated form in both oral SCC tissues and cell lines (Katayama *et al.*, 2007). Knockdown of SUMO-1 in oral SCC cells inhibited cell proliferation. In addition, patients who showed expression of both SUMO-1 and Mdm2 experienced more frequent local recurrence after initial treatments. In this case, one scenario can be considered in conjunction with the observation of Lee *et al.* (2006). According to this scenario, the uncontrolled overexpression of SUMO-1 due to some reason results in enhanced SUMOylation of MDM2 in oral SCC cells; this leads to stabilization of MDM2. Consequently, cellular p53 levels are maintained at low levels probably even under certain damaging conditions; otherwise, p53 levels should be sufficiently high to protect cells from damage. Although it is indirect, this scenario might explain the manner in which SUMOylation is involved in tumor development and progression in relation to p53.

Recent evidences reveal that SUMOylation also contributes to p53-mediated cellular processes, such as senescence and DNA repair. Cellular senescence is recognized as a tumor suppressive mechanism by restricting uncontrolled cell proliferation in response to cellular stresses, such as oxidative stress, activation of oncogenes, or DNA damage (Ben-Porath and Weinberg, 2004; Collado and Serrano, 2005). This stress-induced senescence is controlled by p53 and Rb. Recent studies found that the SUMO system is closely related to p53-mediated senescence. The ectopic expression of SUMO E3 ligase PIASy induces premature senescence in human

primary fibroblasts (Bischof and Dejean, 2007; Bischof *et al.*, 2006). More importantly, PIASy deficiency results in the delayed onset of RAS-induced senescence in murine embryonic fibroblasts (Bischof and Dejean, 2007; Bischof *et al.*, 2006). Mechanism studies revealed that SUMOylation and transcriptional activation of p53 by PIASy induced the expression of downstream target genes involved in senescence, such as p21. It is interesting to note that the viral oncoprotein E6 isolated from high-risk HPV-16 blocks SUMOylation activity of PIASy and PIASy-induced senescence as well; this suggests that PIASy might be a cellular target of viral evading mechanisms to overcome antiviral defenses by hosts and its potential importance in tumorigenesis, such as in cervical cancer. In accordance with the above mentioned results, knockdown of SENP1 resulted in the elevated expression of p53 target genes and premature senescence in human cells (Yates *et al.*, 2008). Overexpression of SUMO-2/-3 also induced premature and stress-induced senescence in a p53-dependent or Rb-dependent manner (Li *et al.*, 2006). In these three reports, the elevation in the rate of SUMOylation on p53 by either SUMO or PIASy overexpression or SENP1 repression in cells appears to be consistently critical in inducing cellular senescence, although the effect of SUMOylation on the activity of p53 was not clear in early studies as described above.

3.2. Regulation of tumor microenvironment by SUMO

3.2.1. Hypoxia response and SUMO

Massive proliferation of tumor cells causes hypoxia, a condition of oxygen shortage (Brahimi-Horn *et al.*, 2007). Tumor cells activate a heterodimeric transcription factor, HIF-1, to overcome the hypoxic condition (Brahimi-Horn *et al.*, 2007). HIF-1-dependent transcriptional events mediate a group of cellular responses such as angiogenesis, determination of cell survival or death, shift to anaerobic metabolism, regulation of pH, and metastasis (Brahimi-Horn *et al.*, 2007). An important regulatory molecule in itself, the α -subunit of HIF-1 (HIF-1 α) is a target of elaborate posttranslational modifications, including SUMOylation. Under normal oxygen supply, cells maintain low levels of HIF-1 α via continuous degradation mediated by the ubiquitin-proteasome system (Huang *et al.*, 1998). Proline hydroxylases (PHD) add hydroxyl groups on the proline residues in the oxygen-dependent degradation (ODD) domain of HIF-1 α ; this domain subsequently serves as a recognition signal for an ubiquitin E3 ligase, von Hippel-Lindau (VHL) complex (Maxwell *et al.*, 1999). Under the hypoxic condition, oxygen depletion inactivates PHD proteins resulting in the loss of mark for degradation on HIF-1 α , and hence enhances the accumulation and translocation to the nucleus of HIF-1 α (Sutter *et al.*, 2000). HIF-1 α forms dimers with HIF-1 β and initiates transcription for target genes containing the hypoxia-response element (HRE) (Schofield and Ratcliffe, 2004).

It has long been suggested that the SUMOylation and deSUMOylation processes are closely involved in the regulation of hypoxia responses. Hypoxic condition increases the expression of SUMO-1 protein (Comerford *et al.*, 2003; Shao *et al.*, 2004). Further, a variety of cellular proteins are subjected to SUMOylation (Nguyen *et al.*, 2006), including HIF-1 α (Bae *et al.*, 2004; Berta *et al.*, 2007; Carbia-Nagashima *et al.*, 2007; Cheng *et al.*, 2007). However, the readouts of SUMOylation on HIF-1 α are somewhat contradictory among research groups. The major concern is whether SUMOylation on HIF-1 α is a stabilization factor or a destabilization signal. The ectopic expression of SUMO-1 enhances the stability and transcriptional activity of HIF-1 α by SUMOylation on Lys391 and Lys477 in human proteins under both normoxic and hypoxic conditions (Bae *et al.*, 2004). In accordance with these observations, the expression of RSUME, a hypoxia-inducible SUMOylation enhancer protein described previously in this review, elevates HIF-1 α SUMOylation, leading to an increase in its stability and transcriptional activity, and expression of HIF-1 target genes, such as VEGF (Carbia-Nagashima *et al.*, 2007). Data from these two groups claim that SUMO plays a positive role in propagation of hypoxic responses by directly modifying and stabilizing HIF-1 α . However, independent research reported conflicting results by examining SUMOylation-defective forms of HIF-1 α (Berta *et al.*, 2007). In the comparison of the stability of wild-type and SUMOylation-defective HIF-1 α , no differences in the half-life were observed. This suggests that SUMOylation in HIF-1 α may not be a direct cause of its stabilization. In addition, SUMOylation-defective HIF-1 α exhibited higher transcriptional activity than wild-type protein, indicating, in retrospect, that SUMOylation on HIF-1 α represses its transcriptional activity.

Critical evidence that SUMO system plays a pivotal role in regulation of hypoxic response is originated from the knockout study of the deSUMOylating enzyme, SENP1, in mice. Deletion of *SENP1* gene in mice caused severe fetal anemia leading to embryonic lethality (Cheng *et al.*, 2007). In the knockout mice, the production of erythropoietin, a major regulator of vascular formation and erythropoiesis, was defective because of reduced levels of HIF-1 α under the hypoxic condition. Interestingly, both protein levels and the half-life of HIF-1 α were increased under hypoxia in wild-type MEFs; however, this increase was much less in SENP1-deficient MEFs, suggesting a protective role of SENP1 against the degradation of HIF-1 α mediated by the ubiquitin-proteasome system. With elaborate mechanism studies, this research group elucidated that SUMOylation in HIF-1 α serves as a binding signal for the VHL component of ubiquitin E3 ligase complex. SUMOylation greatly increased the binding of HIF-1 α to the substrate recognition region of VHL. In summary, according to Cheng *et al.* (2007), SUMOylation provides HIF-1 α with a new interacting motif for binding to VHL ubiquitin E3 ligase complex in the nucleus upon

hypoxic challenges. SUMOylated HIF-1 α is subjected to ubiquitination and thereby degraded by proteasome. In the presence of SENP1, HIF-1 α is protected from degradation by a deSUMOylation process and generates a hypoxic response. Thus, SENP1 deficiency caused a critical defect in response to developmentally induced hypoxia. The effect of SENP1 deficiency on tumor-induced hypoxia has not been studied yet. It will be interesting to analyze whether SENP1 deficiency can similarly restrict tumor-induced hypoxic responses, such as vascular genesis and metastasis. If it is the case, small molecules that can inhibit SENP1 activity might be a good candidate to inhibit cancer spread.

3.2.2. Modulation of inflammatory responses by SUMO

Inflammation is a component of the physiological adaptive response against conditions potentially harmful to the body, such as infection and tissue injury. However, it is now widely accepted that chronic inflammation offers tumor cells favorable environments for their growth and survival, in part, by recruiting immune cells that produce cytokines and growth factors (Lin and Karin, 2007; Mantovani *et al.*, 2008). Many types of cancers, including bladder, cervical, gastric, intestinal, esophageal, ovarian, prostate, and thyroid cancers, have been known to be closely associated with inflammatory diseases (Mantovani *et al.*, 2008). In addition, hyperactivation of key transcription factors involved in inflammation, such as NF- κ B and STATs, has been observed in many types of inflammation-associated cancers (Lin and Karin, 2007; Mantovani *et al.*, 2008). Many evidences presently indicate that SUMOylation regulates cellular function of these transcription factors under various circumstances. The key step of NF- κ B activation upon proinflammatory stimuli is a phosphorylation-dependent degradation of I κ B by the ubiquitin-proteasome system, which allows the nuclear localization of NF- κ B (Karin, 2006a). SUMO-1 modifies I κ B α on the same lysine residue for ubiquitination (Desterro *et al.*, 1998). In this way, SUMOylation stabilizes I κ B α , and thereby suppresses NF- κ B activation (Desterro *et al.*, 1998). Under diverse cellular stress conditions including genotoxic stress, oxidative stress, ethanol exposure, heat shock, and electric shock, SUMO-1 modifies another player of the NF- κ B pathway, that is, the regulatory subunit of IKK called NF- κ B essential modulator (NEMO) with the aid of PIASy (Huang *et al.*, 2003; Mabb *et al.*, 2006; Wuerzberger-Davis *et al.*, 2007). In this case, SUMOylation exerts a favorable effect on NF- κ B activation. SUMOylation induces translocation of IKK-unbound NEMO from cytoplasm to nucleus, which permits subsequent ATM-dependent ubiquitination of NEMO to ultimately activate IKK in the cytoplasm (Huang *et al.*, 2003). Knockout studies on PIAS1 provided critical evidence demonstrating the importance of SUMOylation on the

regulation of inflammatory gene transcription (Liu *et al.*, 2004, 2005). PIAS1 has been well studied as a repressor of cytokine signaling (Shuai and Liu, 2005). PIAS1 deficiency causes severe defects in restricting the expression of a subset of STAT-1 target genes as well as NF- κ B-responsive genes (Liu *et al.*, 2004, 2005). Consequently, mice lacking PIAS1 showed elevated production of proinflammatory cytokines (Liu *et al.*, 2004, 2005). In the short term, enhanced immune responses in PIAS1-knockout mice are beneficial against pathogenic conditions based on increased protection to viral and microbial infections (Liu *et al.*, 2004). However, frequent exposure of these mice to inflammatory stimuli may cause somewhat different results, such as the generation of autoimmune or chronic inflammatory diseases and a high risk of developing inflammation-associated cancer.

SUMOylation is also involved in the transrepression of inflammatory gene expression by nuclear receptors such as PPAR γ , LXR α , and LXR β in macrophages (Ghisletti *et al.*, 2007; Pascual *et al.*, 2005). The transrepression of Toll-like receptor (TLR) signaling by nuclear receptors is one of the key mechanisms that modulate inflammation and immunity in a variety of immune cells. In TLR-4-mediated signaling, signal-responsive gene promoters are repressed by a complex containing TBL1, TBLR1, NCoR, and HDAC3 (Pascual *et al.*, 2005). Lipopolysaccharide (LPS) stimulation induces ubiquitin-dependent degradation of NCoR and HDAC3 followed by replacement of corepressor complexes with coactivator complexes for transcriptional activation. However, when the cells are treated with LPS in the presence of a PPAR γ agonist, transcriptional activation is attenuated by the recruitment of PPAR γ to signal-responsive promoters in a PIAS1-dependent, Ubc9-dependent, and SUMO-1 modification-dependent manner. Further, NCoR and HDAC3 are stabilized on the signal-responsive promoters, explaining the role of SUMOylation in the repression of inflammatory gene expression. Synthetic LXR ligands also inhibit LPS-induced expression of NF- κ B-regulated genes (Joseph *et al.*, 2003) through a SUMOylation-dependent mechanism parallel to that of PPAR γ (Ghisletti *et al.*, 2007). However, SUMO-2/-3 is a key player in LXR-mediated transrepression unlike SUMO-1 in the case of PPAR γ and HDAC4 acts as an E3 ligase mediating LXR SUMOylation (Ghisletti *et al.*, 2007).

From the viewpoint of inflammatory responses, SUMOylation appears to be involved in the restriction of overinflammation by stabilizing I κ B protein, by potentiating transrepression of certain nuclear receptors, or by negatively regulating cytokine signaling via PIAS1 protein, although SUMOylation mediates NF- κ B activation under several stress conditions. Thus, it is possible that the malfunction of the SUMOylation system or overexpression of SENPs in the cancer microenvironment may positively influence cancer growth and propagation.

3.3. SUMO in metastasis regulation

The final stage of cancer progression is metastasis, a multistep process that cancer cells leave from its original sites and then colonize in the secondary region in the body through blood circulation (Gupta and Massague, 2006). The metastatic process includes cancer cell migration and tissue invasion, intravasation into the blood vessels, transit in blood, extravasation, and proliferation in the new environments (Sahai, 2007). Although the role of the SUMO system in the metastatic process has not been widely studied thus far, recent reports indicate strongly that SUMOylation plays critical roles in the expression of metastatic suppressor gene *KAI1/CD82* and in the regulation of TGF- β signaling, which are closely involved in the processes of tumor metastasis (Kang *et al.*, 2008; Kim *et al.*, 2006). KAI1 is a member of tetraspanin family that suppresses tumor metastasis by inhibiting cancer cell motility and invasiveness (Dong *et al.*, 1995; Liu and Zhang, 2006). Overexpression of *KAI1* gene represses tumor metastasis in mouse models (Dong *et al.*, 1996; Kim *et al.*, 2005a). On the other hand, *KAI1* expression is significantly downregulated in a variety of clinically advanced cancers of the prostate, bladder, esophagus squamous cells, and breast (Dong *et al.*, 1996; Uchida *et al.*, 1999; Yang *et al.*, 2000; Yu *et al.*, 1997). The down-regulation mechanisms of KAI1 levels are diverse in metastatic cancer cells, including chromosomal allelic loss, gene mutation, SUMOylation-mediated transcriptional downregulation, and ubiquitin-dependent proteasomal degradation (Dong *et al.*, 1996; Kawana *et al.*, 1997; Kim *et al.*, 2006; Tsai *et al.*, 2007). In nonmetastatic prostate cancer cells, transcriptional coactivator, Tip60, contributes to *KAI1* expression (Kim *et al.*, 2005a). As the Tip60 levels decreases and the nuclear β -catenin levels increases in metastatic cancer cells, β -catenin/reptin corepressor complex replaces Tip60 and represses KAI1 expression (Kim *et al.*, 2005a). SUMOylation of reptin potentiates the association between reptin and HDAC1 that enhances the repressive function (Kim *et al.*, 2006). In addition, overexpression of SENP1 or knockdown of Ubc9, which leads to the reduction of SUMOylation of reptin, suppresses metastatic potential of LNCaP prostate cancer cells (Kim *et al.*, 2006). On the contrary, introduction of SUMOylation-defective mutant form of reptin abolishes the SUMOylation-mediated repressive function of reptin in the cells (Kim *et al.*, 2006). Thus, SUMOylation of reptin positively modulates the invasive activity of cancer cells via downregulation of KAI1 expression. The elevated Ubc9 levels observed in metastatic cancer cells may explain, at least in part, in the downregulation of KAI1 via SUMOylation of reptin (Kim *et al.*, 2006).

The contribution of TGF- β signaling to tumorigenesis and metastasis is well established (Leivonen and Kahari, 2007; Pardali and Moustakas, 2007). TGF- β that performs opposed functions depends on the stage of tumorigenesis and the responsiveness of the tumor cell to tumor suppressive

function of TGF- β . TGF- β inhibits cell proliferation and induces apoptosis in the early stage of tumorigenesis, and thereby, acts as a tumor suppressor (Leivonen and Kahari, 2007; Pardali and Moustakas, 2007). On the other hand, tumor-derived TGF- β promotes tumor cell invasiveness and metastasis via inducing epithelial-mesenchymal transition and angiogenesis, upon loss of the antiproliferative responsiveness to TGF- β in the advanced cancers (Leivonen and Kahari, 2007; Pardali and Moustakas, 2007). TGF- β signals through serine/threonine kinase receptor complexes, which comprise type I (T β RI) and type II (T β RII) receptors (Feng and Derynck, 2005). Upon binding of TGF- β , activated T β RI phosphorylates cytoplasmic mediators, Smad2 and Smad3. Phosphorylated Smad2/Smad3 associates with Smad4, translocates to the nucleus, and initiates transcription of various TGF- β -responsive genes. Interestingly, recent study identified T β RI as a target for SUMOylation (Kang *et al.*, 2008). SUMOylation of T β RI requires the kinase activation of both T β RI and T β RII, and occurs at the same orientation of Smad-binding regions on T β RI. As a consequence, SUMOylation enhances the affinity of T β RI kinase to Smad proteins, allowing more efficient phosphorylation and activation of Smad2/3 in response to TGF- β (Kang *et al.*, 2008). Consistently, Ras-transformed *Tgfb β 1*^{-/-} fibroblasts that express SUMOylation-defective T β RI demonstrate reduced metastatic potential than the same fibroblasts but reconstituted with wild-type T β RI in mouse lung metastasis model. In these two reports, SUMOylation appears to enhance metastatic potentials of malignant cells via the repression of metastatic suppressor KAI1 expression and the elevation of metastatic activity of TGF- β . Thus, the shift to excess SUMOylation in the certain cellular context might contribute to the gain of metastatic ability in malignant cells.

4. CONCLUDING REMARKS

Recent progresses in mouse model studies for genes that participate in SUMOylation and deSUMOylation reveal the importance of SUMOylation in maintaining homeostasis in the mammalian system. The loss of balance between the SUMOylation and deSUMOylation processes results in a variety of cellular defects and thereby, sometimes causes hyperplasia or malignant transformation of cells as we can observe in SENP1 transgenic mice or hypomorphic RanBP2 mice (Cheng *et al.*, 2006; Dawlaty *et al.*, 2008). Although it is unable to study the effect of *Ubc9* knockout on tumorigenesis in mice due to embryonic lethality, it is highly possible that critical reduction of *Ubc9* expression is associated with cancer development in humans. It will be worthwhile to generate a hypomorphic *Ubc9* mouse model for tumorigenesis research.

The mode of contribution of deregulated SUMOylation to tumorigenesis could be indirect via altering tumor microenvironments, such as inflammation and hypoxic responses. The studies on the contribution of *PIAS1* or *SENP1* deficiency to tumor progression will provide us with better understanding about these issues. Finally, excessive SUMOylation appears to enhance metastatic potential of malignant cells. Thus, developing small molecular inhibitors to block SUMOylation, that is, Ubc9 inhibitor, can be considered as a therapeutic approach to restrict tumor metastasis.

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PHOSPHOINOSITIDE SIGNALING PATHWAYS: PROMISING ROLE AS BUILDERS OF EPITHELIAL CELL POLARITY

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Abstract

Polarity is a prerequisite for proper development and function of epithelia in metazoa. The major feature of polarized epithelial cells is the presence of specialized domains with asymmetric distribution of macromolecular contents including proteins and lipids. The apical domain is involved in exchange with the organ lumen, and the basolateral membrane maintains contact with neighboring cells and the underlying extracellular matrix. The two domains are separated by tight junctions, which act as a diffusion barrier to prevent free mixing of domain-specific proteins and lipids. Extensive studies have shed light on the numerous protein families involved in cell polarization. However, many questions still remain regarding the molecular mechanisms of polarity regulation and in particular very little is known about the role of lipids in building polarity. In this chapter,

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essential determinants of epithelial polarity will be reviewed with a particular focus on metabolism and function of phosphoinositides.

Key Words: Epithelium, Phosphoinositides, Cell polarity, Signaling, Trafficking, Morphogenesis. © 2009 Elsevier Inc.

1. INTRODUCTION

Epithelium lines both the outside and the inside cavities and lumen of bodies. It is also the constitutive tissue of many glands. Functions of epithelial cells include secretion, absorption, protection, transcellular transport, sensation detection, and selective permeability. To satisfy their numerous functions, epithelial cells need to be fully polarized. The polarization of epithelial cells leads to the presence of two different domains that are defined by distinct protein and lipid compositions and are separated by tight junctions (Gibson and Perrimon, 2003). The apical surface serves as a barrier to the outside world and is specialized for exchange of materials with the lumen. The basolateral surface is adapted for the interaction with other cells and for exchange with the bloodstream (Nelson, 2003).

Establishment of epithelial polarity involves cell–cell and cell–extracellular matrix interactions, as well as several evolutionary conserved polarity protein complexes including the PAR3–PAR6–aPKC, Scribble–Lgl–Dlg, and Crumbs–Stardust–PATJ Complexes (Macara, 2004; O’Brien *et al.*, 2001; Ohno, 2001; Wodarz, 2002). Polarity also requires trafficking of membrane components to the apical or basolateral domain (Mostov *et al.*, 2003; Rodriguez-Boulan *et al.*, 2005). This involves several distinct trafficking pathways, including direct delivery from the trans-Golgi network (TGN) to the apical or basolateral surface, as well as endocytosis and selective recycling to each domain or transcytosis from the basolateral to the apical surface (Rodriguez-Boulan *et al.*, 2005). The final stage in the polarization process is the outgrowth of a primary cilium from the apical surface (Vieira *et al.*, 2006). There are several other types of cell polarity, including neutrophil and amoeba chemotaxis and neuronal extension (Shi *et al.*, 2003; Sohrmann and Peter, 2003; Weiner, 2002). Our recent data suggested that the underlying molecular mechanisms directing polarization process in these various models of polarity shared common principles (Comer and Parent, 2007; Gassama-Diagne *et al.*, 2006; Martin-Belmonte *et al.*, 2007). Intensive studies have shed light on the numerous protein families involved in cell polarization even though how the integrated action of these different components direct and maintain cell polarity still remains an important question in cell biology. By contrast, until these recent years, only a small number of investigations have concerned lipids. Phosphoinositides have long been

known to have important roles in cell signaling (Berridge and Irvine, 1989). These lipids are regulators of cytoskeletal and membrane dynamics and recently their role has been clearly established in the spatio-temporal regulation of membrane trafficking and accumulating evidence suggest that different organelles contain distinct sets of accessible PIs (De Matteis and Godi, 2004; Di Paolo and De Camilli, 2006; Krauss and Haucke, 2007). How these pools of phosphoinositides are regulated? How are they addressed to a specific biological function? remain important questions, particularly in polarized epithelial cells which have asymmetric distribution of membrane domains. In this review, we summarize data concerning the structure and metabolism of phosphoinositides and discuss their function in epithelial cell polarity.



2. PHOSPHOINOSITIDES: STRUCTURE, METABOLISM, AND FUNCTIONS

2.1. Structure and analysis of phosphoinositides

Although they have acquired various functions through evolution, inositol lipids are found very early in the origins of life (Michell, 2008). Archaea widely use these lipids as membrane constituents, some bacteria also contain inositol lipids and all eukaryotes use them from unicellular to multicellular organisms.

Phosphoinositides are glycerophospholipids accounting for ~15% of total phospholipids in eukaryotic cells. Their metabolism is highly active and accurately controlled by a number of specific kinases, phosphatases, and phospholipases (Michell *et al.*, 1975; Payrastré *et al.*, 2001). Most phosphoinositides are constitutively present in cells (although their level is highly controlled and can rapidly change locally), however some of them, particularly the class I PI 3-kinase products, are considered as second messengers and are detectable in normal cells only in response to membrane receptor stimulation. In terms of structure, the *myo*-inositol moiety of phosphoinositide is connected to a *sn*-1,2-diacylglycerol by a phosphodiester linkage (Fig. 8.1). In higher eukaryotes, the fatty acid composition of phosphoinositides is, for the large majority, 1-stearoyl and 2-arachidonoyl. The polar *myo*-inositol head group contains five free hydroxyls, three of them being phosphorylated/dephosphorylated by specific kinases/phosphatases at positions D-3, D-4, and D-5 (Fig. 8.1). The positions D-2 and D-6 have not been found phosphorylated, probably because of the lack of accessibility by the kinases. Phosphatidylinositol (PtdIns) is the most abundant member of the family and undergoes sequential phosphorylation to generate the seven phosphorylated derivatives also called polyphosphoinositides. Polyphosphoinositides can be rapidly synthesized and degraded in different membranes and have been implicated in the control and integration of

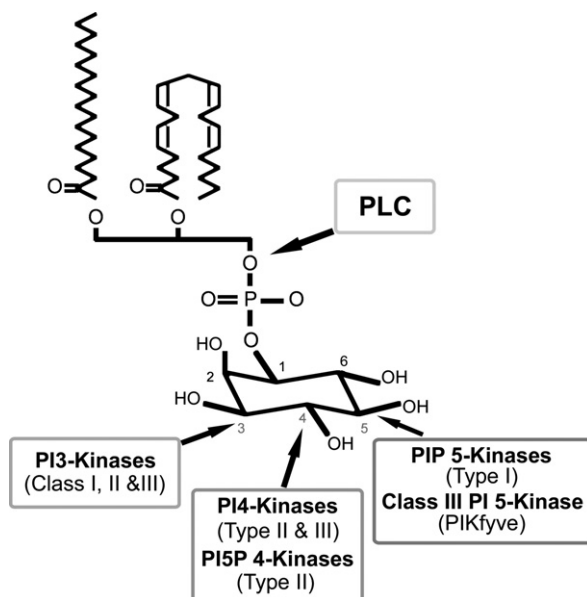


Figure 8.1 Structure of phosphatidylinositol (1-stearoyl, 2-arachidonoyl, sn glycerophospho-D-1- myoinositol), and the phosphorylation sites for the different phosphoinositide (PI) kinases and PLCs.

many dynamic mechanisms of cell regulation. Recent discoveries have brought phosphoinositide metabolizing enzymes to the forefront of biomedical research (Pendaries *et al.*, 2003) and suggest that probably all polyphosphoinositides have distinct biological roles (Di Paolo and De Camilli, 2006).

Several methods allow the detection of phosphoinositides in cells. Although mass spectrometry approaches are under development, at present, most of the available techniques are based on lipid extraction and analysis from ortho[^{32}P]phosphate or [^3H]inositol -labeled cells by a combination of thin layer chromatography and high pressure liquid chromatography. These biochemical techniques allow accurate separation and quantification of the different phosphoinositides (Payrastre, 2004). Moreover, several phosphoinositide binding domains (PH, FYVE, PX, ENTH, etc.) have been identified (Lemmon, 2008) and are used to image these bioactive lipids in living cells (Balla *et al.*, 2000). The results obtained with this attractive detection technique must however be cautiously interpreted since several phosphoinositide binding domains also interact with soluble inositol polyphosphates and with proteins. Moreover, competition with endogenous proteins for binding to phosphoinositides, induction of clustering of phosphoinositides, modification in their turnover or inaccessibility to certain pools of these lipids are among the factors that may bias the results.

2.2. Different phosphoinositides for different functions

PtdIns occurs in all eukaryotes and can be considered as a precursor of the other phosphoinositides. It makes up $\sim 10\%$ of total phospholipids and undergoes sequential and reversible phosphorylations at position D-3, D-4, and D-5 by specific kinases and phosphatases. PtdIns biosynthesis mainly occurs in the endoplasmic reticulum where phosphatidic acid is transformed to cytidine diphosphate-diacylglycerol (CDP-DAG) by the action of a CDP-DAG synthase and the phosphatidyl group of CDP-DAG is transferred to the D1 position of inositol through the action of a PtdIns synthase (Gardocki *et al.*, 2005). This *de novo* synthesis pathway of PtdIns exists in all eukaryotes (Michell, 1975, 2008) and this lipid is essential for normal cell function. PtdIns-transfer proteins are thought to drive the formation of separated phosphoinositide pools by delivering PtdIns to specific places within cells (Cockcroft, 2007).

PtdIns4P, produced by 4-phosphorylation of PtdIns, is the major precursor of PtdIns(4,5)P₂ via the action of type I PtdInsP-kinases. A constant equilibrium between PtdIns, PtdIns4P and PtdIns(4,5)P₂ is maintained by the action of specific 4 and 5-kinases and -phosphatases (Table 8.1). PtdIns4P and PtdIns(4,5)P₂ are present in the plasma membranes of all eukaryotic cells but PtdIns4P is also found in the Golgi complex. Besides being an intermediate metabolite of PtdIns(4,5)P₂ synthesis, PtdIns4P has specific roles. It is an important player in the recruitment of proteins to the Golgi complex such as epsinR, oxysterol binding protein (OSBP), the clathrin adaptor AP-1, and the four-phosphate-adaptor protein 1 and 2 (FAPP 1 and 2) (D'Angelo *et al.*, 2008). PtdIns4P, in cooperation with Arf, targets FAPP 1 and 2 to the TGN *via* interaction with their PH-domains (Godi *et al.*, 2004). FAPP2 is a glucosylceramide-transfer protein playing a role in the synthesis of complex glycosphingolipids. These lipids are made in the Golgi complex and then transported to the plasma membrane. Thus, the level of PtdIns4P in the Golgi complex impacts on the glycosphingolipid synthetic pathway by contributing, together with ARF1, to the recruitment of FAPP2 (D'Angelo *et al.*, 2007).

In addition to its important role in the Golgi complex, PtdIns4P can also bind to cytoskeletal proteins such as talin (Martel *et al.*, 2001). Moreover, like PtdIns(4,5)P₂, PtdIns4P can modulate the profilin-actin complex *in vitro* (Katakami *et al.*, 1992), but its exact contribution to this mechanism *in vivo* remains unclear. In yeast, the p21-activated protein kinase-related kinase Cla 4 has a PH domain interacting with PtdIns4P and this lipid together with the GTPase Cdc42 recruits the kinase to sites of polarized growth (Wild *et al.*, 2004). Thus, PtdIns4P is likely not just an intermediate metabolite in a fixed sequence of reaction (D'Angelo *et al.*, 2008).

PtdIns(4,5)P₂ is mainly present in the plasma membrane although it is also detected in internal membranes and in the nucleus (Irvine, 2006).

Table 8.1 Substrates and products of the best characterized eukaryotic phosphoinositides metabolizing enzymes

Substrates	Metabolizing enzymes	ProProducts
PtdIns	Type II PtdIns 4-Kinases α , β Type III PtdIns 4-Kinases α , β Class III PI 3-kinase	PtdIns4P PtdIns4P PtdIns3P
PtdIns4P	Type I PtdInsP kinases (PIP5K α , β , γ) Class I and II PI 3-Kinases. 4-Phosphatases Sac1 4-Phosphatases synaptojanin 1,2 (via their Sac domain)	PtdIns(4,5)P ₂ PtdIns(3,4)P ₂ PtdIns PtdIns
PtdIns3P	Type III PI 5-kinase (PIKfyve) 3-Phosphatases myotubularins	PtdIns(3,5)P ₂ PtdIns
PtdIns5P	Type II PtdInsP kinases (PIP4K α , β , γ) Class I PI 3-kinases (potentially)	PtdIns(4,5)P ₂ PtdIns(3,5)P ₂
PtdIns(4,5)P₂	PLC Class I PI 3-kinases 5-Phosphatase OCRL 5-Phosphatase INPP5B (or type II p75) 5-Phosphatase Synaptojanin-1 5-Phosphatase PIPP 5-Phosphatase SKIP Type I and II PtdIns(4,5)P ₂ 4-Phosphatases α , β	IP3 + DAG PtdIns(3,4,5)P ₃ PtdIns4P PtdIns4P PtdIns4P PtdIns4P PtdIns4P PtdIns5P
PtdIns(3,5)P₂	3-Phosphatases myotubularins 5-Phosphatase Sac 3	PtdIns5P PtdIns3P
PtdIns(3,4)P₂	3-Phosphatase PTEN Type I and II PtdIns(3,4)P ₂ 4-Phosphatases	PtdIns3P PtdIns4P
PtdIns(3,4,5)P₃	3-Phosphatase PTEN 5-Phosphatases SHIP1 and SHIP2 5-Phosphatase SKIP 5-Phosphatase pharbin (or Type IV 5-Phosphatase)	PtdIns(4,5)P ₂ PtdIns(3,4)P ₂ PtdIns(3,4)P ₂ PtdIns(3,4)P ₂

PI: phosphoinositide, IP3: inositol trisphosphate, DAG: diacylglycerol.

Different pools of this lipid may exist in the inner leaflet of the plasma membrane but little is known about the regulation and the lateral diffusion of these pools. At physiological pH, polyphosphoinositides are negatively charged and can interact with polybasic residues. McLaughlin and colleagues have proposed that a large part of PtdIns(4,5)P₂ may be bound to proteins through electrostatic interactions and thus laterally concentrated via protein translocation (Golebiewska *et al.*, 2008). One of the major function of PtdIns(4,5)P₂ is to serve as a substrate of phosphoinositide-specific phospholipase C (PLC) in response to cell stimulation via a variety of membrane receptors, leading to the production of the well known second messengers inositol (1,4,5) trisphosphate (IP₃) and sn-1,2-diacylglycerol. Following a rapid drop in PtdIns(4,5)P₂ level upon PLC activation there is, classically, an immediate resynthesis of this lipid. PtdIns(4,5)P₂ is also the source of an other critical second messenger, PtdIns(3,4,5)P₃, via the action of class IA phosphoinositide 3-kinases (PI 3-kinases).

Besides its important role as a substrate of PLC or PI 3-kinases, PtdIns(4,5)P₂ acts as a signaling molecule on its own. Lassing and Lindberg (1985) have provided the first indication that PtdIns(4,5)P₂ can interact with actin-binding proteins such as profilin and gelsolin. It can dissociate profilin-actin complexes thereby regulating the free actin monomers concentration. Profilin-bound PtdIns(4,5)P₂ is protected against PLC γ hydrolysis unless this phospholipase becomes tyrosine phosphorylated suggesting a subtle relationship between actin cytoskeleton organization and PtdIns(4,5)P₂ metabolism. Several cytoskeletal anchoring proteins such as vinculin, talin, or ERM family proteins (Ezrin, Radixin, and Moesin) can interact with this phosphoinositide (Payrastre *et al.*, 2001). In this context, Raucher *et al.* (2000) have suggested that PtdIns(4,5)P₂ can control local adhesion energy between the plasma membrane and the underlying cytoskeleton, an important feature of the membrane dynamics linked to cell functions such as cell motility. Once bound to PtdIns(4,5)P₂, ERM proteins can strongly interact with CD44, a cell-surface glycoprotein increasing the adhesion energy between the actin cytoskeleton and the membrane.

Moreover, several reports illustrate the importance of PtdIns(4,5)P₂ as an upstream modulator of actin nucleation through the Arp2/3 complex via its action on regulatory proteins such as N-WASP (Miki *et al.*, 1996, Rohatgi *et al.*, 2000). Local increases in PtdIns(4,5)P₂ concentration through activation of phosphoinositide kinases by small G proteins are thought to occur leading to local regulation of the actin cytoskeleton (Payrastre *et al.*, 2001). These effects of PtdIns(4,5)P₂ in the regulation of the actin cytoskeleton dynamics are particularly important in the context of polarity and cell motility (see below).

Recently, PtdIns(4,5)P₂ has been shown to also regulate integral membrane proteins including ion channels such as inwardly rectifying potassium, voltage gated, cyclic nucleotide-gated, or the transient receptor potential (TRP) channels. The C-terminal PH domain of TRPM4 is implicated in

PtdIns(4,5)P₂ sensing and may be integrated in a complex of proteins including specific lipid phosphatases (Suh and Hille, 2008; Nilius *et al.*, 2006).

Finally, PtdIns(4,5)P₂ is also an essential cofactor for phospholipase D-1 (PLD-1) and PLD-2 activity (Sciorra *et al.*, 2002). The PH domain of PLD-1 is a binding site of PtdIns(4,5)P₂ and is required for both the activity and the distribution of this enzyme. Several other proteins, such the GTPase dynamin have been shown to interact with PtdIns(4,5)P₂. Thus, either directly or indirectly, PtdIns(4,5)P₂ regulates many aspects of cell physiology including signal transduction at the cell surface, cytoskeleton organization, membrane traffic, and nuclear events.

PtdIns(3,4,5)P₃ and **PtdIns(3,4)P₂** are rapidly and transiently produced in response to agonist stimulation via PI 3-kinase activation (Cantley, 2002). Their level is accurately controlled and classically never exceed more than 10% of PtdIns(4,5)P₂. PtdIns(3,4,5)P₃ is a well established signaling molecule able to control the spatio-temporal organization of several critical signaling pathways at the plasma membrane (Cantley, 2002; Vanhaesebroeck *et al.*, 2001). PtdIns(3,4,5)P₃ interacts with several PH-domain containing proteins, including exchange factors for small G proteins that function as regulators of the cytoskeleton, adaptor molecules, or tyrosine and serine/threonine kinases, leading to their membrane localization. Prominent among its described targets is the serine/threonine kinase Akt (PKB) a key enzyme regulating critical cell functions. Following PI 3-kinase activation, PtdIns(3,4,5)P₃ recruits Akt at the plasma membrane by direct interaction with its PH domain leading to a conformational change allowing exposure of the threonine 308 of Akt. PDK-1, which also exhibits a PH-domain with affinity for PtdIns(3,4,5)P₃, is targeted to the same area and phosphorylates the threonine 308 of Akt. Further, phosphorylation of the serine 473 of Akt by PDK-2 (mTORC2) allows full activation of the enzyme which then plays important roles in the regulation of many biological processes including proliferation, apoptosis, growth, and glucose metabolism (Manning and Cantley, 2007). PtdIns(3,4,5)P₃ interacts with a number of signaling proteins, particularly the GEFs and GAPs for small GTPases involved in the actin cytoskeleton organization. Moreover, like PtdIns(4,5)P₂, PtdIns(3,4,5)P₃ can efficiently interact with basic protein sequences via electrostatic interactions as shown for neurogranin, neuromodulin, or centaurin α and also cytoskeletal proteins. Recently, PtdIns(3,4,5)P₃ was shown to be critical during mitosis as this lipid is required for a correct spindle orientation, parallel to the substratum in nonpolarized cells (Gachet and Tournier, 2007). Localized accumulation of PtdIns(3,4,5)P₃ to the midsection at the cortex is important for regulating the recruitment of dynactin allowing dynein–dynactin complexes-dependent pulling forces to take place in order to obtain the proper spindle orientation. Thus, the regulatory functions of PtdIns(3,4,5)P₃ are fundamental in physiology since this it is a crucial lipid mediator of major cell functions including proliferation, survival, growth, differentiation, migration, chemotaxis, and metabolic changes.

PtdIns(3,4,5)P₃ can be transformed to **PtdIns(3,4)P₂** by the SH2-domain containing inositol 5-phosphatases SHIP1 and SHIP2 (Astle *et al.*, 2007). Although the targets of this phosphoinositide remain poorly identified, PtdIns(3,4)P₂ has been shown to interact with the PH-domain of Akt and of lamellipodin (Krause *et al.*, 2004). Thus, PtdIns(3,4)P₂ has certainly specific roles which remain to be clearly identified.

PtdIns3P is constitutively present in cells where it represents about 15–20% of total PtdIns monophosphates. It is found in the plasma membrane but appears to be enriched in early endosomes. The major part of PtdIns3P is produced by the phosphorylation of PtdIns by the class III PI 3-kinase VPS34, a kinase involved in vacuolar sorting in yeast. This lipid interacts with FYVE domain which are a type of zinc finger motif present in several proteins involved in membrane trafficking both in yeast and in mammals (Hayakawa *et al.*, 2007). PtdIns3P in cooperation with Rab GTPases play major role in the assembly of proteins complexes at the membrane–cytoplasmic interface thus controlling endosome sorting. The PtdIns3P signal can be terminated by the action of 3-phosphatases such as myotubularins (Tronchère *et al.*, 2003; Robinson and Dixon, 2006). PtdIns3P can also be phosphorylated to **PtdIns(3,5)P₂** by Fab1p/PIKfyve, a FYVE domain -containing 5-kinase. The functions of this lipid are still hill defined but it seems to play an important role in the recycling of vacuoles membranes (Odorizzi *et al.*, 2000). PtdIns(3,5)P₂ can bind to proteins of the PROPIN family of seven-bladed β -propellers which are involved in the retrieval of membrane and proteins from the vacuole to the late endosome in yeast (Dove and Johnson, 2007). PtdIns(3,5)P₂ can be degraded by a 5-phosphatase such as Fig4p in yeast or by 3-phosphatases such as myotubularins providing then a way to produce **PtdIns5P** (Tronchère *et al.*, 2004). PtdIns5P can also be produced by dephosphorylation of PtdIns(4,5)P₂ by 4-phosphatases including the bacterial phosphatase IpgD (Niebuhr *et al.*, 2002; Pendaries *et al.*, 2006) and the human type II phosphatases (Ungewickell *et al.*, 2005). The roles of this lipid, generally present in small amounts in cells ($\sim$ 1–10% of total PtdInsP), are still poorly known. It has been shown that PtdIns5P can be used by the type II PtdIns5P 4-kinase to produce little amounts of PtdIns(4,5)P₂ (Rameh *et al.*, 1997). Moreover, recent data suggest that this lipid may play important functions in different cell compartment (Coronas *et al.*, 2007; Jones *et al.*, 2006) and its concentration has been shown to increase during agonist or oncogene-mediated mammalian cell activation (Coronas *et al.*, 2008; Morris *et al.*, 2000).

2.3. Phosphoinositide metabolizing enzymes

The enzymatic machinery involved in the maintenance of phosphoinositide homeostasis is composed of highly efficient, specific and accurately regulated and localized phosphoinositide-kinases, phosphoinositide-phosphatases, and phospholipases (Table 8.1). The turnover rate of the monoester

phosphates of polyphosphoinositides is rapid (they are therefore the first phospholipids to be labeled in cells incubated with ^{32}Pi), thus a change in kinase or phosphatase activity can rapidly modify the local concentration of these lipids. Recent discoveries indicate that mutations in several phosphoinositide kinases and phosphatases take part in the development of human diseases including cancer, X-linked myotubular myopathy, fleck corneal dystrophy, or Lowe syndrome (Blero *et al.*, 2007; Pendaries *et al.*, 2003).

It is widely accepted that these phosphoinositides are kept in a steady state in the membranes through continuous phosphorylation/dephosphorylation reactions by specific 4 and 5 -kinases and -phosphatases (Michell, 1975). Two families of PtdIns 4-kinases (type II and III) and type I PtdInsP-kinases are involved in the control of this turnover as well as 4 and 5-phosphatases such as Sac1, synaptojanin, or OCRL (Astle *et al.*, 2007; Blero *et al.*, 2007). Upon cell stimulation via membrane receptors triggering, PtdIns(4,5) P_2 is hydrolyzed by PLC isoforms (Fukamy, 2002) and/or phosphorylated by class I PI 3-kinases (Cantley, 2002; Vanhaesebroeck *et al.*, 2001). The level of the second messenger PtdIns(3,4,5) P_3 , the product of class I PI 3-kinases, is highly controlled by several phosphatases (Astle *et al.*, 2007) including the 3-phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10), a tumor suppressor (Downes *et al.*, 2007), and polyphosphate 5-phosphatases Pharbin, SKIP and SHIP1 and SHIP2 (Blero *et al.*, 2007; Gratacap *et al.*, 2008). Interestingly, the PtdIns(3,4,5) P_3 degradation pathway involving PTEN and the pathway involving SHIP1 are not redundant probably due their different products (Table 8.1) and respective docking properties. Indeed, in contrast to PTEN, which transforms PtdIns(3,4,5) P_3 into PtdIns(4,5) P_2 , SHIP1 generates PtdIns(3,4) P_2 from PtdIns(3,4,5) P_3 and is thus a regulator of the balance between these two lipids. As mentioned above, several lines of evidence indicate that PtdIns(3,4) P_2 may have important and specific roles.

The PtdIns/PtdIns3P/PtdIns(3,5) P_2 pathway which is constitutively detectable in cells is also highly controlled by specific kinases and phosphatases including class III PI 3-kinase, PIKfyve, myotubularins, and Sac3 (Table 8.1). The mechanisms of regulation of these enzymes are still poorly known but they likely involve players coordinating cell signaling and metabolism to the intracellular trafficking machinery.

Although key advances have been made these last years, the extraordinary complexity in the control of the phosphoinositide metabolism leaves several areas still poorly understood such as the localization of phosphoinositide metabolizing enzymes and of their products or the role of the continuous phosphoinositide turnover and of its fluctuation in the function of these lipids.

The remarkable feature of phosphoinositides, which can be rapidly synthesized and degraded in discrete membrane domains places them as ideal regulators and integrators of very dynamic mechanisms of cell regulation such as motility but also of cell orientation including spindle orientation during mitosis or polarization.

3. PHOSPHOINOSITIDES AS KEY DETERMINANTS IN BUILDING POLARITY

3.1. Phosphoinositides, cell shape, and morphogenesis

3.1.1. Role of PtdIns(3,4,5)P₃ in the formation of protrusion: A parallel with migrating cells

Cell migration is an essential event in several physiological processes which include cell movements during development, chemotaxis, wound healing, and metastasis (Franz *et al.*, 2002; Nabi, 1999; Webb *et al.*, 2002). Migration is initiated by protrusion of the leading edge and cells change their shape to become polarized in the direction of their motion. How cells initially become polarized to migrate remains a major question. These recent years, this polarization process has been mainly studied during chemotaxis using the neutrophils or the amoeba *Dictyostelium discoideum* as model systems (Firtel and Chung, 2000; Van Keymeulen *et al.*, 2006; Weiner, 2002; Wong *et al.*, 2006). Studies with these models have shed light on the dynamic role of phosphoinositides and their metabolizing enzymes during cell polarization. In resting cells, there is an homogenous distribution of PtdIns(4,5)P₂ in the plasma membrane. In response to chemoattractant there is an activation of PI 3-kinase and a rapid accumulation of PtdIns(3,4,5)P₃ at the leading edge where it induces the formation of pseudopodia (Wang *et al.*, 2002). This localization of PtdIns(3,4,5)P₃ is accompanied by a recruitment of PtdIns(3,4,5)P₃ binding proteins such as protein kinase B (Akt) and GEF of the Rho family of small GTPases (Devreotes and Janetopoulos, 2003; Weiner *et al.*, 2002; Willard and Devreotes, 2006). This results in the propagation of polarized signals by regulation of actin/myosin cytoskeleton dynamics (Etienne-Manneville and Hall, 2002; Jou and Nelson, 1998), a positive feedback loop amplifying, and maintaining the polarization signal and directional chemotaxis (Weiner *et al.*, 2002). Conversely, PTEN is restricted to the sides and back of cells together with an actomyosin network containing myosin II that provides the contractile force which helps release the cell from the substratum and allows the posterior to move forward (Comer and Parent, 2002; Funamoto *et al.*, 2002; Kolsch *et al.*, 2008). Myosin II is also found along the lateral sides of cells, where it increases the cortical tension in these regions, providing a physical barrier to restrict lateral pseudopod formation. Cells lacking myosin II or components required for myosin II assembly produce lateral pseudopodia, cannot properly retract the uropod, and consequently exhibit chemotaxis defects (Chung *et al.*, 2001; Wessels *et al.*, 1988). Nishio *et al.* show that SHIP1 is a key regulator of neutrophil migration and polarization. They also indicate that, in contrast to SHIP1, loss of PTEN had no impact on neutrophil chemotaxis (Nishio *et al.*, 2007). By contrast, it is recently demonstrated that

PTEN is necessary for cells to focus on their moving direction, avoiding lateral pseudopodes formations (Heit *et al.*, 2008; Wessels *et al.*, 2007). An explanation about this apparent controversy in PTEN function could come from the important function of PTEN as a docking protein, independently of its phosphatase activity.

These concepts concerning the role of phosphoinositides in polarity of migrating cells have emerged these last years and, interestingly, we have recently demonstrated that similar concepts may apply to epithelial cell polarity. Indeed, when MDCK cells are polarized, PtdIns(3,4,5)P₃ is stably localized at the basolateral membrane and is excluded from the apical plasma membrane, as shown by the localization of fluorescent probes for PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ (i.e., the PH domain of Akt fused to GFP, GFP-PH-Akt) (Watton and Downward, 1999; Yu *et al.*, 2003). Based on these observations, we hypothesized that PtdIns(3,4,5)P₃ itself could be a key determinant of epithelial cell polarity. To test this, we used a lipid shuttling system to deliver exogenous PtdIns(3,4,5)P₃ to the apical surface of polarized MDCK cells stably transfected with PH-Akt-GFP. Within 30 s of addition of synthetic PtdIns(3,4,5)P₃ to polarized MDCK cells, some cells formed PtdIns(3,4,5)P₃-rich protrusions extending above the apical surface normally devoid of this lipid product (Gassama-Diagne *et al.*, 2006). We have examined if other species of phosphoinositides could stimulate protrusion formation and demonstrated that this effect is induced specifically by PtdIns(3,4,5)P₃ and at a lesser extent by PtdIns(3,4)P₂. Both phosphoinositides are produced upon PI 3-kinase activation and bind to the PH domain of Akt (Scheid *et al.*, 2002). The other species of phosphoinositides such as PtdIns4P and PtdIns(4,5)P₂ were unable to stimulate apical protrusion formation (Gassama-Diagne *et al.*, 2006). Membrane protrusion and generation of PtdIns(3,4,5)P₃ are events often associated with actin rearrangement (Pollard and Borisy, 2003). In our experiments, we observed that the protrusions were rich in F-actin and in the GTPases Rac1 and cdc42. Their formation was completely blocked by latrunculin B, indicating that their formation requires F-actin (Gassama-Diagne *et al.*, 2006). By contrast, when MDCK cells stably expressing GFP-PH-PLC, a fusion with the PH domain of phospholipase C δ which binds to PtdIns(4,5)P₂ (Balla and Varnai, 2002; Varnai *et al.*, 2002), were used for the experiment, apical addition of PtdIns(3,4,5)P₃ did not result in GFP-PH-PLC labeling of the protrusions, indicating that PtdIns(4,5)P₂ is not concentrated there, thus confirming the specificity of the PI 3-kinase lipid products. Together, these results indicate that PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ are able to rapidly form transient membranous protrusions in the absence of growth factors or other stimuli. The presence of PI 3-kinase itself was observed in the protrusions and we propose that exogenous PtdIns(3,4,5)P₃ somehow acts by recruiting PI 3-kinase and leads to a positive feedback loop required to maintain the protrusions. The recruitment of PI 3-kinase downstream of

PtdIns(3,4,5)P₃ addition is confirmed by the dramatic reduction of the number of protrusions when MDCK cells were pretreated with a inhibitor of PI 3-kinase prior to PtdIns(3,4,5)P₃ addition. These data suggest that *de novo* synthesis of endogenous PtdIns(3,4,5)P₃ is required to amplify the initial signal provided by exogenous PtdIns(3,4,5)P₃ (Gassama-Diagne *et al.*, 2006). This positive feedback loop is similar to that operating in the generation of polarity in chemotaxing cells (Weiner *et al.*, 2002), suggesting that this signal amplification mechanism may be a conserved strategy used in many systems (Huynh and St Johnston, 2004).

3.1.2. Role of phosphoinositides in tubulogenesis

Tubules are the architectural hallmark of epithelia such as kidney, lung, and mammary gland. During embryogenesis, tubules develop in response to morphogenetic growth factors (Costantini and Shakya, 2006; Hogan and Kolodziej, 2002; Lu *et al.*, 2006). Such factors induce a wide range of cellular activities to create a tubular architecture. These included proliferation, migration, differentiation, and polarization. The mechanisms involved in these events are poorly understood. *In vitro* cell culture models, in particular MDCK cells have provided significant mechanistic insights into the processes involved in tubule formation (Debnath and Brugge, 2005; Lubarsky and Krasnow, 2003; O'Brien *et al.*, 2002). When MDCK cells are grown in a collagen gel in the absence of tubulogenesis induced- growth factors, they spontaneously form cysts that consist of a monolayer of cells surrounding a central lumen. In such condition, addition of HGF directs the tubule development from cysts.(O'Brien *et al.*, 2004) HGF-induced MDCK tubulogenesis is the best understood of the 3D-tubulogenetic systems and much progress has been made with regard to signaling downstream of the HGF receptor Met and related tyrosine kinases receptor (Birchmeier and Gherardi, 1998). It was speculated that HGF initiates new tubule development by inducing a partial, transient epithelial-mesenchymal transition (EMT) that temporarily overrides the drive for three surfaces. EMT would cause cells to migrate out of the cyst wall and invade the surrounding matrix during extension and chain formation. After chain formation, downregulation of HGF signaling causes cells to cease EMT and to redifferentiate to polarize. This switch would allow chains to progress to cords and, ultimately, to mature tubules (O'Brien *et al.*, 2002). Using this 3D MDCK model, authors have also shown that matrix degradation is important in the process of tubulogenesis: while p-EMT is ERK dependent and MMP independent, redifferentiation is MMP dependent and ER independent (O'Brien *et al.*, 2004).

In a study focusing on how MDCK transit to EMT under HGF stimulation, it was found that PtdIns(3,4,5)P₃ is concentrated to the leading edge in extensions meaning from the basolateral membrane and PI 3-kinase activity was required for extensions formation (Yu *et al.*, 2003). These

studies evoked the role of PtdIns(3,4,5)P₃ in extensions formation. Thus, following our demonstration that PtdIns(3,4,5)P₃ induces protrusions formation in MDCK cells, we employed cells grown as 3D cysts in a ECM gel, as an additional biological system to show that PtdIns(3,4,5)P₃ itself contributed to form extension and thus is involved in tubulogenesis. When exogenous PtdIns(3,4,5)P₃ was added to cysts that had been grown in Matrigel, within 30 min of PtdIns(3,4,5)P₃ addition, extensions were observed growing from the BL surface (Gassama-Diagne *et al.*, 2006). These were highly enriched in GFP-PH-Akt and morphologically strongly resembled the extensions produced by treatment with HGF (Yu *et al.*, 2003). This finding indicates that addition of PtdIns(3,4,5)P₃ alone is sufficient to mimic the early steps of HGF treatment and demonstrated that PtdIns(3,4,5)P₃ is important for initiation of tubulogenesis.

The involvement of PI 3-kinase /PTEN in branching morphogenesis of kidney was also revealed by others in different set of publications. Larsen *et al.* (2003) found that inhibitors of PI 3-kinase, wortmannin, and LY294002, substantially inhibited branching morphogenesis in submandibular gland. They also report that PI 3-kinase inhibitors act directly on the epithelium and that PtdIns(3,4,5)P₃ added exogenously to mouse submandibular gland via a membrane-transporting carrier in the presence of PI 3-kinase inhibitors, stimulates cleft formation, indicating a role for PI 3-kinase and PtdIns(3,4,5)P₃ in the first steps of branching morphogenesis. Other published data indicate that PI 3-kinase signaling is also essential in the branching morphogenesis induced by the activation of the tyrosine kinase receptor RET (Tang *et al.*, 2002), the product of the *c-ret* gene. RET is essential for the development of the kidney and enteric nervous system. Activation of RET requires the secreted glial derived neurotrophic factor (GDNF), a chemoattractant for RET expressing epithelial cells (Kim and Dressler, 2007; Tang *et al.*, 1998). RET activation was shown to result in asymmetric localization of PtdIns(3,4,5)P₃ and consequently PTEN was shown to suppress this RET-mediated cell migration and chemotaxis in cell culture assays. Together, these data suggest a critical role for the PI 3-kinase /PTEN signaling in shaping the pattern of epithelial branches in response to RET activation (Kim and Dressler, 2007).

A critical event in cysts formation and tube morphogenesis is the central lumen development which is a polarization event involving reorganization of cytoskeleton, formation of the cell-cell junctions and the apical membrane formation (Hogan and Kolodziej, 2002). Using the 3D MDCK epithelial system, we revealed the dynamic involvement of the lipid phosphatase PTEN both in lumen and apical membrane formation (Martin-Belmonte *et al.*, 2007). Depletion of PTEN using specific RNAi of PTEN resulted in the lack of central lumen. We suggest a molecular mechanism underlying this process which involved PTEN, PtdIns(4,5)P₂, Annexin2,

Cdc42, and Par6/aPKC. This role of PTEN in apical membrane determination will be described in more details in the next paragraph.

Our different contributions and data from others, pinpoint PI3K/PTEN and their lipid products/substrate as key determinants for epithelial cells morphogenesis and ongoing researches should give more on the downstream signaling pathway leading to branching and tube formation.

3.2. Phosphoinositides as determinants of apico-basal polarity

A key feature of polarized epithelial cells is the ability to maintain the specific biochemical composition of the apical and basolateral plasma membrane domains. Different mechanisms contribute to generation and maintenance of this surface polarity. It is generally accepted that the initial polarity cue is generated by cell–cell and cell–ECM interactions mediated by E-cadherin and integrins, respectively (Adams *et al.*, 1998; Ehrlich *et al.*, 2002). These spatial cues induce localized assembly of networks of specialized cytoskeletal and signaling proteins at contacting membranes. The cytoskeleton assembly serves as a scaffold for recruitment and binding of signaling proteins that further define the different membrane domains (Jacobson and Mostov, 2007; Jaulin *et al.*, 2007; Miyoshi and Takai, 2008). This classical and largely accepted model highlighted the function of basolateral membrane in epithelial cell polarization. Recent studies indicate that cadherins function as a scaffold for the establishment of cell polarity but that they are not essential for the maintenance of preformed cell–cell junctions and apical/basal polarity (Capaldo and Macara, 2007; Theard *et al.*, 2007).

These last years, the identification of these well conserved protein complexes between mammals, *Drosophila*, and *Caenorhabditis elegans* (Bilder, 2004) have brought new concepts in our interpretation of cell polarization cues. If such an important work is done to define the role of proteins in the polarity process, the literature concerning the role of lipids is very sparse.

3.2.1. PtdIns(3,4,5)P3 defines the basolateral surface

Previous studies of signaling downstream of E-cadherin have focused on a potential role of PI 3-kinase as a signaling intermediate (Noren *et al.*, 2001; Pece *et al.*, 1999; Woodfield *et al.*, 2001). Activation of PI 3-kinase at sites of cell–cell contact could increase activity of Rac1 because many Rho family GEFs contain pleckstrin homology (PH) domains that are activated by binding to the lipid products of PI 3-kinase. However, using the GFP-PH-Akt as a molecular probe in a study using live MDCK cells (Ehrlich *et al.*, 2002), an enrichment of PI 3-kinase lipid products was observed at cell–cell contact only at the initial protrusive stage of lamellipodia extension and this signal decreased when lamellipodia became stable.

Our recent finding that the formation of the protrusion at the apical surface normally devoid of components involved in cell migration was a forward-looking observation suggests a real change in apical membrane composition. Indeed, basolateral plasma membrane protein determinants, such as the glycoprotein p58, syntaxin 4, and the exocyst component Sec8, were present in the PtdIns(3,4,5)P₃-induced apical membrane protrusions (Gassama-Diagne *et al.*, 2006). Strikingly, gp135/podocalyxin, which is normally restricted to the apical membrane (Ojakian *et al.*, 1997), was absent from the protrusions. These remarkable changes in membrane composition led to the conclusion that the presence of PtdIns(3,4,5)P₃ is able to transform an apical membrane into a basolateral one. Thus, PtdIns(3,4,5)P₃ acts as a compass to identify the basolateral domain. Most likely, PtdIns(3,4,5)P₃ is downstream of the extracellular signals provided by cell–cell and cell–substrate interactions, but is apparently upstream of the other components of the vesicular traffic machinery since our data provided evidence that this prompt recruitment of basolateral proteins to the basolateral side is guided by vesicular movement and not by *de novo* synthesis.

To address further this new role of PtdIns(3,4,5)P₃ in epithelial cell polarity, we exploited *Pseudomonas aeruginosa* a gram-negative pathogen which is a leading cause of nosocomial infections in hospitalized patients (Engel, 2002). In a previous study, we have revealed that activation of PI 3-kinase and Akt are necessary for *P. aeruginosa* entry into polarized MDCK cells (Kierbel *et al.*, 2005). In tissue culture models, *P. aeruginosa* is observed to preferentially bind to and enter the cells at basolateral surfaces (Geiser *et al.*, 2001). Upon binding to the apical surface of MDCK cells, we demonstrated that *P. aeruginosa* recruits PtdIns(3,4,5)P₃, normally found only at the BL membrane, activates Akt, and induces the formation of large protrusions at the point of attachment. We also insert exogenous PtdIns(3,4,5)P₃ into the apical membrane and this ectopically localized apical PtdIns(3,4,5)P₃ enhanced bacterial entry. Later on, we could show that in response to *P. aeruginosa* binding to the apical surface, many basolateral proteins, as well as PI 3-kinase, are rapidly redistributed to the regions of bacterial binding at the apical surface. Concurrently, proteins normally resident at the apical surface are removed from the regions of bacterial attachment, resulting in the transformation of apical membrane into basolateral membrane. These studies suggest that the bacteria are able to transform an apical surface into one with basolateral characteristics, creating a local microenvironment that facilitates colonization and entry into to mucosal barrier. These events associated to bacteria entry recapitulates those observed with apical addition of exogenous PtdIns(3,4,5)P₃ and demonstrate that this lipid is central to epithelial cell polarity and that this process can be subverted by microbial pathogens to cause disease.

Takahama *et al.* recently reported that aPKC is necessary for the restriction of PtdIns(3,4,5)P₃ to the basolateral membrane during MDCK cell

polarization (Takahama *et al.*, 2008). Actually, aPKC is part of the Par3/Par6/Cdc42/aPKC complex, a central regulator of polarity. Thus, these new reports reinforce the importance of PtdIns(3,4,5)P₃ to identify the basolateral membrane and suggest a new pathway for regulation of cell polarity by Par complex.

Other biological conditions where PtdIns(3,4,5)P₃ has a different polarized localization have been reported. For instance, in *Drosophila* photoreceptors, PtdIns(3,4,5)P₃ is more accumulated in the apical domain. It was also shown that stimulation of kidney epithelial cells by insulin binding to the basolateral surface leads to accumulation of PtdIns(3,4,5)P₃ at the apical surface, followed by insertion of an epithelial Na channel into the apical membrane (Blazer-Yost *et al.*, 2004). These differences could be due to a reduced activation of PTEN in the apical region compared to MDCK cells.

3.2.2. PtdIns(4,5)P₂ is a key determinant of the apical surface

Using the MDCK 3D culture system, we recently reported the key function of PtdIns(4,5)P₂ in the formation of the apical surface. Indeed, in nonpolarized MDCK cells, both PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ are distributed along the unique existing membrane domain, indicating an activation of PI 3-kinase at this early steps of cell growth and polarization. Afterwards, when cells start polarization, we observed a recruitment of PTEN and most of the PtdIns(4,5)P₂ is accumulated at the apical membrane, setting the limit of the newly formed lumen, while PtdIns(3,4,5)P₃ remains localized to the basolateral membrane and was excluded from the apical membrane (Gassama-Diagne *et al.*, 2006). These data clearly indicate that PTEN is essential for the segregation of the phosphoinositides and pinpoint PtdIns(4,5)P₂ as a determinant of apical surface. Furthermore, the ectopic insertion of exogenous form of PtdIns(4,5)P₂ into the basolateral domain of mature MDCK cysts relocate apical and tight junction proteins to the insertion domain. To exert its effects PtdIns(4,5)P₂ interacts with annexin 2, a protein that binds anionic phospholipids in a calcium-dependent manner. The binding to PtdIns(4,5)P₂ is specific and induced its clustering (Martin-Belmonte and Mostov, 2007). Cdc42 is an other protein acting downstream of PtdIns(4,5)P₂. In metazoa one of the targets of Cdc42 is Par6, part of the Par3/Par6/aPKC complex. We observed that activated Cdc42 relocalized from cell-cell contacts to the apical pole as the cysts become polarized and develop a lumen. PtdIns(4,5)P₂ ectopically implanted into the basolateral surface relocalized also Cdc42 from the apical to the basolateral plasma membrane. The reduction of the normal levels of Cdc42 inhibited the fusion of intracellular vesicles containing the apical proteins with the plasma membrane causing the formation of intracellular small lumens and the malformation of the central lumen. These morphological effects related to the down regulation of Cdc42 were not previously observed using polarized MDCK cells in 2D-monolayers. Thus, this

confirms the relative sensitivity of the 3D culture system. Taken together, these results lead us to speculate that PTEN is needed for segregation of $\text{PtdIns}(4,5)\text{P}_2$ to the apical plasma membrane, from where it recruits annexin 2, which in turn causes localization of Cdc42.

In the *Drosophila* epithelium, $\text{PtdIns}(4,5)\text{P}_2$ is found at the apical surface and adherens junctions, where it is needed for the recruitment of Bitesize (Pilot *et al.*, 2006). Interestingly, here again, the bacteria inositol 4 phosphatase (*Salmonella*, SigD) is exploit to demonstrate the role of $\text{PtdIns}(4,5)\text{P}_2$ in maintaining epithelial cell polarity. Indeed, depletion of $\text{PtdIns}(4,5)\text{P}_2$ by expressing SigD in epithelial cells was associated with marked alterations in the actin cytoskeleton. The junctional complexes gradually opened and the $\text{PtdIns}(4,5)\text{P}_2$ -depleted cells eventually detached from the monolayer (Mason *et al.*, 2007). This effect seems to be a broad mechanism since the data are observed both with intestinal and kidney cell. These data concerning the role of phosphoinositides in cell polarity are summarized in Fig. 8.2.

3.3. PI and polarized trafficking in epithelial cells

The maintenance of polarity requires continuous active sorting and selective delivery of newly synthesized proteins and lipids to apical and basolateral plasma membrane domains. The groundbreaking discovery that in polarized MDCK cells, influenza hemagglutinin virus (HA) assembles from the apical surface while vesicular stomatitis virus (VSV) assembles from the basolateral surface guided by the polarized distribution of their envelope

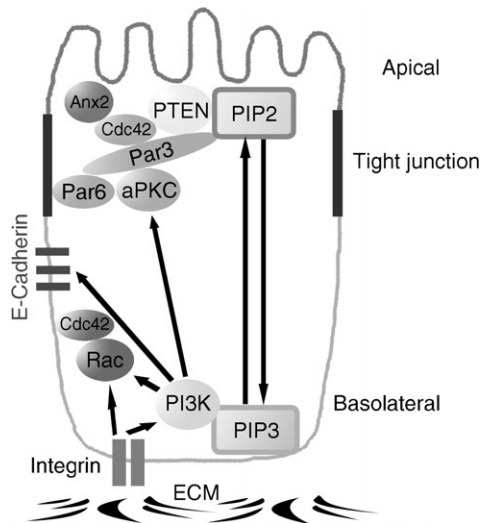


Figure 8.2 Illustration of the critical role of phosphoinositides in regulation of epithelial cell polarity.

glycoproteins provide an important experimental advantage to study polarized trafficking by using virus-infected MDCK cells (Boulan and Pendergast, 1980; Rodriguez-Boulan and Sabatini, 1978). There are two basic pathways described by which proteins or lipids reach the correct surface of polarized epithelial cells; the direct biosynthetic delivery and the indirect selected recycling/transcytosis delivery. In the biosynthetic route, newly synthesized proteins are inserted into the rough endoplasmic reticulum and transported through the TGN into carriers that take them specifically and move along microtubules to fuse with the apical or the basolateral membrane (Rodriguez-Boulan and Nelson, 1989; Simons and Wandinger-Ness, 1990). It is likely that there are different pathways from the TGN to each surface. From the indirect route, proteins are sent to one surface which is usually the basolateral. From there they can either recycle to the surface of origin, be degraded in late endosomes and lysosomes or transcytosed to the opposite surface. The relative importance of these two pathways depends on the protein and the cell type. Transcytosis has been most extensively studied through analysis of the polymeric immunoglobulin receptor (pIgR), which transports polymeric immunoglobulin A (pIgA) across epithelia. Number of these studies used hepatocytes, enterocytes, and mainly Madin–Darby Canine Kidney (MDCK) cells stably expressing pIgR (Mostov *et al.*, 2003; Rojas and Apodaca, 2002).

The basolateral sorting information is mostly associated with the cytoplasmic tail of proteins in the form of either tyrosine- or di-leucine-based motifs, which are recognized by cytosolic adaptor proteins (Folsch, 2005; Folsch, 2008). In general, these cytosolic adaptors are heterotetrameric and interact with clathrin. There are four major classes of adaptors (AP-1 through AP-4) and AP-1B plays an important function in basolateral sorting from recycling endosomes (Perret *et al.*, 2005). A recent report indicate a selective requirement for clathrin in the biosynthetic sorting of basolateral plasma membrane proteins in epithelial cells. These data positioned biosynthetic sorting as the main determinant of basolateral route and predicts within AP-1 the participation of other clathrin adaptors in basolateral polarity (Deborde *et al.*, 2008).

Apical targeting signals included glycosylation, as well as signals in the cytoplasmic and luminal domains of the protein (Nelson and Yeaman, 2001; Rodriguez-Boulan and Gonzalez, 1999). Apical traffic also involves Annexin 13b (Lafont *et al.*, 1998) and MAL1/MAL2 (De Marco *et al.*, 2002; Puertollano *et al.*, 1999). Transport through lipid rafts cargos is established as a major route (Simons and Ikonen, 2000; Weimbs *et al.*, 1997) for apical sorting. Nevertheless, some traffic to the apical surface occurs independently of rafts, and conversely, not all raft-associated molecules are sent via an apical route (Sarnataro *et al.*, 2002; Weimbs *et al.*, 1997). Some proteins enter rafts, either because their transmembrane segments partition into rafts or because the proteins are anchored to the external

leaflet by glycosylphosphatidylinositol anchors, which associate with rafts. The importance of this glycolipid anchorage as an apical delivery signal is largely documented but will not be considered in this review.

In recent years, a growing literature has implicated phosphoinositides and their metabolizing enzymes as key players in membrane trafficking (Di Paolo and De Camilli, 2006; Krauss and Haucke, 2007). Even though these extensive investigations, little is known about how they regulate traffic in polarized epithelial cells.

PtdIns4P is the main phosphoinositide in the Golgi complex and has been reported to play multiple roles in transport of cargo from the TGN to the plasma membrane. This critical function of PtdIns4P has been first described in Yeast (Hama *et al.*, 1999; Walch-Solimena and Novick, 1999) and appears to be preserved in mammalian cells as well. So far, two PI 4-kinase in mammalian cells have been reported to contribute to the production of PtdIns4P pool in the Golgi complex, PI4KIII β and PI4KII α (Wang *et al.*, 2003; Weixel *et al.*, 2005).

The recruitment and activity of PI4-kinase (PI4K) on Golgi membranes is controlled by ARF 1 (Bruns *et al.*, 2002; Godi *et al.*, 1999; Wang *et al.*, 2003). Overexpression of frequenin, a positive modulator of PI4K β activity in polarized MDCK cells indicated a selective inhibition of apical delivery from TGN (Weisz *et al.*, 2000). Moreover, expression of a catalytically inactive mutant of PI4KIII β inhibited intra-Golgi transport, but inconsistently stimulated TGN to cell surface delivery of the apical marker HA in these cells. By contrast, overexpression of wild-type PI4KIII β had no effect on early Golgi transport but inhibited TGN-to-apical membrane delivery. PI4KII α seems to have a broader distribution in the cell and has been shown to be associated with several membrane bound organelles, including endosomes, a subcompartment of the endoplasmic reticulum, synaptic vesicles, and the Golgi complex (Bruns *et al.*, 2002; Weixel *et al.*, 2005).

PtdIns4P is specifically required for AP-1 and EpsinR recruitment to the TGN (Mills *et al.*, 2003). EpsinR ENTH domain binds specifically PtdIns4P (Hirst *et al.*, 2003) as well as the Golgi AP-1 adaptor complex (Wang *et al.*, 2003). The functional role of PtdIns4P has also been addressed by studying the role of PtdIns4P binding proteins FAPP2 and FAPP1 in the delivery of biosynthetic cargo from the Golgi to the cell surface in polarized MDCK cells (Vieira *et al.*, 2005, 2006). These data demonstrate that only the knock-down of FAPP2 and not FAPP1 delay the apical delivery of different cargos including HA and GPI-anchored proteins. FAPP2 and not FAPP1 is involved in the post golgi transport and apical delivery machinery despite their shared similarity. It has been speculated that FAPP2 contributes to the apical delivery process by stabilization of the raft cluster. Interestingly, FAPP2 also contains a glycolipid-transfer-homology domain, and it seems that FAPP2 is necessary for transport of glucosylceramide from the cis-Golgi (D'Angelo *et al.*, 2007). Therefore, it has been speculated that FAPP2 might

coordinate glycosphingolipid synthesis with apical transport. Furthermore, FAPP2 knock down disrupted apically enriched staining for galectin-3. Galectin-3 is a member of a new lectin family for apical trafficking and is required for intracellular sorting and correct targeting of non-raft-associated glycoproteins (Delacour *et al.*, 2006, 2007). Thus, the authors speculated that FAPP2 may also be involved in sorting of non raft vesicles.

Together, these data suggest that PtdIns4P is central in delivery of the apical cargo.

PtdIns(4,5)P₂ is involved in endocytosis by mediating the activity of different proteins important for assembling a clathrin-coated vesicles including AP-2 and Epsin. The three isoforms of PI5K (α , β , γ) involved in generation of PtdIns(4,5)P₂ appear to be all involved in endocytosis as mentioned above. However, the main part of these studies is realized in non polarized cells and the role of these PtdIns(4,5)P₂ metabolizing enzymes in polarized cells is less known. Overexpression of PI5K α in MDCK cells were shown to selectively stimulate the TGN to apical delivery of a raft-associated protein without affecting the overall polarity of delivery (Guerriero *et al.*, 2006). This also resulted in increase of actin comet frequency. Moreover, the effect of PtdIns(4,5)P₂ appears to involve an Arp2/3-dependent pathway, as a dominant-negative inhibitor of Arp2/3 function selectively inhibited biosynthetic transport of this protein (Guerriero *et al.*, 2006). Together, these data suggest that apical transport of raft-dependent and -independent cargo is differentially regulated, and that raft-dependent cargo are transported via a protein kinase C, PIP2- and Arp2/3-dependent pathway consistent with the involvement of actin comets.

Recently, the role of PI5K α on the expression of the epithelial sodium channel (ENAC), was analyzed in cortical collecting duct cells (Weixel *et al.*, 2007). ENAC is an apical membrane transporter and its expression level is regulated by clathrin mediated endocytosis after ubiquitination. Expression of PI5K α decreased the surface expression of ENAC, while the catalytically inactive mutant had no effect. These data also show an apical localization of PIP5K and by contrast PI5K γ is localized to the basolateral domain and regulates the endocytosis of basolateral protein as transferring receptor (Bairstow *et al.*, 2006), indicating that the different isoforms of PI5K are segregated to regulate PIP2 level at distinct membrane domain in polarized cells. Finally, the same authors have shown recently that PI4phosphate-5 kinase γ modulates the basolateral delivery of E-cadherin by the generation of PtdIns(4,5)P₂ (Ling *et al.*, 2007). However, it is not known, whether this function is specific to E-cadherin sorting or if it is due to a general role in the AP-1B/basolateral pathway.

PtdIns(3,4,5)P₃ regulates transcytosis of basolateral membrane components. Sparse previous reports suggested the requirement of PI 3-kinase for transcytosis. Rat thyroid (FRT) cells treated with wortmannin, resulted in a 50% reduction in basolateral to apical as well as apical to basolateral

transcytosis of ricin, a toxic lectin which binds to terminal galactose residues on glycoproteins and glycolipids and thus serves as an effective marker of bulk membrane transport (Hansen *et al.*, 1995; Zurzolo *et al.*, 1992). Wortmannin had no effect on endocytosis from the basolateral membrane domain of FRT cells, while some reduction in apical endocytosis was detected.

In MDCK cells expressing the pIgR, basolateral to apical transcytosis of both ricin and dimeric IgA (dIgA) was reduced in the presence wortmannin, showing that bulk membrane and receptor-mediated transcytosis are affected similarly (Cardone and Mostov, 1995; Hansen *et al.*, 1999). It was proposed therefore that PI 3-kinase plays an essential role in regulating the transcytosis.

Recently, using MDCK cells polarized on filter as a model system, we selectively biotinylated the basolateral molecules in a pulse-chase experiment (Gassama-Diagne *et al.*, 2006) and observed that a large amount of biotinylated proteins appears at the apical surface within 5 min suggesting that biosynthetic contributions are probably small. The apical recruitment of basolateral proteins upon addition of exogenous PtdIns(3,4,5)P₃ is totally abolished in the presence of a dominant negative form of dynamin (Altschuler *et al.*, 1998), indicating that these processes require endocytosis. Many basolateral plasma membrane components are known to be rapidly endocytosed and recycled to the basolateral surface (Mostov *et al.*, 1992). It is likely that apical PtdIns(3,4,5)P₃ causes these recycling vesicles to be redirected to the apical surface.

Altogether, these data show up the dynamic role of phosphoinositides in vesicles trafficking, but more investigations are needed to precise the function in epithelial polarized transport (see summarize in Fig. 8.3).

4. CONCLUDING REMARKS

Evidence reviewed here clearly indicated a critical role of phosphoinositides in both the establishment and the maintenance of cell polarity in epithelial cells. The spatial and temporal segregation of specific phosphoinositide pools seems to be an essential feature for polarization. There is a rapid turnover of these lipids and complex interconversions between them. Important development is required to elucidate the mechanism underlying these interconnections and to identify the different actors involved.

Evidence is also emerging that phosphoinositides can control the activities of PAR complex which are currently the master regulators of epithelial cell polarity. The biological significance of this interaction needs to be clarified regarding the involvement of phosphoinositides in the different aspects of cellular biology.

This review uncovered the tubulogenesis as an important point in the development of epithelium. In the future, it will be interesting to identify

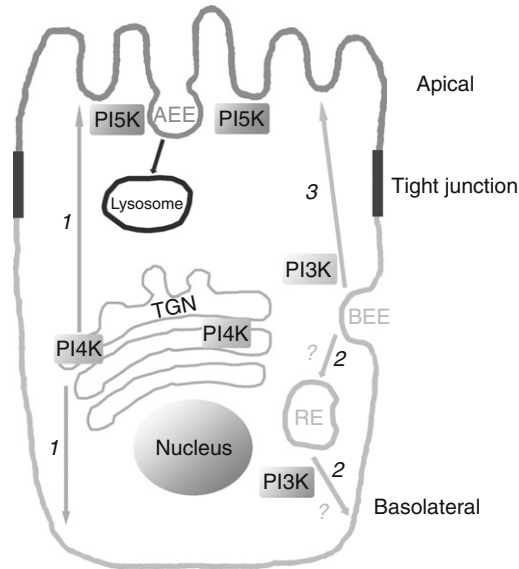


Figure 8.3 Phosphoinositides and their metabolizing enzymes in polarized trafficking of epithelial cells. (1) Biosynthetic pathways to the apical and basolateral surfaces, (2) Recycling pathways at the basolateral surface, (3) Pathway for transcytosis from the basolateral to the apical surface. For each lipid product, the metabolizing enzymes and the involved pathways are indicated in the same color: blue for PtdIns(4,5)P₂, red for PtdIns(4,5)P₂, and green for PtdIns(3,4,5)P₃. AEE, apical early endosome; BEE, basolateral early endosome; PI3K, PI 3-kinase, PI4K, PtdIns 4-kinase; PI5K, PtdIns 5-kinase.

the downstream mediators of PI 3-kinase/PTEN signaling and the other phosphoinositides metabolizing enzymes most likely involved in the extensions and branching formation. The function of polarity during development is an emerging field and the opening interface between development and cell biology is an important direction for research in different pathologies such as cancer. The relationship between loss of epithelial polarity and progression toward malignancy has long been known, but the precise correlation remains a fundamental question. In this context, exploring the phosphoinositides signaling pathways for studying cell polarity is a promising step in cancer research.

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