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Sumitra Deb
Swati Palit Deb *Editors*

p53 Protocols

Second Edition

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Preface

We are proud to present the second edition of p53 protocols to keep up with the progress in p53 research since the publication of its first edition. The present edition includes 19 chapters delineating state-of-the art methods to address the challenging questions of the p53 field such as identification of the target genes and binding partners of gain of function p53 mutants, methods to determine stress response, autophagy or senescence induced by p53, cell cycle analysis, analysis of different phases of DNA replication modified by p53, and generation of pluripotent stem cells.

As in the earlier version, the primary focus of p53 protocols was to include methods that are important for research on p53 or other tumor suppressors. We have also attempted to discuss the problems and to provide tips for troubleshooting. We owe thanks to Professor John Walker for giving us the opportunity and encouragement to design and edit the second edition. We also thank the staff of Humana Press for their help in compiling the book. We sincerely thank all the contributing authors for sharing their invaluable experience to the research community by writing the high-quality chapters. We would also like to thank Dr. Rebecca Frum and our students Catherine Vaughan, Isabella Pearsall, Shilpa Singh, and Olivia Bouton who enthusiastically acted as foot soldiers assisting us with grammatical editing, communicating with the authors, and organizing the manuscript.

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Chapter 1

Detecting and Quantifying p53 Isoforms at mRNA Level in Cell Lines and Tissues

Marie P. Khoury*, Virginie Marcel*, Kenneth Fernandes, Alexandra Diot, David P. Lane, and Jean-Christophe Bourdon

Abstract

The *TP53* gene expresses at least nine different mRNA variants (p53 isoform mRNAs), including the one encoding the canonical p53 tumor suppressor protein. We have developed scientific tools to specifically detect and quantify p53 isoform expression at mRNA level by nested RT-PCR (reverse transcription-polymerase chain reaction) and quantitative real-time RT-PCR (RT-qPCR using the TaqMan® chemistry). Here, we describe these two methods, while highlighting essential points with regard to the analysis of p53 isoform mRNA expression.

Key words: p53 isoforms, Cancer, Detection, Quantification, mRNA, Splicing, Nested PCR, Quantitative real-time RT-PCR (TaqMan® chemistry), p53 tumor suppressor protein

1. Introduction

The *TP53* gene expresses at least nine different mRNA variants (p53 isoform mRNAs), due to alternative splicing and internal promoter usage (see Fig. 1) (1). The dual promoter structure of the *TP53* gene results in the production of two subclasses of mRNA variants which differ in their 5′UTR (TAp53 and Δ133p53 subclasses) (2). A third subclass of mRNA variant (Δ40p53 subclass) retains the entire intron 2 by alternative splicing (3). These three different subclasses can be combined to three distinct subclasses of mRNA variants differing by their 3′UTR due to alternative splicing of intron 9 (α, β, and γ subclasses) (2). Indeed, complete "splicing out" of intron 9 generates the α subclass, while

*Both authors have contributed equally to this work.

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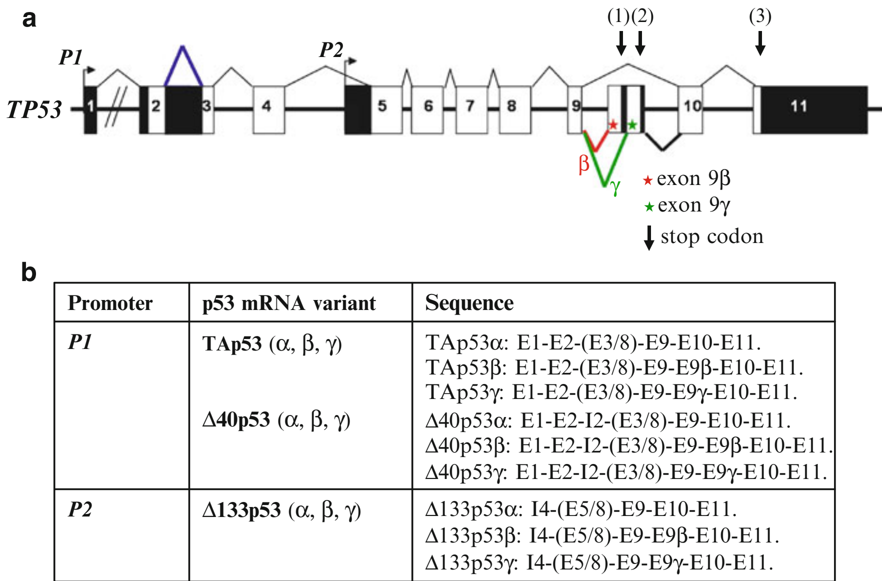

Promoter	p53 mRNA variant	Sequence
P1	**TAp53** (α, β, γ) **Δ40p53** (α, β, γ)	TAp53α: E1-E2-(E3/8)-E9-E10-E11. TAp53β: E1-E2-(E3/8)-E9-E9β-E10-E11. TAp53γ: E1-E2-(E3/8)-E9-E9γ-E10-E11. Δ40p53α: E1-E2-I2-(E3/8)-E9-E10-E11. Δ40p53β: E1-E2-I2-(E3/8)-E9-E9β-E10-E11. Δ40p53γ: E1-E2-I2-(E3/8)-E9-E9γ-E10-E11.
P2	**Δ133p53** (α, β, γ)	Δ133p53α: I4-(E5/8)-E9-E10-E11. Δ133p53β: I4-(E5/8)-E9-E9β-E10-E11. Δ133p53γ: I4-(E5/8)-E9-E9γ-E10-E11.

Fig. 1. (**a**) The genomic sequence of the human *TP53* gene spans 19,200 bp and contains 11 exons. The dual promoter structure of the *TP53* gene (promoters (*P1*) and (*P2*)) allows the production of several p53 mRNA variants due to alternative splicing and internal promoter usage (2). (**b**) Alternative splicing of intron 9 generates the β and γ subclasses (designated by exon 9β and exon 9γ). As highlighted in the text, the three different subclasses of p53 isoform mRNAs differing by their 5′UTR (TAp53, Δ40p53, and Δ133p53) can be combined to three different subclasses of mRNA variants differing by their 3′UTR due to alternative splicing of intron 9 (α, β, and γ subclasses). Of note, while nine different mRNA variants can be obtained in total, 12 different p53 isoform proteins can be generated from the *TP53* gene (due to alternative initiation of translation (see Chapter 2, Fig. 1)). Exons (E) are numbered, non-coding exons are represented by *black boxes*, coding exons by *white boxes* and introns (I) are marked with a *bold straight line*. The stop codons in the β, γ, and α subclasses of p53 mRNA variants are indicated with an *arrow* with the numbers (1), (2), and (3), respectively. E3/8: exon 3 to exon 8; E5/8: exon 5 to exon 8.

retention of different parts of intron 9 produces the β and γ subclasses (see Fig. 1). The canonical p53 protein (encoded by the p53 mRNA variant retaining all exons 1–11 correctly spliced) belongs to the TAp53 subclass. Thus, p53 mRNA variants have distinct nucleotide sequences allowing the design of primers and probes specific for p53 isoforms. Nested PCRs allow the detection of each p53 mRNA variant (see Fig. 2), while RT-qPCR enables the quantification of subclasses of p53 mRNA variants (i.e., TAp53, Δ40p53, Δ133p53, α, β, and γ subclasses), since it is not technically possible to perform quantitative PCR on DNA fragments larger than 200 bp (see Fig. 3).

Using these methods, we and others have shown that p53 isoforms are differentially expressed in human tissues at mRNA level (2, 4–10). For instance, while the TAp53 mRNA subclass is the most abundant one in most human cell lines and tissues, the Δ133p53 subclass is present at about 1–30% of the TAp53

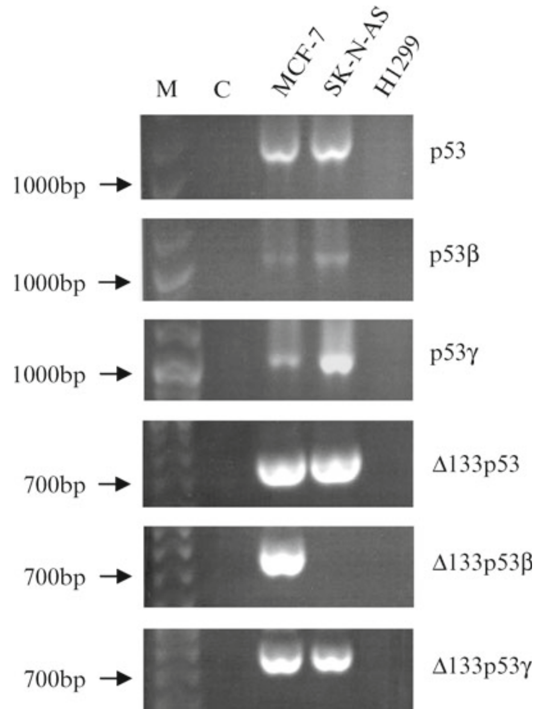

Fig. 2. Example of amplification of p53 isoform mRNAs by nested PCR in human cell lines (MCF-7: human breast adenocarcinoma cell line, wild-type p53; SK-N-AS: Human neuroblastoma cell line, mutant p53: R342X; H1299: human non-small cell lung carcinoma cell line, p53-null). Specific primers were used for amplifying p53, p53β, p53γ, Δ133p53, Δ133p53β, or Δ133p53γ by nested RT-PCR (see Table 1). 20 μL of the PCR products were migrated on 1% agarose gel. M: Marker (100 bp DNA ladder, Promega), C: negative control (see Note 8). The Sanger Method was used for DNA sequencing of the bands obtained (see Note 10).

mRNA subclass. Despite their low expression level, p53 isoforms have been shown to be potent regulators of p53 transcriptional activity and tumor suppressor functions (2, 9, 11–13). In human cancers, several studies have shown that p53 isoform mRNA variants are abnormally expressed. Indeed, comparison of expression of p53 mRNA variants in normal and tumor tissues revealed that the β and γ subclasses tend to be lost in human tumors, while others (Δ133p53 subclass) tend to be overexpressed (1, 10). Furthermore, we have recently reported that the p53γ isoform is associated with cancer prognosis in a cohort of primary breast tumors (10, 13). Given the important role of p53 isoforms in the modulation of p53 tumor suppressor activity and their differential expression in human cancer, future studies will require a systemic characterization of their expression in cell lines and tumor tissues in order to decipher their response to cellular stress.

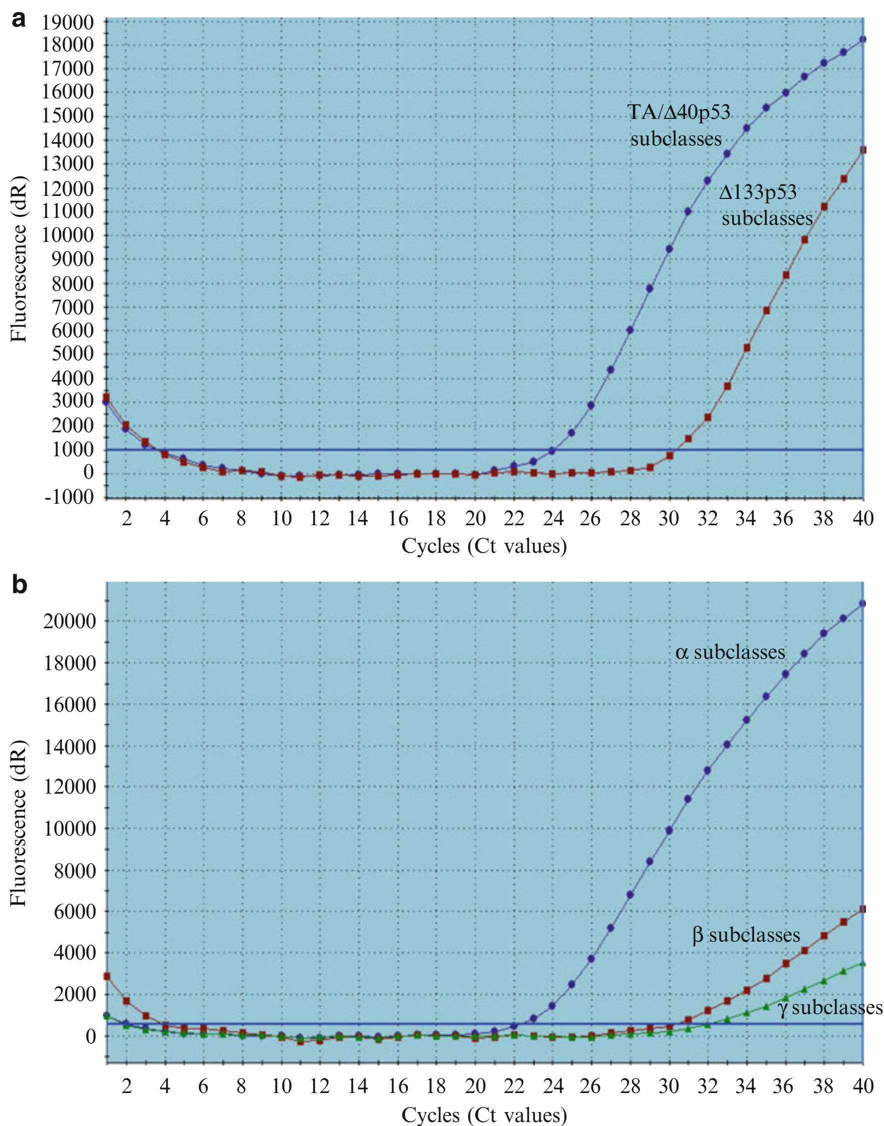

Fig. 3. Example of amplification plots for p53 isoform cDNA standards by the Stratagene Mx3005P™ Real-Time PCR system. In this figure are shown examples of Ct values (or number of PCR cycles required for specific amplification) for amplification of (**a**) TAp53, Δ40p53, and Δ133p53 isoforms and (**b**) α, β, and γ isoforms. Of note, the reverse transcription for this experiment was performed with 1 μg of RNA and then diluted to 200 μL of final volume with water (see Subheadings 2 and 3).

Up until now, while some of the available antibodies allow the detection/identification of all p53 isoforms but with a different accuracy (see Chapter 3), the identification and quantification of p53 isoforms can only be achieved by nested PCRs and RT-qPCR, respectively, and it is therefore crucial to standardize the techniques used to characterize p53 mRNA variants.

2. Materials

2.1. RNA Extraction

1. Clean, RNase-free area (see Note 1).
2. RNeasy® Mini Kit (Qiagen, West Sussex, UK, catalog number 74104).
3. RNase-free DNase Set (Qiagen, catalog number 79254).

2.2. Nested Reverse Transcription-Polymerase Chain Reaction

2.2.1. Reverse Transcription

1. Clean, RNase-free area.
2. Random primers (stock solution at 3 μg/μL, Invitrogen, catalog number 48190-011). Dilute in sterile, distilled water to obtain a final concentration of 0.5 μg/μL (see Note 5).
3. Set of dATP, dCTP, dGTP, dTTP (stock solution at 100 mM each, Promega, catalog number U1240). Prepare 10 mM dNTP Mix by diluting 10 μL of each dATP, dCTP, dGTP, dTTP to 100 μL of final volume with sterile, distilled water.
4. RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, Paisley, UK, catalog number 10777-019).
5. Cloned AMV Reverse Transcriptase (15 U/μL, Invitrogen, catalog number 12328-019) which includes the Cloned AMV Reverse Transcriptase, 5× cDNA Synthesis Buffer, and 0.1 M DTT.
6. Sterile, distilled water.

2.2.2. Polymerase Chain Reaction

1. PCR SuperMix (Invitrogen, catalog number 10572-063).
2. Primers (Forward and Reverse) specific for p53 isoforms were designed in our laboratory and purchased from MWG-Biotech (UK). Primers are used at 10 μM and stored at –20°C. The list of primers used for amplification of human p53 isoforms by nested PCR is provided (see Table 1).
3. 5× TBE (Tris-borate-EDTA): Prepare a concentrated (5×) stock solution of TBE by dissolving 30 g Tris-base, 15.4 g Boric Acid, 1.86 g EDTA-Na_2-$2H_2O$ in water for a final volume of 1 L. The solution can then be stored at room temperature. Use a diluted (0.5×) TBE solution for agarose gel preparation.
4. Ultrapure™ Agarose (Invitrogen, catalog number 16500-500).
5. UltraPure™ 10 mg/mL Ethidium Bromide (Invitrogen, catalog number 15585-011).
6. 100 bp DNA ladder (Promega, catalog number G2101).
7. Prepare 1% agarose gels by boiling agarose in 0.5× TBE buffer. Add 1 μL of ethidium bromide (10 μg/μL, Invitrogen) per 100 mL of buffer.

Table 1
Primers for amplification of human p53 isoform and actin mRNAs by RT-PCR (nested PCR)

mRNA (PCR fragment size)	PCR	Primer name and targeted region	5′–3′ sequence
p53 (1,130 bp)	I	**e2.1 (F)** (exon 2)	GTCACTGCCATGGAGGAGCCGCA
		RT1 (R) (exon 11)	GACGCACACCTATTGCAAGCAAGGGTTC
	II	**e2 (F)** (exon 2)	ATGGAGGAGCCGCAGTCAGAT
		RT2 (R) (exon 11)	ATGTCAGTCTGAGTCAGGCCCTTCTGTC
p53β (1,120 bp)	I	**e2.1 (F)** (exon 2)	GTCACTGCCATGGAGGAGCCGCA
		RT1 (R) (exon 11)	GACGCACACCTATTGCAAGCAAGGGTTC
	II	**e2 (F)** (exon 2)	ATGGAGGAGCCGCAGTCAGAT
		p53b (R) (exon 9β)	TTTGAAAGCTGGTCTGGTCCTGA
p53γ (1,120 bp)	I	**e2.1 (F)** (exon 2)	GTCACTGCCATGGAGGAGCCGCA
		RT1 (R) (exon 11)	GACGCACACCTATTGCAAGCAAGGGTTC
	II	**e2 (F)** (exon 2)	ATGGAGGAGCCGCAGTCAGAT
		p53g (R) (exon 9γ)	TCGTAAGTCAAGTAGCATCTGAAGG
Δ133p53 (770 bp)	I	**i4F1 (F)** (intron 4)	TAGACGCCAACTCTCTCTAG
		RT1 (R) (exon 11)	GACGCACACCTATTGCAAGCAAGGGTTC
	II	**i4F2 (F)** (intron 4)	CTAGTGGGTTGCAGGAGGTGCTTACAC
		RT2 (R) (exon 11)	ATGTCAGTCTGAGTCAGGCCCTTCTGTC
Δ133p53β (760 bp)	I	**i4F1 (F)** (intron 4)	TAGACGCCAACTCTCTCTAG
		RT1 (R) (exon 11)	GACGCACACCTATTGCAAGCAAGGGTTC
	II	**i4F2 (F)** (intron 4)	CTAGTGGGTTGCAGGAGGTGCTTACAC
		p53b (R) (exon 9β)	TTTGAAAGCTGGTCTGGTCCTGA
Δ133p53γ (760 bp)	I	**i4F1 (F)** (intron 4)	TAGACGCCAACTCTCTCTAG
		RT1 (R) (exon 11)	GACGCACACCTATTGCAAGCAAGGGTTC
	II	**i4F2 (F)** (intron 4)	CTAGTGGGTTGCAGGAGGTGCTTACAC
		p53g (R) (exon 9γ)	TCGTAAGTCAAGTAGCATCTGAAGG
Actin (one PCR)	Actin (F)		ATCTGGCACCACACCTTCTACAATGAGCTGCG
	Actin (R)		CGTCATACTCCTGCTTGCTGATCCACATCTGC

The specific region (exon or intron) that each of the primers target is indicated. (F): Forward, (R): Reverse. PCR fragment sizes (bp) corresponding to p53 isoforms are also indicated. cDNA quality is confirmed by PCR amplification of actin. Of note, the primers corresponding to Δ40p53 isoforms (Δ40p53α, Δ40p53β, and Δ40p53γ) are not listed in this table, as we have not studied the Δ40p53 isoforms in our laboratory

8. 10× DNA loading buffer: Prepare a concentrated (10×) stock solution of DNA loading buffer by dissolving 125 mg of bromophenol blue in 16.5 mL of Tris Buffer (150 mM, pH 7.6). Add 30 mL of glycerol and 3.5 mL of water. The solution can then be stored at room temperature. Use the solution of DNA loading buffer at 1×.
9. Sterile, distilled water.

2.3. Quantitative Real-Time RT-PCR (Real-Time PCR Using the TaqMan® Chemistry)

2.3.1. Reverse Transcription (RT)

1. Clean, RNase-free area.
2. Random primers (stock solution at 3 μg/μL, Invitrogen, catalog number 48190-011). Prepare as in Subheading 2.1 (see Note 5).
3. Set of dATP, dCTP, dGTP, dTTP (Promega, catalog number U1240). Prepare as in Subheading 2.1.
4. RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, catalog number 10777-019).
5. Superscript™ II reverse transcriptase (200 U/μL, Invitrogen, catalog number 18064-014) which includes the Superscript™ II reverse transcriptase, 5× First-Strand Buffer, and 0.1 M DTT.
6. Prepare a fresh 1:3 dilution of the reverse transcriptase by diluting the Superscript™ II reverse transcriptase (200 U/μL) in 1× First-Strand Buffer (for example, 10 μL of Superscript™ II reverse transcriptase is added to 20 μL of 1× First-Strand Buffer, for a final volume of 30 μL), as recommended by the manufacturer. 1× First-Strand Buffer is diluted in sterile, distilled water from the 5× stock solution (5× First-Strand Buffer).
7. Sterile, distilled water.

2.3.2. RT-qPCR (Quantitative Real-Time RT-PCR Using the TaqMan® Chemistry)

1. Clean, RNase-free area.
2. A set of pipettes dedicated to RT-qPCR experiments only.
3. TaqMan® Universal PCR Master Mix, No AmpErase® UNG (2×, Applied Biosystems, catalog number 4324020).
4. Primers and probes specific for each subclass of p53 isoform mRNAs were designed in our laboratory and purchased from MWG-Biotech (UK). Primers and probes are used at 10 μM and stored at −20°C. The list of primers and probes used for amplification of human p53 isoforms by RT-qPCR is provided (see Table 2, Notes 13 and 14).
5. MicroAmp™ Optical 96-Well Reaction Plate (Applied Biosystems, catalog number N8010560).
6. A rack to support the 96-well reaction plate.
7. MicroAmp™ Optical 8-Cap Strip (Applied Biosystems, catalog number 4323032).

3. Methods

3.1. RNA Extraction

1. For cell harvesting, remove medium from wells/dishes then wash cells twice with ice-cold 1× PBS.
2. Cells are lysed by directly adding onto wells/dishes Buffer RLT supplemented with β-Mercaptoethanol (β-Mercaptoethanol is

Table 2
Primers and probes for quantitative amplification of human p53 isoforms by RT-qPCR (Real-Time PCR using the TaqMan® chemistry)

p53 isoform subclass	Primer name	Targeted region	5′–3′ sequence
TA/Δ40p53	(F)	Exon 4	CAGCCAAGTCTGTGACTTGCA
	(R)	Exon 5	GTGTGGAATCAACCCACAGCT
	(Pr)	Exon 5	TCCCCTGCCCTCAACAAG ATGTTTTGCC
Δ133p53	(F)	Intron 4	ACTCTGTCTCCTTCCTCTTCCTACAG
	(R)	Exon 5	Same as TAp53 (R)
	(Pr)	Exon 5	Same as TAp53 (Pr)
α (alpha)	(F)	Exon 9	AACCACTGGATGGAGAATATTTCAC
	(R)	Exon 10	CAGCTCTCGGAACATCTCGAA
	(Pr)	(Exon 9/10)	TCAGATCCGTGGGCGTGAGCG
β (beta)	(F)	Exon 9	Same as p53α (F)
	(R)	(Exon 9β)	TCATAGAACCATTTTCATGCTCTCTT
	(Pr)	(Exon 9/exon 9β)	CAGGACCAGACCAGCTTTCAAAAAGAAAATTGTT
γ (gamma)	(F)	Exon 9	Same as p53α (F)
	(R)	(Exon 9γ)	TCAACTTACGACGAGTTTATCAGGAA
	(Pr)	(Exon 9/exon 9γ)	TTCAGATGCTACTTGACTTACGATGG
E8/9	(F)	Exon 8	GAAGAGAATCTCCGCAAGAAAGG
	(R)	Exon 9	TCCATCCAGTGGTTTCTTCTTTG
	(Pr)	Exon 8/9	AGCACTAAGCGAGCACTGCCCAACA

The specific region (exon or intron) that each of p53 primers target is indicated. (F): Forward, (R): Reverse, Pr: Probe. The TaqMan® probe that we use contain the reporter dye (Fam) and the quencher dye (Tamra). The p53 isoform primers/probe sets listed in this table are specific for more than one p53 isoform mRNA (a subclass of p53 isoform mRNAs): "TA/Δ40p53" primers/probe quantify TAp53α (also named p53 or p53α), TAp53β (p53β), TAp53γ (p53γ), Δ40p53α, Δ40p53β, and Δ40p53γ. "Δ133p53" primers/probe quantify Δ133p53α, Δ133p53β, and Δ133p53γ. "E8-9" primers/probe targeting exons 8/9 (as indicated below) quantify all p53 isoform mRNAs. The reference gene used for normalization is the TATA box-binding protein (*TBP*) (see Note 14)

added to Buffer RLT under a chemical hood), according to the manufacturer's instructions.

3. Cells are scrapped off with a cell scraper and collected into a tube.
4. Lysate is passed at least five times through a 23-G needle fitted to an RNase-free syringe (see Note 2).
5. Extract RNA from cells with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions (see Note 3).
6. In addition, on-column DNase digestion is carried out using RNase-free DNase Set (Qiagen) during RNA extraction (between Buffer RW1 wash and first Buffer RPE wash), according to the manufacturer's instructions (see Note 4).

7. Elute RNA in 50 μL of final volume.
8. Concentration of RNA can be determined, for example, by measuring the absorbance at 260 nm using a spectrophotometer.
9. RNA samples should be stored at −80°C.

3.2. Nested RT-PCR (Reverse Transcription-Polymerase Chain Reaction)

3.2.1. Reverse Transcription (RT)

(Per reaction)

1. Mix 0.5 μL of random primers (0.5 μg/μL) with 2 μL of dNTP Mix (10 mM) and 300 ng of total RNA (see Notes 6 and 7).
2. Add sterile, distilled water to 15 μL.
3. Heat the mixture at 65°C for 5 min (using, for example, a heating block), then chill on ice for 5 min.
4. Collect the content of the tube by brief centrifugation.
5. Add 4 μL of cDNA Synthesis Buffer (5×), 1 μL of DTT (0.1 M), 1 μL of RNaseOUT™ (40 U/μL), and 1 μL of Cloned AMV Reverse Transcriptase (15 U/μL). Mix the contents of the tube gently (see Note 8).
6. Incubate at 45°C for 1 h using, for example, a heating block (see Note 9).
7. Reverse transcription product is then stored at −20°C.

3.2.2. PCR (Polymerase Chain Reaction)

(50 μL of final volume for each PCR)

Nested PCR assays are performed as two consecutive PCRs with two separate primer pairs for p53 isoforms (see Table 1).

1. For the first PCR, mix 45 μL of PCR SuperMix (Invitrogen) with 2 μL of cDNA (undiluted), 2 μL of p53 isoform specific primers (1 μL of Forward primer (10 μM) and 1 μL of Reverse primer (10 μM)) (see Table 1), and dilute to 50 μL of final volume with sterile, distilled water.
2. Perform PCR amplification using, for example, the Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Thermal cycling conditions for PCR amplification (first and second PCRs) for p53 isoforms are as follows: [94°C for 3 min (1 cycle)], [94°C for 30 s, 60°C for 45 s, 72°C for 1 min 30 s (30 cycles)], and finally [72°C for 8 min (1 cycle)].
3. For the second PCR, mix 45 μL of PCR SuperMix (Invitrogen) with 1 μL of product of first PCR and 2 μL of p53 isoform specific primers (1 μL of Forward primer (10 μM) and 1 μL of Reverse primer (10 μM)) (see Table 1), and dilute to 50 μL of final volume with sterile, distilled water.
4. Perform PCR amplification as in step 2.
5. PCR products are then analyzed by agarose gel electrophoresis (1% gel, see Subheading 2). For 20 μL of final volume, add

2 μL of 10× DNA loading buffer to 18 μL of DNA (DNA from samples or from 3 μL of 100 bp DNA ladder (Promega) diluted in 15 μL of water), then load onto gels.

6. The bands obtained are visualized using, for example, the Syngene UV transilluminator (see Note 10, Fig. 2).

3.3. Quantitative Real-Time RT-PCR (Real-Time PCR Using the TaqMan® Chemistry)

Prior to reverse transcription, extract total RNA as in Subheading 1 (see Notes 1–4, 11).

3.3.1. Reverse Transcription (RT)

(Per reaction, for 20 μL of final volume) (see Note 1).

1. Prepare Mix 1 by adding 0.5 μL of random primers (0.5 μg/μL), 1 μL of dNTP Mix (10 mM), and 1 μg of total RNA (see Notes 6 and 7).
2. Add sterile, distilled water to Mix 1–12 μL.
3. Heat the mixture at 70°C for 10 min (using, for example, a heating block) then chill on ice for 5 min.
4. Collect the content of the tube by brief centrifugation.
5. Prepare Mix 2 by adding 4 μL of 5× First-Strand Buffer, 1 μL of DTT (0.1 M), and 1 μL of RNaseOUT™ (40 U/μL). Add contents of Mix 2 to Mix 1. Mix the contents of the tube gently.
6. Incubate at 25°C for 5 min.
7. Add 2 μL of the diluted solution of Superscript™ II reverse transcriptase in 1× First-Strand Buffer (see Subheading 2) to each tube (see Note 8).
8. Reverse transcription is then performed under the following thermal conditions: 25°C for 10 min, 42°C for 50 min, and 70°C for 15 min, using, for example, the Applied Biosystems 2720 Thermal Cycler (Applied Biosystems).
9. Dilute cDNA reaction to a final volume of 200 μL by adding 180 μL of sterile, distilled water (to obtain about 5 ng of total RNA per μL) (see Note 12).

3.3.2. RT-qPCR (Quantitative Real-Time RT-PCR Using the TaqMan® Chemistry)

(Per reaction) (see Note 1).

1. Prepare a Master Mix by adding 5 μL of TaqMan® (2×) Universal PCR Mastermix (final concentration of 1×), 1 μL of Forward primer (10 μM), 1 μL of Reverse primer (10 μM), 0.5 μL of probe (10 μM), and 2.5 μL of sterile, distilled water (for 10 μL of final volume per well) (see Notes 7, 8, 14–17).
2. Mix the contents of the tube gently then collect the content of the tube by brief centrifugation.

3. Thaw cDNA sample, mix the contents of the tube gently, then collect the content of the tube by brief centrifugation.
4. Add to a MicroAmp™ Optical 96-Well Reaction Plate, 10 μL of Master Mix (per well) (see Notes 17 and 18).
5. Then add to the plate 2 μL of cDNA (per well). Use fresh sterile tips for each well, even for the triplicate from the same sample (see Notes 8, 16–19).
6. Gently and carefully fit lid strips into position and firmly secure into position (see Notes 20 and 21).
7. Centrifuge the plate at +4°C for 30 s (1,334 × *g*).
8. Real-time quantitative PCR is carried out using, for example, the Stratagene Mx3005P Real-Time PCR system (Stratagene, La Jolla, CA, USA). The thermal cycling conditions are used: [95°C for 10 min (1 cycle)] and [95°C for 15 s and 60°C for 1 min (40 cycles)] (see Note 22). An example of amplification for p53 isoform cDNA is provided (see Fig. 3).

4. Notes

1. In order to prevent RNA degradation, it is very important to work in an RNase-free environment. Therefore, the area where RNA is handled (the bench, racks, pipettes) should be cleaned before and after each experiment (with 70% ethanol or other commercially available RNase decontamination solutions) and located away from microbiological work stations. Work in a quiet environment. The cleaned area can then be covered by clean aluminum foil. Clean, RNase-free, disposable gloves must be worn at all times and changed frequently (and when a contamination is suspected). All solutions, pipettes, tubes, tips, sterile distilled water (or RNase-free water), and lab coats used should be for RNA only. For qPCR analysis, the centrifuge used for spinning down the qPCR plate should be cleaned prior to use.
2. The experiment can be stopped at this stage and samples should be kept at −80°C.
3. RNA extraction from tissues is performed as following: tissues are isolated from different organs and kept overnight at +4°C in 1 mL of RNAlater RNA Stabilization Reagent (Qiagen, catalog number 76106). Samples are transferred the following day to −80°C. Total RNA is then extracted from tissues using the RNeasy Mini Kit (Qiagen) and treated with DNase (Qiagen) prior to reverse transcription, according to the

manufacturer's instructions. For total RNA extracted from tissues, it is important to confirm their integrity prior to reverse transcription, using, for example, the BioAnalyzer 2100™ (Agilent Technologies, Palo Alto, CA, USA) and all samples with a ratio of 28S/18S < 1.2 should be discarded.

4. DNase digestion can also be performed after RNA extraction (prior to reverse transcription), using RQ1 RNase-Free DNase from Promega (catalog number M6101), according to the manufacturer's instructions.
5. Alternatively, random primers from another supplier can be used, for example (500 μg/mL, Promega, Southampton, UK, catalog number C1181). Dilute Random Primers (500 μg/mL, Promega) by mixing 3 μL of Random Primers with 7 μL of sterile, distilled water (for 10 μL of final volume).
6. RNA samples should be kept on ice during the experiment.
7. In order to minimize pipetting errors, it is always important to make a master mix, depending on the number of reactions needed (always prepare 10% extra).
8. The negative control needed for reverse transcription (prior to PCR or qPCR analysis) is where water is used instead of the reverse transcriptase. If amplification is obtained by PCR or qPCR analysis, this may be indicative of DNA contamination. For qPCR analysis, it is also recommended to do another negative control (see Note 16).
9. cDNA synthesis can also be performed at higher temperatures (for example, at 60°C to denature secondary structures).
10. If DNA sequencing is required, the bands obtained by PCR can then be cut and purified using the Qiaquick® Gel Extraction Kit (Qiagen, catalog number 28704), according to the manufacturer's instructions. DNA is then sequenced by the Sanger method (Bigdye terminators, ABI 3730 Genetic Analyzer). The primers used for sequencing are the same as the ones used in the second PCR (see Table 1, Fig. 2).
11. To avoid contamination, complete DNA removal (DNase treatment) is recommended prior to RT-qPCR analysis, as this technique is very sensitive to very small amounts of DNA (DNA contamination could severely alter the results).
12. Depending on the initial RNA concentration used and p53 isoform RNA expression level in the samples, cDNA reaction can be more or less diluted by adding more or less sterile, distilled water.
13. Since the PCR fragments corresponding to p53 isoforms are all around 800–1,000 bp long and as mRNA quantification by

RT-qPCR is not reliable for fragments larger than 200 bp, it is not technically possible to specifically quantify each of the human p53 isoform mRNAs. Therefore, for p53 isoform mRNAs, we have designed in our laboratory primers/probe sets that specifically amplify a subclass of p53 isoform mRNAs. For more information about the primers/probe sets that we use, as well as the isoforms they amplify, see Table 2.

14. In our laboratory, the reference gene often used for normalization of qPCR experiments is the TATA box-binding protein (*TBP*). The (5′-3′) sequence of primers/probe used is the following: Forward primer: (CACGAACCACGGCACTGATT); Reverse primer: (TTTTCTTGCTGCCAGTCTGGAC); Probe: (Fam-5′-TGTGCACAGGAGCCAAGAGTGAAGA-3′-Tamra).
15. Prior to using the primers/probe for the first time, it is important to test their efficiency. Therefore, a 1:3 serial dilution of cDNA is performed (encompassing a range of at least five different concentrations including undiluted samples) and run in triplicate with the new primers/probe set. Primer efficiency (E) is then determined (standard curve) (14).
16. The second type of negative control needed for qPCR is the non-template control (NTC), where for each set of primers/probe, water is added to the qPCR plate instead of cDNA. This control should not show any amplification and if it does, it may indicate that the water used is contaminated. The qPCRs corresponding to the negative control are also run in triplicate.
17. Samples should be run in triplicate, including the negative controls (see Notes 8 and 16).
18. As RT-qPCR is a highly sensitive technique, pipetting should be as accurate as possible.
19. Apply the cDNA mid-way down the plate well to prevent losing cDNA when cap strips are applied later on.
20. A cap sealer may be used to apply the cap strips. Use new clean gloves when applying cap strips.
21. An adhesive film can be used instead of the cap strips to cover the qPCR plate.
22. Important notes regarding qPCR result analysis: After primers/probe efficiency (E) is determined, this value is then taken into consideration to obtain the relative expression of each of the genes analyzed. Relative expression is determined by the comparative $(1+E)^{-\Delta\Delta Ct}$ method. Therefore, the relative quantification is obtained by normalizing the Ct (Cycle threshold) values to the reference gene (ΔCt) and to the calibrator (ΔΔCt) (14).

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Chapter 2

Detecting p53 Isoforms at Protein Level

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Abstract

The human p53 protein isoforms are expressed in several cell lines and modulate p53 tumor suppressor activity, mainly through modulation of gene expression (1–4). Thus, identifying the pattern of p53 isoforms expression in cell lines is a key step for future studies of the p53 network (5). At the moment, the detection of p53 protein isoforms is based on the use of a panel of antibodies allowing their identification by comparing their molecular weights and their detection pattern by different antibodies (6). Here, classical protocols supplemented with technical know-how are described to detect p53 protein isoforms at protein level by Western blotting and immunoprecipitation. Furthermore, a simple method to study the impact of p53 protein isoforms on p53 transcriptional activity through luciferase reporter gene assays is provided.

Key words: p53 protein isoforms, Antibodies, Western blotting, Immunoprecipitation, Luciferase reporter gene assays, Protein detection, p53 tumor suppressor protein, Animal model

1. Introduction

The human tumor suppressor *TP53* gene expresses several p53 transcripts due to the use of alternative promoters, splicing sites, and/or internal initiation sites of translation (1, 7–9). The different p53 transcripts encode at least 12 protein isoforms produced by the combination of four distinct N-terminal domains (TA, Δ40, Δ133, and Δ160) and three distinct C-terminal domains (α, β, and γ) (see Fig. 1) (5). In the N-terminus, the TA forms (initiation site of translation: ATG-1) exhibit a transactivation domain (TAD) and a DNA-binding domain

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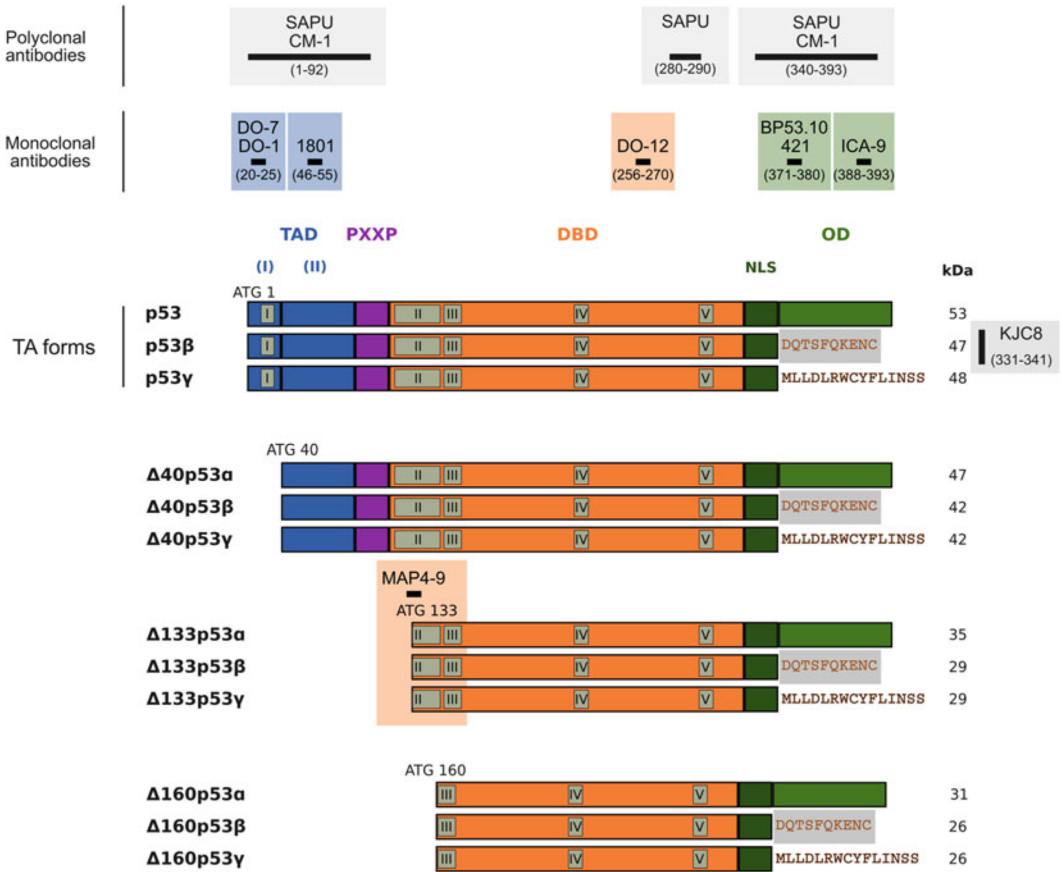

Fig. 1. The human p53 protein isoforms. At least 12 p53 protein isoforms are expressed by the *TP53* gene. Four N-truncated forms (TA, Δ40, Δ133, and Δ160) can be combined to three distinct C-terminal forms (α, β, and γ). As compared to the canonical p53 protein (or TAp53α), the Δ40 forms lack the transactivation domain I (TADI), while the Δ133 and Δ160 forms lack the entire TAD and part of the DNA-binding domain (DBD). In the C-terminus, the α forms contain an oligomerization domain (OD), whereas β and γ forms include novel residues instead of the OD. By Western blotting, the detection of p53 protein isoforms is based on their individual molecular weight (*right panel*) and their distinct recognition pattern by a panel of monoclonal and polyclonal anti-p53 antibodies (*upper panel*). TAD: transactivation domain, *blue*; PXXP: proline-rich domain, *violet*; DBD: DNA-binding domain, *orange*; NLS: nuclear localization signal, *dark green*; OD: oligomerization domain, *light green*; *gray boxes*: conserved domains; ATG: codon used to initiate each N-truncated p53 protein isoform; kDa: kilo Dalton, theoretical protein molecular weight; *colored boxes*: monoclonal and polyclonal anti-p53 antibodies; *numbers within brackets*: epitopes.

(DBD), while the Δ40 forms (ATG-40) lack part of the TAD and the Δ133/Δ160 forms (ATG-133 and ATG-160, respectively) lack the entire TAD and part of the DBD. In the C-terminus, the α forms contain an oligomerization domain (OD), which is replaced by 10 or 15 new residues in β and γ forms, respectively. Among the p53 isoforms, the canonical p53 protein corresponds to the largest protein expressed by *TP53* with a TAD, a DBD, and a C-terminal domain (OD) and thus termed p53α or TAp53α (see Fig. 1). Several studies have reported the interplay between p53 protein isoforms, resulting in the modulation of p53 tumor suppressor activity mainly through regulation of gene expression (2–4, 7).

Table 1
Panel of commercially and non-commercially available primary antibodies to detect p53 protein isoforms (see Note 9)

Species	Primary antibody	Source	Western blotting (μg/ml)	Immuno-precipitation (μg)	Immunostaining (μg/ml)	Ex. supplier/catalog number	Ref.
Human	DO-1	Mouse	1	5	1	Santa Cruz Biotechnology/ sc-126	
	DO-12	Mouse	5	5	5		(1, 8)
	CM1	Rabbit	1	5	1	Convance/SIG-3520	
	SAPU	Sheep	1	5	1		(1)
	KJC8	Rabbit	1	10	1		(1)
	MAP4-9	Rabbit	3	nd	nd		(3)
Drosophila	anti-Dmp53	Rabbit					(11)
	PolyCt	Rabbit					(12)
Zebrafish	5.1	Mouse					(13)
	9.1	Mouse					(13)

nd not determined, *ex.* example, *ref.* reference

The human p53 protein isoforms have been detected at endogenous level in several human cell lines (1, 7, 8). Using the current available antibodies (see Table 1), the detection of p53 isoforms is mainly based on the comparison of signal pattern given by a panel of antibodies, since p53 protein isoforms share a common amino-acid sequence and thus similar epitopes (see Fig. 1). For example, the detection of p53 protein isoforms by Western blotting is based on (1) a different reactivity to a panel of several antibodies recognizing distinct epitopes and (2) a different electrophoresis motility (see Fig. 2a). Interestingly, new antibodies have been developed to specifically recognize some subclasses of p53 protein isoforms. Such antibodies allow immunoprecipitation and immunofluorescence experiments (i.e., MAP4-9 recognizes the Δ133 forms, including Δ133p53α, Δ133p53β, and Δ133p53γ; KJC8 is specific for the β forms, including TAp53β, Δ40p53β, Δ133p53β, and Δ160p53β) (see Figs. 1 and 2). In addition, it should be kept in mind that one could compare the expression levels of each p53 isoform only when the antibody recognizes an identical epitope per isoform. The polyclonal antibodies (SAPU or CM1), which recognize epitopes in both N- and C-terminal p53 domains, do not detect the p53 isoforms with the same affinity since some isoforms lack the N- and/or the C-terminus.

The best described biochemical activity of p53 protein isoforms is their ability to modulate p53 transcriptional activity, as described using luciferase reporter gene assays (1, 7, 9, 10). However, luciferase reporter gene assays performed to assess the impact of one protein on the transcriptional activity of a second one, requires the user to establish appropriate experimental conditions. Of note, the activity of p53 isoform on transcription can be detected and quantified even at low expression level of p53 protein isoforms, below the detection threshold by Western blotting. This suggests that the p53 isoforms are potent regulators of p53 transcriptional activity in human cells (see Fig. 3) (1, 10).

Detecting p53 isoforms at protein level remains to be improved, mainly by increasing the panel of antibodies specific to p53 isoforms. The development of these tools has been initiated with the generation of antibodies recognizing some subclasses of p53 isoforms (i.e., KJC8 or MAP4.9) and has been extended to p53 isoforms expressed in animal models, in particular, in *Drosophila melanogaster* and *Danio rerio* models (see Table 1, Fig. 4) (5).

2. Materials

2.1. Western Blotting

1. NP40 extraction buffer: 50 mM Tris–HCL pH 7.5; 10% glycerol; 0.1% "NP-40 Alternative" (Calbiochem); 100 mM NaCl; 0.2 mM EDTA. Store at 4°C. The buffer is supplemented extemporaneously with 1× Complete™ Protease Inhibitor Cocktail (Roche).

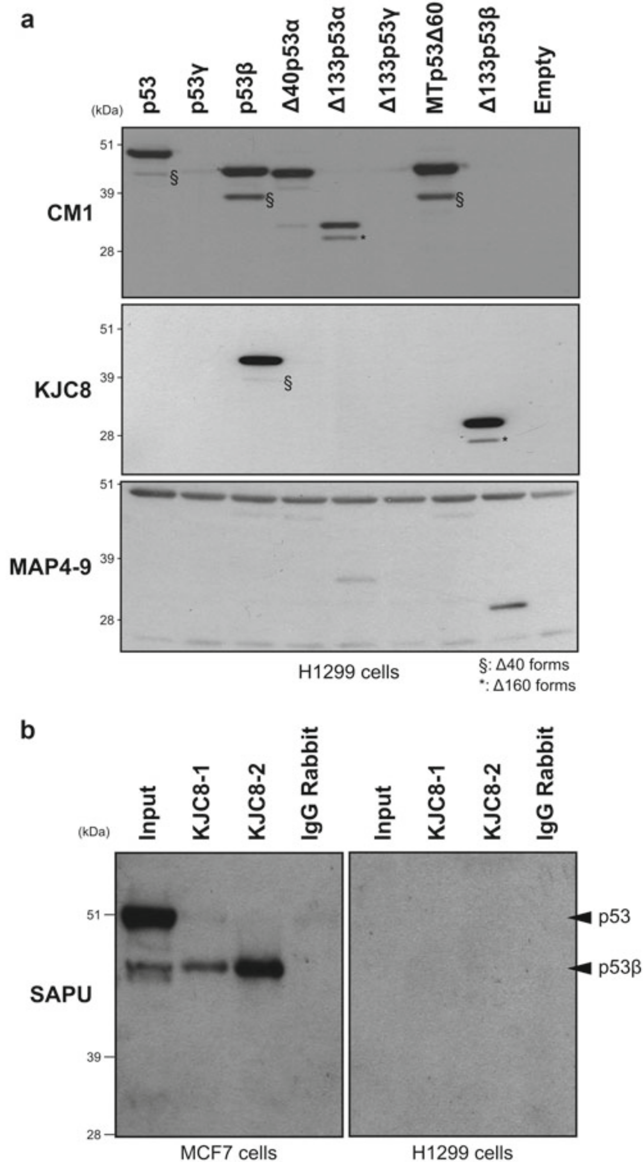

Fig. 2. Detection of human p53 protein isoforms. (**a**) Detection of ectopic p53 protein isoforms by Western blotting. p53-null H1299 cells were transfected with vectors expressing different p53 protein isoforms. Expression of ectopic p53 isoforms was verified by Western blotting using three different antibodies (see Subheading 2). The interpretation of a Western blot for p53 isoform expression is based on (1) distinct migration profile and (2) distinct recognition pattern given by several antibodies. For example, the band corresponding to p53β protein isoform (1) is located at around 47 kDa and (2) is detected by both CM1 (all isoforms) and KJC8 (β forms) but not by MAP4-9 (Δ133 forms). MTp53Δ60: truncated mutant p53 lacking the last 60 residues; Empty: empty-expression vector; kDa: kilo Dalton; §: Δ40 forms; *: Δ160 forms. (**b**) Detection of endogenous p53 protein isoforms by immunoprecipitation. Wild-type p53 MCF7 cells were used to immunoprecipitate endogenous p53β protein isoform using the rabbit polyclonal KJC8 antibody specific for the β forms, then revealed by Western blotting using the sheep polyclonal SAPU antibody recognizing all p53 protein isoforms (see Subheading 3). Immunoprecipitation of β forms allows a simple interpretation as compared to Western blotting. p53-null H1299 cells were used as negative control. Input: positive control (unprecipitated protein extract); IgG Rabbit: negative control (immunoprecipitation using nonspecific antibody); KJC8-1/2: 2 different batches of purified KJC8 antibodies with distinct affinity for p53β protein isoform.

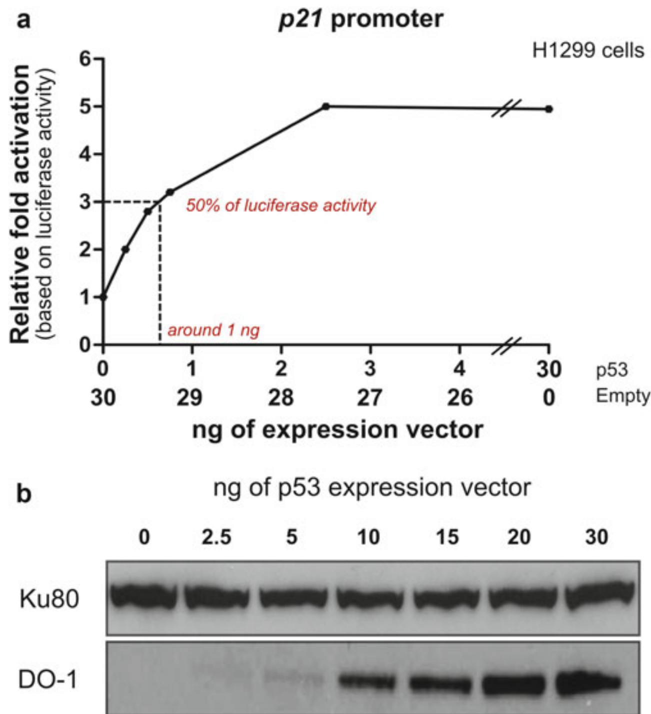

Fig. 3. Luciferase reporter gene assays to study the impact of p53 protein isoforms on p53 transcriptional activity. (**a**) Determination of the amount of p53 expression vector to transfect to obtain 50% of maximum promoter activity. The p53-null H1299 cells were co-transfected (see Subheading 4) with (1) a constant amount of Firefly luciferase reporter plasmid driven by the promoter of interest, (2) a constant amount of the control *Renilla* luciferase reporter plasmid, driven by the SV40 promoter, and (3) an increasing amount of p53 expression vector balanced with the corresponding amount of Empty-expression vector. The Firefly luciferase activity driven by the *p21* promoter is increased by a low amount of ectopic p53 protein barelly detectable by western blotting (**b**) and reaches a plateau starting from 2.5 ng of transfected p53 expression vector. To be able to detect either an increase or a decrease of p53 transcriptional activity on the *p21* promoter, we transfected the amount of p53 expression vector that leads to 50% of maximum the *p21* promoter activity (here, 1 ng of pcDNA3-p53 expression vector). Empty: Empty-expression vector; Ku80: loading control.

2. 1× LDS buffer: dilution from the 4× stock solution NuPAGE® LDS Sample Buffer (Invitrogen). Store at room temperature (see Note 1).
3. DTT 1 M: 1.54 g in a final volume of 10 ml milli-Q water. Store at −20°C.
4. Electrophoresis components:

   XCell SureLock® Mini-Cell Electrophoresis System (Invitrogen).

   Pre-casted NuPAGE® Novex® 10% Bis-Tris Mini Gels (Invitrogen).

   1× Running buffer prepared from 20× NuPAGE® MOPS SDS Running Buffer (Invitrogen).

   NuPAGE® Antioxidant (Invitrogen).

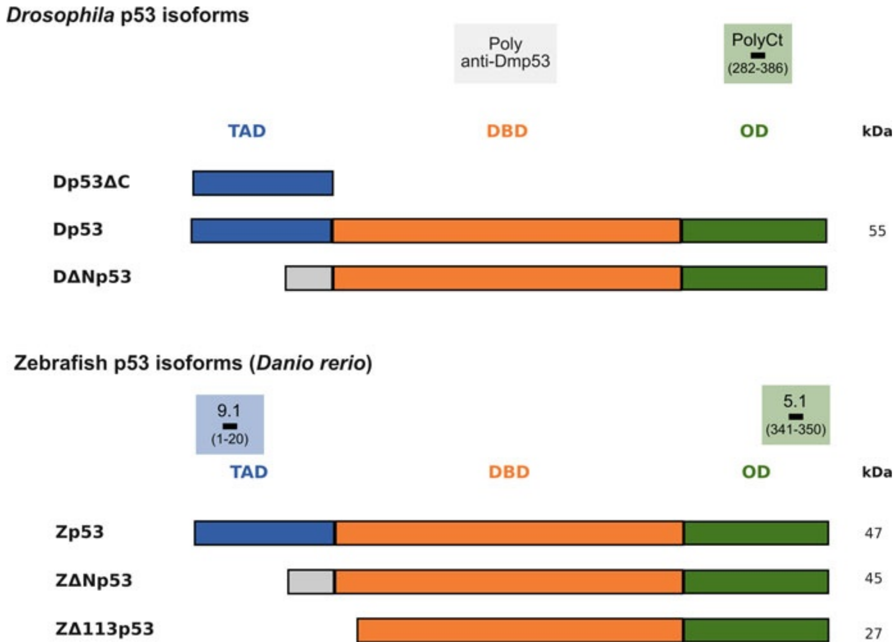

Fig. 4. p53 protein isoforms in animal models. The *TP53* gene expresses several protein isoforms conserved in different animal models. Up until now, three p53 protein isoforms have been identified in *Drosophila melanogaster* and in *Danio rerio* (zebrafish) that correspond mainly to N-truncated p53 protein isoforms. Specific antibodies have been developed to recognize *Drosophila* and zebrafish p53 isoforms. TAD: transactivation domain, *blue*; DBD: DNA-binding domain, *orange*; OD: oligomerization domain, *green*; *gray box*: residues not present in the other protein isoforms; D: *Drosophila* p53 protein; Z: zebrafish (*Danio rerio*) p53 protein; kDa: kilo Dalton, observed protein molecular weight; *colored boxes*: anti-p53 antibodies; Poly: polyclonal antibody; *numbers within brackets*: epitopes.

5. Transfer components:

   Mini Trans-Blot® Cell (Biorad).

   1× Transfer buffer prepared from 10× Wet blot solution (144 g glycine—30 g Tris-Base—in a final volume of 1 L milli-Q water) supplemented with 20% methanol.

   BA83 Protran® Nitrocellulose Membrane (Whatman).

6. Immunoblotting components:

   Wash solution-1: 1× PBS prepared using 10 Dulbecco "A" tablets in a final volume of 1 L milli-Q water (Oxoid).

   Wash solution-2: 1× PBS—0.1% Tween 20.

   Blocking solution: 5% nonfat milk—1× PBS—0.1% Tween 20.

   Primary antibodies are diluted in 5% milk—1× PBS—0.1% Tween 20, stored at −20°C and used up to three times (Table 1, Fig. 1).

   Secondary Horse Radish Peroxidase (HRP)-conjugated antibodies (IgG) are purchased from Jackson Immuno

Research Laboratories, Inc., used at 1:10,000 dilution (in 5% milk—1× PBS—0.1% Tween 20). Dilutions are stored at −20°C and used only 1 time to improve detection.

7. Detection reagents:

   Amersham ECL™ Western Blotting Detection Reagents (GE Healthcare).

   SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific).

   Amersham Hyperfilm ECL (GE Healthcare).

2.2. Immuno-precipitation

1. Materials described in Subheading 2.1.
2. RQ1 RNase-free DNase (Promega); RNase A (Qiagen).
3. *Preparation of protein G coupled to beads*: Dynabeads® Protein G (Invitrogen) according to the manufacturer's protocol ("Washing/Preparation Dynabeads").

2.3. Luciferase Reporter Gene Assays

1. Transfection reagents:

   FuGENE® 6 Transfection Reagent (Roche).

   OptiMEM (Gibco™, Invitrogen).

2. Luciferase assays:

   White 96-well microplates (Greiner Bio-One).

   Dual Luciferase® Reporter Assay System (Promega) (see Note 19).

3. Methods

3.1. Western Blotting

1. Wash cells twice with cold 1× PBS, add about 100 μl of NP40 extraction buffer directly into dishes, keep on ice, and scrape off the cells with a cell scraper (see Notes 1 and 2).
2. Collect cell lysate into a tube, keep on ice (see Note 3).
3. Pass cell lysate at least five times through a syringe with a 23 G needle to fragment cell membranes (see Note 3).
4. Spin cell lysate for 10 min, 13,800 × *g*, at 4°C.
5. Conserve the supernatant in a fresh tube (see Note 3).
6. Determine protein concentration (see Note 4).
7. Prepare 10–30 μg of protein sample supplemented with 0.1 M DTT and 1× LDS buffer (Invitrogen) (see Note 5).
8. Heat protein samples at 95°C for 5 min.
9. Load between 10 and 30 μg of protein sample per well on pre-casted NuPAGE 10% Bis-Tris polyacrylamide gels (Invitrogen) and separate proteins using 1× MOPS Running Buffer

supplemented with 0.2% Antioxidant as described by the manufacturer (Invitrogen) at 80 V for 2–3 h (see Note 6).

10. Transfer proteins overnight (25 mA, 11 V, about 14 h) onto the nitrocellulose membrane using 1× Transfer buffer supplemented with 20% methanol (see Note 7).
11. Block membrane with 5% nonfat milk—1× PBS—0.1% Tween 20 for 20 min by gentle shaking (see Note 8).
12. Place the membrane on the bench protected with a piece of saran film and add directly the primary antibody onto the membrane (1 ml per 50 cm^2) (see Note 9).
13. Cover to avoid evaporation and incubate for 1 h (see Note 10).
14. Wash with 5% nonfat milk—1× PBS—0.1% Tween 20 for 10 min by gentle shaking.
15. Repeat steps 12 and 13 for the secondary antibody (see Table 1).
16. Wash the membrane twice with 1× PBS—0.1% Tween 20 for 7 min, followed by an additional 7 min wash with 1× PBS.
17. Incubate membrane with Amersham ECL Western blotting detection system (GE Healthcare) or with SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific), according to the manufacturer's recommendations, by directly adding the reagent onto the membrane placed on the bench (see Note 11).
18. Exposure time of Hyperfilm to membrane varies according to the p53 protein isoform of interest (see Note 12).
19. Development (for example, using the Konica Medical Film Processor).
20. Analysis of p53 protein isoform expression (see Note 13) (see Fig. 2a).

3.2. Immunoprecipitation

1. Seed cells at least 24 h prior to immunoprecipitation to obtain up to 80% confluent cells the day of harvest (e.g., 2×10^7 of MCF-7 cells in a 125 ml flask).
2. Wash cells twice with cold 1× PBS.
3. Add 3 ml of NP40 extraction buffer extemporaneously supplemented with 1× Complete Mini protease inhibitor and 1 mM DTT directly onto cells, keep on ice.
4. Scrape off cells from flask and leave flask to rock gently for 15 min at 4°C.
5. Transfer cell lysate to 5 ml tubes, keep in ice (see Note 3).
6. Syringe the cell lysate at least ten times through a 23 G needle (see Note 3).
7. Transfer into several 1.5 ml tubes, keep in ice, and spin at $11{,}800 \times g$ for 10 min at 4°C.

8. Aliquot 500 μl of supernatant per tube and treat with 10 U of DNase RQ (Promega) and 35 U of RNase A (Qiagen) for 20 min at room temperature on a rotating wheel (see Note 3).
9. Pre-clear the lysate by adding 50 μl of prepared protein G coupled to beads for 15 min at 4°C on a rotating wheel.
10. Using the magnet, transfer the lysate into a new tube (see Note 3).
11. Determine protein concentration (see Note 4).
12. Use 5 mg of total protein lysate concentrated in 1 ml per immunoprecipitation (see Notes 14 and 15).
13. Add the required amount (suggested up to 10 μg) of primary antibody to the total protein lysate and incubate for 2 h at 4°C on a rotating wheel (see Note 16) (see Table 1).
14. Add 100 μl of prepared protein G coupled to beads for 2 h at 4°C on a rotating wheel.
15. Using the magnet, discard the supernatant.
16. Wash beads four times with 500 μl of NP40 extraction buffer extemporaneously supplemented with 1× Complete Mini protease inhibitor using the magnet (see Note 17).
17. Wash beads twice with 500 μl 1× PBS using the magnet.
18. Suspend the beads in 20 μl of 1× LDS Buffer (Invitrogen) and heat for 10 min at 70°C.
19. Using the magnet, transfer the supernatant in a fresh tube (see Note 5).
20. Perform Western blot as described in Subheading 2.2 starting from step 9 using the totality of protein samples.
21. Analysis of immunoprecipitation (see Note 18) (see Fig. 2b).

3.3. Luciferase Reporter Gene Assays

1. Seed 3×10^4 cells per well in 24-well plates 24 h prior to transfection in a final volume of 500 μl.
2. Prepare a mix of plasmids containing the amount of plasmids determined during the pre-experimental process of titration (see Fig. 3). For example, using the *p21*-luciferase reporter gene in p53-null H1299 cells, mix 2 ng/well of *Renilla* luciferase reporter plasmid, 200 ng/well of Firefly luciferase reporter plasmid, and 1 ng/well of expression vector (see Note 20).
3. Transfect plasmid mix using FuGENE® 6 Transfection Reagent (Roche) with OptiMEM® (Invitrogen) according to the manufacturer's protocol (ratio FuGENE:DNA = 3:1) using a final volume of 100 μL. Prior to transfection, replace media with 400 μL of fresh complete media.
4. Incubate cells under normal conditions for 24 h.
5. Wash cells twice with cold 1× PBS and measure luciferase activity as described by the manufacturer using 10 μL of the whole

cell lysate and 30 μL of both luciferase substrates (5 s time delay and 10 s measurement, for example, using the Luminometer (EG&G BERTHOLD, Microplate Luminometer LB 96 V)).

6. Perform analysis (see Note 21).

4. Notes

1. Cells can be directly lysed in 1× LDS buffer (Invitrogen) instead of using the NP40 extraction buffer (no need for items 4 and 5 of Subheading 2.2). However, detection of p53 protein isoforms can be less efficient using this alternative extraction method because of post-translational modifications on some epitopes.
2. Use one volume of NP40 extraction buffer corresponding to cell confluence (i.e., add 80 μl of buffer onto 80% confluent cells).
3. Experiment can be stopped at this stage and samples can be stored at −80°C.
4. Different protocols can be used. We recommend the Qubit® 2.0 Fluorometer (Invitrogen) using the Qubit Protein Assay (Invitrogen), which provides a sensible and accurate detection.
5. Experiment can be stopped at this stage and samples can be stored at −20°C.
6. Detection of p53 protein isoforms requires sufficient migration to dissociate proteins with closed molecular weights (see Fig. 1). We recommend to stop the running process when the migration front has just dissolved in the lower buffer chamber (keep the 15 kDa band of the protein molecular weight marker in the gel).
7. Alternatively, transfer can be performed for 1 h 30 min at 200 mA at 4°C.
8. The blocking process may vary from 20 min to 1 h without any alteration of the detection.
9. Addition of antibodies directly onto the membrane, rather than using the classical "bath" process, limits antibody consumption and improves the immunoreaction. Usually, use 1 μg of antibody per ml diluted in 5% milk—1× PBS—0.1% Tween 20.
10. Alternatively, membranes can be incubated within a bath of 5 ml primary antibody overnight at 4°C under gentle shaking.
11. In most cell lines, Amersham ECL Western blotting detection system (GE Healthcare) is sufficient to detect N-terminal TA

and Δ40 forms (see Fig. 2a, CM1 antibody), while SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific) is required to detect Δ133 and Δ160 forms (see Fig. 2a, MAP4-9 antibody).

12. Time of exposure using Amersham Hyperfilm varies according to cell lines and to the p53 protein isoform of interest and has to be determined by the user. Exposure time may vary from 1 min to 1 h.

13. Regarding the analysis of p53 protein isoforms, the interpretation of Western blotting is based on (a) the molecular weight and (b) the detection pattern of distinct antibodies (see Fig. 2a, e.g., p53β at about 47 kDa and detected with CM1 (specific for all isoforms) and KJC8 (specific for β forms) but not with MAP4-9 (specific for Δ133 forms)). However, comparison of expression levels between p53 isoforms requires the use of an antibody, which recognizes an identical epitope, at a given time exposure using the same detection reagent (Fig. 2a, e.g., comparison of p53β and Δ133p53β expression levels using KJC8 (β forms) but not CM1 (all isoforms)). To assess the specificity of the antibody, it is necessary to first compare the detection of protein issued from cells transfected with vector expressing ectopic p53 and with an Empty-expression vector. Of note, internal initiations of translation have been described for TAp53 forms and Δ133 forms leading to Δ40 and Δ160 forms, respectively (7, 8). Thus, ectopic expression of TA and Δ133 forms results in the detection of a doublet (see Fig. 2a). The clear identification of a p53 protein isoform by Western blotting requires at least one of the following controls: (a) use of a panel of distinct antibodies; (b) loading of proteins extracted from cells transfected with p53 isoform expression vector; and (c) use of siRNA specifically targeting p53 isoforms.

14. To immunoprecipitate p53 isoforms, 1–5 mg of total protein lysate is required and is prepared in a final volume of 500–1,000 μl.

15. For each sample, use the same amount of protein extract to perform an immunoprecipitation using your antibody of interest and an immunoprecipitation using a control antibody (i.e., IgG mouse if the primary antibody of interest is a mouse antibody, IgG rabbit if using a primary rabbit antibody, etc). About 50 μg of protein extract is stored at −80°C and used as the input control (see Fig. 2b).

16. The amount of primary antibody varies according to the expression level of the p53 isoform of interest, the number of epitopes per isoform for one considered antibody, and the specificity of the antibody (see Table 1, Fig. 1).

17. Do not add DTT at this step.

18. If the right controls are performed, interpretation of immunoprecipitation is easier than the one of Western blotting since analysis is based only on the distinct molecular weight of p53 protein isoforms. Immunoprecipitation using two different anti-p53 antibodies is an ideal experiment to identify p53 isoforms. However, immunoprecipitation cannot be used to compare protein expression levels under different experimental conditions.
19. As compared to other reporter gene assays (i.e., Chloramphenicol Acetyl Transferase assays), the luciferase reporter gene assay is highly recommended to study promoter activity since the luciferase enzymes have a short half-life, as well as a rapid enzymatic activity, and current luminometer kits allow the detection of small amounts of luciferase enzyme (from 10^{-20} molecule).
20. The amount of plasmids (Basic luciferase, Firefly luciferase, *Renilla* luciferase, and expression vector) should be carefully predetermined prior to experimentation, because of the specificity of each cell line, each promoter, and each transcription factor. First, the amount of the promoterless Basic luciferase reporter plasmid, used to normalized the experiments (see Note 21), should not yield ten times more luciferase activity than the background light given by non-transfected cells. Second, the optimal amount of Firefly luciferase reporter plasmid driven by the promoter of interest should yield two to three times more luciferase activity than the promoterless Basic luciferase reporter plasmid. Third, the amount of *Renilla* luciferase reporter plasmid, which is driven by the constitutive promoter SV40, corresponds to 1/100 of the amount of Firefly luciferase reporter plasmid. We do not recommend the use of the thymidine kinase promoter as in internal control since it contains some p53 responsive elements. Finally, the amount of p53 expression vector should be carefully determined. To study the regulation of p53 transcriptional activity on a promoter of interest, the experiment should be performed at 50% of the maximum promoter activity allowing thus the detection of both decreased and increased p53 transcriptional activity. Usually, a promoter activity in response to increasing amount of p53 has two phases (see Fig. 3): a "linear" phase, where the promoter activity is proportional to the amount of p53; and a "plateau" phase, corresponding to the maximal activity of a given promoter in response to p53. A titration experiment allows the determination of the amount of p53 expression vector to transfect to obtain 50% of maximum promoter activity. Importantly, it is crucial to balance the amount of p53 expression vector with the corresponding Empty-expression vector in order to transfect a constant amount of

DNA expression vector (e.g., pcDNA3-p53 + pcDNA3-Empty = 30 ng) (see Fig. 3).

21. Results of luciferase assays are usually presented as an average of at least three independent experiments performed in triplicate. In a first step, each independent experiment should be carefully normalized. This "intra-"normalization using the *Renilla* luciferase activity avoids misinterpretation due to variation in pipetting, transfection efficiency, or cell death (Firefly/*Renilla*). In addition, each condition is expressed as a ratio compared to the promoterless Basic luciferase reporter plasmid ($[\text{Firefly}/\textit{Renilla}]_{\text{condition1}}/[\text{Firefly}/\textit{Renilla}]_{\text{Basic}}$). As for the "intra-"normalization, this "inter-"normalization avoids misinterpretation due to variation in cell passage, transfection efficiency, etc. In a second step, average can be calculated from the normalized values. Important controls to consider are (1) "Basic" condition (co-transfection of promoterless Basic luciferase reporter plasmid and Empty-expression vector) to allow the "inter-"normalization; (2) "Empty" condition (co-transfection of the Firefly luciferase reporter plasmid and Empty-expression vector) to allow determination of the intrinsic promoter activity and to be used as a negative control when an ectopic protein is co-expressed; (3) "Plateau" condition (co-transfection of the Firefly luciferase reporter plasmid with the amount of expression vector reaching the plateau of fold-activation, see Note 20) to be used as a positive control when an ectopic protein is co-expressed. Of note, when transfecting increasing amount of expression vector, the total amount of DNA corresponding to the expression vector should be kept constant by balancing with Empty-expression vector (e.g., pcDNA3-p53 + pcDNA3-Empty = 30 ng) (see Fig. 3).

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Chapter 3

Autophagy, Senescence, and Apoptosis

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Abstract

This chapter presents methods for interrogating the involvement of p53 in signaling to apoptosis, autophagy, and senescence. The well-known association of p53 with the stress response to chemotherapy and radiation is the basis for presenting these approaches. The development of quantitative and efficient in vitro assays has enabled researchers to overcome the limitations of previous methodologies. This chapter provides up-to-date procedures relating to the molecular networks in which the p53 protein has been shown to play a central role that allows damaged cells either to adapt to stress (autophagy and/or senescence) or to progress towards programmed cell death (apoptosis).

Key words: Apoptosis, Senescence, Autophagy, Flow cytometry, Western immunoblotting, Fluorescent microscopy, Acridine orange, β-Galactosidase, Annexin V, Propidium iodide

1. Introduction

1.1. p53 and Autophagy

The p53 tumor suppressor pathway serves to protect genomic stability and suppress tumor formation. Cellular stress can induce autophagy in a p53-dependent manner; however, p53 can play dual roles in the control of autophagy. Whereas nuclear p53 can induce autophagy through transcriptional effects, cytoplasmic p53 may act as a repressor of autophagy (1).

Autophagy reflects a cellular response to stress in which cytoplasmic constituents, including organelles, are sequestered into double-membraned autophagosomes, which subsequently fuse with lysosomes, allowing for degradation of the sequestered components to generate energy and metabolic precursors; accordingly, there is evidence that autophagy can serve either as a cytoprotective or cytotoxic mechanism, depending on the cells and the nature of the stress-promoting challenge (2–6).

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Standard methods utilized for detection of autophagy include conventional electron microscopy for assessing the appearance of double-membraned autophagosomes that encapsulate various cytoplasmic components as well as acridine orange staining of acidic vacuoles. In addition, the molecular basis of autophagosome formation and degradation provides useful marker proteins for monitoring autophagic activity (7).

1.2. p53 and Senescence

The p53 protein, also known as the guardian of the genome, is a transcription factor which plays a role in DNA damage, cellular senescence, cell cycle progression, and, when damage is irreparable, executing cell death (8–10). With regard to cellular senescence, p53-dependent senescence is triggered by a wide spectrum of stimuli. In normal cells, replicative senescence is associated with telomere shortening, which limits their proliferative potential. In addition, non-telomeric signals, such as DNA damage (stress-induced senescence—SIS), oncogenic signaling (oncogene-induced senescence—OIS), oxidative stress, HDAC inhibitors, and depletion of heat shock proteins have all been found to induce p53-dependent senescence (11–13). Since p53-induced senescence is a route taken by cells to escape malignancy, understanding how p53 exerts these activities is of utmost importance.

In this chapter, we describe methods for studying p53-mediated senescence. Until recently, the most well-known methods to analyze senescence have been beta-galactosidase staining and western immunoblotting for "key players" in the senescent state. Here, we describe the common methods along with a more updated flow cytometric assay for the measurement of p53-mediated senescent activity. With the use of a fluorescently labeled p53 construct (GFP, RFP), the exongenously labeled versus non-transfected p53 protein can be detected by flow cytometry. The extent of p53-induced senescence is determined by the detection of senescence-associated beta-galactosidase (SA-βgal) activity using flow cytometry.

1.3. p53 and Apoptosis

Apoptosis is a caspase-dependent programmed cell death pathway (14). It is a normal physiological process that occurs during cellular development and serves to maintain cellular homeostasis and protect against abnormal cell division (15). It can also be activated following extreme cellular stress conditions. Morphological features include cell shrinkage, membrane blebbing, and nuclear fragmentation (16).

Apoptosis is regulated by many genes, one of which is p53. P53-dependent apoptosis can occur in response to cellular stressors such as DNA damage or hypoxia (17). Upon activation, p53 activates genes which are involved in both extrinsic and intrinsic apoptotic pathways (18). Both pathways result in the cleavage of caspases and subsequent cell death.

There are numerous assays to measure apoptosis. These include visualizing nuclear morphology by 4′,6-diamidino-2-phenylindole (DAPI) staining, DNA fragmentation by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), cell cycle analysis for the percentage of cells in the sub-G1 population, western blot analysis for cleavage of caspase-3 or PARP, and fluorescence-activated cell sorting (FACS) analysis for the apoptosis marker phosphotidylserine using annexin V, which is frequently coupled with propidium iodide. In-depth protocols for apoptosis detection are described below.

2. Materials

2.1. p53 and Autophagy

2.1.1. Detection of Autophagic Cells by Transmission Electron Microscopy

1. Permanox Petri dishes.
2. Phosphate-buffered saline (PBS).
3. 0.1 M sodium cacodylate buffer, pH 7.4.
4. 2% Paraformaldehyde/2% glutaraldehyde in 0.1 M sodium cacodylate buffer.
5. 1% Osmium tetroxide in 0.1 M cacodylate buffer.
6. Graded ethanol series: 50%, 70%, 80%, 95%, 100%.
7. Propylene oxide (3×).
8. PolyBed 812 resin (Polysciences, Inc.).
9. 50/50 Mix of propylene oxide and PolyBed 812 resin.
10. Leica EM UC6i Ultramicrotome (Lecia Microsystems).
11. Hexagonal Grids, Copper.
12. 5% Uranyl acetate and Reynold's lead citrate.
13. JEOL JEN-1230 transmission electron microscopy (TEM) (JEOL USA, Inc.).
14. Gatan Ultrascan 4000 digital camera (Gatan Inc., Pleasanton, CA).

2.1.2. Detection of Autophagic Cells by Acridine Orange Staining

1. Cells and suitable medium, serum, and other relevant supplements (depending on the cell system of choice).
2. Trypsin solution (0.02% ethylenediamine tetraacetic acid [EDTA], 0.25% trypsin) for the dissociation of cells from the culture substrate.
3. 0.1 μg/mL acridine orange (Invitrogen, cat. no. A-3568).
4. Fluorescence microscope (e.g., Olympus) or similar microscope.

2.1.3. Quantification of Acridine Orange Stained Cell via FACS Analysis

1. Cells and suitable medium, serum, and other relevant supplements (depending on the cell system of choice).
2. Trypsin solution (0.02% EDTA, 0.25% trypsin) for the dissociation of cells from the culture substrate.
3. Phosphate buffer solution.
4. Tissue culture grade dishes.
5. Acridine orange staining solution (Invitrogen, cat. no. A3568).
6. 5 mL Polystyrene round-bottom tubes with cell-strainer cap for the use of the flow cytometer (BD Falcon, cat. no. 352235). *Note*: For cell types that do not "clump," purchase tubes without the cell strainer (BD Falcon, cat. no. 352058).
7. Flow cytometer (e.g., FACSCalibur (Becton Dickinson) or FC500 MPL (Beckman Coulter)) or a similar flow cytometer. Appropriate software for analysis (e.g., Cellquest (Becton Dickinson)).

2.1.4. Autophagic Initiation and Flux by Western Immunoblotting Analysis

1. Lysing reagent, e.g., RIPA Buffer; M-PER mammalian protein extraction reagent (Thermo Scientific #78501).
2. Protease Inhibitor Cocktail (Roche, 11836145001).
3. Phosphatase Inhibitor Cocktail (Sigma, cat. no. 040M4005).
4. Bovine serum albumin (BSA): Albumin Standard (2 mg/mL, PIERCE, cat. no. 23210).
5. Lowry reagents: Solution A: 2% sodium carbonate (FW: 106, Sigma, cat. no. S-6139) in 0.1 M NaOH; Solution B: 1% cupric sulfate (FW: 249.7, $CuSO_4 \cdot 5H_2$); Solution C: 2% potassium sodium tartrate (Na-K Tartrate, FW: 282.2); Solution D: (Solution A + B + C:A:B:C = 100:1:1); Solution E: Folin phenol (folin and ciocalteu's phenol reagent, Sigma, cat. no. F-9252) diluted 1:1 with dH_2O.
6. Standard loading buffer (5×): (60 mM Tris-base (pH 6.8), 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue).
7. Benchmark pre-stained molecular weight standard (Invitrogen).
8. Long Shelf Life Precast Gels for use with Tris/Glycine Buffers (Mini-PROTEAN TGX, 4–15%, 10-well comb, 30 μL, BIO-RAD).
9. BIO-RAD Western Apparatus.
10. Western Blot Running Buffer: 25 mM Tris-base, 190 mM glycine, 0.1% SDS.
11. Nitrocellulose membranes.
12. Western blot transfer buffer: 0.025 M Tris, 0.192 M glycine, and 20% methanol.

13. Tris buffered saline (TBS; 10×): 1.5 M NaCl, 0.1 M Tris–HCl, pH 7.4.
14. TBS containing 0.05% Tween-20 (TPBST).
15. Blocking solution: 5% milk in TPBST. Store at 4°C.
16. Plastic container.
17. Immunoblotting components: Carry out all procedures at room temperature unless otherwise.
18. Primary antibodies used: anti-p62 (SQSTM1) (Santa Cruz, cat. no. sc-28359) anti-LC3 (Novus Biologicals, cat. no. NB100-2220).

2.2. p53 and Senescence

2.2.1. Measurement of Senescence-Associated Beta-Galactosidase Activity by Beta-Galactosidase Staining

1. Cells and suitable medium, serum, and other relevant supplements (depending on the cell system of choice).
2. Tissue culture grade dishes (92 mm).
3. Trypsin solution (0.02% EDTA, 0.25% trypsin) for the dissociation of cells from the culture substrate.
4. Fixing buffer components:
   (a) Formaldehyde.
   (b) Glutaraldehyde.
5. Phosphate-buffered saline (PBS).
6. Staining buffer components:
   (a) Citric acid/Sodium phosphate buffer, pH 6.0.
   (b) Potassium ferrocyanide.
   (c) Potassium ferricyanide.
   (d) Sodium Chloride.
   (e) Magnesium Chloride.
   (f) X-gal.
   (g) Dimethylformamide.

2.2.2. Measurement of Senescence-Associated Beta-Galactosidase Activity by FACS Analysis

1. Cells and suitable medium, serum, and other relevant supplements (depending on the cell system of choice).
2. Tissue culture grade dishes (92 mm).
3. Trypsin solution (0.02% EDTA, 0.25% trypsin) for the dissociation of cells from the culture substrate.
4. Transfection reagent such as Effectene (Qiagen, cat. no. 301425) or Lipofectamine (Life Technologies, cat. no. 18292-011).
5. 15 mL high-clarity polypropylene conical centrifuge tubes for centrifugation.
6. 5 mL polystyrene round-bottom tubes with cell-strainer cap for the use of the flow cytometer (BD Falcon, cat. no. 352235). *Note*: For cell types that do not "clump," purchase tubes without the cell strainer (BD Falcon, cat. no. 352058).

7. Phosphate-buffered saline (PBS).
8. Bafilomycin A1 (Sigma, cat. no. B1793) *Caution*! Irritant. It is recommended that one wears suitable protective clothing (e.g., gloves, lab coat, goggles) when handling the solution. Use a fume hood.
9. C_{12}FDG (Molecular Probes, Invitrogen, cat. no. D-2893). This is a β-galactosidase substrate which has been covalently modified to include a 12-carbon lipophilic moiety. Once the substrate is in the cells, it is cleaved by β-galactosidase producing a fluorescent product that is well-retained by the cells.
10. Flow cytometer (e.g., FACSCalibur (Becton Dickinson) or FC500 MPL (Beckman Coulter)) or a similar flow cytometer. Appropriate software for analysis (e.g., Cellquest (Becton Dickinson)).

2.2.3. Measurement of SA-β-Gal Activity by Fluorescent Microscopy

1. Cells and suitable medium, serum, and other relevant supplements (depending on the cell system of choice).
2. Tissue culture grade dishes (92 mm).
3. Trypsin solution (0.02% EDTA, 0.25% trypsin) for the dissociation of cells from the culture substrate.
4. Chamber slides.
5. Phosphate-buffered saline (PBS).
6. Bafilomycin A1 (Sigma, cat. no. B1793) *Caution*! Irritant. It is recommended you wear suitable protective clothing (e.g., gloves, lab coat, goggles) when handling the solution. Use a fume hood.
7. C_{12}FDG (Molecular Probes, Invitrogen, cat. no. D-2893). This is a β-galactosidase substrate which has been covalently modified to include a 12-carbon lipophilic moiety. Once the substrate is in the cells, it is cleaved by β-galactosidase producing a fluorescent product that is well-retained by the cells.
8. Triton X-100 (Invitrogen, cat. no. HFH-10) *Caution*! Harmful. It is recommended you wear suitable protective clothing (e.g., gloves, lab coat, goggles) when handling the solution.
9. BSA (Fisher Scientific, cat. no. BP1600100).
10. ProLong Gold anti-fade mounting medium (Invitrogen, cat. no. P36934).
11. Coverslips (Corning, cat. no. 2740-225).

2.3. p53 and Apoptosis

2.3.1. Terminal Deoxynucleotidyl TUNEL/DAPI Assay for Apoptosis

1. In situ Cell Death Detection kit (Roche, cat. no. 11373242910, 033335660001).
2. Phosphate buffered saline.
3. DAPI.
4. 1 mg/mL BSA in PBS.
5. Cold ethanol.
6. Cold acetic acid.

7. Vectashield.
8. 4% Formaldehyde in PBS.
9. Glass slides.
10. Coverslips.
11. Immunopen (Calbiochem, cat. no. 402176).
12. Humidified chamber.
13. Aluminum foil.

2.3.2. Cell Cycle Analysis by Propidium Iodide Staining

1. 70% Cold ethanol.
2. Phosphate buffered saline.
3. 1 mg/mL Propidium Iodide. *Caution*! Light sensitive. Vial should be protected from light and stored at 4°C (Sigma, cat. no. 81845).
4. DNase-free RNase A.
5. 0.1% Triton X-100 in PBS (v/v).
6. Flow tubes (BD Falcon, cat. no. 352235).

2.3.3. FACS Analysis for Annexin V and Propidium Iodide Positive Cells to Monitor Apoptosis and Necrosis

1. Annexin V-FITC (BD Pharmingen, cat. no. 556420).
2. Propidium Iodide at a 10 μg/mL stock solution (BD Pharmingen, cat. no. 556463).
3. Phosphate buffered saline.
4. Flow tubes.

3. Methods

3.1. p53 and Autophagy

3.1.1. Detection of Autophagic Cells by TEM

1. Plate approximately 1×10^5 cells on Permanox Petri dishes.
2. Perform desired treatments to promote autophagy.
3. At selected times, wash cells with 1× PBS and then fix cells with 2% paraformaldehyde/2% glutaraldehyde in 0.1 M sodium cacodylate buffer. *Caution!* Irritant. It is recommended you wear suitable protective clothing (e.g., gloves, lab coat, goggles) when handling the solution. Use a fume hood.
4. After 1 h at room temperature, remove fixative and replace with 0.1 M sodium cacodylate buffer, pH 7.4.
5. Fix plates in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h, after which they are rinsed in 0.1 M cacodylate buffer for 5–10 min.
6. Plates are then dehydrated in graded ethanol series: 50%, 70%, 80%, and 95% for 5–10 min each.
7. Plates are dehydrated in 100% ethanol 3×, 10–15 min each, followed by propylene oxide 3×, 10–15 min each.

8. Infiltrate cells with 50/50 mix of propylene oxide and PolyBed 812 resin mix; leave overnight.
9. Cells are infiltrated with pure PolyBed 812 resin (Polysciences, Inc.) mix; leave overnight.
10. Place cells in embedding molds containing PolyBed 812 and allow to polymerize overnight in a 60°C oven.
11. Sectioning is performed using a Leica EM UC6i Ultramicrotome (Leica Microsystems) or similar system. For electron microscopy, 70–90 nm thick sections are collected on copper mesh grids and stained with 5% uranyl acetate and Reynold's lead citrate. Images are captured using a JEOL JEM-1230 TEM (JEOL USA, Inc.) with Gatan Ultrascan 4000 digital camera (Gatan Inc, Pleasanton, CA).

3.1.2. Detection of Autophagic Cells by Acridine Orange Staining

As a marker of autophagy, the volume of the cellular acidic compartment can be visualized by acridine orange staining (19) as demonstrated in Fig. 1.

1. Cells are harvested from culture and 1×10^5 cells are plated per well in six-well tissue culture dishes and treated with agents of interest.
2. At various time points following treatment, cells are incubated with medium containing 0.1 μg/mL acridine orange for 15 min at 37°C; the acridine orange is removed, cells are washed once with PBS, fresh medium is added, and fluorescent micrographs are taken using an Olympus inverted fluorescence microscope. The number of cells with increased acidic vesicular

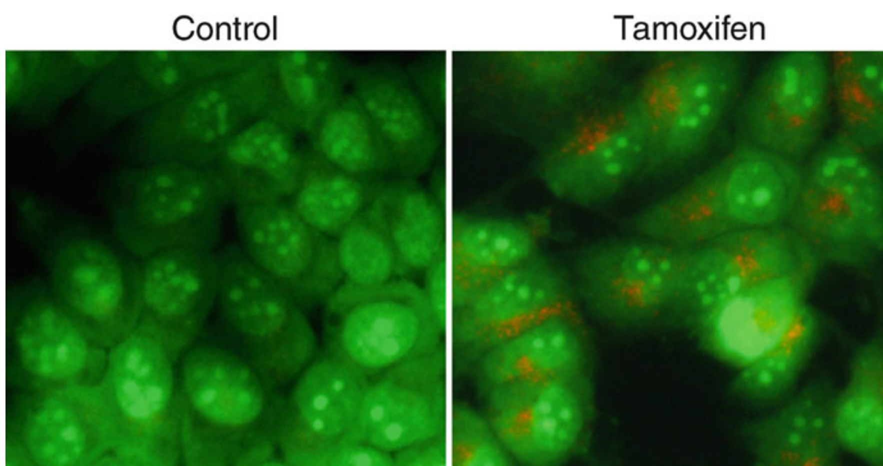

Fig. 1. Tamoxifen induces acidic vesicular organelles in MCF7 cells. MCF7 cells were exposed to Tamoxifen (2.5 μM) for 4 days and stained with acridine orange. Examples of acridine orange staining associated with autophagy can be found in Goehe et al (2012) The autophagy-senescence connection in chemotherapy; must tumor cells (self) eat before they sleep? J Pharm Exp Ther.

organelles is determined by counting all vesicles per cell in at least three representative fields per treatment condition. *Caution!* Light Sensitive. Be sure to perform staining in the dark and protect plates from light.

3.1.3. Quantification of Acridine Orange Stained Cell via FACS Analysis

In addition to visualizing cells with acridine orange by a fluorescence microscope, the measurement of the acidic component can also be quantitatively measured by FACS analysis (19, 20). This protocol identifies the intensity of the red (FL-1) fluorescence in comparison to the intensity of the green (FL-3) fluorescence. Cells undergoing autophagy will fluoresce with an intense red or orange color; alternatively, cells not undergoing autophagy will demonstrate low red and green intensity levels. Green fluorescence indicates unacidified compartments.

1. Cells are plated at a semi-confluent density (~50%), 24 h before transfection and incubated at 37°C, 5% CO_2.
2. Treatments of choice plus a positive (e.g., serum starvation) and negative (e.g., Bafilomycin) control.
3. Once treatments are completed, remove the solution and wash the cell monolayers twice with PBS at RT.
4. Harvest the cells by trypsinization.
5. Pellet the cells by centrifuging at 1,000 rpm in a tabletop centrifuge for 5 min at 4°C.
6. Discard the supernatant and resuspend cells in either medium or PBS at a concentration of $\sim 1 \times 10^6$ cells/mL. It should be noted that a minimum of 0.4 mL cell suspension is required for analysis.
7. Add acridine orange to the cell suspensions at a final dilution of 1:10,000.
8. Immediately run the cell suspension in a BD Canto or similar flow cytometer. Acquire and analyze the data using FCS Express or similar software.
9. Data processing to analysis acridine orange staining: on a two-parameter display of FSC versus SSC set up an analysis region that excludes dead cells and subcellular debris. Depict the events within this region in a histogram where the *y*-axis indicates green fluorescence intensity (FL-1) and the *x*-axis indicates red fluorescence intensity (FL-3). On this histogram, estimate the intensity of the red fluorescence (compared with positive, negative, and control vehicle cells) using the mean fluorescence intensity (MFI) of the population.

3.1.4. Autophagic Initiation and Flux by Western Immunoblotting Analysis

LC3 is a novel marker for autophagy. Newly synthesized LC3 is immediately processed by mammalian Atg4 and is present in the cytosol as LC3-I. When autophagy is induced, LC3-I is converted into LC3-II, which migrates faster on SDS-PAGE (7). Alternatively,

it is now recognized that although autophagosome formation is a necessary component of the autophagic process, autophagosome formation can occur without completion of autophagy and degradation of the autophagosomal content; consequently, it becomes necessary to also assess autophagic flux (21). To evaluate autophagic flux, levels of the p62 protein are monitored by Western blotting. As a positive control, a decline in p62 levels is typically evident when the cells are serum starved.

1. Protein Isolation
   - (a) Cells are typically plated at a density of 50,000–100,000 cells per 10 cm tissue culture dish.
   - (b) After the indicated treatments and at appropriate time points, media is aspirated, 1.5 mL cold 1× PBS added per flask, cells are scraped from the surface of the flask, and transferred to microfuge tube.
   - (c) Spin 1 min at 13,000 rpm to pellet cells.
   - (d) Aspirate PBS and lyse using 100–500 μL lysing reagent (e.g., RIPA Buffer containing protease and phosphatase inhibitors).
   - (e) Vortex to dislodge pellet and boil at 95–100°C for 5 min.
   - (f) Place cells on ice. Sonicate on ice for 18 s to shear DNA then centrifuge for 10 min at 10,000 rpm at 4°C. Transfer supernatant to new microfuge tube and store at −80°C until day of western.
2. Lowry Assay for Protein Concentration
   - (a) Protein concentrations are determined by the Lowry method (22). Dilutions of isolated proteins are compared to a standard curve using various doses of BSA. Absorbance at 750 μm is then determined using an Ultraspec 300 UV/Visible Spectrophotometer.
3. Electrophoresis
   - (a) Concentrations determined by the Lowry assay allow for equal aliquots of protein (35–50 μg) added to 5× sample buffer (60 mM Tris-base (pH 6.8), 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue) and diluted to equal volumes (30 μl) water.
   - (b) The samples are then loaded onto a polyacrylamide gel and separated using SDS-PAGE running buffer for 0.5–2 h with 60 milliamps constant current.
   - (c) Proteins are then transferred onto a nitrocellulose membrane electrophoretically for 1 h at 100 V in transfer buffer at 4°C.

4. Immunoblotting
   (a) The membrane is blocked in TBS-Tween 20 buffer containing 5% nonfat dry milk for 1 h.
   (b) After removal of the blocking buffer, membranes are immunoblotted with the primary antibody of choice overnight at 4°C with an orbital shaker.
   (c) Primary antibody is then removed and the blot is washed five times, 5 min each in blotto wash.
   (d) The membranes are then incubated with the respective horseradish peroxidase-conjugated secondary antibodies for 30 min.
   (e) Proteins are visualized using an enhanced chemiluminescence kit from Pierce (Thermo Scientific #34080) and the light emission from the oxidation reaction exposed to audioradiography film.

3.2. p53 and Senescence

3.2.1. Measurement of Senescence-Associated Beta-Galactosidase Activity by Beta-Galactosidase Staining

The following assay is most commonly used in determining whether the senescent phenotype is occurring. This protocol is a cytochemical assay using the chromogenic substrate 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-gal), which yields an insoluble blue compound when cleaved by β-galactosidase as demonstrated in Fig. 2.

1. Seed the cells at 50% confluency into each chamber 24 h before staining. Culture cells for 24 h at 37°C, 5% CO_2.
2. Wash the cell monolayers twice with PBS.
3. Make a solution containing 2% formaldehyde + 0.2% glutaraldehdye. Add 1–3 mL of this fixing buffer to your cells at RT for 5 min. *Caution*! Irritant. It is recommended you wear suitable

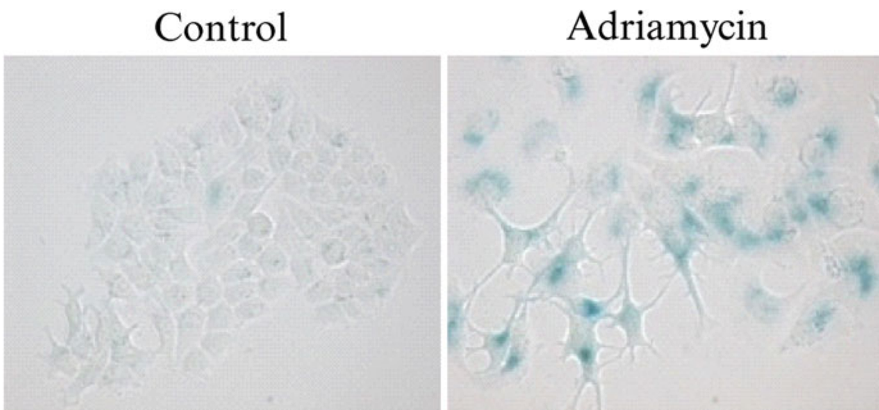

Fig. 2. Adriamycin induces senescence in MCF-7 cells. Cells were exposed to 1 μM of Adriamycin for 2 h followed by removal of drug and replacement with fresh medium. Cells were staining for β-Gal and imaged.

protective clothing (e.g. gloves, lab coat, goggles) when handling the solution. Use a fume hood.

4. Wash the cell monolayers twice with PBS.
5. Add 1–3 mL of staining buffer to the cells 37°C for 2–24 h. If the cells are undergoing senescence a blue color will be detectable. The time frame for development of senescence is different for each cell system. Staining buffer:
   (a) 20 mg/mL X-gal in dimethylformamide
   (b) 0.2 M citric acid/sodium phosphate buffer at pH 6.0
   (c) 100 mM potassium ferrocyanide
   (d) 100 mM potassium ferricyanide
   (e) 5 M sodium chloride
   (f) 1 M magnesium chloride
   (g) H_2O
6. After incubation, wash staining solution off cell monolayers and replace with PBS. Store at 4°C.
7. Visualize cells underneath an electron microscope.

3.2.2. Measurement of Senescence-Associated Beta-Galactosidase Activity by FACS Analysis

Replicative senescence is a phenomenon which occurs naturally in multicellular organisms when diploid cells are no longer able to divide due to the shortening of telomeres. DNA damage and oncogenic signaling can induce SIS or OIS, respectively. The senescent phenotype is characterized by morphological and biochemical features that include an enlarged cell size, flattened morphology, the inability to synthesize DNA, and expression of senescence-associated β-galactosidase (SA-β-gal) (23, 24). SA-β-gal activity has been reported to reflect an increase in lysosomal enzyme (25). Since acidic SA-β-gal activity is detected at pH 4.0, we take advantage of the inhibitors Chloroquine and Bafilomycin A1 to induce lysosomal alkalization. Chloroquine is a weak base that concentrates in lysosomes, raising the pH to approximately 6 (26–28). Bafilomycin A1 is a specific inhibitor of vacuolar-type H+-ATPase and inhibits lysosomal acidification (29). Cells are incubated with C_{12}FDG, a substrate of β-gal that fluoresces upon hydrolysis.

p53-transfected cells can be detected by anti-p53 antibodies or by a tag, such as green fluorescent protein (GFP). In the following section, we describe the senescence-associated beta-galactosidase activity (SA-βgal) by flow cytometry following transient transfection with a GFP-p53 plasmid. The use of naturally fluorescent proteins, such as GFP, avoids the potential problem of background staining and saves time and reduces the costs of reagents.

1. Plate cells at a semi-confluent density (~50%), 24 h before transfection and incubate at 37°C, 5% CO_2.
2. Transfect cells with p53 expression plasmid (between 0.5 μg and 1 μg/sample). Transfection reagents such as Lipofectamine

or Effectene, which tend to not cause much cell death and yet achieve high efficiency, are suitable. The use of electroporation is not suitable because of the high levels of cell death resulting from the transfection process.

3. *Optional*: At 24–72 h post-transfection, induce lysosomal alkalinization by pretreating subconfluent cells with 100 nM Bafilomycin A1 for 1 h in fresh cell culture medium at 37°C, 5% CO_2. *Caution*! Irritant. It is recommended you wear suitable protective clothing (e.g., gloves, lab coat, goggles) when handling the solution. Use a fume hood.
4. After 1 h, add C_{12}FDG to the cell culture medium to a final concentration of 30 μM and continue to incubate for 1–2 h at 37°C, 5% CO_2.
5. Remove the solution and wash the cell monolayers twice with PBS at RT.
6. Harvest the cells by trypsinization.
7. Pellet the cells by centrifuging at 1,000 rpm in a tabletop centrifuge for 5 min at 4°C.
8. Discard the supernatant and resuspend cells in ice-cold PBS at a concentration of $\sim 1 \times 10^6$ cells/mL. It should be noted that a minimum of 0.4 mL cell suspension is required for analysis.
9. Immediately run the cell suspension in a BD Canto or similar flow cytometer. Acquire and analyze the data using FCS Express or similar software.
10. Data processing to estimate relative βgal activity: on a two-parameter display of FSC versus SSC set up an analysis region that excludes dead cells and subcellular debris. Depict the events within this region in a green fluorescence histogram where the *y*-axis indicates cell number and the *x*-axis indicates C12-fluorescein fluorescence intensity in log scale. On this histogram, estimate the relative β-galactosidase activity (compared with positive or negative control cells) using the MFI of the population.

3.2.3. Measurement of SA-β-Gal Activity by Fluorescent Microscopy

In addition to using C_{12}FDG for β-gal via FACS, it is also a suitable fluorescent substrate for fluorescence microscopy.

1. Depending on the chamber slides used, seed the cells at 50% confluency into each changer 24 h before staining. Culture cells for 24 h at 37°C, 5% CO_2.
2. To induce lysosomal alkalinization, remove the culture medium and pretreat cells with 100 nM bafilomycin A1 for 1 h in fresh cell culture medium at 37°C, 5% CO_2.
3. After 1 h incubation with bafilomycin A1, add C_{12}FDG to the cell culture medium to give a final concentration of 30 μM and continue to incubate for 1–2 h at 37°C, 5% CO_2.

4. After 1–2 h, remove the solution and wash the cell monolayers twice with PBS at RT.
5. Permeabilize the cells in 1% (v/v) Triton X-100 for 5 min at RT. *Caution*! Harmful. It is recommended you wear suitable protective clothing (e.g., gloves, lab coat, goggles) when handling the solution.
6. Remove the Triton X-100 solution and wash the cells twice with 2–4% (w/v) BSA at RT.
7. Mount the cells onto the slides by dispensing a drop (~25 μL) of ProLong Gold anti-fade reagent containing DAPI mounting media onto the slides. Add coverslips and allow the mounting medium to disperse over the entire slide.
8. Observe with a confocal microscope, such as Leica.

3.3. p53 and Apoptosis

3.3.1. Terminal Deoxynucleotidyl TUNEL/ DAPI Assay for Apoptosis

The TUNEL assay uses a terminal transferase (TdT) to label free 3′OH ends in genomic DNA with fluorescein-dUTP. Upon labeling, cells with free 3′OH ends will fluorescence green and can be visualized using a fluorescence microscope as demonstrated in Fig. 3a.

1. Treated cells are harvested (both adherent and in suspension). Approximately 20,000 cells should be collected for each treatment.
2. Transfer harvested cells to glass slides by cytospin.
3. Cells are then washed in PSB for 5 min.
4. Fix cells with 4% formaldehyde in PBS for 10 min at RT.
5. Wash slides two times for 5 min each with PBS.
6. Fix cells with a 1:2 dilution of cold acetic acid in ethanol for 5 min at RT.
7. Wash slides two times for 5 min with PBS.
8. Using an immunopen, draw a tight circle around the cells to contain solutions.
9. Block with BSA for 30 min at RT (50–100 μL BSA per slide). Blocking should be performed in a humidified chamber covered with aluminum foil.
10. Wash slides two times for 5 min with PBS.
11. Stain slides with Roche enzyme mixture (see below) for 1 h at 37°C. *Caution*! Light Sensitive. Be sure to perform staining in the dark and protect slides from light.
    (a) *Note*: For ten slides, prepare a staining solution containing 40 μL of 5× reaction buffer, 2 μL of terminal transferase, 20 μL of 25 nM $CoCL_2$, 124 μL of H_2O, and 4 μL of Fluorescein-12-dUTP which should be added last. Per slide, 20 μL of the staining solution should be added.

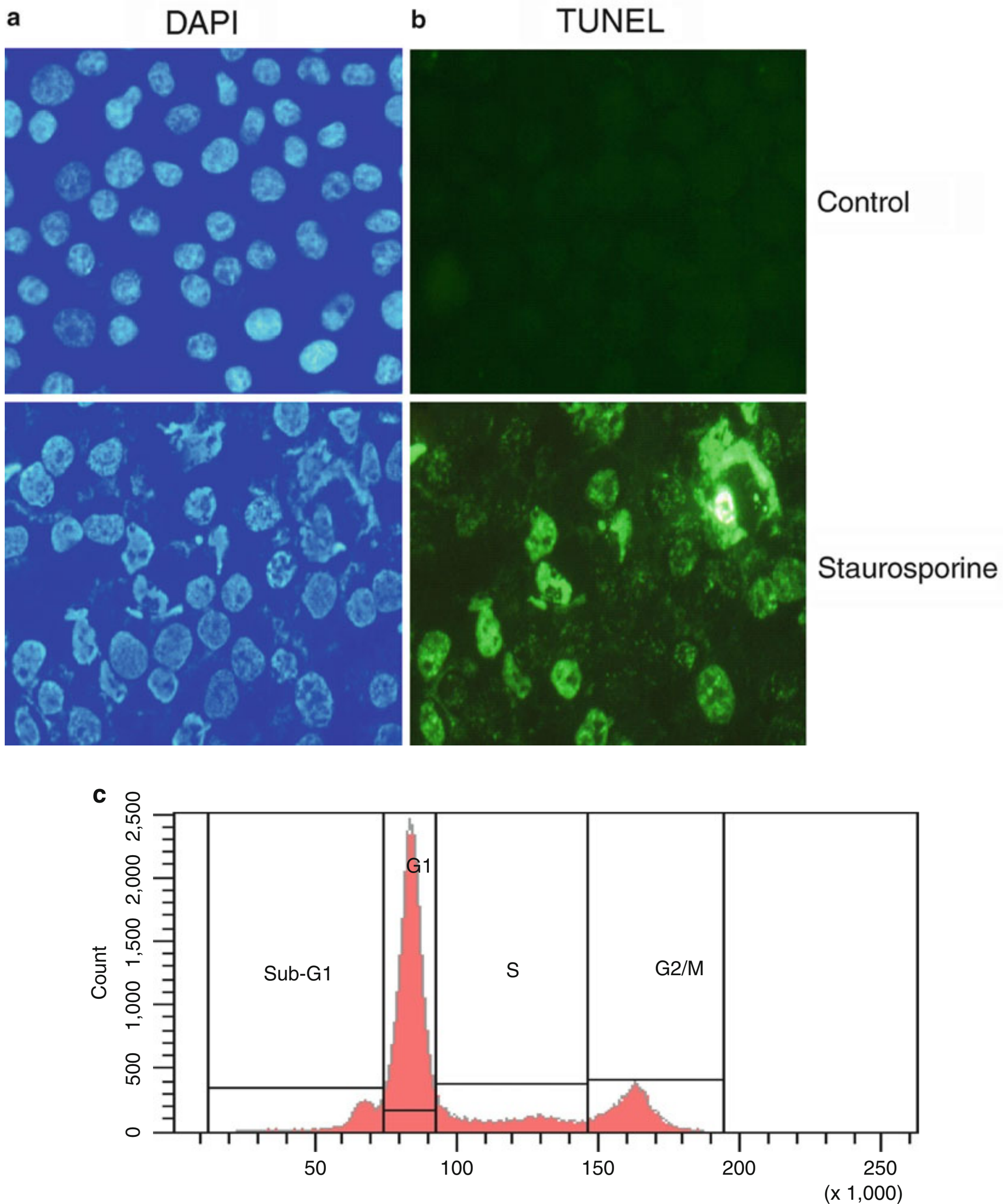

Fig. 3. (**a**) ZR75-1 cells were treated with 1 μM Staurosporine for 24 h before being visualized by a fluorescent microscope. Cells were counterstained with DAPI to visualize nuclear morphology. (**b**) Cell cycle analysis by flow cytometry. The *x-axis* indicates the intensity of cell propidium stained cells while the *y-axis* indicates the cell count. (**c**) Flow cytometric analysis of apoptotic subpopulations utilizing annexin V and PI in ZR-75 breast cancer cells. The *right panel* is a diagrammatic representation that identifies the quadrant subpopulations. *Left panel* demonstrates histograms of either control cells or cells treated with staurosporine with the indicated percentages of necrotic or apoptotic subpopulations. Control cells (*dots*) represent the majority of the population in quadrant 3 (Q3) or viable cells. In comparison, ZR-75-1 cells pretreated with staurosporine (a well-known positive control for apoptosis) demonstrate a high percentage of cells in Q2 and Q4, representing early and late apoptotic cells.

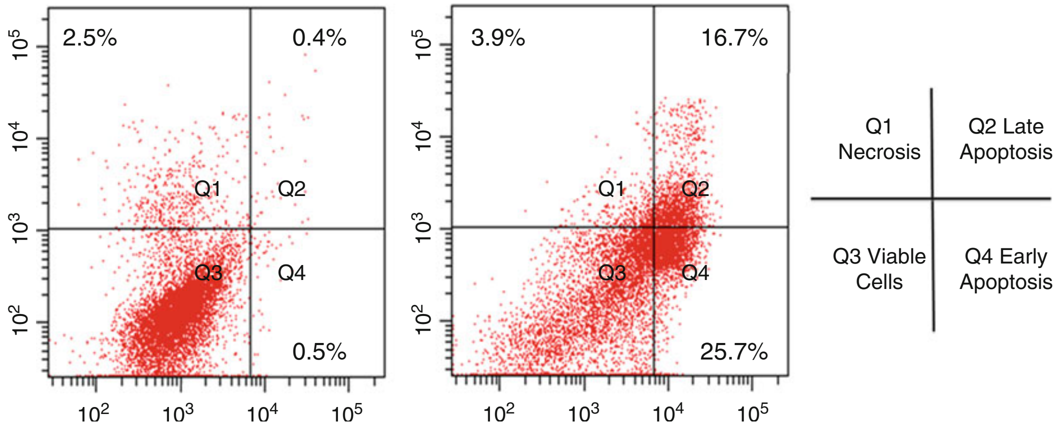

Fig. 3. (continued)

12. Wash slides two times for 5 min with PBS in the dark.
13. Prepare a 1:1,000 solution of DAPI in vectashield and add 10–20 μL per slide and mount with coverslips.
14. Immediately visualize slides using a fluorescent microscope.

3.3.2. Cell Cycle Analysis by Propidium Iodide Staining

Cells undergoing *apoptosis* will demonstrate DNA fragmentation in late *apoptosis* and those cells may be detected as having sub-G1 DNA content. A representation of cell cycle analysis is demonstrated in Fig. 3b.

1. Harvest treated cells (a total of 10^6–10^7 cells for each condition).
2. Pellet cells by centrifugation and resuspend in 0.2 mL of PBS.
3. To fix the cells, add 1.8 mL of cold ethanol dropwise while vortexing to obtain a single cell suspension.
4. Incubate cell suspension on ice for 30 min or store at 4°C until ready for staining.
5. Prepare staining solution. For 10 mL of staining solution, add 2 mg of DNAse-free RNase A and 0.2 mL of propidium iodide stock (1 mg/mL) to 0.1% (v/v) Triton X-100.
6. To proceed with staining, cells should be centrifuged to remove ethanol and resuspended in PBS.
7. Repeat centrifugation step and resuspend pellet in 1 mL of propidium iodide/RNase/Triton X-100 staining solution. Cells should be left in the staining solution 30 min to 24 h before analysis by flow cytometer.
8. Analyze samples. Propidium iodide is excited by 488 nm light and fluoresces with a broad spectrum from about 550 nm to 700 nm. A minimum of 20,000 events should be recorded.

3.3.3. FACS Analysis for Annexin V and Propidium Iodide Positive Cells to Monitor Apoptosis and Necrosis

Annexin V/PI staining can be used to monitor cells undergoing early and late apoptosis. Annexin V binds to cells that have expressed phosphatidylserine on the cell surface, a feature found in apoptosis (30). Upon binding, Annexin V fluoresces and can be measured by FACS analysis (Fig. 3c).

1. Treated cells are harvested (a total of 10^6–10^7 cells for each condition).
2. Pellet cells by centrifugation and resuspend in 0.2 mL of PBS. Repeat one more time to wash pellet of any trypsin or media.
3. Resuspend cell pellets with 100 μL of 1× binding buffer.
4. Add 5 μL of Annexin V-FITC to each sample.
5. Add 5 μL of the 10 μg/mL propidium iodide stock solution to each sample.
6. Vortex samples and incubate at room temperature in the dark for 15 min.
7. Immediately before analysis add 400 μL of 1× binding buffer to yield a total volume of 500 μL. The number of cells with increased Annexin V-FITC/PI staining can be determined by flow cytometry. To set compensation and voltages there should be a sample with no stain, Annexin V-FITC alone, and PI alone. A minimum of 10,000 events should be recorded.

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Chapter 4

p53 and Cell Cycle Effects After DNA Damage

Emir Senturk and James J. Manfredi

Abstract

Flow cytometry, a valuable technique that employs the principles of light scattering, light excitation, and emission of fluorochrome molecules, can be used to assess the cell cycle position of individual cells based on DNA content. After the permeabilization of cells, the DNA can be stained with a fluorescent dye. Cells which have a 2N amount of DNA can be distinguished from cells with a 4N amount of DNA, making flow cytometry a very useful tool for the analysis of cell cycle checkpoints following DNA damage. A critical feature of the cellular response to DNA damage is the ability to pause and repair the damage so that consequential mutations are not passed along to daughter generations of cells. If cells arrest prior to DNA replication, they will contain a 2N amount of DNA, whereas arrest after replication but before mitosis will result in a 4N amount of DNA. Using this technique, the role that p53 plays in cell cycle checkpoints following DNA damage can be evaluated based on changes in the profile of the G1, S, and G2/M phases of the cell cycle.

Key words: Flow cytometry, Fluorescence, Dual parameter, Propidium iodide, BrdU, Phosho-histone H3, p53, DNA damage, DNA damage checkpoints, G1 arrest, G2/M arrest, Apoptosis

1. Introduction

Genomic stability is a critical requirement for cell survival and the prevention of tumorigenesis. In order to ensure that mutations that result from DNA damage are not passed on to daughter generations, the cell must pause and repair the damage. The cellular response pathway is a network that involves sensors of damage that ultimately transmit signals to mediator proteins that regulate the transcription of effector proteins that play an important role in arresting the cell cycle. In the cell cycle, transitions (G1/S, intra S, G2/M) that lead from DNA replication to mitosis are monitored for successful completion. In the event of DNA damage, genotoxic stress, or ribonucleotide depletion, cell cycle checkpoints prevent

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progression to the next phase of the cell cycle until the damage is repaired, the stress is removed, or nutrients are replenished. Other pathways may be activated that result in programmed cell death if the damage is irreparable (1). When there are defects in the cell cycle checkpoints, gene mutations, chromosome damage, and aneuploidy can result and ultimately, cell transformation can be a consequence of such defects (2).

p53, a transcription factor (3, 4) and tumor suppressor protein (5), can regulate the expression of proteins that play critical roles in growth arrest and apoptosis (programmed cell death) (6). p53 plays a critical role both in the G1/S checkpoint, in which cells arrest prior to DNA replication and have a 2N content of DNA, and in the G2/M checkpoint, in which arrest occurs before mitosis and cells have a 4N content of DNA. The activation of p53 following DNA damage results in the expression of many proteins which are important in cell cycle arrest, repair, and apoptosis (7).

The cyclin-dependent kinase inhibitor, p21, accomplishes cell cycle arrest by inhibiting cyclin–cdk complexes that phosphorylate cell cycle proteins that mediate the passage from G1 to S (8–10). As a result of inhibition, the retinoblastoma protein (pRB) remains hypophosphorylated, E2F remains bound to pRB, and arrest occurs at the G1/S boundary. Proliferating cell nuclear antigen (PCNA), a protein that plays a role in both DNA replication and repair, is a component of the cyclin–cdk complex. p21 binds to and inhibits PCNA from mediating elongation during replication thereby preventing replication in cells that have already entered S phase (11).

Although the G1/S checkpoint is recognized as being entirely p53 dependent, the G2/M checkpoint can be accomplished as a result of multiple pathways. In the presence of DNA damage, p53-dependent and -independent pathways converge to inhibit the activities of cyclin B and Cdc2, proteins that play a role in promoting mitosis (12, 13). Activation of the ATM/CHK2/cdc25 or ATR/CHK1/cdc25 pathways (14) results in the inactivation of phosphatases in the cdc25C family by downregulation and cytoplasmic sequestration. Additionally, p53, itself, is phosphorylated by the kinases in these pathways and in turn becomes stabilized and active. p53 contributes to the maintenance of the G2/M checkpoint by transcriptional repression of both cdc25C and cyclin B (15), upregulation of p21 that can inhibit cyclin B–cdk1 complexes (16), 14-3-3 sigma proteins that target cdc25C proteins to the cytoplasm (17), and GADD45, a protein that can inhibit cyclin B–Cdc2 complexes (18).

Cells in which p53 is deleted or mutated lose the G1 checkpoint and no longer arrest at the G1/S transition. Although they maintain a G2 arrest, this arrest can decay over time thus allowing cells to enter mitosis with unrepaired DNA damage and mutations

that increase the risk of progression to malignancy. People who have Li–Fraumeni syndrome, a cancer prone condition in which one allele of the p53 gene is mutated, are susceptible to sarcomas, leukemias, brain and adrenal tumors. In these tumors the remaining allele of p53 is often deleted (loss of heterozygosity) highlighting the importance of the role of p53 in genomic stability (19).

Flow cytometry is a valuable technique used for the analysis of cell cycle checkpoints after DNA damage. Using the principles of light scattering, light excitation, and light emission, fluorescent compounds can be incorporated into the DNA of cells and emit a signal that is detectable and proportional to the number of fluorochrome molecules. A unique feature of flow cytometry is that fluorescence is measured on a per cell basis and not as a bulk volume as in spectrophotometry. In the following method, propidium iodide, a fluorescent compound, is used to intercalate into DNA of cells that have been treated with the DNA damaging agent, doxorubicin. This property can be used to evaluate the DNA content of cells because cells will have a 2N content of DNA prior to cell replication, followed by a 4N amount of DNA after replication.

Briefly, a sample containing stained cells in suspension is taken up by the flow cytometer and hydrodynamically focused through a small nozzle so that single cells pass before a laser, one cell at a time. An argon laser is commonly used in flow cytometry because the 488 nm light that it emits can excite more than one fluorochrome. Therefore multiple fluorochromes can be incorporated simultaneously, provided that their emission wavelengths are far enough apart for detection. In the first protocol, a single parameter assay is performed in which only propidium iodide is incorporated and detected (see Fig. 1). Propidium iodide and a fluorescein isothiocyanate (FITC) conjugated antibody with emissions of 575 nm and 520 nm, respectively, are used to create a dual parameter assay in the second and third protocol. In the second protocol, bromodeoxyuridine (BrdU), a synthetic analog of thymidine, is incorporated into replicating DNA and the antibody that detects BrdU is conjugated to the fluorescent compound (see Fig. 2). In the third protocol, an antibody to phosphorylated histone H3, a marker for mitotic cells, is used which is bound by a secondary antibody that is conjugated to the FITC molecule (see Fig. 3). The light that is emitted is sent to different detectors via band filters and converted to electrical pulses that ultimately are amplified and processed so that the data can be plotted on a graph as digital events. Histograms, which are dot plots of each event, can be constructed to illustrate both parameters. In the second protocol, actively replicating cells can be distinguished because of the BrdU uptake. Cells in G1 (2N content of DNA) and G2/M (4N content of DNA) can be easily discerned because all the cells

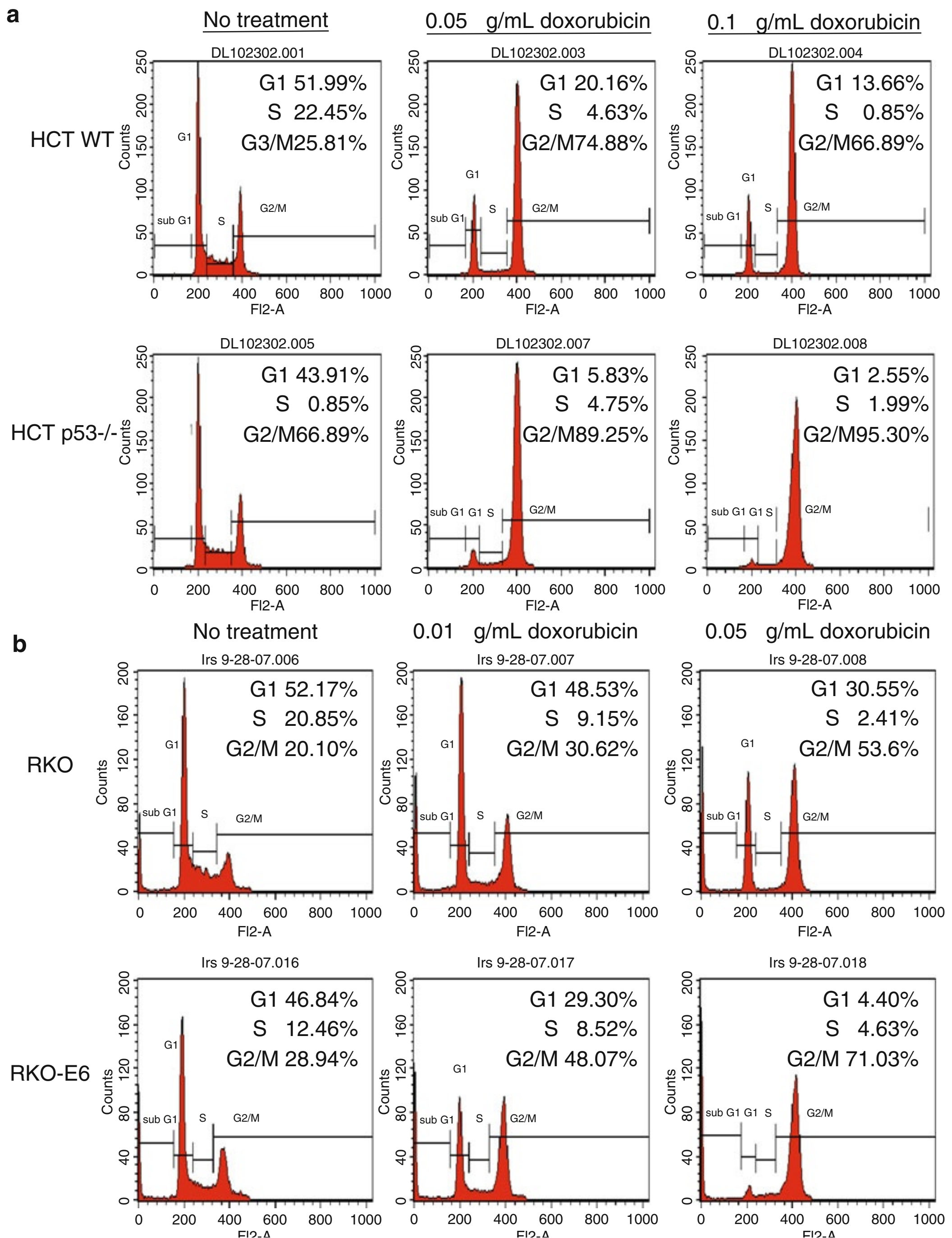

Fig. 1. Wild-type p53 cells maintain a G1 and G2/M arrest after treatment with doxorubicin, whereas p53−/− cells primarily arrest in G2/M. (**a**) HCT 116 WT and p53−/− colorectal carcinoma cells were treated with 0.05 and 0.1 μg/ml doxorubicin for 24 h before harvesting for flow analysis. (**b**) RKO (wild-type p53) and RKO E6 (p53 is targeted for degradation by HPV E6) colorectal carcinoma cells were treated with 0.01 and 0.05 μg/ml doxorubicin for 24 h before harvesting for flow analysis. The analysis cell profiles are shown for untreated and treated cells. The percentage of cells in G1, S, and G2/M are indicated in the upper right hand corner of each histogram.

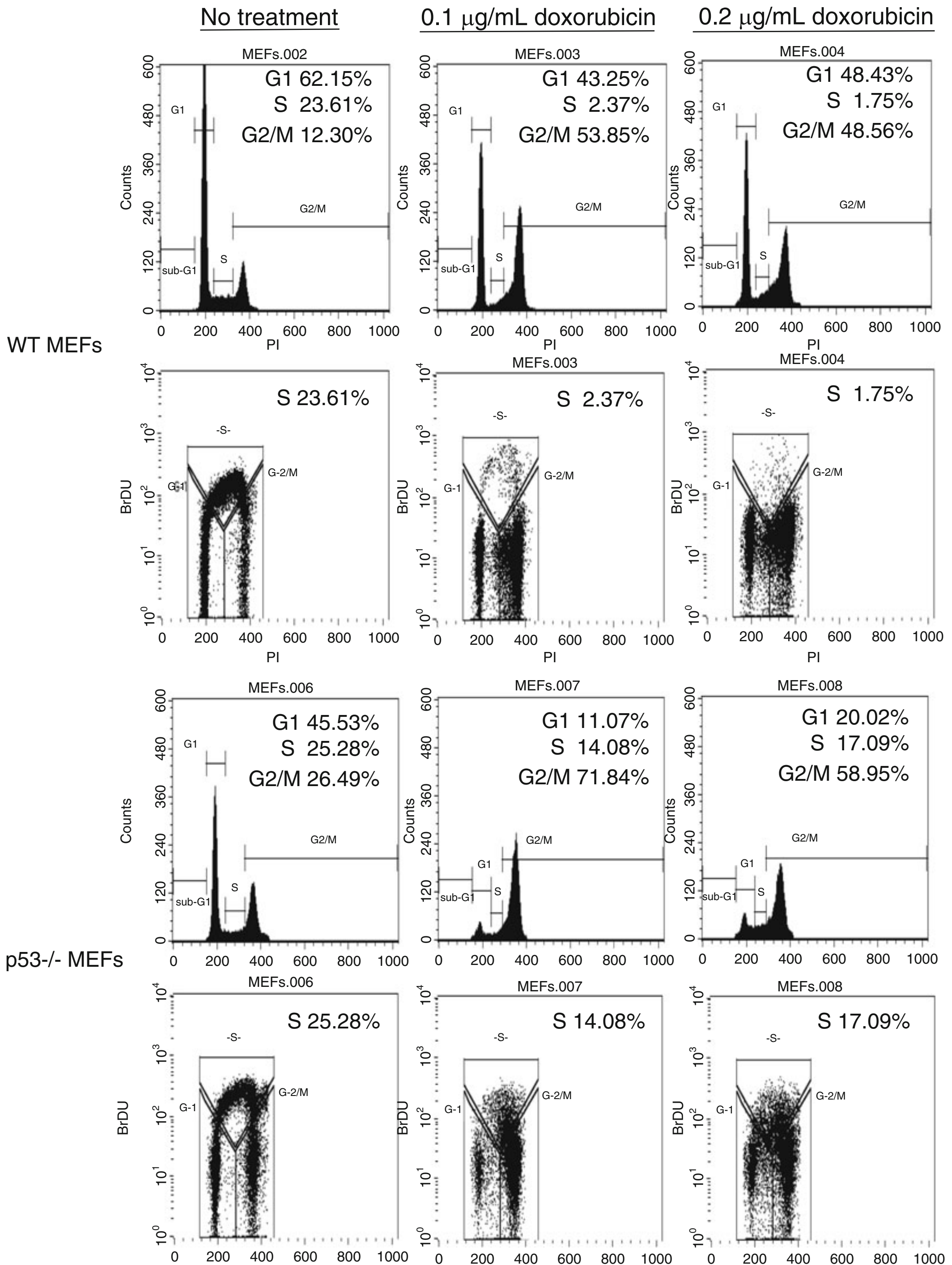

Fig. 2. Treatment with doxorubicin in WT p53 mouse embryonic fibroblasts (MEFs) results in a significant reduction of S phase cells. Wild-type and p53−/− MEFs were treated with 0.1 and 0.2 μg/ml doxorubicin for 24 h prior to being pulsed with 10 μM BrdU for 4 h. Following antibody treatment and propidium iodide staining the cells were analyzed by dual parameter flow cytometry. Both single and dual parameter histograms are shown for each condition. The percentage of cells in G1, S, and G2/M are indicated in the upper right hand corner of each histogram.

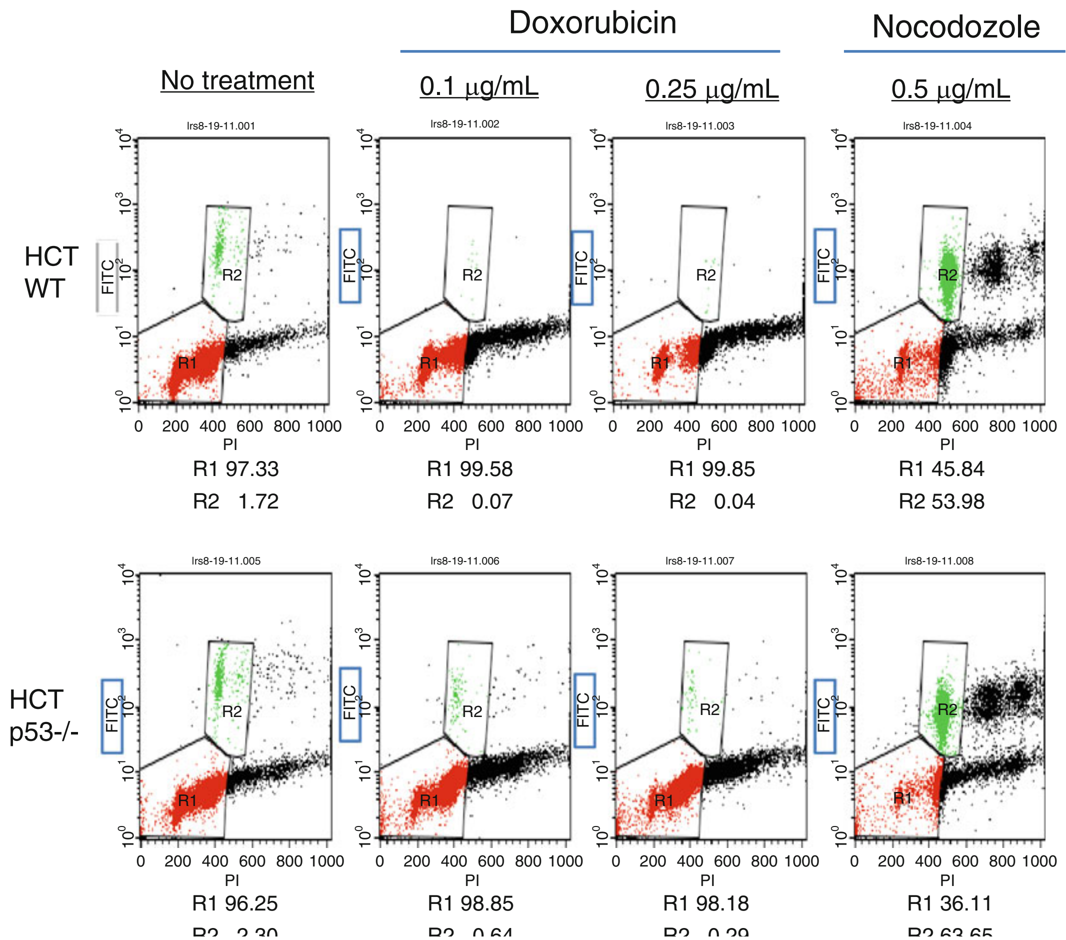

Fig. 3. Phosphorylation of histone H3 is diminished after DNA damage with doxorubicin in WT p53 cells. HCT 116 p53 WT and –/– cells were treated with 0.1 and 0.25 μg/ml doxorubicin or 0.5 μg/ml nocodozole for 24 h and stained for Ser10-phosphorylated histone H3 and DNA content (propidium iodide). The dual parameter flow analysis is shown. Phosphorylated histone H3 positive cells are *boxed* and denoted as R2, whereas cells in G1 and G2 are grouped in R1.

have incorporated the PI stain. When using the PI stain, however, one cannot distinguish between G2 and mitotic cells since both contain a 4N amount of DNA. The third technique broadens the cell cycle information because mitotic cells are specifically detected using an antibody to phosphorylated histone H3. Once cells exit mitosis, H3 becomes dephosphorylated and is no longer recognized by the antibody.

Although the focus of this chapter is the use of flow cytometry to determine cell cycle position, it should be noted that other methods that include western analysis and immunofluorescence may be used to detect the expression of specific proteins that play roles in DNA damage and cell cycle arrest and apoptosis.

2. Materials

2.1. Cell Culture, Lysis, DNA Damaging Agent, and Mitotic Tubule Inhibitor

1. Dulbecco's modified Eagle's medium (DMEM with High Glucose) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals) that has been heat inactivated at 56°C for 30 min.
2. 1× Dulbecco's phosphate buffered saline without calcium and magnesium (DPBS).
3. 0.05% Trypsin–ethylenediamine tetraacetic acid (EDTA).
4. Doxorubicin (Sigma-Aldrich, St. Louis, MO) is dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) to yield a concentration of 1 mg/ml. A sterile solution is prepared by passage through a 0.2 μm filter and aliquots are stored at −20°C. A subsequent dilution of the stock in sterile H_2O, 0.1 mg/ml, may be prepared and also stored at −20°C. This can be added directly to tissue culture cells at the appropriate concentration.
5. Nocodozole (Sigma-Aldrich, St. Louis, MO) is dissolved in distilled water to yield a 500 μg/ml stock that is passed through a 0.2 μm filter and stored at −20°C.

2.2. Propidium Iodide Staining for FACS Analysis

1. Dulbecco's phosphate buffered saline without calcium and magnesium (1× DPBS).
2. Dulbecco's modified Eagle's medium (DMEM with High Glucose) is supplemented with 10% FBS (Atlanta Biologicals) that has been heat inactivated at 56°C for 30 min.
3. 0.05% Trypsin–EDTA.
4. 70% Ethanol (ETOH) is prepared from reagent grade, 190 proof (95%) ethanol and diluted with distilled water.
5. Propidium iodide (PI) (Sigma-Aldrich, St. Louis MO). A 100× stock (2 mg/ml in PBS) is prepared and stored at 4°C in a foil-covered tube for protection from the light.
6. Bovine pancreatic ribonuclease A (RNase A) (Sigma, St. Louis, MO) is stored at −20°C and for each use an appropriate amount is added fresh to PI/PBS solution.
7. Polystyrene round-bottom 12×75 mm (5 ml) Falcon tubes (BD Falcon, Franklin Lakes, NJ).

2.3. (+)-5-Bromo-2-Deoxyuridine (BrdU) Incorporation

1. BrdU (Sigma-Aldrich, Fairlawn, NJ) is prepared at a concentration of 10 mM in distilled H_2O and stored at −20°C.
2. 1× DPBS.
3. 1% Bovine serum albumin (BSA)/DPBS.
4. 70% ETOH is prepared from reagent grade, 190 proof (95%) ethanol and diluted with distilled H_2O.

5. FITC-conjugated Anti-BrdU antibody (Becton Dickenson).
6. PI (Sigma Chemical Co.).
7. 2 N HCl with 0.5% Triton-X-100 (v/v).
8. 0.1 M Sodium tetraborate ($Na_2B_4O_7.10\ H_2O$), pH 8.5.
9. 0.5% Tween-20/1% BSA/DPBS.
10. 12 × 75 Polystyrene Falcon tubes (BD Falcon, Franklin Lakes, NJ).

2.4. Phosphorylated Histone H3 Assay

1. 0.5% Formaldehyde in 1× DPBS.
2. 90% Methanol is prepared with distilled H_2O.
3. Anti-phosphorylated ser 10 histone 3 antibody (Cell Signaling) is diluted in DPBS.
4. RNase A (Sigma, St. Louis, MO) is stored at –20°C and for each use an appropriate amount is added fresh to antibody/PBS solution.
5. FITC-conjugated secondary antibody (Jackson Laboratories) is appropriately diluted in DPBS.
6. BSA.

3. Methods

3.1. Treatment with DNA Damaging Agent

3.1.1. Day 0

In preparation for treatment with specific DNA damaging agents, cell lines containing wild type, mutant, or no p53 are trypsinized and plated at an appropriate number into 60 mm or 100 mm tissue culture dishes to yield dishes that will be subconfluent the following day. It is best to start by seeding a sufficient number of dishes in order to do a dose–response curve for your drug.

3.1.2. Day 1

Doxorubicin (0.1 mg/ml) is thawed and added directly to cell cultures at a broad range of concentrations (0.01–0.5 μg/ml) to establish a dose that is suitable. The cell cultures are maintained for an additional 24 h at 37°C before harvesting (see Note 1).

3.2. Staining with Propidium Iodide and Preparation for FACS Analysis

3.2.1. Day 0 and Day 1

Follow the above given protocol.

3.2.2. Day 2

1. The treated and untreated cells are harvested by trypsinization, making sure to retain the medium from each dish. This prevents the loss of dying cells that lifted off of the dish as a result

of the drug treatment. This medium can be used to neutralize the trypsin.

2. The cell suspensions are pelleted by centrifugation at 1,000 rpm for 5 min at 4°C.
3. The cell pellets are washed by resuspension in 1× DPBS and spun again at 1,000 rpm for 5 min at 4°C.
4. The supernatant is carefully removed and the pellet is gently dislodged. 1 ml of 70% ETOH is added dropwise to the cells while vortexing in order to disrupt the clumping of the cells (see Notes 2 and 3).
5. Once the cells are fixed they can be stored at –20°C for a minimum of 12 h or up to 2 weeks prior to PI staining.

3.2.3. Day 3

1. The fixed cells are centrifuged at 1,000 rpm for 5 min at 4°C.
2. The cells are rehydrated with DPBS and spun again at 1,000 rpm for 5 min at 4°C.
3. The pellet is resuspended in 20 μg/ml PI in PBS containing 1 mg/ml of RNase A and the cells are transferred to polystrene 12×75 mm Falcon tubes. Generally, 1–2 ml of the PI stain is adequate for 10^6 cells. The PI is prepared from a 100× stock (2 mg/ml) but the RNase is added fresh each time. The tubes are placed in the dark at room temperature for 2 h prior to FACS analysis. They may also be stored overnight at 4°C in the dark.
4. A BD BioSciences flow cytometer (FACSCalibur) is used to analyze samples for cell cycle position. Acquisition and analysis plots of 10,000 cells are generated using CellQuest software. The FL2 laser is employed for detection of the propidium iodide staining of the DNA.

3.3. Dual Parameter, BrdU Incorporation Assay, and Propidium Iodide Staining

3.3.1. Day 0

An appropriate number of cells are seeded for optimal growth in 100 mm tissue culture dishes.

3.3.2. Day 1

The cells are treated with doxorubicin. The stock (0.1 mg/ml) is thawed and added directly to cell cultures. A dose–response curve for each cell line should be performed in order to attain a dose that produces a p53-dependent response. This can be done as a single parameter experiment using PI before setting up a BrdU incorporation assay.

3.3.3. Day 2

1. BrdU is added directly to the culture medium to attain a final concentration of 10 μM. Washing the cells prior to addition of BrdU is not recommended because it might slow the growth of

the cells and the incorporation of the analog. Plan on including a dish of cells that will not be pulsed with BrdU (see Note 4).

2. Incubate cells at 37°C for 45–60 min, but note that this time will vary depending on the doubling time of the cells.
3. The BrdU pulsed cells are trypsinized, making sure to save the medium so as not to lose cells that are no longer adhered to the tissue culture dish and spun for 5 min at 1,000 rpm.
4. The number of cells per sample is adjusted to approximately 10^6 by resuspension of the cell pellet in 1× PBS and spun again for 5 min at 1,000 rpm.
5. The pellet is retained and resuspended in 1 ml 70% ETOH. It is important to prevent clumping of cells by gently vortexing the pellet while adding ETOH.
6. The cells can be stored overnight at −20°C before proceeding. This step can be shortened but usually a minimum of 12 h in fixative is standard before the denaturation step.
7. The cells are centrifuged at 1,000 rpm for 5 min. 1 ml of 2 N HCL/0.5%Triton X-1000 (v/v) is slowly added to the pellet, a few drops at a time while maintaining a vortex. This will disrupt clumps of cells that may result from addition of the HCL/Triton X mixture. This treatment will denature the DNA and create single-stranded molecules.
8. Incubate at room temperature for 30 min.
9. The cell suspension is spun at 1,000 rpm for 5 min. The pelleted cells are resuspended in 1 ml of 0.1 M $Na_2B_4O_7 \cdot 10H_2O$ pH 8.5, a sodium tetraborate solution that neutralizes the acid.
10. You can pause at this point and store the BrdU-labeled cells at −20°C. If you choose to do this, the cells are centrifuged at 1,000 rpm for 5 min and the pellet is resuspended in 70% ETOH. Otherwise you can proceed directly from steps 9 to 11.
11. The cells are spun down at 1,000 rpm for 5 min and the pellet is resuspended in 500 μl 0.5% Tween-20/1% BSA/PBS containing an appropriate concentration of anti-BrdU-FITC secondary antibody (Becton Dickinson).
12. The cells are maintained at room temperature (RT) and protected from the light for 30 min.
13. The cells are centrifuged at 1,000 rpm for 5 min, followed by resuspension of the pellet in 1 ml of PBS containing 10 μg/ml propidium iodide. The labeled cells are transferred into 12×75 mm polystyrene Falcon tubes and stored at RT in the dark for 2 h.
14. The samples are analyzed for cell cycle position using a BD BioSciences flow cytometer (FACSCalibur). Although the

excitation, 488 nm, is the same for both stains, the FL1 and FL3 detection lasers can discriminate between the emission of the green fluorescent FITC stain and the red propidium iodide staining of the DNA. CellQuest software is used to generate the acquisition and analysis plots of 10,000 cells.

3.4. Phosphorylated Histone H3 Assay

3.4.1. Day 0

An appropriate number of cells are seeded for optimal growth in 100 mm tissue culture dishes.

3.4.2. Day 1

The cells are treated with doxorubicin. The stock (0.1 mg/ml) is thawed and added directly to cell cultures. A dose–response curve for each cell line should be performed previously in order to attain a dose that produces a p53-dependent response. To aid in analysis, a dish of cells should also be treated with nocodozole at a final concentration of 500 ng/ml (see Note 5). This agent is an inhibitor of microtubules and will result in a population of cells that are arrested in M phase. The cell cultures are maintained for an additional 24 h at 37°C.

3.4.3. Day 2

1. The cells are trypsinized, making sure to save the medium that may contain cells that are no longer adhered to the tissue culture dish, and centrifuged for 5 min at 1,000 rpm.
2. The pellets are resuspended in PBS and spun down for 5 min at 1,000 rpm.
3. The pelleted cells are fixed in 1 ml of 0.5% formaldehyde for 10 min at 37°C.
4. The fixed cells are spun out at 1,000 rpm for 5 min and washed in 1 ml PBS to dilute out the fixative.
5. The cells are spun again at 1,000 rpm for 5 min and permeabilized using 1 ml of 90% methanol.
6. The cells are incubated on ice for 30 min or can be stored at –20°C for up to 10 days.
7. The cells are centrifuged and resuspended in PBS.
8. Once again the cells are centrifuged at 1,000 rpm for 5 min followed by resuspension in the primary antibody, anti-phosphorylated Ser 10 histone 3. The antibody is diluted according to the manufacturer's suggestion in PBS that contains 100 μg/ml RNase A.
9. The cells are incubated for 1 h with occasional shaking at RT.
10. The cells are pelleted and subsequently washed twice with 1% BSA/PBS.

11. After centrifugation the cells are taken up in the secondary, anti-FITC-conjugated antibody that is diluted accordingly in PBS.
12. The cells are incubated for 1 h at RT in the dark.
13. The samples are centrifuged for 5 min at 1,000 rpm and resuspended 1% BSA/PBS.
14. Once again the cells are centrifuged and resuspended in PBS containing 20 μg/ml propidium iodide and transferred into polystyrene 12×75 mm Falcon tubes.
15. The stained cells are incubated in the dark at RT for 30 min.
16. A FACS brand flow cytometer can be used for the analysis of the labeled samples. FL1 and FL3 detectors are selected to distinguish between the emission of PI and FITC. CellQuest software is used to generate the acquisition and analysis histograms of labeled cells.

4. Notes

1. The cellular response to a particular DNA damaging drug will vary for individual cell lines. Therefore it is important to empirically derive a concentration that will produce a biologic effect that is p53 dependent. It is important to establish a concentration curve of each drug for each cell line. Moreover it is a good idea to use parallel dishes to harvest for protein. An SDS-PAGE gel can be run, blotted, and probed for p53 and its target genes. One can then determine at which dose p53 is being induced and at what levels the response remains p53 dependent.
2. Cells are impermeable to PI. Therefore for this assay, the cells must be fixed so that the PI stain can penetrate the cells and intercalate into the DNA. Although ETOH is used, paraformaldehyde can also be used and might be preferable, when you want to prevent transfected proteins, like green fluorescent protein (GFP) from leaking out of the cell.
3. Some cell lines tend to be very sticky and are prone to clumping. Use of 12×75 mm Falcon polystyrene tubes with strainer caps will filter out clumps of cells from the final cell suspension.
4. A sample should be included to which BrdU has not been added. This control will be helpful when gating cells in the analysis portion of this assay.
5. Nocodozole-treated cells will serve as a positive control for the mitotic population that contains phosphorylated histone H3. This control will aid in proper gating in the analysis.

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Chapter 5

p53 Ubiquitination and Proteasomal Degradation

Ian M. Love, Dingding Shi, and Steven R. Grossman

Abstract

p53 levels and activity are controlled in large part through regulated ubiquitination and subsequent destruction by the 26S proteasome. Monoubiquitination of p53 is mediated primarily by the RING-finger E3 ubiquitin ligase MDM2 and impacts p53 activity through modulation of p53 localization and transcription activities. Recently, several E4 ubiquitin ligases (E4s) have been identified which serve to extend these monoubiquitin chains. The ubiquitin ligase activity of these factors toward p53, and their contribution to p53 degradation, can be studied using a variety of in vitro and in vivo methods and reagents which will be described in this chapter. These methods include in vivo ubiquitination of p53 using HA-ubiquitin or his-ubiquitin; the in vitro E3 ubiquitin ligase assay, in which ubiquitin reaction components (URC) are incubated with a purified E3 or E4 ligase; a one-step E4 assay, in which URC are incubated with a substrate, E3, and E4; and a two-step E4 assay in which p53 is monoubiquitinated in an E3 reaction, and subsequently purified and incubated with an E4. Finally, we will describe an in vitro degradation assay in which ubiquitinated p53 is incubated with purified 26S proteasomes. Together, these assays can be used to provide insight into the biochemical nature of p53 ubiquitination and degradation.

Key words: p53, MDM2, Ubiquitination, 26S proteasome, Ubiquitin ligase

1. Introduction

Homeostatic control of p53 levels is primarily accomplished through its regulated ubiquitination and subsequent degradation by the 26S proteasome. Potent cell-cycle inhibitory and apoptotic activities necessitate that p53 levels be kept under tight control in normal cells. Upon genotoxic or other stresses, however, p53 levels need to be rapidly upregulated to generate a quick response, and the inhibition of its constitutive ubiquitin/proteasome degradation after stress allows for a suitably rapid response. Ubiquitination of p53 occurs through the ATP-dependent transfer of a 76-residue ubiquitin moiety to a target lysine via an E3

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ubiquitin ligase (1), which then serves as a substrate for extension of ubiquitin chains by an E4 ubiquitin ligase (1–3). A sufficient number of ubiquitin molecules composing a chain (often four or more) (4), when linked by K48 to G76 of a preceding ubiquitin, serve as a proteasome recognition signal which directs degradation of the covalently attached substrate. In addition to the K48 linkage, a number of other physiologically relevant linkages exist (K6, K11, K29, and K63) (5).

The most well-characterized p53 ubiquitin ligase is the proto-oncogene MDM2, which directs the monoubiquitination of a cluster of C-terminal lysines of p53 and is critical for the proper regulation of p53 stability (6–8). In vitro, however, MDM2 in limiting physiologically relevant quantities is insufficient to facilitate p53 polyubiquitination and degradation (2, 9). This additional function is provided by the activity of an E4 ubiquitin ligase, of which the transcriptional coactivators p300 and CBP have been identified to harbor this activity toward p53. In recent years, additional p53 ubiquitin ligases have been identified (10–13). It is likely that a number of additional p53 E3 and E4 ligases will be uncovered in the future, and the methods described below will greatly aid in the investigation of their activities.

We will first describe two commonly used methods to sensitively detect ubiquitin chains on substrates in cells by utilizing exogenously expressed histidine (his) or HA-tagged ubiquitin. By using various ubiquitin lysine mutants in place of wild-type tagged ubiquitin, these methods also allow the study of ubiquitin chain linkages that might be preferentially assembled by a specific E3 or E4. The HA-ubiquitin method exhibits greater sensitivity for polyubiquitin chain conjugates than the his-tagged ubiquitin and Ni-NTA pull down method. However, the his-Ub method provides more consistent results when detecting monoubiquitinated substrates and short-chain conjugates.

Next, we will describe in vitro methods of studying p53 ubiquitination using purified components. These methods facilitate the study of ubiquitin chain formation, stability, and specificity in the presence of specific, purified components, allowing the contribution of specific factors or reaction conditions to these processes to be defined. These methods have been useful in the identification of novel E4 activities, where native proteins do not serve as substrates unless monoubiquitinated (2, 14), as well as in the identification of structural and functional elements required for substrate ubiquitination.

A p53 proteasome degradation assay will be described last, which involves the incubation of ubiquitinated p53 with purified 26S proteasomes. This assay can inform upon the contribution of factors or conditions which regulate the degradation of p53 by the proteasome after ubiquitination, and allow the probing of structural and functional elements of the substrate, ubiquitin chains, proteasome adapters, and even the proteasome itself, necessary to direct p53 degradation.

2. Materials

2.1. In Vivo Ubiquitination of p53 with HA-Tagged Ubiquitin

1. Transfection competent cell line(s) (suggestions: U2OS [wild-type p53] for study of native p53; H1299 [p53-null] for study of exogenous p53).
2. Lipophilic transfection reagent.
3. HA-ubiquitin expression vector (and p53 expression vector where indicated).
4. Proteasome inhibitor MG132 (Sigma-Aldrich).
5. RIPA buffer (50 mM Tris–HCl, pH 7.4, 250 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.5% deoxycholate (DOC)).
6. *N*-Ethyl maleimide (NEM, Sigma).
7. Protease inhibitor cocktail (Complete-mini EDTA-free, Roche).
8. Protein A (FL-393) or Protein G (DO-1) agarose beads (Upstate).
9. Antibodies: α-HA F-7 for detection of ubiquitin conjugates by western blot (Santa Cruz); p53 antibody (DO-1 or FL-393) for immunoprecipitation of p53, and blotting of IPs as a control and to detect short chain and native p53 in the IPs.

2.2. In Vivo Ubiquitination of p53 with His-Tagged Ubiquitin

1. Transfection competent cell line(s).
2. Lipophilic transfection reagent.
3. His-ubiquitin expression vector.
4. Proteasome inhibitor MG132 (Sigma-Aldrich).
5. Guanidinium lysis buffer (6 M guanidinium-HCl, 100 mM Na_2HPO_4/NaH_2PO_4 pH 8.0, 10 mM Tris–HCl pH 8.0, 5 mM imidazole, 10 μM β-mercaptoethanol).
6. Urea buffer (8 M urea, 100 mM Na_2HPO_4/NaH_2PO_4 pH 8.0, 10 mM Tris–HCl pH 8.0, 10 μM β-mercaptoethanol).
7. Wash buffer A (8 M urea, 100 mM Na_2HPO_4/NaH_2PO_4 pH 6.3, 10 mM Tris–HCl pH 8.0, 10 μM β-mercaptoethanol).
8. Triton X-100 (to supplement washes).
9. Imidazole elution buffer (200 mM imidazole, 5% SDS, 150 mM Tris–HCl pH 6.7, 30% glycerol, 720 mM β-mercaptoethanol).
10. *N*-Ethyl maleimide (NEM, Sigma).
11. Protease inhibitor cocktail (Complete-mini EDTA-free, Roche).
12. Ni^{2+}-NTA-agarose beads (follow manufacturer instructions to be sure beads are properly loaded with Ni^{2+}; Sigma-Aldrich).
13. Antibodies: α-His antibody for immunoprecipitation of ubiquitin conjugates; p53 antibody (DO-1 or FL-393) for detection of ubiquitinated p53.

2.3. One-Step E4 Assay

1. NP-40 lysis buffer (150 mM NaCl, 25 mM Tris–HCl, pH 8.0, 1% NP-40, 5 mM EDTA).
2. RIPA lysis buffer (50 mM Tris–HCl, pH 7.4, 250 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.5% DOC, 1 μM $ZnCl_2$).
3. Ubiquitin reaction buffer (URB): 25 mM Hepes pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.05% Triton X-100, 0.5 mM DTT.
4. Mg^{2+}-ATP (Boston Biochem).
5. FLAG peptide (Sigma-Aldrich).
6. URC: E1 enzyme, E2 enzyme (UbcH5a), ubiquitin (Ub), Me-Ub (all from Boston Biochem).
7. Proteasome inhibitors: MG132 (Sigma-Aldrich), lactacystin, clasto-lactacystin-β-lactone.
8. Sf9 insect cells (see Notes 1 and 2).
9. HA-p53, FLAG-MDM2, and FLAG-tagged E4 recombinant baculoviruses (p53 and MDM2 baculoviruses available on request from Grossman laboratory after execution of MTA agreements—srgrossman@vcu.edu) or plasmids encoding truncated or full length FLAG-tagged E4 polypeptides for transfection into human cells.
10. 37°C Water bath or heat block.

2.4. Two-Step E4 Assay

1. URB: 25 mM Hepes pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.05% Triton X-100, 0.5 mM DTT.
2. Mg^{2+}-ATP (Boston Biochem).
3. URC: E1 enzyme, E2 enzyme (UbcH5a), ubiquitin (Ub), Me-Ub (all from Boston Biochem).
4. Proteasome inhibitors: MG132 (Sigma-Aldrich), lactacystin, clasto-lactacystin-β-lactone.
5. Sf9 insect cells (see Notes 1 and 2).
6. HA-p53, FLAG-MDM2, and FLAG-tagged E4 baculovirus.
7. 37°C Water bath or heat block.
8. (Optional) Nutlin-3a or racemic Nutlin-3 (Sigma-Aldrich).

2.5. In Vitro Proteasomal Degradation Assay

1. Purified human 26S proteasomes (BIOMOL or Boston Biochem).
2. URB.
3. In vitro-ubiquitinated p53 reactions from Subheading 3.2 or Subheading 3.3.
4. Proteasome inhibitors: MG132 (Sigma-Aldrich), lactacystin, clasto-lactacystin-β-lactone.
5. Mg^{2+}-ATP (Boston Biochem).
6. A 30°C water bath or heat block.

3. Methods

3.1. In Vivo Ubiquitination of p53 with HA-Tagged Ubiquitin

In this assay, HA-ubiquitin is transfected into cells and the substrate is immunoprecipitated after lysis. After separation by SDS-PAGE, ubiquitin conjugates can be visualized by western blot using HA or p53-specific antibodies.

1. Plate cells on 10 cm^2 dishes at a suitable density for next-day transfection (cells should be approximately 60% confluent at the time of transfection).
2. Using an appropriate transfection reagent (we recommend Lipofectamine 2000 at a ratio of 2 μL:1 μg DNA into antibiotic-free media; Fugene will work if Lipofectamine is too cytotoxic), transfect cells with 2 μg HA-ubiquitin construct, (optionally) 0.1–0.5 μg of p53 expression vector as appropriate, and additional factors (E3 ubiquitin ligases or modifiers of substrate ubiquitination, for example) as the experiment requires.
3. 32–40 h after transfection, treat cells with 10–20 μM MG132 proteasome inhibitor for 8 h (range of 6–12 h depending on sensitivity to MG132 causing cytotoxicity) to promote the accumulation of polyubiquitinated p53 species.
4. Following MG132 treatment (40–48 h post-transfection), harvest cells in 500 μl RIPA buffer freshly supplemented with NEM (see Note 3; 5 mM) and complete EDTA-free tablets (Roche) and incubate for 30 min on ice.
5. Perform immunoprecipitations on ~0.5 mg (for transfected p53) to ~1 mg (endogenous p53) cleared lysate (14,000 rpm or 19,000 RCF for 10 min) in lysis buffer by adding 1 μg p53 antibody (FL-393, Santa Cruz) to lysates and incubating overnight, rotating at 4°C.
6. Add 20 μL RIPA-washed Protein A or G agarose beads (slurry 20 μl, dry beads in 10 μl) to immunoprecipitation reactions, rotating at 4°C for 2 h.
7. Wash immunoprecipitation reactions five times in cold RIPA lysis buffer supplemented with fresh 5 μM NEM, removing the supernatant after the final wash.
8. Elute bound protein off Protein A or G beads by boiling for 3–5 min using an SDS or LDS sample buffer.
9. Separate eluted protein by SDS-PAGE and transfer as normal (if detection of high molecular weight species is problematic then add 0.01–0.05% SDS to the transfer buffer).
10. Immunoblot using an anti-HA antibody to detect immunoprecipitated high molecular weight ubiquitin conjugates and image. The blot can be reprobed with p53 antibody (DO-1 mouse monoclonal or FL-393 rabbit polyclonal) to detect

short chain or monoubiquitinated p53 species. Detection of high molecular weight polyubiquitin conjugates may require higher exposure times than detection of other proteins.

3.2. In Vivo Ubiquitination of p53 with His-Tagged Ubiquitin

In this assay, his-ubiquitin is transfected into cells, and his-ubiquitin conjugated proteins are pulled down with Ni-NTA beads after lysis. After elution from Ni-NTA beads with imidazole, his-ubiquitin conjugates are separated by SDS-PAGE, and p53-his-ubiquitin conjugates can be visualized by western blot using p53 antibody.

1. Plate cells on 10 cm dishes at a suitable density for next-day transfection (cells should be approximately 60% confluent at the time of transfection).
2. Using an appropriate transfection reagent (we recommend Lipofectamine 2000 at a ratio of 2 μL:1 μg DNA and transfection into antibiotic-free media; Fugene if Lipofectamine is too cytotoxic), transfect cells with 2 μg his-ubiquitin construct, p53 expression vector as appropriate (0.1–0.5 μg), and (optionally) additional factors (E3 ubiquitin ligases or modifiers of substrate ubiquitination, for example) as the experiment requires.
3. 32–40 h after transfection, treat cells with 10–20 μM MG132 proteasome inhibitor for 8 h to promote the accumulation of polyubiquitinated p53 species.
4. Following MG132 treatment (40–48 h post-transfection), wash cells twice with cold PBS and harvest cells in 6 ml (per 100 cm dish) guanidinium lysis buffer supplemented with freshly prepared NEM (5 mM; see Note 3) and complete EDTA-free tablets (Roche). Allow lysis to occur 30 min on ice.
5. Perform Ni-NTA pulldowns on 1 mg of cleared lysate (spin 14,000 rpm or 19,000 RCF for 10 min) by adding 75 μL Ni^{2+}-NTA-agarose bead slurry to lysates and incubating for 2 h, rotating at room temperature.
6. Wash pulldowns successively using 1 ml of the following buffers one time each: guanidinium lysis buffer, urea buffer, wash buffer A supplemented with 0.2% Triton X-100, buffer A alone, and buffer A supplemented with 0.1% Triton X-100. At the last wash, carefully remove as much of the supernatant as possible from the beads.
7. Elute bound protein from Ni^{2+}-NTA-agarose beads by adding 2–3× bed volume of imidazole elution buffer, incubating 30 min on ice.
8. Dilute eluate with an appropriate SDS-PAGE sample buffer to a final concentration of 1× SDS-PAGE sample buffer; boil samples for 5 min.

9. Separate eluted protein by SDS-PAGE and transfer using standard separation and transfer conditions.
10. Immunoblot using an anti-p53 antibody to detect immunoprecipitated ubiquitin conjugates and image. The blot can be reprobed with anti-his antibody to detect total his-ubiquitin conjugates.

3.3. One-Step E4 Assay

The one-step E4 ubiquitin ligase assay allows for the analysis of E4 activity toward a substrate while in the presence of an E3 ligase and all ubiquitination reaction components. p53/MDM2 complexes and E4 truncated or full-length polypeptides affinity purified from Sf9 insect cell lysates and incubated with URC to generate ubiquitinated p53 species in vitro (see Note 4).

3.3.1. Production of Protein from Sf9 Cells

1. Split Sf9 cells at 50% confluence into 8 ml SF900 II SFM media (Life Technologies).
2. After cells are attached, add 200 μl of passage 2 (P2) baculovirus. If p53/Mdm2 complexes are being purified, the 2 separate viruses are coinfected to facilitate complex formation of p53 and MDM2, with different baculovirus ratios recommended depending on the application: 1:1 p53:Mdm2 is recommended for assays where p53 needs to be efficiently polyubiquitylated, while 10:1 p53:Mdm2 ratio is recommended to produce mono-/oligoubiquitylated p53. For assistance with cloning or producing baculovirus for serial infections, see http://tools.invitrogen.com/content/sfs/manuals/bactobac_man.pdf.
3. After 40–48 h, collect cells and transfer into falcon tubes. Centrifuge 5 min at 500 RCF. Wash cells with ice-cold PBS. Centrifuge again at 500 RCF.
4. Resuspend cells in lysis buffer supplemented with 1 mM PMSF, 1 mM DTT, and phosphatase inhibitors. Use 1 ml of lysis buffer for cells from two 10 cm plates. Lyse 30 min at 4°C with end-over-end rotation. *Note*: For purification of p53/Mdm2 complexes, use NP-40 lysis buffer. For purification of CBP or p300 polypeptides, use RIPA lysis buffer.
5. Centrifuge 15 min, 4°C, 14,000 rpm (19,000 RCF).
6. Transfer supernatant to a new tube.
7. Add pre-washed FLAG M2-agarose beads (Sigma)—for purification of FLAG-tagged proteins.
8. Incubate at 4°C 2 h—overnight (an overnight incubation will improve the yield) with end-over-end rotation.
9. Wash beads three times with lysis buffer, then two times with URB.
10. Aspirate liquid with a flat tip. Add 2–3 bed volumes of URB supplemented with FLAG-peptide at 200 ng/μl.
11. Incubate 2–3 h at 4°C with end-over end rotation.

12. Collect eluate by transferring to fresh tubes and measure protein concentration. Use this purified protein fresh, or freeze in small aliquots (add 15% glycerol prior to flash freezing in dry ice-ethanol) and store at −80°C.

3.3.2. Ubiquitin Ligase Assay

1. Combine URC (100 ng rabbit E1, 50 ng human E2 UbcH5a, 10 μg ubiquitin (Boston Biochem)) in 20 μL URB supplemented with 3 mM fresh Mg^{2+}-ATP.
2. Add 10–100 ng insect cell purified p53/MDM2 complex to the ubiquitination reaction.
3. Incubate for 30 min at 37°C.
4. Add 1–100 ng insect cell purified E4 ubiquitin ligase (E4 activity should be experimentally determined in this assay).
5. Incubate the reaction further for 60 min at 37°C.
6. Add a suitable SDS or LDS sample buffer to each reaction and briefly boil for 5 min.
7. Separate the p53-ubiquitin conjugates by SDS-PAGE and immunoblot reaction products using an appropriate p53 or ubiquitin antibody.

3.4. Two-Step E4 Assay

The two-step E4 ubiquitin ligase assay allows the analysis of E4 ligase activity toward a substrate in the absence of an E3 ubiquitin ligase. While requiring an additional purification step, this method provides the added advantage of allowing the distinct contribution of an E4 to substrate ubiquitination to be assessed in the absence of other ubiquitin ligase activity in the reaction. Therefore it is critical to differentiate the specific ubiquitin signal on substrate from auto-ubiquitination of E3 and E4 ubiquitin ligases. As in the one-step assay, p53, MDM2, and E4 peptides or full-length proteins are affinity purified from Sf9 insect cell lysates. p53 and MDM2 are incubated with URC to generate monoubiquitinated p53, which is then re-purified and incubated with purified E4 protein in an additional ubiquitination reaction.

3.4.1. Production of Protein from Sf9 Cells

1. Split Sf9 cells at 50% confluence into 8 ml SF900 II SFM media (Life Technologies).
2. After cells are attached, add 200 μl of P2 baculovirus. Use a 10:1 p53:Mdm2 ratio to allow production of monoubiquitinated p53 which will serve as an efficient substrate for E4 activity.
3. Refer to Subheading 3.3.1 above, steps 3–12, to complete protein isolation.

3.4.2. Two-Step E4 Assay

1. Combine URC (100 ng rabbit E1, 50 ng human E2 UbcH5a, 10 μg ubiquitin (Boston Biochem) in 20 μL URB supplemented with 3 mM fresh Mg-ATP.
2. Add 10–100 ng insect-cell purified p53 and MDM2 to the ubiquitination reaction.

3. Incubate for 30 min at 37°C.
4. To immunoprecipitate monoubiquitinated p53, add 0.2 μg p53 FL-393 antibody conjugated agarose beads for 2 h, rocking at 4°C.
5. Wash beads three times with 200 μL lysis buffer (optional: with 20 μM Nutlin-3 or 10 μM Nutlin-3a included; see Note 5), then two times with 200 μL URB to remove residual ubiquitin and MDM2.
6. Add a fresh set of ubiquitination reaction components (100 ng E1, 50 ng UbcH5a, 10 μg FLAG-tagged ubiquitin (Boston Biochem), and 10–100 ng CBP or other E4 purified in the last step) to the bead-immobilized p53.
7. Incubate at 37°C for 60 min.
8. Wash beads three times with RIPA buffer to remove any CBP auto-ubiquitination products or untethered polyubiquitin conjugates.
9. Add a suitable SDS or LDS sample buffer to each reaction and briefly boil for 5 min.
10. Separate the p53-ubiquitin conjugates by SDS-PAGE and immunoblot reaction products using an appropriate p53 or ubiquitin antibody.

3.5. In Vitro Proteasome-Dependent Degradation Assay

1. After performing in vitro p53 (poly)ubiquitination reactions as described above (see Note 6), preincubate 1 μg purified 26S proteasomes (see Note 7) with 10 μM each of MG132, lactacystin, and clasto-lactacystin-β-lactone proteasome inhibitors vs. vehicle for 30 min at 4°C in 15 μl URB (use of proteasome inhibitors in control reactions allows confirmation that effects seen are due to proteasome degradation and not other effects; see Note 8).
2. Add the ubiquitinated substrate (10–50 ng) in a minimal volume to the pre-incubated proteasome cocktail see Note 9, add fresh 3 mM Mg^{2+}-ATP (maximum total volume 20–25 μl), and incubate for 5 h at 37°C see Note 10).
3. Stop the reaction by adding EDTA to a final concentration of 5 mM if not loading on gel immediately, and/or add SDS or LDS sample buffer and boil 5 min.
4. Analyze the degradation reactions by SDS-PAGE and immunoblot with appropriate p53 antibody (DO-1 preferred due to sensitivity required to detect low abundance high molecular weight species). Quantitate the extent of degradation by densitometry or other method comparing the ratio of p53 species above 110 kDa to the abundance of the native band +/− monoubiquitinated bands below 110 kDa.

4. Notes

1. The baculovirus expression system is highly recommended to produce recombinant E3/E4 ubiquitin ligases (MDM2, p300 and CBP). This system boasts a number of advantages in protein production. Most importantly, the system is eukaryotic, producing high-yield recombinant proteins with high solubility, as well as proper post-translational modifications including phosphorylation, acetylation, and glycosylation. These modifications may be prerequisites for ubiquitin ligase enzymatic activity in the assay. Additionally, expression of protein complexes can be achieved by simultaneously infecting cells with two or more baculoviruses.
2. Mammalian expression systems *can* be used, if necessary, to produce recombinant protein, but yields will generally be much lower as compared to the baculovirus/Sf9 system, and must be scaled up accordingly.
3. NEM can be made fresh immediately before use or can be aliquoted from 500 mM stock solution in 100% EtOH stored at –80°C.
4. As an alternative to insect cell production of E4 polypeptides, FLAG-tagged E4 polypeptides (truncated or full-length) can be purified from transfected human cells though with a lower yield than would be expected using the Sf9 insect cell system. Transfection conditions are as described in 3.1, while purification of the FLAG-tagged polypeptides is performed as described in 3.3.1 steps 3–12.
5. Nutlin-3, a potent nanomolar inhibitor of the p53-MDM2 interaction, can be used to assist in the removal of MDM2 from ubiquitinated p53 that is to be used as a substrate in the two-step E4 assay. Nutlin-3a is the active enantiomer, so the concentration of racemic nutlin should be doubled relative to nutlin-3a.
6. To achieve optimal degradation in the in vitro proteasome degradation assay, ubiquitinated protein substrates from a prior ubiquitination reaction and a saturating concentration of proteasomes should be used in the assay. Also, in the ubiquitination assay, the protein substrate should be ubiquitinated to the maximum extent in the reaction.
7. The degradation activity of proteasome is a key factor in the in vitro proteasome degradation assay. Therefore, it is critical to preserve the enzymatic activity of the proteasome. When ready for use, the proteasome should be thawed by standing on ice and should be used immediately after thawing, as the proteasome complex is labile. Multiple freeze–thaws dramatically decrease enzymatic activity and should be avoided.

8. When assessing the impact of small molecules or other regulatory proteins on in vitro proteasomal degradation, incubate these molecules in URB for 30 min at 4°C (with or without MDM2 or p53 depending on the nature of these modifiers) with proteasomes.

9. Ubiquitin aldehyde (2–5 μM) can also be added to the in vitro proteasome degradation assay (added at the same time as proteasome inhibitor or vehicle as described in Subheading 3.5.1) to enhance degradation by preserving polyubiquitin conjugates. This modified ubiquitin is a DUB inhibitor like NEM but not toxic to proteasomes; therefore it is useful in preserving ubiquitin chains after synthesis during subsequent enzymatic reactions.

10. Fresh Mg^{2+}-ATP (added 1–3 mM) can be added halfway through the degradation reaction (after 2–2.5 h) to ensure maximum activity of proteasomes through the entire reaction.

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Chapter 6

Identification of p53 in Mitochondria

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Abstract

p53 is a master regulator of cell death pathways and has transcription-dependent and transcription-independent modes of action. Mitochondria are major signal transducers in apoptosis and are critical for p53-dependent cell death. Our lab and others have discovered that a fraction of stress-induced wild-type p53 protein rapidly translocates to mitochondria upon various stress stimuli and exerts p53-dependent apoptosis. Suborganellar localization by various methods shows that p53 localizes to the surface of mitochondria. Direct targeting of p53 to mitochondria is sufficient to induce apoptosis in p53-null cells, without requiring further DNA damage. Recently, p53 has been also shown to localize to other mitochondrial compartments such as the mitochondrial matrix where it plays a role in maintaining mitochondrial genome integrity. Here, we describe subcellular fractionation as a classic technique for detecting mitochondrial p53 in cell extracts. It consists of cell homogenization by hypo-osmotic swelling, removal of nuclear components by low-speed centrifugation, and mitochondrial isolation by a discontinuous sucrose density gradient. Additionally, we describe a method for submitochondrial fractionation, performed by phosphate buffer mediated swelling/shrinking. p53 and other mitochondrial proteins can then be detected by standard immunoblotting procedures. The quality of mitochondrial isolates/subfractions can be verified for purity and intactness.

Key words: p53, Mitochondria purification, Submitochondrial fractionation

1. Introduction

p53 kills tumor cells by transcriptionally activating gene products that have various roles in the cell death pathway. However, it has long been observed that p53 has a transcription-independent way of killing cells, although no mechanism was known (1–3). Mitochondria are major signal transducers in apoptosis and are critical for p53-dependent cell death. Mitochondria can trigger cell death by releasing cytochrome c and other potent apoptosis activators stored in their intermembraneous space that, once released,

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activate effector caspases and break down nuclear chromatin (4, 5). Our group and others had discovered that a fraction of stress-induced wild-type p53 protein rapidly translocates to mitochondria during p53-dependent apoptosis induced by, e.g., γ-irradiation (IR), topoisomerase inhibitors, and hypoxia. This phenomenon is widespread and occurs in human and mouse cultured cells (6–9) (see Note 1). The translocation of p53 to mitochondria is rapid (within 1 h after p53 activation) and precedes changes in mitochondrial membrane potential, cytochrome c release, and procaspase-3 activation. In contrast, p53 does not translocate during p53-independent apoptosis or p53-mediated cell cycle arrest. Importantly, bypassing the nucleus by targeting p53 to mitochondria is sufficient to induce apoptosis in p53-null tumor cells and xenografts, without further DNA damage (10–12). Thus, p53 protein is sufficient to launch apoptosis directly from the mitochondria. Although, initially it was considered that the predominant amount of p53 is located at the surface of mitochondria, recently it has been shown that p53 protein can be detected in mitochondrial matrix as well (13–15). This matrix associated subfraction of mitochondrial p53 might have a role in normal mitochondrial homeostasis and/or non-apoptotic outcomes after stress; however, the exact role or mitochondrial matrix associated p53 still remains to be determined.

Subcellular fractionation for mitochondrial enrichment is a major analytical tool for studying mitochondrial translocation of p53. While this method can also be adapted to isolate mitochondria from rat or mouse liver (16), bovine heart, skeletal muscle (17), or yeast (18), we focus here on cultured tumor cells, because they are easy to grow and respond well to various treatments with drugs, IR, UV, hypoxia, or transfection. The protocol for isolation of mitochondria is a discontinuous sucrose gradient and follows largely the method described by Bogenhagen and Clayton (19). We also describe a fast and easy method for separation of submitochondrial compartments—outer membrane (OM), intermembrane space (IMS), inner membrane (IM), and matrix (MA). The method involves consecutive swelling and shrinking of isolated mitochondria by phosphate buffers and several steps of differential centrifugation as described by Hovius et al. (20) and others (21, 22) with minor modifications.

2. Materials

2.1. Mitochondria Isolation

1. Confluent suspension or adherent cultured cells, treated or untreated with drugs, IR, hypoxia, and/or transfection.
2. TD washing buffer: 135 mM NaCl, 5 mM KCl, 25 mM Tris–HCl, pH 7.6, ice-cold. To make a 10× solution: 39.15 g NaCl,

1.85 g KCl, 15.15 g Tris–HCl, 3.485 g Tris-base, 0.5 g Na_2HPO_4. Add water up to 500 mL. Store at 4°C.

3. CaRSB buffer: 10 mM NaCl, 1.5 mM $CaCl_2$, 10 mM Tris–HCl, pH 7.5, ice-cold. Mix together 1 mL 5 M NaCl, 750 μL 1 M $CaCl_2$, 5 mL 1 M Tris–HCl, pH 7.5. Add water up to 500 mL.
4. MS buffer: 210 mM mannitol, 70 mM sucrose, 5 mM ethylenediamine tetraacetic acid (EDTA), 5 mM Tris, pH 7.6, ice-cold. To make a 2.5× solution: 38.26 g mannitol, 23.96 g sucrose, 5 mL 1 M Tris–HCl, pH 7.6, 20 mL 0.25 M EDTA, pH 7.4. Add water up to 400 mL. Store at 4°C.
5. Sucrose buffer: 1 M or 1.5 M sucrose, 2 mM dithiothreitol (DTT), 5 mM EDTA, 10 mM Tris, pH 7.6. Mix together 34.24 or 51.34 g sucrose, 200 μL 1 M DTT, 2 mL 0.25 M EDTA, 1 mL 1 M Tris–HCl, pH 7.6. Add water up to 100 mL. Store at 4°C.
6. Protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN, USA). Should be made fresh and added to each solution. Store as recommended by company.
7. Glass Dounce homogenizer (Kimble/Kontes, Vineland, NJ, USA), fit A.
8. Low-speed centrifuge with swinging bucket rotor.
9. Table-top refrigerated microcentrifuge (e.g., Eppendorf® microcentrifuge).
10. Ultracentrifuge with swinging bucket rotor (e.g., Rotor type SW41; Beckman Coulter, Fullerton, CA, USA: cat. no. L8-80M).
11. 10% Sodium dodecyl sulfate (SDS) acrylamide gel.
12. Tris-buffered saline with Tween® 20 (TBST) buffer. For 8 L of stock solution: 80 mL 1 M Tris–HCl, pH 8.0, 20.4 g NaCl, 4 mL 20% Tween 20. Add distilled water (dH_2O) up to 8 L. Store at room temperature.

2.2. Submitochondrial Fractionation

1. Swelling buffer: 10 mM KH_2PO_4, pH 7.4. Dissolve 680 mg KH_2PO_4 in water up to 500 mL, adjust pH to 7.4.
2. Shrinking buffer: 10 mM KH_2PO_4, pH 7.4, 32% sucrose, 30% glycerol, 10 mM $MgCl_2$. Mix together 16 g sucrose, 15 mL glycerol, 476 mg $MgCl_2$, add 10 mM KH_2PO_4, pH 7.4 up to 500 mL.
3. Protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN, USA). Protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN, USA).
4. Ultracentrifuge with swinging bucket rotor (e.g., Rotor type SW41; Beckman Coulter, Fullerton, CA, USA: cat. no. L8-80M).

5. Table-top refrigerated microcentrifuge (e.g., Eppendorf® microcentrifuge).
6. Refrigerated centrifugal concentrator (or centrifugal filters, e.g., Amicon Ultra centrifugal filters for protein purification and concentration).

3. Methods

3.1. Isolation of Mitochondria

3.1.1. Suspension Culture Cells

Prepare Cells

1. Pellet suspension cells by spinning for 5 min at 1,000–3,000 × *g* at 4°C in low-speed centrifuge with swinging rotor (see Note 2).
2. Aspirate media completely.
3. Resuspend cells from 100 mL culture into 50 mL 1× TD buffer, ice-cold (see Note 3).
4. Pellet cells by spinning for 5 min at 1,000–3,000 × *g* at 4°C.

Homogenize Cells

1. Discard supernatant completely and resuspend cells into CaRSB buffer containing protease inhibitors. Use approximately 10× volume of packed cells.
2. Leave on ice for 10 min to let cells swell; monitor swelling under microscope.
3. Transfer cells to ice-cold glass Dounce homogenizer and homogenize on ice with frequent monitoring under microscope (see Note 4).
4. Quickly add 2 mL of 2.5× MS buffer containing protease inhibitor per 3 mL homogenate. Transfer an aliquot to another tube for Western blotting to check for nuclear contamination. Another aliquot is used for protein determination (see Note 5).
5. Transfer the rest of the homogenate to centrifuge tubes and spin down nuclei for 5 min at 1,000–3,000 × *g* at 4°C. Decant supernatant into new clean tube and repeat again (see Note 6).

Isolate Mitochondria

1. Make a discontinuous sucrose gradient in a ultracentrifuge tube (Polyallomer tubes; Beckman Coulter; cat. no. 331372) by carefully layering 3 mL of 1 M sucrose buffer on top of 3 mL of 1.5 M sucrose buffer (each containing protease inhibitors) (see Note 7).
2. After spinning, take supernatant of homogenate (from Subheading "Homogenize Cells," step 5) and apply carefully on top of the sucrose gradient.
3. Ultracentrifuge the gradient in a SW41 swinging bucket rotor for 35 min at 26,000 rpm (85,000 × *g*) at 4°C.

4. Mitochondria will collect at the 1–1.5 M interphase of the sucrose gradient and appear as a clearly visible white band. Carefully aspirate the band with an 18-G needle using a 3-mL syringe. Be careful not to suck off buffer. Mix with four times the volume of 1× MS buffer containing protease inhibitors (see Note 8).
5. Aliquot into microcentrifuge tubes and spin them for 10–15 min at 16,000 × *g* at 4°C in a table-top microcentrifuge.
6. Aspirate supernatant and pool all pellets in 100 μL of 1× MS (with protease inhibitors) and spin again (see Note 9).
7. Resuspend in a final 200 μL of 1× MS buffer (with protease inhibitors). This sample will be used for all subsequent assays (see Note 10).
8. Measure protein concentration. Add SDS/β-mercapto-ethanol sample buffer for electrophoresis.

3.1.2. Adherent Cells

Prepare Cells

1. Aspirate media completely and rinse quickly once with phosphate-buffered saline (PBS).
2. Scrape cells into PBS and pellet for 5 min at 1,000–3,000× *g* at 4°C.
3. Aspirate media completely. Resuspend cells into 50 mL of 1× TD buffer, ice-cold.
4. Pellet cells for 5 min at 1,000–3,000 × *g* at 4°C.
5. Follow the steps in Subheading "Prepare Cells," steps 1–4, Subheading "Homogenize Cells," steps 1–5, and Subheading "Isolate Mitochondria," steps 1–8.

3.2. Western Blot Analysis

1. Prepare a 10% SDS polyacrylamide gel.
2. The same amount of mitochondrial and crude lysates are run (1–10 μg protein per lane).
3. Transfer proteins to nitrocellulose membrane with wet transfer apparatus at 100 V for 1 h, during transfer keep buffer ice-cold.
4. Block in 5% nonfat dry milk in TBST for at least 20 min at room temperature.
5. Incubate for 2 h at room temperature with a cocktail of anti-p53 antibody, antiproliferating cell nuclear antigen (PCNA) antibody (nuclear contamination marker), and anti-mitochondrial (mt) heat-shock protein (HSP) 70 antibody (mitochondrial enrichment marker) properly diluted in blocking buffer (see Note 11).
6. Wash with TBST 3× for 15 min.
7. Incubate for 1 h at room temperature with secondary antibody.

8. Wash with TBST 3× for 15 min.
9. Develop with enhanced chemiluminescence reagent (Luminol Reagent; Pierce Chemical, Rockford, IL, USA).

3.3. Submitochondrial Fractionation

3.3.1. Determine Mitochondrial Protein Concentration

1. Obtain mitochondria suspension in 1×MS buffer, prepared following the procedure above. Take a small aliquot, lyse the mitochondria, and determine protein concentration using, for example, Bradford assay.

3.3.2. Separate Outer Membrane, Intermembrane Space, Inner Membrane, and Matrix

1. Pellet mitochondria by spinning the mitochondrial suspension from step 1 of Subheading 3.3.1 for 10 min at 16,000 × *g* using table-top centrifuge at 4°C. Aspirate completely all traces of MS buffer.
2. Resuspend the mitochondrial pellet in swelling buffer at a concentration 200 μg mitochondrial protein/mL. The volume of swelling buffer to use should be calculated using the concentration of mitochondrial protein obtained in step 1 of Subheading 3.3.1.
3. Incubate the mitochondria in swelling buffer for 20 min at 4°C. Mix gently every 5 min.
4. Add the same volume of shrinking buffer (see Note 12).
5. Spin down at 10,000 × *g* for 10 min (see Note 13).
6. Transfer the supernatant containing outer membrane and intermembrane space fractions into fresh tube. Label the tube "OM&IMS."
7. Wash the mitoplasts pellet from step 5 by adding 1:1 mixture of swelling and shrinking buffer and spin down 10,000 × *g* for 10 min. Repeat at least one more time (see Note 14).
8. After washing, resuspend the mitoplasts in swelling buffer and incubate for 20 min at 4°C. Mix gently every 5 min. Use the same volume of buffer as used in step 2. Label the tube "IM&MA" (see Note 15).
9. Spin down tubes "OM&IMS" and "IM&MA" at 14,000 × *g* for 60 min, 4°C, using ultracentrifuge.
10. Transfer supernatants into fresh tubes and label the tubes "IMS" and "MA." The pellets represent OM and IM.
11. Concentrate the IMS and MA fractions using refrigerated centrifugal concentrator or centrifugal filters. If using centrifugal filters, follow the manufacturer instructions.
12. Resuspend the OM and IM pellets in 1× SDS/β-mercaptoethanol sample buffer, add up to 1× sample buffer to the concentrated IMS and MA, and boil 5 min 100°C (see Note 16).
13. Perform Western blotting as described in Subheading 3.2 (see Note 17 and Table 1).

Table 1
Antibodies

	Name	Origin	Dilution	Company
Anti-p53	DO-1	Mouse IgG_{2a}	1:1,000	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Anti-PCNA	PCNA (PC10)	Mouse IgG_{2a}	1:1,000	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Anti-mt hsp70	Mitochondrial heat-shock protein 70	Mouse IgG_3	1:1,000	Affinity Bioreagents (Golden, CO, USA)
Anti-mt hsp60	Mitochondrial heat-shock protein 60	Mouse IgG_{2a}	1:1,000	Affinity Bioreagents (Golden, CO, USA)
Anti-cytochrome c	Cytochrome c	Mouse $IgG_{2b}\kappa$	1:1,000	BD Biosciences (San Jose, CA, USA)
Anti-VDAC	Voltage-Dependent Anion Channel	Mouse IgG_{2b}	1:1,000	Calbiochem
Anti-cyclophilin D	Cyclophilin D	Mouse IgG_1	1:1,000	Calbiochem
Anti-CoxIV	OxPhos Complex IV subunit I	Mouse $IgG_{2a}\kappa$	1:1,000	Invitrogen (Camarillo, CA, USA)

4. Notes

1. A death stimulus is required to induce endogenous wild-type p53 to translocate to mitochondria. Mitochondria from unstimulated cells have small levels of mitochondrial p53, which are sometimes hard to detect by western blotting. γ-IR, DNA damaging agents (e.g., 5 μM camptothecin for 6 h), or hypoxia (125 μM desferoxamine for 5 h or GasPak pouches; Becton Dickinson, Franklin Lakes, NJ, USA) induce mitochondrial p53 (6, 7). Cancer cell lines with mutant p53 exhibit mitochondrial p53 constitutively without prior DNA damage (8). When mitochondrially targeted p53 fusion protein is transfected (the LFp53wt plasmid used in refs. 10–12), mitochondrial p53 is clearly detectable by immunofluorescence analysis.
2. First resuspend pellet in about 5 mL of CaRSB buffer by pipetting up and down, then add the rest of the buffer. For some cell types, it is difficult to ascertain whether they undergo swelling within 10 min. In that case, it is acceptable to keep

cells in TD buffer or in growth medium and only remove small aliquots to try out the proper swelling time. Compare with cell shape prior to swelling and observe enlargement under the microscope. ML-1 cells (human chronic myelogenous leukemia line) are the standard cell line for verifying swelling.

3. How many strokes are needed? It really depends on the type of cells. Homogenize to the point where ideally 70–90% of the cells are broken, but the nuclei are still intact (they appear as smooth round small spheres under the phase optic microscope). The nuclei of some cell lines are very fragile. Some cell lines need 100 strokes, others need only 10. To determine the proper number of strokes, visually monitor after every 5 strokes.
4. Usually 100 μL of crude cell extract is enough.
5. To avoid nuclear contamination, the second centrifugation is critical. A third spin is even recommended if the second spin still produces a significant nuclear pellet. Although 5 min is enough for centrifugation, it can be extended until 15 min. Do not disturb the pellet when removing the supernatant. Decanting is better than pipetting. Although pure mitochondria will be isolated with the following sucrose gradient, washing the nuclear pellet to obtain a higher yield of mitochondria is not recommended, because some cell lines have very fragile nuclei that may release nuclear proteins.
6. In preparation for the experiments, the rotor and rotor buckets should be placed at 4°C and –20°C, respectively.
7. Do not disturb the mitochondrial shelf. Another white ring on top of the sucrose gradient contains smaller organelles (e.g., lysosomes, peroxisomes).
8. Pipette very gently up and down as to not break the mitochondria.
9. After this step, the mitochondria can also be resuspended in cell lysis buffer for subsequent co-immunoprecipitation experiments.
10. An example of the antibody combination is given in Table 1. To verify the absence of nuclear contamination, we found that PCNA is highly sensitive.
11. For all the antibodies described in Table 1, we use sheep anti-mouse immunoglobulin (IgG) (peroxidase-linked, species-specific whole antibody) (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Use 1:2,000 dilution in blocking buffer.
12. During the incubation with swelling buffer, the outer mitochondrial membrane bursts, while inner membrane stays intact. Shrinking buffer is added to prevent bursting of the inner mitochondrial membrane during the subsequent spinning.

13. During this spin the intact mitoplasts are pelleted down, while the supernatant contains OM and IMS.
14. Washing the mitoplast pellet will eliminate contamination from OM and IMS. Second wash is necessary for obtaining clean fractions.
15. During this step the inner membrane (IM) bursts and the following centrifugation separates IM from MA. Alternatively, one can sonicate to disrupt the IM: sonication twice at 40 W for 15 s with a 1 min interval is sufficient.
16. If determining protein concentration is necessary, one can resuspend the OM and IM pellets in RIPA buffer—25 mM Tris, pH 8.2, 50 mM NaCl, 0.5% NP40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitors.
17. To verify the purity of the fractions, protein markers for each fraction are needed. For OM we use VDAC, for IMS—cytochrome C, for IM—coxIV and for MA—cyclophilin D. See Table 1 for antibody information.

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Chapter 7

Identification of Novel Mutant p53 Interacting Proteins by Proteomic Analysis

Sumitra Deb and Paul R. Graves

Abstract

Protein–protein interaction studies can provide valuable insight into protein function. One of the most practical and high-yielding approaches is immunoprecipitation of a bait protein followed by mass spectrometry to identify co-precipitating proteins. Here we describe an effective and simplified version of this method that can be performed in most laboratories using standard laboratory equipment (apart from the mass spectrometer). We further demonstrate the utility of this method to identify proteins that specifically interact with mutant forms of the tumor suppressor protein, p53.

Key words: Proteomics, Mutant p53, Mass spectrometry, Co-precipitation

1. Introduction

Protein–protein interactions are essential to the function of all proteins and help define their biological roles. Therefore, identification of protein binding partners can yield important information about protein functions in normal or diseased cellular processes. To identify protein interactions, a wide variety of approaches have been devised that can be broadly grouped into two different categories. One, in vitro interaction studies, and two, those performed in intact cells. In vitro protein interaction studies include the use of proteins immobilized on various surfaces (protein chips) (1) and have the advantage that both low and high abundant proteins can be presented for binding studies in equimolar amounts. This allows the large bias against low abundant proteins to be overcome. However, in vitro studies may also result in binding artifacts as cellular constituents and cellular organization is absent. In contrast, protein interaction techniques conducted in vivo to identify protein interactions, such as bimolecular

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fluorescence complementation (2), provide a method to measure protein interactions within intact cells under physiological conditions (provided the proteins are not greatly overexpressed). These studies are most likely to reflect the true biological function of proteins as cells are not disrupted; however, due to the labor involved, this technique is not well suited for proteome-wide screening.

As a compromise between these two techniques, protein immunoprecipitation of a bait protein expressed within cells coupled with mass spectrometry to identify associated proteins has become a versatile and practical approach. The main advantage to this technique is that protein interactions are studied under conditions that are more biologically relevant than in vitro studies and it can be used to screen for proteome-wide interactions. The main disadvantages to this technique are that protein binding artifacts can still occur as cells are lysed during the co-immunoprecipitation step and it may be difficult to identify low abundant proteins as high abundant proteins tend to dominate the analysis. However, this technique is relatively inexpensive and technically feasible for most laboratories using standard laboratory equipment. Although mass spectrometry is required, if one is not available, there are many research cores that can provide protein identification as a service.

Here we illustrate how protein immunoprecipitation can be used to identify mutant p53-specific binding proteins. In this approach, we utilize a stable cell line that expresses mutant p53 and perform large-scale p53 immunoprecipitation to identify novel interacting proteins. As a control, we use a cell line that does not express any form of p53 and cells that express wild-type p53. The proteomic analysis identified two novel proteins that co-precipitated with mutant p53 but not wild-type p53, Tim50 and MCM7 (Fig. 1). Of these two proteins, we have

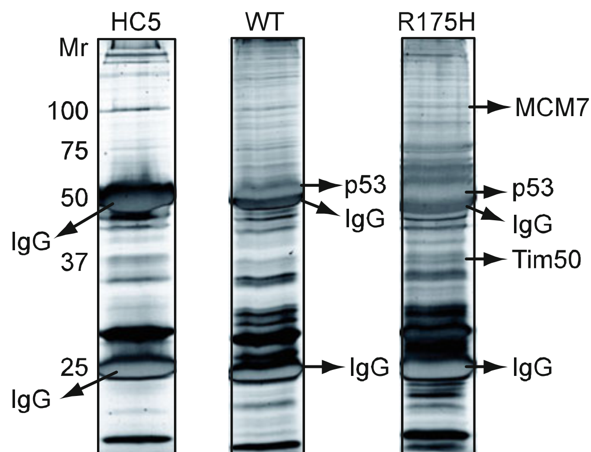

Fig. 1. Identification of mutant p53-specific interacting proteins. Large-scale p53 immunoprecipitations were performed from HC5, H-p53-R175H, or H1299 cells transfected with WT-p53 and proteins resolved on 12% polyacrylamide gels. A silver-stained gel is shown. *Arrows* indicate the identity of some proteins determined by mass spectrometry.

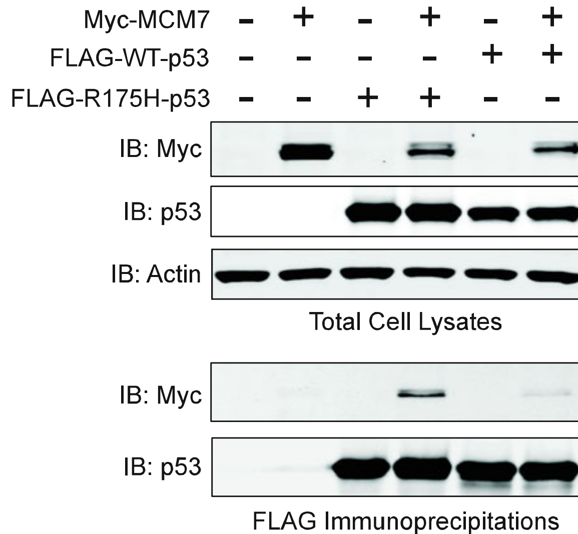

Fig. 2. Confirmation of the specific interaction between mutant p53 and MCM7. H1299 cells were transfected with Myc-MCM7 alone or together with FLAG-WT-p53 or FLAG-R175H-p53 and FLAG immunoprecipitations performed. Although all proteins were expressed to relatively equal levels (see total lysates), only FLAG-R175-p53 immunoprecipitants contained significant amounts of MCM7 protein (see FLAG immunoprecipitations).

confirmed that MCM7 does indeed show specific binding to mutant p53 but not WT p53 upon co-expression in H1299 cells (Fig. 2). The other protein identified, Tim50, was shown to be upregulated by mutant p53 at the transcriptional level (3). Thus, these results illustrate the practical feasibility of this method to identify novel protein–protein interactions or targets of mutant p53.

2. Materials

2.1. Cell Growth, Maintenance, and Transfection

All media components for the growth of cells must be sterile and preparation of media conducted in a sterile environment. Tissue culture maintenance is conducted in a sterile tissue culture hood and cells grown at 37°C in a CO_2 incubator.

1. Cell media: DMEM (Dulbecco's modified Eagle's medium) supplemented with fetal bovine serum (FBS) and Gentamicin. A gentamicin stock solution of 10 mg/ml was generated, sterile-filtered, and stored in aliquots at −20°C.
2. Cell passaging and harvesting: Trypsin–EDTA and PBS (phosphate-buffered saline).
3. Cell transfection: Lipofectamine-2000 and highly purified DNA stock (see Note 1).

2.2. Immunoprecipitation of the Bait Protein, Mutant p53

1. Mammalian cell lysis buffer: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 2 mM EDTA, and 1 μM EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN).
2. Antibody to bait protein (p53): The p53 DO-1 antibody, conjugated to agarose, was used for p53 immunoprecipitation (#sc-126AC, Santa Cruz, Biotech).
3. SDS sample buffer: 8.75 ml 1 M Tris–HCl, pH 6.8, 7.5 ml glycerol, 2.5 g SDS.
4. 2.33 g DTT, 0.1 ml Bromophenol blue solution (100 mg Bromophenol blue in 5 ml water).
5. Q.s. to 25 ml. Aliquot into 1.5 ml tubes and store at −20°C.
6. Epitope-tagged protein immunoprecipitation: Anti-myc and Anti-FLAG agarose (Sigma) (see Note 2).

2.3. SDS-Polyacrylamide Electrophoresis and Silver Staining

1. Running gel: 30:0.8% polyacrylamide/bis-acrylamide solution, 1.5 M Tris–HCl, pH 8.8, 10% SDS, 10% ammonium persulfate, TEMED. Stacking gel: 0.5 M Tris–HCl, pH 6.8. Molecular weight markers (Bio-Rad).
2. SDS-PAGE running buffer: 1× stock (1 L): 3 g Tris, 14 g glycine, and 1 g SDS.
3. Silver staining of protein gels: sodium thiosulfate, silver nitrate, 37% formaldehyde, methanol, glacial acetic acid, potassium carbonate.

2.4. Excision of Protein Bands, Silver Stain Removal, and Trypsin Digestion

1. Excision of protein bands: clean glass plate, razor blades, and siliconized tubes.
2. Silver stain removal: sodium thiosulfate, potassium ferricyanide, ammonium bicarbonate, acetonitrile, formic acid.
3. In-gel digestion of proteins with trypsin: ammonium bicarbonate, trypsin resuspension buffer (Promega, #V5111), and sequencing grade modified porcine Trypsin (Promega, #V5111).

2.5. Mass Spectrometry

1. Peptide purification: Poros 20 R2 reverse phase packing (Applied Biosystems, Foster City, CA), NanoES spray capillaries (Proxeon, Odense, Denmark), methanol, formic acid (see Note 3).

2.6. Confirmation by Western Blotting

1. Protein transfer: Western transfer buffer, 1× (1 L): 14.4 g glycine, 3 g Tris, 0.2 g SDS. Dissolve components and q.s. to 1 L with water but add 200 ml of methanol (20% methanol final). Nitrocellulose, Western blocking reagent in TBS-T (Bio-Rad), TBS-T (1×): 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20.

2. Myc-agarose and FLAG-agarose were obtained from Sigma. Primary antibodies for detection of proteins included: Anti-FLAG and anti-Myc antibodies obtained from Sigma and Santa Cruz Biotech, respectively. Anti-actin antibodies (#1615) and anti-p53 antibodies (#9282) were obtained from Santa Cruz Biotech and Cell Signaling, respectively. Secondary antibodies included IRDye800-conjugated affinity-purified rabbit anti-IgG (Rockland Immunochemicals, Gilbertsville, PA), Alexa Fluor 680-conjugated goat anti-mouse IgG antibody (Molecular Probes, Invitrogen, Carlsbad, PA), and Alexa Fluor 680-conjugated rabbit anti-goat IgG antibody (Invitrogen, Carlsbad, CA) (see Note 4).

3. Methods

3.1. Cell Growth and Harvesting

The cell lines used in this study were derived from H1299 cells which is a p53 null, non-small cell lung carcinoma cell line. Clones of H1299 cells were isolated that stably maintained the control vector (designated HC5 cells) or a vector expressing the p53 gain of function mutant, R175H (designated here as H-p53-R175H) as previously described (4–6). To express Wild-type (WT) p53, H1299 cells were transfected with DNA encoding WT p53 using Lipofectamine-2000 according to the manufacturer's instructions.

1. Cell lines were grown in DMEM supplemented with 10% (v/v) FBS and 400 μg/ml Gentamicin to maintain selection for stable clones. Approximately, hundred 10-cm dishes of HC5 or H-p53-R175H cells, respectively, were grown to ~80% confluence and harvested by trypsinization to collect adherent cells. The trypsin was inactivated by addition of fresh media to the plates and cells were subsequently collected by centrifugation (5 min at 1,000 rpm). The supernatant was discarded and the resultant cell pellet was washed 2× with 1× PBS and the cell pellet (approximately 5 g) was stored at −80°C.

3.2. Immuno-precipitation of the Bait Protein, Mutant p53

1. The cell pellet was lysed in mammalian cell lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 2 mM EDTA, and 1 μM EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN)). Cell lysates were clarified by centrifugation at 13,000 × *g* for 20 min and the resultant supernatant mixed with 250 μg of p53 DO-1 antibody conjugated agarose (#sc-126AC, Santa Cruz, Biotech) and left on a rotator overnight at 4°C.
2. The next day, the antibody-agarose conjugate was collected by gentle centrifugation (1,000 rpm for 2 min), and then washed

four times with cold lysis buffer (1 ml each wash), once with cold PBS, the supernatant removed, and the beads incubated for 5 min at 100°C in 2× SDS sample buffer.

3.3. SDS-Polyacrylamide Electrophoresis and Silver Staining

1. Fresh running and stacking gels were poured the day of the experiment. For two 12% mini-gels, 10 ml of running gel solution was prepared: (2.5 ml of 1.5 M Tris–HCl, pH 8.8, 0.1 ml of 10% SDS, 4 ml of 30:0.8 acrylamide:bis-acrylamide solution, and q.s. to 10 ml with water). Gel polymerization is initiated by the addition of 100 μL of 10% ammonium persulfate and 10 μL TEMED. A solution of 0.1% SDS in water was overlaid on the running gel to allow for gel polymerization. Then, 5 ml of stacking gel was prepared: (1.25 ml of 0.5 M Tris–HCl, pH 6.8, 50 μL of 10% SDS, 1 ml of 30:0.8 acrylamide:bis-acrylamide solution, and q.s. to 5 ml with water). Stacking gel polymerization is initiated by the addition of 50 μL of 10% ammonium persulfate and 5 μL TEMED and a 10-well gel comb was immediately inserted into the stacking gel.
2. Approximately 50 μL of sample in SDS sample buffer was loaded per lane along with protein molecular weight standards separated by several empty wells. Samples were resolved using the Bio-Rad Mini-Protean II electrophoresis system and electrophoresis carried out for approximately 45 min at 200 V, constant voltage.
3. After the electrophoresis was completed, the gels were disassembled and placed in a plastic tray in fixing solution consisting of 10% acetic acid/10% methanol/water on a table-top shaker at slow speed so as not to damage the gels. The gels were fixed for a minimum time of 30 min or left overnight on the shaker.
4. After fixing, the fixing solution was removed and gels were washed 4× in water. Each wash lasted at least 5 min and after each wash, the water was replaced with distilled, purified water.
5. Gels were then incubated in a solution of sodium thiosulfate (0.18 g/500 ml of water) for 90 s. The sodium thiosulfate solution must be made fresh each time and some of the solution (~20 ml) is saved for the developing step.
6. At the end of the 90 s, the sodium thiosulfate solution is discarded and the gels are washed again with water 3× in rapid succession (gels are covered with water and then wash water instantly discarded).
7. After the washes, the gels are stained in solution of silver nitrate (0.9 g silver nitrate in 500 ml water) for at least 10 min, with no maximum time limit. The gels should turn slightly yellow. At the end of the staining period, the silver nitrate is recovered

for re-use and the gels are washed with water 3× in rapid succession (gels are covered with water and then water instantly discarded).

8. The gels are developed by addition of developing solution: 10 g potassium carbonate, 20 ml sodium thiosulfate solution (from the first step), and 250 μL 37% formaldehyde in a total volume of 500 ml, q.s. with de-ionized, purified water. The developer amount can be scaled according to how many gels need to be stained and 50 ml is enough for one mini-gel. When the darkness of the silver stain is determined to be satisfactory, the developer is stopped by addition of fixing solution (10% MeOH/10% acetic acid/water). Gels are then scanned using a desktop scanner for permanent recording of the stained image (Fig. 1).

3.4. Excision of Protein Bands, Silver De-staining, and Trypsin Digestion

Throughout the gel band cutting and digestion procedure, care should be taken to work in a clean environment free of dust to avoid excess keratin introduction to the samples. Therefore, all surfaces should be washed prior to use and gloves worn throughout the procedure.

1. Using a light box to visualize silver-stained protein bands of interest, place the gel on a clean glass plate to allow cutting of gel bands. Gel bands should be excised with a clean razor blade, chopped into four to five pieces, and transferred to clean siliconized, eppendorf tubes. Excess unstained gel pieces should be avoided. If combining samples, no more than ~2–3 gel bands per sample should be collected to avoid excess gel matrix.
2. The method for de-stain and trypsin cleavage of proteins is adapted from earlier work (7). For each sample, add ~200 μL of a 1:1 mixture of 100 mM sodium thiosulfate and 30 mM Potassium Ferricyanide (or enough liquid to completely cover the gel bands) to the eppendorf tube. The stock solutions of 100 mM thiosulfate and 30 mM Potassium Ferricyanide can be stored for several weeks at 4°C. Incubate gel bands for at least ~10 min in the solution at room temperature with occasional agitation. After 10 min, the silver stain should disappear and the gel pieces will become yellow in color.
3. Remove the excess liquid with a pipetman, being careful not to remove the gel pieces, and wash the gel pieces 3× with ~500 μL of purified water at ~10 min per wash. At the end of the wash steps, the yellow color should be gone and the gel pieces should be colorless.
4. Add ~500 μL of 50% acetonitrile/50% 100 mM NH_4HCO_3 and incubate for 10 min at room temperature. Repeat the wash with this solution 2×.

5. Add 500 μL of 100% acetonitrile for 15 min. Gel pieces will become dehydrated and turn white. Completely remove acetonitrile with a pipetman.
6. For trypsinization, use sequencing grade modified porcine Trypsin from Promega (catalog #V5111). Promega Trypsin is packaged at 20 μg per glass vial. Add 40 μL of ice-cold trypsin resuspension buffer (included) to give a concentration of 0.5 μg/μL. Then, dilute trypsin to 0.02 μg/μL with ice-cold 50 mM NH_4HCO_3 and add 30 μL of this solution to each sample and store tube on ice for ~1 h. The trypsin will become absorbed into the gel pieces but the digestion will not begin. The final amount of trypsin is 600 ng or 0.6 μg per sample.
7. After 1 h on ice, pipette away excess liquid from gel pieces and start trypsin digestion by addition of 30 μL of 50 mM NH_4HCO_3 to each tube and incubate overnight at 37°C.
8. The next day, spin down tubes briefly in a microfuge (1,500 rpm) to collect condensation and transfer the supernatant (containing the tryptic peptides) to new siliconized eppendorf tubes. Add 30 μL of 20 mM NH_4HCO_3 to the gel pieces, incubate 15 min at room temperature, and combine the supernatant from this step with the previous supernatant.
9. Add 30 μL of 50% acetonitrile/5% formic acid/water to the gel pieces, let stand 15 min, and combine supernatant with the previous. Repeat and then discard the gel pieces.
10. Reduce the volume of samples in a speed-vacuum (~20 μL) but do not dry down samples completely. Samples can be stored at –20°C until mass spectrometry is performed.

3.5. Mass Spectrometry

1. Tryptic peptides are purified with Poros 20 R2 reverse phase packing (Applied Biosystems, Foster City, CA) and subjected to direct infusion nanospray using NanoES spray capillaries (Proxeon, Odense, Denmark) on an Applied Biosystems QSTAR pulsar XL mass spectrometer.
2. MS spectra is collected both in data-dependent acquisition mode and in manual mode using an ion spray voltage of 800 V, a curtain gas of 20, a declustering potential of 75 V and a focusing potential of 280 V.
3. For data-dependent acquisition, MS data were collected for a mass range of 400–2,000 m/z with a charge state of 2–5 which exceed one count. MS/MS (tandem MS) data were acquired for ions from a mass range of 60–2,000 m/z with a dwell time of 15 s per ion.
4. Identification of peptides is achieved by manual interpretation of MS/MS spectra with the aid of Analyst QS 1.1 (MDS Sciex, Concord, ON, Canada) and by searching the nonredundant protein database with the aid of Mascot (Matrix Science, Boston, MA) (see Note 5).

3.6. Confirmation by Western Blotting

1. To confirm the specificity of the interaction between mutant p53 and MCM-7, MCM-7 was engineered to contain a Myc epitope at the amino-terminus and p53 a FLAG epitope. Purified DNA preparations of the respective plasmids was performed and the DNA co-transfected using Lipofectamine-2000 in H1299 cells according to the manufacturer's instructions.
2. Following transfection for 48 h, H1299 cells were harvested and FLAG immunoprecipitations performed overnight at 4°C.
3. Protein immunoprecipitants were resolved by SDS-PAGE (8 or 12% gels) and transferred to nitrocellulose membranes. Following blocking of the membranes with casein blocking reagent (Bio-Rad) the membranes were incubated with anti-p53 or anti-Myc antibodies overnight at 4°C at a 1:1,000 dilution in TBS-T with casein blocking reagent.
4. Following washing of the nitrocellulose membranes to remove unbound primary antibody (4 washes, 10 min each wash with 1× TBS-T), primary antibodies were detected with secondary mouse or rabbit antibodies at a 1:7,000 dilution in TBS-T with blocking reagent. Following a similar washing step to remove unbound secondary antibodies, proteins were visualized using the Odyssey system (Li-COR) and where indicated, relative amounts of immunoreactive protein in each band were determined by densitometric analysis and normalized to the level of actin (Fig. 2).

4. Notes

1. Although we prefer Lipofectamine-2000 (Invitrogen) for transfections, there are a large number of lipid-based transfection reagents that will suffice. DNA for transfections should be of high purity as produced using commercial plasmid DNA preparation kits (for example, Qiagen maxi-preps).
2. A wide variety of epitope tags are available for validation of protein interactions, although we prefer Myc and FLAG for this purpose. For proteomics applications, FLAG-agarose is very useful as the FLAG epitope can be easily added to a bait protein of interest, generally does not interfere with protein function, and the protein complexes can be eluted with FLAG-peptide.
3. Peptide purification for mass spectrometry with Poros 20 R2 reverse phase packing and NanoES spray capillaries may not be necessary depending upon the mass spectrometry core service used. The investigator should determine the type of mass spectrometry that will be performed before investing in these materials.

4. The secondary antibodies listed here were used in conjunction with the Li-COR imaging system that allows fluorescent detection of a signal. As an alternative, standard ECL reagents can be used with HRP-conjugated secondary antibodies.
5. Although this is the mass spectrometric method we utilized to identify the proteins in hand, the type of mass spectrometer available and core services offered will ultimately determine the methodology used for mass spectrometry.

Acknowledgments

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Chapter 8

Identification of Small Molecules Affecting p53-MDM2/MDMX Interaction by Fluorescence Polarization

Qi Zhang and Hua Lu

Abstract

Fluorescence polarization (FP) has become a powerful technique to quantitatively analyze the binding of a small soluble fluorescence-labeled probe to a larger soluble protein and its displacement by other molecules. Here, we describe a detailed protocol to identify small molecules capable of targeting p53-MDM2/MDMX interactions using a fluorescence polarization assay with Rhodamine-labeled p53 peptides.

Key words: Fluorescence polarization, p53, MDM2, MDMX, Small molecules

1. Introduction

MDM2 (HDM2 in human) and MDMX (also known as MDM4) are two chief monitor proteins of the tumor suppressor p53. In a feedback fashion, they directly bind to p53, inhibit its transcriptional activity, and mediate its ubiquitination and degradation. The interactions between p53 and MDM2 or MDMX are mediated mainly by three key residues (Phe19, Trp23, and Leu26) of p53 and the hydrophobic pocket in the N-terminal domain of MDM2 or of MDMX (1–3). Despite the similarity in the p53 recognition surface of MDM2 and MDMX, a number of MDM2 inhibitors have been reported to show significantly less affinity to MDMX (1, 4, 5). Given that both MDM2 and MDMX are overexpressed in many cancers and function as the major suppressors of p53 in cells, working independently or synergistically, the identification and development of p53-MDM2/MDMX dual inhibitors are appealing and highly desirable, but still remain challenging (2, 6, 7).

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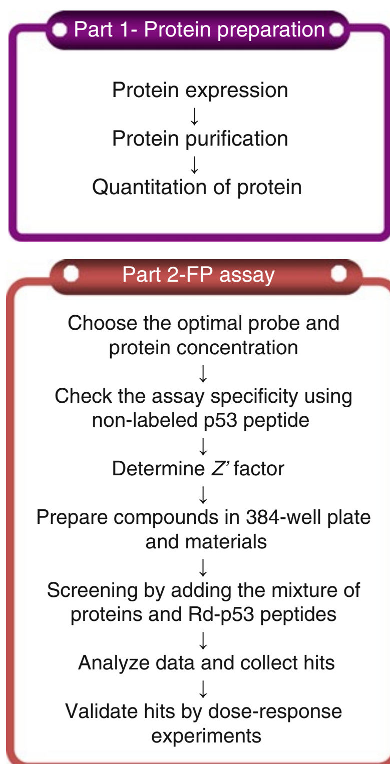


Fig. 1. Experimental workflow chart. The protocol is divided into two parts: (1) protein preparation and (2) identification of p53-MDM2/MDMX inhibitors by fluorescence polarization.

Here, we describe a step-by-step fluorescence polarization (FP) protocol to identify MDM2/MDMX dual inhibitors based on our own experience. This protocol is divided into two parts: (1) preparation of MDM2 or MDMX proteins and (2) compound identification including screening and hit validation by fluorescence polarization (FP) and data analysis (see Fig. 1). The first part includes the expression of MDM2 or MDMX proteins in bacteria, protein purification, and quantification. The second part deals with the screening of a library consisting of 900 highly soluble α-helix mimetic small molecules (7). The FP assay is based on the high affinity binding of the N-terminal domain of MDM2 or MDMX to a specific 15-amino acid sequence derived from p53 (see Fig. 2). After absorbing polarized light, unbound Rhodamine-labeled p53 peptides of relatively small molecular weight emit light in all directions due to the fast tumbling rate, resulting in low polarization. Upon binding to the target protein MDM2 or MDMX, the labeled peptides rotate slower due to the larger combined molecular size of the complex. As a consequence, they emit radiation in the same direction as that of the incident light, exhibiting higher polarization.

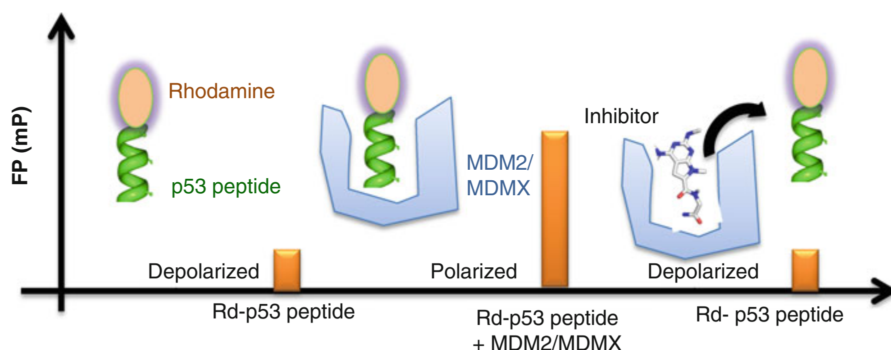


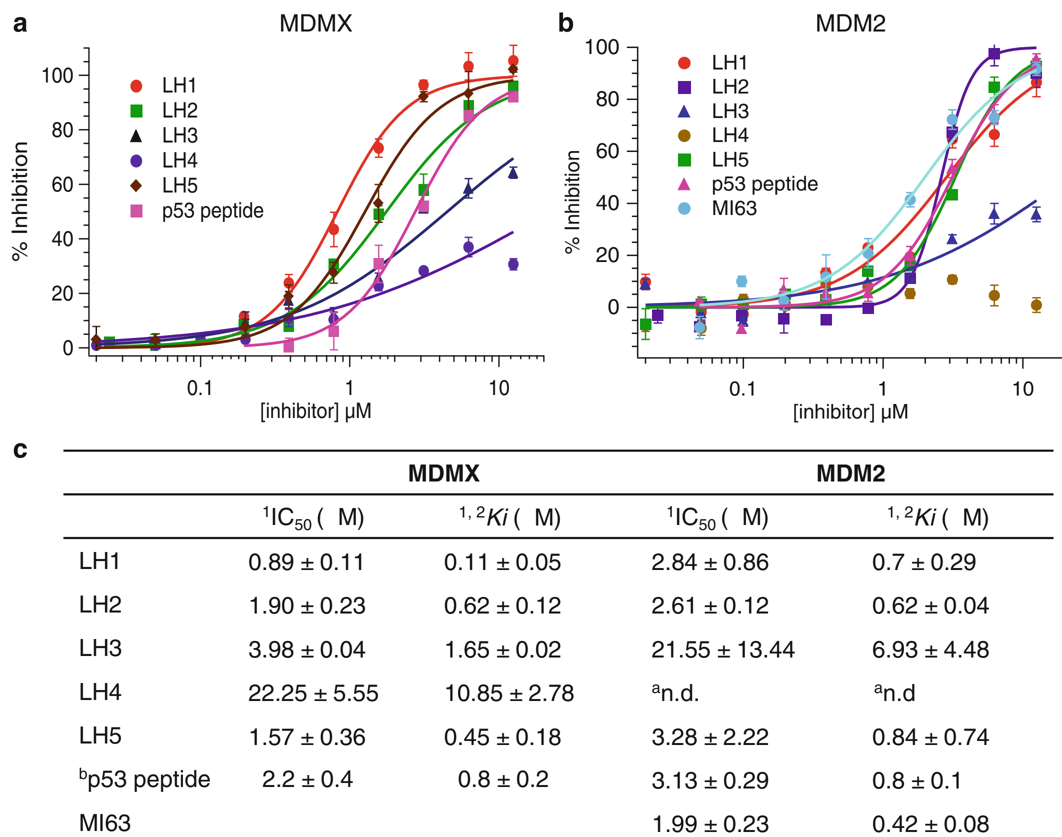
Fig. 2. A schematic representation of FP assays used to monitor the interactions between Rd-p53 peptide (SQETFSDLWKLLPEN-NH-Rhodamine) and MDMX or MDM2 protein and displacement of the peptide by small molecules.

If compounds displace the Rhodamine-labeled p53 peptides from MDM2 or MDMX, the disruption of the binding between the peptides and the protein can be identified by decreased polarization. The specificity of this FP assay is confirmed by the competitive displacement of unlabeled p53 peptides and the specific MDM2 inhibitor MI-63 (4). The primary screen of the 900-compound library at 40 μM yielded seven putative hits that inhibited the p53-MDM2/MDMX interaction by at least 50%. By subsequent dose-dependent validation experiments with these compounds, we verified the top three hits, denoted as LH1, 2, and 5, which acted as dual inhibitors of MDMX- and MDM2-p53 interactions at *Ki* values lower than 3 μM (see Fig. 3). Two of them were evaluated for their ability to trigger apoptosis through a p53-dependent pathway by binding to MDMX and MDM2 and inhibiting their function toward p53 (7). Thus, this FP assay can be used for identification of small molecule inhibitors that disrupt the MDM2-p53 or MDMX-p53 interactions in vitro.

2. Materials

2.1. Reagents

1. 1 M Dithiothreitol (DTT): Dissolve 1.54 g in 10 ml water.
2. Protease inhibitor cocktail: leupeptin 1.42 mg, pepstain A 6.85 mg, Benzamidine 1.65 g, and PMSF 0.85 g. Dissolve in 50 ml 100% EtOH and stored in −20°C.
3. 1 M Isopropyl-β-D-thiogalactoside, dioxin-free (IPTG): dissolve 2.38 g in 10 ml water.
4. BSA (Bovine serum albumin, EMD Chemicals, cat. no. 2930).
5. PreScission protease (GE Healthcare, cat. no. 27-0843-01).



	MDMX		MDM2	
	[1]IC_{50} (M)	[1,2]*Ki* (M)	[1]IC_{50} (M)	[1,2]*Ki* (M)
LH1	0.89 ± 0.11	0.11 ± 0.05	2.84 ± 0.86	0.7 ± 0.29
LH2	1.90 ± 0.23	0.62 ± 0.12	2.61 ± 0.12	0.62 ± 0.04
LH3	3.98 ± 0.04	1.65 ± 0.02	21.55 ± 13.44	6.93 ± 4.48
LH4	22.25 ± 5.55	10.85 ± 2.78	[a]n.d.	[a]n.d
LH5	1.57 ± 0.36	0.45 ± 0.18	3.28 ± 2.22	0.84 ± 0.74
[b]p53 peptide	2.2 ± 0.4	0.8 ± 0.2	3.13 ± 0.29	0.8 ± 0.1
MI63			1.99 ± 0.23	0.42 ± 0.08

1. IC_{50} and Ki value represent the average of three replicates alongside the standard error.
2. Calculation of Ki assumes competitive inhibition

Fig. 3. Validation of hits obtained from the primary screen for the competition of Rd-p53 binding to human MDMX (amino acids 1–137) (**a**) and human MDM2 (amino acids 1–118) (**b**) by fluorescence polarization. (**c**) IC_{50} and *Ki* values of inhibitors. [a]Not determined. [b]Unlabeled 15-mer p53 peptide (SQETFSDLWKLLPEN).

6. LB medium: dissolve 25 g of LB broth Miller in ~800 ml distilled water (dH_2O). Add dH_2O to a final volume of 1 L. Autoclave using liquid cycle. Tighten lid and store at room temperature.
7. LB agar plates: add 15 g of LB agar Miller and 25 g of LB broth Miller to 1 L of dH_2O. Cover flasks tightly with foil. Autoclave them using the liquid cycle, then allow to cool to 50°C. Add 1 ml of a 100 mg/ml ampicillin stock to obtain a final concentration of 100 μg/ml. Pour a thin layer of LB agar into each petri dish (10-cm diameter) and cover with lid immediately. Let plates cool for a few hours or overnight. Store the plates in a plastic bag at 4°C.
8. PBS: dissolve 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2PO_4 \cdot 2H_2O$, and 0.24 g KH_2PO_4 in 1 L of deionized H_2O, adjust pH 7.4, and then store at 4°C.

9. Glutathione agarose beads (Thermo Scientific Pierce, cat. no. 15160).
10. Millipore-Amicon ultra-15 device.
11. Poly-Prep Chromatography Columns (Bio-Rad Laboratories, cat. no. 731-1550).
12. 5× FP Assay Buffer: 5× PBS, 0.025% Tween-20, pH 7.5, 0.5% BSA.
13. 384-well black plates (cat. no. 262260, Nalge Nunc International).
14. pGEX- 6P-1 plasmid coding p53-binding domain of human MDMX (a.a. 1–137) or human MDM2 (a.a. 1–118) was developed in the Lu laboratory (Indiana University School of Medicine, IN).
15. p53 peptides (SQETFSDLWKLLPEN-NH_2) and N-terminally labeled Rhodamine p53 peptides (SQETFSDLWKLLPEN-NH-Rhodamine) were synthesized by Antagene Inc.

2.2. Equipment

1. Sonicator (e.g., Fisher Scientific Model 100).
2. Mini-PROTEAN Tetra Cell (Bio-Rad, cat. no. 165-8000).
3. Incubator shaker (e.g., Excella E25, New Brunswick Scientific).
4. End-over-end rotation apparatus (e.g., Rotospin test tube rotator, Tarsons).
5. Nanodrop 2000c spectrophotometer (or other standard protein assay kit).
6. Benchtop centrifuge for 96-well plates (e.g., ALC PK120 plate centrifuge, DJB Labcare).
7. Plate reader capable of FP measurements (e.g., SpectraMax M5[e] (Molecular Devices)).

3. Methods

3.1. Protein Preparations

3.1.1. Expression and Purification of MDMX (a.a. 1–137) and MDM2 (a.a. 1–118) Proteins

1. Transform pGEX-6P1 plasmid coding p53-binding domain of human MDMX (a.a. 1–137) or human MDM2 (a.a. 1–118) into competent *E. coli* BL21 (DE3) cells by a standard heat-shock method (8).
2. Prepare LB agar plates containing 100 μg/ml ampicillin.
3. Plate the cells on the agar plates using a sterile loop and incubate overnight at 37°C.
4. Prepare liquid LB medium with 100 μg/ml ampicillin.

5. Using a sterile pipette tip, touch a single colony of bacteria from your agar plate. Inoculate the liquid LB by swirling the tip in it.
6. Incubate the culture in an orbital shaker at 250 rpm overnight (~12 h) at 37°C.
7. Prepare 1 L LB medium with 100 μg/ml ampicillin.
8. Inoculate the 1 L LB medium with 5 ml of the overnight culture.
9. Grow the cultures in an orbital shaker, at 200 rpm and at 37°C until the OD_{600} reaches about 0.6–0.8 (after ~4 h) (see Note 1).
10. Add 1 ml of 0.5 M IPTG (final concentration, 0.5 mM) to induce the cells.
11. Incubate induced cultures at 28°C with orbital shaking at 200 rpm (see Note 1).
12. At 4–5 h after induction, centrifuge the cells at 6,000 × *g* for 5 min at 4°C (see Note 2).
13. Discard the supernatants, wash the pellets with 10 ml cold PBS by gently pipetting the cells, and then spin down at 6,000 × *g* for 5 min.
14. Re-suspend the pellets in 50 ml PBS lysis buffer (freshly add 50 μl protease inhibitor cocktail solution and 200 μl of 1 M DTT (final concentration, ~4 mM) to 50 ml of ice-cold PBS, pH 7.5, 10% glycerol) (see Note 3). Vortex until the pellets are fully re-suspended.
15. Incubate the suspension with lysozyme (50 μL of a solution of 100 mg/ml; final concentration, 100 μg/ml) on ice for 30 min.
16. Sonicate lysates on ice for six-ten 20s bursts at 300W with a 20s cooling period between each burst and centrifuge at 10,000 × *g* for 30 min at 4°C.
17. Collect the supernatants. Take 10–100 μl of the supernatants to quantify protein expression by electrophoresis and brilliant coomassie blue staining.
18. Transfer 4 ml 50% slurry of glutathione agarose beads to a 50-ml tube. Sediment the slurry by centrifugation at 400 × *g* for 5 min. Aspirate the supernatants carefully and discard them.
19. Wash glutathione agarose beads by adding 20 ml of cold PBS and separate the beads by centrifugation at 400 × *g* for 5 min. Aspirate the supernatants carefully and discard. Repeat three times.
20. Add 50 ml of the cleared lysates to the beads and mix by gentle inversion.
21. Incubate the mixture for 2–3 h at 4°C with gentle end-over-end rotation.

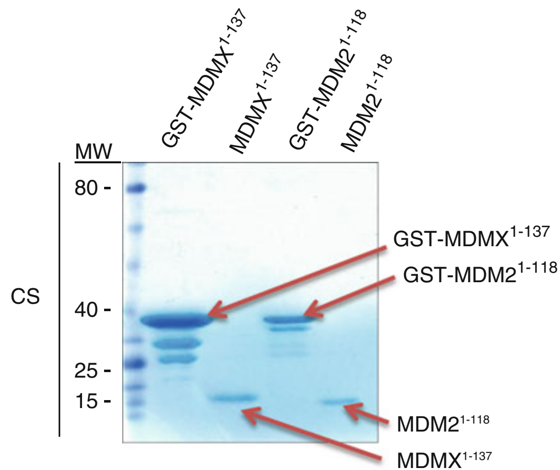


Fig. 4. Coomassie-stained gel showing the purified recombinant MDM2$^{1–118}$ and MDMX$^{1–137}$ with or without GST tag produced in *E. coli* BL21-(DE3).

22. Pour the mixture into an empty Poly-Prep Chromatography column. Tap the column and allow the beads to settle.
23. Open the column outlet and collect the flow-through until the column completely drains. Take an aliquot (~20 μl) of the flow-through for analysis of nonbound proteins by electrophoresis and coomassie staining.
24. Close the column outlet and add 10 ml of cold PBS lysis buffer to the column. Incubate for 10 min with gentle end-over-end rotation (see Note 4). Open the column outlet and allow the column to drain. Repeat at least five times (see Note 5). Take some beads (~20 μl) for analysis by electrophoresis and coomassie staining (see Fig. 4).
25. Prepare the PreScission protease mix by adding 200 μl (400 U) of the PreScission protease to 5 ml of cold PBS lysis buffer.
26. Load the PreScission protease mixture onto the column and incubate with gentle end-over-end rotation for ~12 h at 4°C (see Note 6).
27. Open the column outlet and collect eluates, which contain non-GST-tagged of purified MDM2 or MDMX proteins. The PreScission enzyme contains a GST tag, so it remains bound to the column.
28. Add 4 ml PBS lysis buffer to the column and rotate for 10 min at 4°C. Collect the eluates, which also contain purified MDM2 or MDMX proteins. Repeat five times. Save aliquots (~10 μl) of each elute for analysis by the Bradford assay, with subsequent electrophoresis analysis with coomassie staining (see Fig. 4).

29. Collect the elution and transfer it to a pre-cooled 50 ml ultrafiltration tube (Millipore-Amicon ultra), centrifuge at 2,000 × *g* until the protein concentration goes to 2 mg/ml (see Note 7). Save a small aliquot (~10 μl) for protein quantification by the Bradford assay, electrophoresis, and coomassie staining using BSA as a standard.
30. Aliquot purified proteins into small volumes (~100 μl) and freeze them in liquid nitrogen or store them in a −80°C freezer. The purified proteins are stable at −80°C for several months.

3.1.2. Quantification of the Concentration of MDM2 and MDMX Proteins

1. Determine the total protein concentration using Nanodrop 2000c spectrophotometer (or other standard protein assays), according to the manufacturer's instructions.
2. Load 1–10 μg per lane (at least 2 lanes per protein) of purified proteins, and 1, 2, 4, 10, 20 μg per lane of BSA and molecular weight markers onto a 15% SDS-PAGE gel. Run the gels with the Mini-PROTEAN Tetra Cell, following the manufacturer's instructions. Perform EZ-Run Protein Gel Staining according to the manufacturer's instructions.
3. Quantify the intensity of all bands in each lane for each protein corresponding to BSA (~67 kDa), MDM2 (~13 kDa), or MDMX (~15 kDa). Use ImageJ (freely available from: http://rsbweb.nih.gov/ij/download.html) or custom software provided by the manufacturer of the gel quantification software.
4. Establish the linear relationship between the amounts of BSA loaded and quantification of the detected band intensity.
5. Using the established BSA amount-band intensity, calculate the percentage of total MDM2 or MDMX proteins in each preparation.

3.2. Fluorescence Polarization Assay

3.2.1. Choosing the Working Probe and Protein Concentration

1. Dilute 10 μl 1 mM DMSO stock of Rhodamine-labeled p53 peptides (Rd-p53 peptide) 1:100 by adding 990 μl FP assay buffer (final concentration: 10 μM) (see Notes 8 and 9).
2. Prepare 12 dilutions at twofold dilution of the Rd-p53 peptides in a 384-well plate: dispense 60 μl FP assay buffer to wells A2–A12. Pipette 60 μl of 10 μM Rd-p53 to A1 and A2. After mixing A2, transfer 60 μl from A2 to A3, and continue this twofold dilution all the way to well A12. Discard the excess 60 μl from A12. In this way, there are 12 dilutions 1:2 serial dilution of the peptides at the highest concentration of 10 μM (A1).
3. Centrifuge the plate for 2 min at 200 × *g* (see Note 10).
4. Place the plate into a SpectraMax M5^e plate reader (Molecular Devices). Open the Softmax Pro software and select the FP option. Set the excitation at 531 nm and emission at 595 nm. Read fluorescence polarization at room temperature (RT,

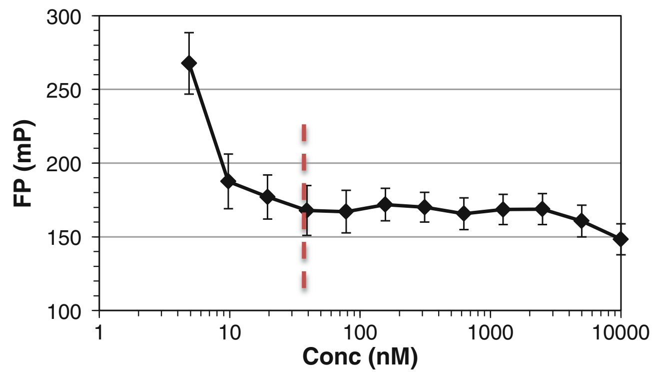


Fig. 5. The optimal concentration of the free probe for FP assay is determined by varying the concentration of the free probe and identify the lowest concentration that starts to give a stable polarization signal.

19–25°C). Select the fluorescence option and read the total fluorescence.

5. Transfer the Softmax Pro data to Microsoft Excel. Plot the data points (fluorescence polarization as a function of probe concentration) and identify the optimal concentration of the probe (Rd-p53 peptide) which starts to show stable FP signal (see Fig. 5) (see Note 11).
6. Prepare 12 dilutions at twofold dilution of MDM2/MDMX proteins in a 384-well plate. The final volume of diluted proteins in each well is 20 μl.
7. Add 40 μl of 75 nM Rd-p53 peptide in each well for a final concentration of 50 nM Rd-p53 peptide per well.
8. Spin down the plate for 2 min at 200 × *g* then cover the plate, incubate for 30 min and then measure the fluorescence polarization and total fluorescence in the SpectraMax plate reader (see Note 12).
9. Transfer the Softmax Pro data to Microsoft Excel. Using any curve fitting analysis software (e.g., Igor Pro), plot the mP values versus the concentrations of the proteins. Calculate the K_D values using the Hill equation (see Fig. 6).

3.2.2. Specificity of FP Assay

1. Prepare 12 dilutions at twofold dilutions of non-labeled p53 peptides in 20 μl FP assay buffer in the 384-well plate. The final volume of diluted peptides in each well is 20 μl.
2. Dispense 40 μl assay solution containing 1.5 μM of MDM2 or MDMX and 75 nM of the Rd-p53 peptide. The final protein concentration is 1 μM and the probe concentration is 50 nM in a final volume of 60 μl per well.

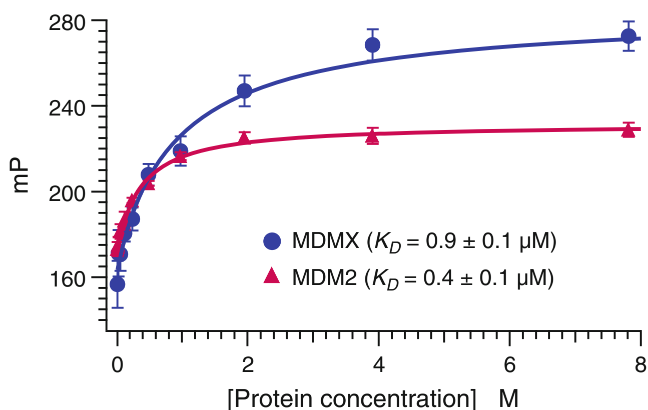


Fig. 6. The saturation curve of Rd-p53 peptide to recombinant $MDMX^{1-137}$ and $MDM2^{1-118}$ proteins.

3. Spin down the plates for 2 min at 200 × *g* following incubation at room temperature for 10 min.
4. Measure the fluorescence polarization signal with excitation at 531 nm and emission at 595 nm using a SpectraMax plate reader.
5. Analyze the data according to Protocol 3.2.7.

3.2.3. Effect of DMSO Concentration on p53-MDM2/MDMX Binding

1. Prepare 12 dilutions at twofold dilutions of DMSO in the 384-well plate in 20 μl assay buffer.
2. Add 40 μl FP assay buffer containing 75 nM Rd-p53 peptide and 1.5 μM MDM2/MDMX proteins. The final highest concentration of DMSO is 33.3% in a final volume of 60 μl per well.
3. Spin down the plates for 2 min at 200 × *g* following incubation at RT for 30 min.
4. Determine the fluorescence polarization in the SpectraMax plate reader.
5. Transfer the Softmax Pro data to Microsoft Excel. Plot fluorescence polarization signal as a function of the % DMSO to find the minimum DMSO concentration which does not interfere with the binding (see Fig. 7).

3.2.4. Determining Z′-Factor

1. Prepare 1,200 μl of the probe solution containing 50 nM Rd-p53 peptides in FP assay buffer.
2. Prepare 1,200 μl of the protein solution containing 1 μM of the MDM2 or MDMX proteins and 50 nM Rd-p53 peptides in FP assay buffer.

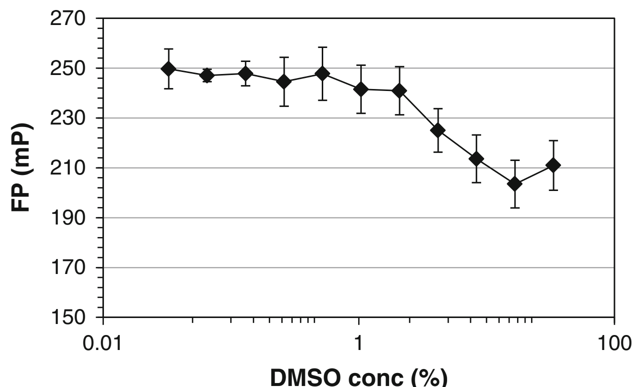


Fig. 7. The effect of DMSO concentration on the stability of FP binding experiments. The measurements were carried out by titration of DMSO into the mixture of Rd-p53 (50 nM) and MDMX proteins (1 μM). A stable FP was observed at lower than 4% DMSO, suggesting that the assay is safe for screening compounds within 4% DMSO.

3. Dispense 60 μl/well of the probe solution (no protein) to wells A24–P24 (totally 16 wells). These are positive controls (100% inhibition).
4. Dispense 60 μl/well of the protein solution to wells A23–P23 (totally 16 wells). These are negative controls (0% inhibition).
5. Centrifuge the plate for 2 min at 200 × *g* then cover the plate.
6. Incubate for 30 min and then determine the fluorescence polarization using the SpectraMax plate reader.
7. Calculate *Z*′-factor according to Protocol 3.2.7.

3.2.5. Screening

1. Prepare compounds in 100% DMSO in 96-well plates at 2 mM concentration. Each plate should have non-labeled p53 peptides and MI63 as controls. Add 100% DMSO into the wells of the last column (column 12) for the negative controls. Thus, each plate contains 88 compounds. This is the parent plate (see Fig. 8) (see Note 13).
2. Transfer the compounds from the parent plates, in duplicate, to 384-well plates. Pipette 3 μl of each compound from the 96-well parent plates into 47 μl of FP assay buffer in the 384-well plates according to the plate map in Fig. 9. For example, the compound is transferred from the well A1 in the 96-well plates to the wells A1 and A2 in the 384-well plates. Thus, each 384-well plate contains the compounds from two 96-well parent plates. The last two columns (Columns 23 and 24) correspond to 6% DMSO without compounds. This is the dilution plate (see Notes 14–16).

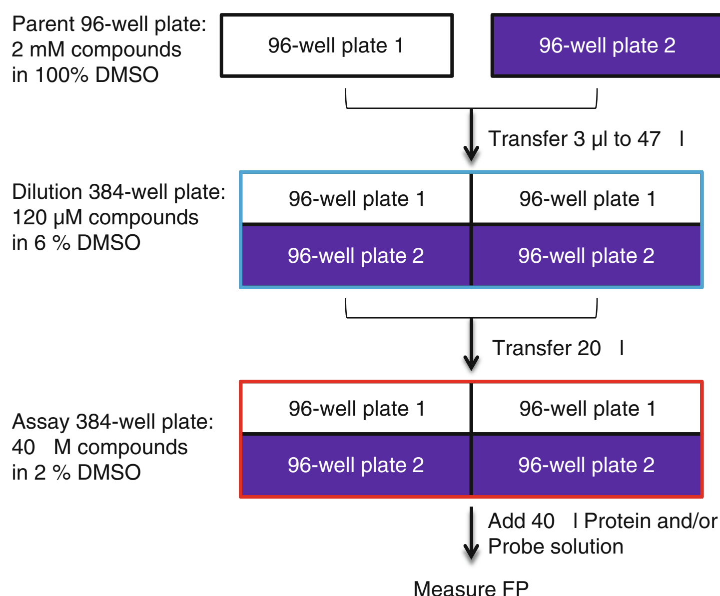


Fig. 8. Workflow chart: preparation of plates for the screening by FP assay. A single well of the parent 96-well plate is repeated in duplicate in the 384-well plate.

3. Pipette 20 μl of 6% DMSO compound stocks to a 384-well plate, this is the assay plate.
4. Thaw a sufficient amount of MDM2 or MDMX proteins on ice.
5. Prepare solution 1 containing 75 nM Rd-p53 peptides and 1.5 μM MDM2 or MDMX proteins (1.5× solution) and solution 2 containing only 75 nM Rd-p53 peptides (1.5× solution), which are sufficient for the whole plate reactions.
6. Dispense 40 μl protein/probe solution 1 to all wells except the last column to the assay plate.
7. Add 40 μl of probe/buffer solution 2 to the last column: positive control (no proteins) wells.
8. Spin down the plate for 2 min at 200 × *g*.
9. Incubate for 30 min at room temperature. Keep plates covered and in the dark.
10. Measure the fluorescence polarization using a SpectraMax plate reader (see Note 17).
11. Analyze data according to Protocol 3.2.7.

3.2.6. FP Dose-Response Validation Assay

1. To each hit compound to be tested, prepare 11 dilutions at twofold serial dilution in 100% DMSO at 2 mM concentration in a 384-well plate. Add 100% DMSO into the wells of the last two columns of the 384-well plate (columns 23 and 24) for the controls. This is the parent plate.

	Rd-p53 + MDM2/MDMX proteins																							Rd-p53
	Compounds																						Negative	Positive
	Duplicate																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	A1	A1	A2	A2	A3	A3	A4	A4	A5	A5	A6	A6	A7	A7	A8	A8	A9	A9	A10	A10	A11	A11	DMSO	DMSO
B	A1	A1	A2	A2	A3	A3	A4	A4	A5	A5	A6	A6	A7	A7	A8	A8	A9	A9	A10	A10	A11	A11	DMSO	DMSO
C	B1	B1	B2	B2	B3	B3	B4	B4	B5	B5	B6	B6	B7	B7	B8	B8	B9	B9	B10	B10	B11	B11	DMSO	DMSO
D	B1	B1	B2	B2	B3	B3	B4	B4	B5	B5	B6	B6	B7	B7	B8	B8	B9	B9	B10	B10	B11	B11	DMSO	DMSO
E	C1	C1	C2	C2	C3	C3	C4	C4	C5	C5	C6	C6	C7	C7	C8	C8	C9	C9	C10	C10	C11	C11	DMSO	DMSO
F	C1	C1	C2	C2	C3	C3	C4	C4	C5	C5	C6	C6	C7	C7	C8	C8	C9	C9	C10	C10	C11	C11	DMSO	DMSO
G	D1	D1	D2	D2	D3	D3	D4	D4	D5	D5	D6	D6	D7	D7	D8	D8	D9	D9	D10	D10	D11	D11	DMSO	DMSO
H	D1	D1	D2	D2	D3	D3	D4	D4	D5	D5	D6	D6	D7	D7	D8	D8	D9	D9	D10	D10	D11	D11	DMSO	DMSO
I	E1	E1	E2	E2	E3	E3	E4	E4	E5	E5	E6	E6	E7	E7	E8	E8	E9	E9	E10	E10	E11	E11	DMSO	DMSO
J	E1	E1	E2	E2	E3	E3	E4	E4	E5	E5	E6	E6	E7	E7	E8	E8	E9	E9	E10	E10	E11	E11	DMSO	DMSO
K	F1	F1	F2	F2	F3	F3	F4	F4	F5	F5	F6	F6	F7	F7	F8	F8	F9	F9	F10	F10	F11	F11	DMSO	DMSO
L	F1	F1	F2	F2	F3	F3	F4	F4	F5	F5	F6	F6	F7	F7	F8	F8	F9	F9	F10	F10	F11	F11	DMSO	DMSO
M	G1	G1	G2	G2	G3	G3	G4	G4	G5	G5	G6	G6	G7	G7	G8	G8	G9	G9	G10	G10	G11	G11	DMSO	DMSO
N	G1	G1	G2	G2	G3	G3	G4	G4	G5	G5	G6	G6	G7	G7	G8	G8	G9	G9	G10	G10	G11	G11	DMSO	DMSO
O	H1	H1	H2	H2	H3	H3	H4	H4	H5	H5	H6	H6	H7	H7	H8	H8	H9	H9	H10	H10	H11	H11	DMSO	DMSO
P	H1	H1	H2	H2	H3	H3	H4	H4	H5	H5	H6	H6	H7	H7	H8	H8	H9	H9	MI63	MI63	Peptide	Peptide	DMSO	DMSO

Fig. 9. 384-well plate layout for the screening of MDM2/MDMX inhibitors by FP. All wells in columns 1–24 contain Rd-p53 (50 nM). Columns 1–23 include the proteins (1 μM) and compounds (40 μM) or DMSO (column 23).

2. Add 3 μl of each compound from the parent plates to 47 μl of FP assay buffer in a 384-well plate. This is the dilution plate and these intermediate stocks are at 6% DMSO.
3. Pipette 20 μl diluted compounds from the dilution plate to a new 384-well microplate, this is the assay plate. The final DMSO concentration is 2%.
4. Thaw a sufficient amount of MDM2 or MDMX proteins on ice.
5. Prepare solution 1 containing 75 nM Rd-p53 peptide and 1.5 μM MDM2 or MDMX proteins (1.5× solution) and solution 2 containing only 75 nM Rd-p53 peptides (1.5× solution), which are sufficient for the whole plate reactions.
6. Dispense 40 μl protein/probe solutions to all wells of the assay plates except the last column.
7. Add 40 μl of probe/buffer to the last column: positive control (no proteins) wells.
8. Spin down the plate for 2 min at 200 × *g*.
9. Cover the plate with a new plate seal. Incubate for 30 min at room temperature. Keep plates covered and in the dark.
10. Measure the fluorescence polarization using a SpectraMax plate reader.
11. Analyze the data according to Protocol 3.2.7.

3.2.7. Data Analysis

1. Transfer the Softmax Pro data to Microsoft Excel.
2. Calculate the average and the standard deviations of the negative control samples (column 23).
3. Calculate the average and the standard deviations of the positive control samples (column 24).
4. Calculate the Z′ factor for each plate:

$$Z'\text{factor} = 1 - \frac{3 \times (\text{SD}_+ + \text{SD}_-)}{|\mu_+ - \mu_-|},$$

where μ_+ and μ_- represent the means of the positive and negative control signals, respectively, and SD_+ and SD_- are standard deviations of the mean values for the positive and negative controls, respectively (see Note 18).

5. Calculate the percent inhibition of each sample as follows:

$$\text{Inhibition\%} = \frac{\text{Negative} - \text{Sample}}{\text{Negative} - \text{Positive}} \times 100\%$$

Inhibitory activity is calculated as the mean FP value of negative controls minus the sample FP value divided by the mean FP value of negative controls minus the mean FP value of positive controls, multiplied by 100.

6. For each compound, percentage inhibitions were plotted against the compound concentration (see Fig. 3). Compounds greater than a certain cutoff are considered hits. For example, compounds with greater than 50% inhibition of MDM2/MDMX-p53 binding at 40 μM are defined as active hits of the primary screening. The primary screening active hits proceed to the dose-response confirmation stage.
7. For the dose-response confirmation, using suitable nonlinear regression analysis software such as Igor Pro, plot the calculated percent inhibition values versus the log of the concentrations of the test compound. Calculate the IC_{50} values using Hill equation.
8. *Ki* values were calculated by a web-based computer program developed for FP-based binding assays (http://sw16.im.med.umich.edu/software/calc_ki/) (9).

4. Notes

1. Expression of GST-MDM2 or GST-MDMX at higher cell densities (A_{600} > 0.5) and reduced temperature (28°C) results in greater yields of intact forms of the soluble proteins.
2. All procedures hereafter should be performed at 4°C to minimize protein degradation.
3. Add glycerol and DTT (1–10 mM) into the lysis buffer. Glycerol and DTT prevent degradation of proteins and increase the binding of proteins to glutathione agarose beads.
4. Each washing of the glutathione agarose beads after protein binding at slow speed or longer incubation time (10–30 min) is important to ensure the purity of GST fusion proteins.
5. After the final wash, drain the column completely to avoid dilution of the eluates.
6. The conditions for cleavage need to be optimized (e.g., amount of protein, time, and temperature).
7. Concentrate proteins to a concentration below 2 mg/ml because they may precipitate at higher concentrations.
8. Keep in mind that fluorophores are light-sensitive and pH-sensitive. Prepare the stock of Rhodamine-labeled p53 peptides in 100% DMSO, aliquot, and keep them from light.
9. High concentration of peptide stock (>10 mM) may lead to poor solubility when dissolved in the buffer.
10. Make sure to centrifuge the plate to remove bubbles and keep the reaction solution on the bottom.

11. Choose the lowest concentration (to keep it below its K_D) that gives minimal variations of FP signal. We use 50 nM Rd-p53 peptides for the FP assay (protocols 3.2.5 and 3.2.6).
12. Determine the total fluorescence to assess whether the fluorescence of Rd-p53 changes when it binds to the protein. If binding affects the fluorescent properties of the fluorophore, the latter should be conjugated through another position, or the use of another fluorescent ligand and/or protein fragment should be explored.
13. For each compound to be screened, to make sure that the compounds do not interfere with the polarization assay, determine their fluorescence in the working fluorescence wavelength range and eliminate those that show fluorescence.
14. For all solutions in the reaction plates, mix the reaction solution well using the pipette, a pipetting system (e.g., Precission from BioTek Instruments, Inc.), an orbital shaker at 500–700 rpm, or shaking in the plate reader to ensure homogeneity.
15. Change the pipette tips or thoroughly rinse the tips if you are using a pipetting robot with permanent tips.
16. This preparation of plates for the FP assay involves systems that employ pipette tips. For systems that use the pintool device, the protocol described here needs to be adjusted accordingly.
17. It takes about 10–15 min to measure the fluorescent polarization of the entire plate depending on the plate readers. Consider intervals and arrange the time if you have more than one plate to measure.
18. The Z′ factor, indicating the quality of a FP assay, is expected to be higher than 0.7, based on negative (containing Rd-p53 peptides with MDMX or MDM2 proteins) and positive (containing Rd-p53 peptides only) controls (16 data points per positive and negative controls). If the Z′-factor is below 0.5, the plate has to be rescreened in order to increase the assay quality. The concentration of the probe and protein need to be optimized to improve the Z′-factor.

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Chapter 9

Determine the Effect of p53 on Chemosensitivity

Emir Senturk and James J. Manfredi

Abstract

The p53 tumor suppressor protein plays a central role in mediating the cellular response to a variety of stresses. Activation of p53 signaling will trigger cell cycle arrest or apoptosis in normal cells, depending on such factors as cell type and genetic context. The ability of a cell to circumvent either of these p53-directed outcomes leads to inappropriate proliferation, thereby contributing to the development of cancer. As such, tumors frequently escape the apoptotic pathway in response to cell stress. DNA-damaging agents, however, achieve significant tumor cytotoxicity in spite of this hallmark characteristic. Tumors treated with DNA-damaging drugs often undergo alternate forms of cell death, such as senescence or mitotic catastrophe, in addition to apoptosis that may ultimately lead to regression. Although not a predictor of chemotherapy response in patients per se, p53 status in tumor-derived cells is frequently a determinant of the death pathway promoted by these agents. The cytotoxic effects of DNA-damaging agents can be readily appreciated using such tools as cell cycle analysis, phopsho-$H3^{Ser10}$ immunoblotting, and annexin V detection.

Key words: p53, Tumor cells, DNA damage, Chemotherapy, Chemosensitivity, Drug response, Cell cycle, Apoptosis, Senescence, Mitotic catastrophe

1. Introduction

The p53 tumor suppressor protein is responsible for impeding the proliferation of cells in response to a variety of stresses, including genotoxic stress (1). In normal, healthy cells, p53 achieves this outcome by triggering either cell cycle arrest at the G1/S and G2/M checkpoints or apoptosis (2). Cells are thus able to prevent the propagation of potentially oncogenic mutations or other genetic aberrations. A testament to the critical role of p53 in protecting against such genomic instability is the observation that p53 is the most commonly mutated gene in human malignancies (2). Furthermore, p53-null mice develop tumors at an accelerated

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rate, often succumbing to disease by 6 months (3). Loss of p53 function constitutes a mechanism of checkpoint bypass and escape from apoptosis, the latter of which is a well-established hallmark of cancer (4).

Initial studies of cellular response to antineoplastic agents put forth that p53-directed apoptosis was the common underlying mechanism of chemotherapy and radiation response in tumors (5, 6). Indeed, certain tumors, particularly hematologic malignancies, undergo a robust, p53-dependent apoptotic response to extrinsic DNA damage (7). These findings further suggested that loss of p53 by mutation or deletion would render a given tumor resistant to treatment with genotoxic agents. Subsequent work in p53-null cells and animal models, however, revealed that DNA-damaging treatments are able to achieve significant cytotoxicity in a p53-independent manner (8–10) (Fig. 1). Moreover, epithelial tumor lines and solid tumor models have been observed to respond to DNA damage despite not undergoing frank apoptosis (11). As the overwhelming majority of human malignancies are epithelial in origin, these alternate forms of DNA damage-induced tumor cell death are of particular interest in assessing tumor response to conventional cancer treatments, including topoisomerase inhibitors, cross-linking agents, and ionizing radiation.

Tumors in which the wild-type p53 gene remains expressed respond to DNA damage in a cell type- and genetic context-dependent manner, as discussed elsewhere (2). Under such circumstances, p53 and its downstream effectors may remain intact and responsive to DNA damage but have become insensitive to other oncogenic stresses (e.g., signaling stress) as a result of mutations in genes encoding upstream p53 activators (e.g., $p14^{ARF}$). Treatment of many epithelial neoplasms with DNA-damaging agents in cell culture, for example, will often provoke p53-dependent arrest at the G1/S and G2/M checkpoints that will prevent further progression through the cell cycle until the damage is repaired (12, 13). Removal of the offending compound will lead to eventual resolution of these checkpoints and resumption of the cell cycle (12). If DNA damage persists, perhaps by means of an effective dosing strategy, cells arrested in the cell cycle may ultimately senesce, as discussed below. Alternatively, tumors that arise from tissues known to be exquisitely sensitive to DNA damage, such as bone marrow or thymus, forego cell cycle arrest in favor of robust p53-dependent apoptosis (14, 15). In these tumors, loss of p53 has been associated with resistance to DNA-damaging therapies (5) (Fig. 1). The mechanistic basis of these divergent p53-dependent outcomes in response to DNA damage is a subject of continued investigation.

More recently, sustained p53 activation in the setting of DNA damage has also been shown to trigger accelerated cell senescence (16). Senescence refers to a permanent growth-arrested state in

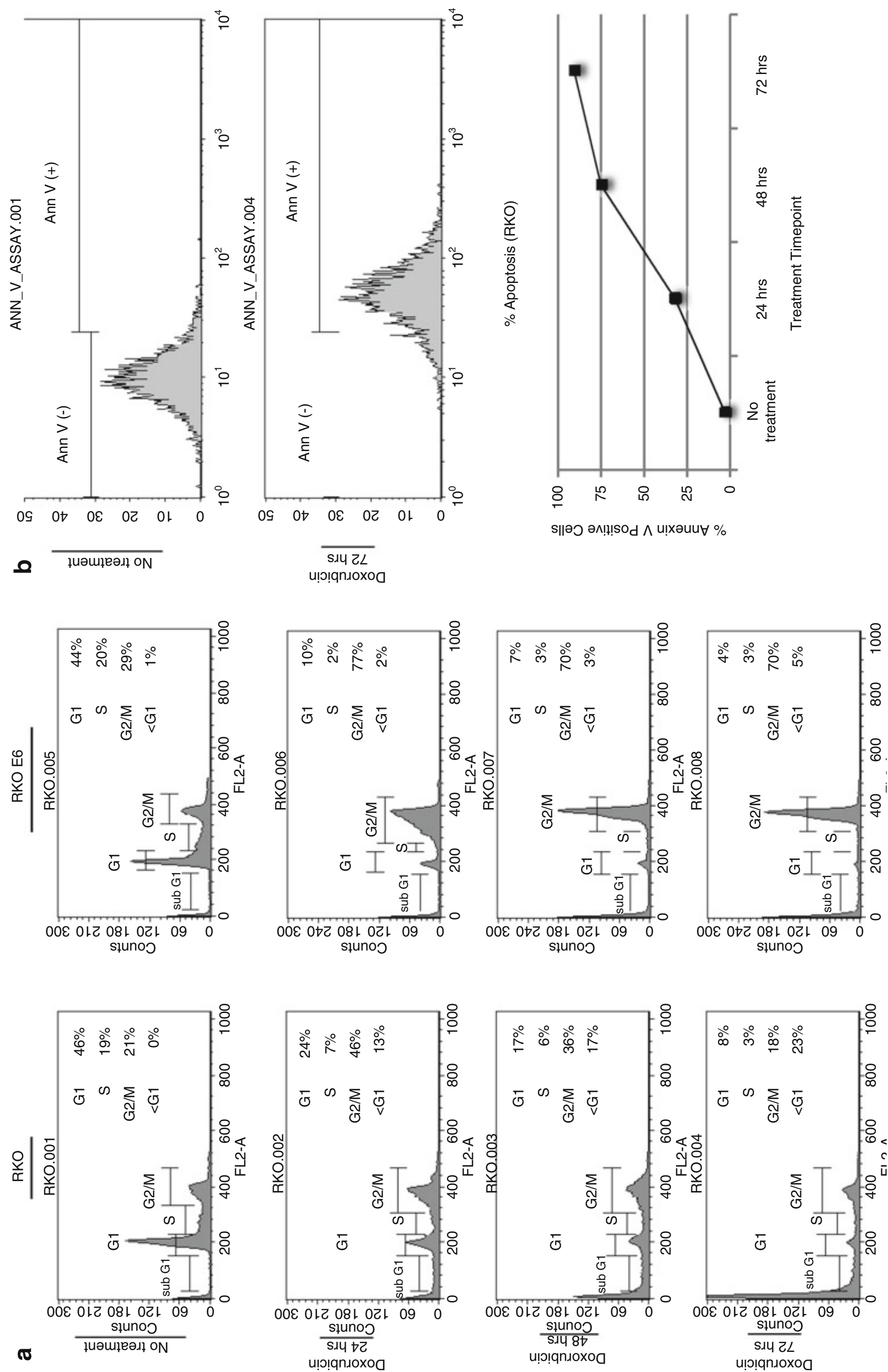

Fig. 1. DNA damage-induced apoptosis in human colorectal carcinoma cell line RKO and derivative line RKO E6, in which stable expression of the HPV E6 protein has been shown to promote the degradation of p53 (26). RKO cells respond to sustained DNA damage by undergoing p53-dependent apoptosis, revealed by an increase in the hypodiploid population of cells after treatment by PI staining and flow cytometry (**a**, *first column*). The same assay reveals RKO E6 cells, on the other hand, to be resistant to DNA damage-induced apoptosis (**a**, *second column*). Apoptosis in RKO cells can be quantified by way of annexin V immunolabeling and detection by flow cytometry, as shown in (**b**).

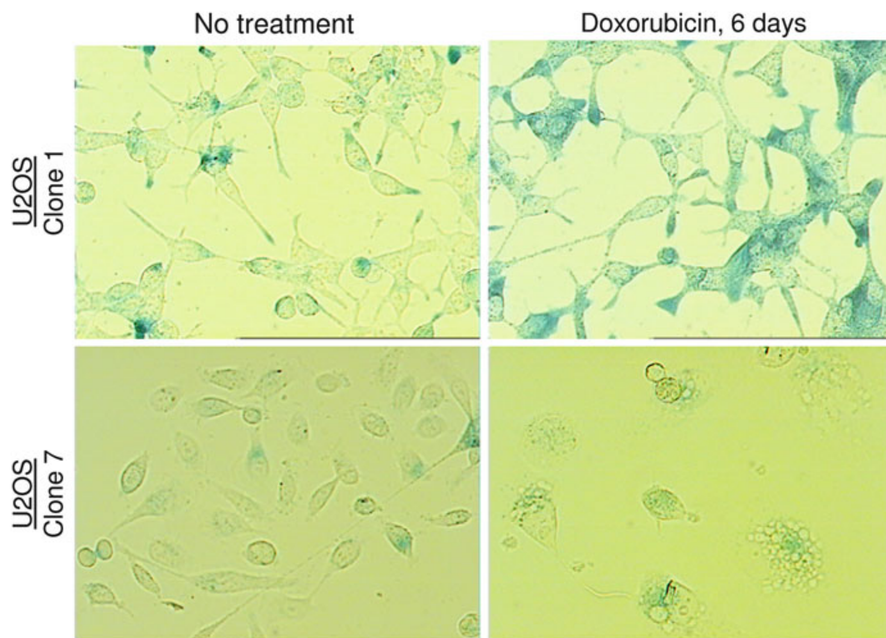

Fig. 2. Accelerated senescence in response to DNA damage occurs in a p53-dependent manner. U2OS clone 1 cells, which overexpress a control shRNA, accumulate SA-β-gal and acquire the typical flatterend morphology of senescent cells after 6 days of doxorubicin treatment. Conversely, U2OS clone 7 cells, which stably express shRNA to p53, demonstrate blebbing of their plasma membranes, a characteristic of p53-independent, apoptosis-like cell death (27).

normal cells that have achieved their replicative lifespan, at which point telomere attrition has progressed so far as to expose chromosome ends (16, 17). Cells interpret the latter lesion as a double-stranded DNA break, driving sustained p53 signaling that yields viable, metabolically active but growth-arrested cells with readily discernible characteristics (16). These features include a flattened morphology, densely vacuolated cytoplasm, and strong staining for senescence-associated (SA) β-galactosidase (Fig. 2) (16). Although cell senescence does not eradicate tumor cell populations in culture, animal models have suggested that senescent tumors are cleared by the innate immune system in vivo (18).

Despite the high frequency of p53 mutations in cancer, the absence of p53-mediated outcomes does not correlate with a failure of solid tumors to respond to DNA-damaging treatments (19). Cells that have lost p53 are deficient in G1/S arrest but remain capable of p53-independent arrest at the G2/M checkpoint, albeit transiently (20). Under conditions of sustained DNA damage, these cells escape G2 arrest with unrepaired DNA (Fig. 3). The resulting abnormal mitosis gives way to chromosome missegregation, cell fusion, micronuclei formation, and multinucleated cells in a process termed "mitotic catastrophe" (21, 22). Mitotic catastrophe eventually triggers cell death that may occasionally share some features of apoptosis (e.g., caspase activation)

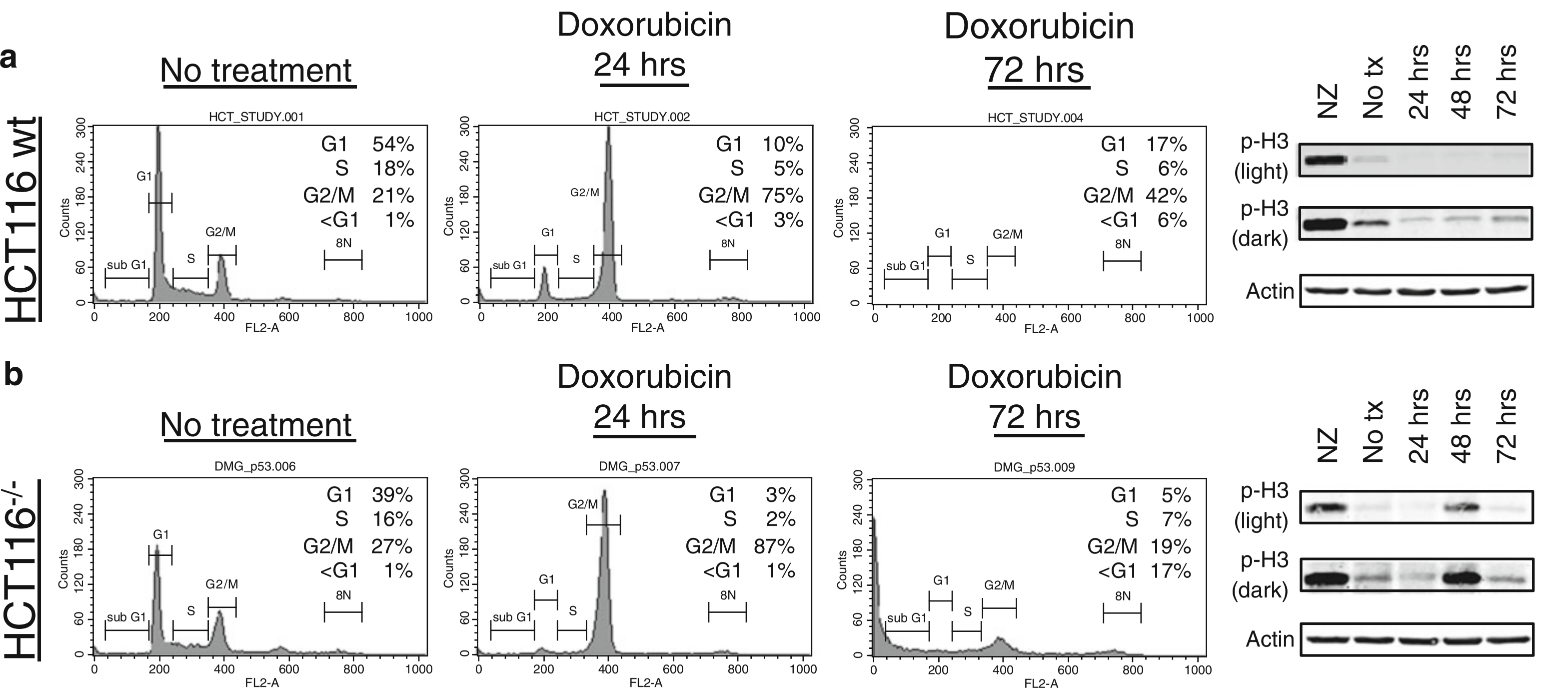

Fig. 3. Response to sustained DNA damage in paired HCT116 cell lines. HCT116 is a readily available, wild-type p53-expressing colorectal carcinoma cell line that has been subject to homologous recombination so as to produce an isogenic $p53^{-/-}$ derivative (28). The parental line demonstrates sustained cell cycle arrest with decreased phospho-$H3^{Ser10}$ detection. The $p53^{-/-}$ line, however, achieves only transient cell cycle arrest at the G2/M checkpoint with eventual reentry into mitosis, evidenced by a detectable rise in phospho-$H3^{Ser10}$ by immunoblotting. This rise in phospho-$H3^{Ser10}$ accompanies the observed decay in the G2/M peak and an increase in the sub-G1 population of cells, indicative of mitotic catastrophe. At 72 h, phospho-$H3^{Ser10}$ levels are seen to drop again as cells die. A sample of cells treated with 0.5 μg/mL nocodazole is a useful positive control (lanes labeled "NZ"). This agent arrests cells in prometaphase owing to its interference with the polymerization of microtubules.

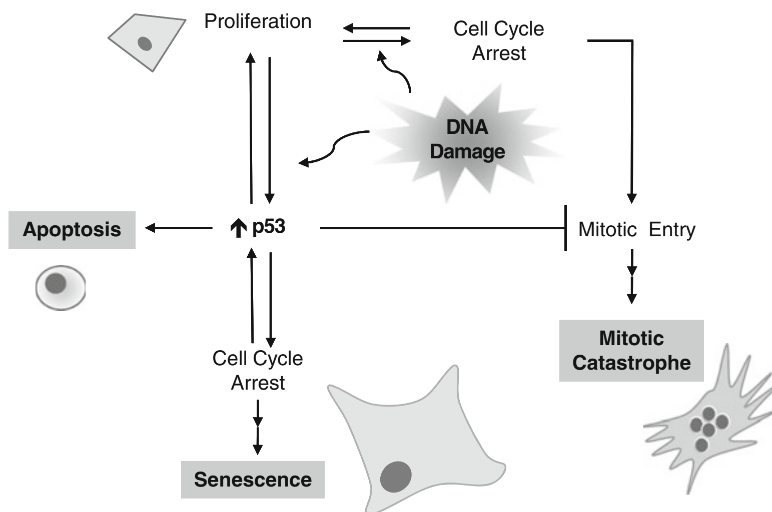

Fig. 4. p53 status as a determinant of cell outcome in response to DNA damage. In tumors harboring wild-type p53, exposure to DNA damage results either p53-dependent apoptosis or cell cycle arrest at the G1/S and G2/M checkpoints. If the latter outcome persists, these cells may senesce. Senescent cells in vivo have been shown to be cleared by the innate immune system, ultimately leading to tumor regression (18). Tumors that have lost p53, on the other hand, will respond to DNA damage by transiently arresting at the G2/M checkpoint but eventually re-entering mitosis. The ensuing process, often referred to as mitotic catastrophe (21), results in a p53-independent form of cell death that follows chromosome mis-segregation, cell fusion, micronuclei formation, and the presence of multinucleated cells.

but is nevertheless distinct in that it is preceded by the above-captioned atypia (23).

The effect of p53 status on DNA damage-directed cell fate can be readily assessed by way of propidium iodide staining and flow cytometry. This technique is complemented by immunoblotting for Serine 10-phosphorylated histone H3, an epigenetic alteration that accompanies chromosome condensation and segregation during mitosis (24) (Fig. 3). Apoptotic cell death can also be assayed by way of flow cytometry-directed detection of immunolabeled annexin V. Annexin V binds with high affinity to phosphatidylserine, which is known to be exposed on the outer leaflet of the plasma membrane of cells undergoing apoptosis (25) (Fig. 4).

2. Materials

2.1. Cell Culture, Drug Treatment, and Harvest

1. Dulbecco's modified Eagle's medium (DMEM) with High Glucose, supplemented with 10% fetal bovine serum (FBS).
2. 0.05% Trypsin–ethylenediaminetetraacetic acid (EDTA).

3. Doxorbucin HCl (Sigma-Aldrich, St. Louis, MO), dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO). 1 mg/mL stocks should prepared and aliquoted after filtration through a 0.2-μm filter under a laminar flow hood and maintained at −20°C thereafter. Working dilutions can also be maintained at −20°C after 1:10 dilution in sterile water.

2.2. Propidium Iodide Staining for FACS Analysis

1. DMEM with High Glucose, supplemented with 10% FBS.
2. 0.05% Trypsin–EDTA.
3. 70% Ethanol (EtOH).
4. 100× Stock propidium iodide (Sigma-Aldrich, St. Louis, MO) prepared by dissolving available powder in PBS. Stock is stored in the dark at 4°C.
5. Ribonuclease A (RNAse A) powder, prepared from bovine pancreas (Sigma-Aldrich, St. Louis, MO). Powder is stored as-is at −20°C.
6. Conical-bottom 15 mL Falcon tubes, 17 × 120 mm style (BD Falcon, Franklin Lanes, NJ).
7. Round-bottom 12 × 75 mm Falcon tubes, polystyrene (BD Falcon, Franklin Lanes, NJ).

2.3. SDS-PAGE, Electrophoretic Transfer, and Immunoblotting for Serine 10-Phoshorylated Histone H3

1. Tetramethylethylenediamine (TEMED) (Bio-Rad Laboratories, Rockaway, NJ).
2. 10% Ammonium persulfate (Sigma-Aldrich, St. Louis, MO) dissolved in distilled water. Store at 4°C for up to 1 month.
3. 30% Acrylamide/Bis solution, 37.5:1 (2–8°C) (Sigma-Aldrich, St. Louis, MO).
4. 10% Sodium-dodecyl sulfate (SDS) (Invitrogen, Carlsbad, CA) in distilled water.
5. 12% Resolving gel mix: Add 3 mL 10% SDS to 75 mL 1.5 M *tris*(hydroxymethyl)aminomethane (TRIS) (Sigma-Aldrich, St. Louis, MO), diluted in distilled water, and pH adjusted to 8.8 with HCl. Bring final volume to 200 mL with distilled water. Store at 4°C.
6. 5% Stacking gel mix: Add 3 mL 10% SDS to 75 mL 0.5 M TRIS (Sigma-Aldrich, St. Louis, MO), diluted in distilled water, and pH adjusted to 6.8 with HCl. Bring final volume to 250 mL with distilled water. Store at 4°C.
7. 1× SDS-PAGE running (Tris/Glycine/SDS) buffer (Bio-Rad Laboratories, Rockaway, NJ).
8. 0.1% Polyethylene glycol sorbitan monolaurate (TWEEN®20) dissolved in 1× PBS (Sigma-Aldrich, St. Louis, MO).

9. 3MM blotting paper (Whatman, Piscataway, NJ).
10. Nitrocellulose membranes (Bio-Rad Laboratories, Rockaway, NJ).
11. Gel transfer buffer. Prepare 1 L using 100 mL 10× Tris/Glycine buffer (Bio-Rad Laboratories, Rockaway, NJ), 200 mL 195-proof EtOH, and 700 mL distilled water.
12. Blocking solution: 2.5% nonfat dry milk (LabScientific, Livingston, NJ) dissolved in 1× PBS/0.1% TWEEN®20.
13. Rabbit polyclonal Anti-Phospho-Histone $H3^{Ser10}$ antibody (Millipore, Billerica, MA), diluted 1:10,000 in blocking solution.
14. Stock lysis buffer reagents:

    Lysis buffer

    (a) 10% Triton-X-100 (FisherScientific, Fair Lawn, NJ).

    (b) 1 M HEPES pH 7.5 (FisherScientific, Fair Lawn, NJ).

    (c) 5 M NaCl (FisherScientific, Fair Lawn, NJ).

    (d) 1 M $MgCl_2$ (FisherScientific, Fair Lawn, NJ).

    Stock Triton-X-100-base may be prepared with final concentrations of 1% Triton-X-100, 50 mM HEPES pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$ in an appropriate volume of distilled water. The following inhibitors are added to this buffer base immediately prior to use.:

    Phosphatase inhibitors

    (a) 0.1 M Sodium orthovanadate (Sigma-Aldrich, St. Louis, MO). Store stock at –20°C. Use 10 mL per mL of lysis buffer.

    (b) 0.5 M Sodium fluoride (Sigma-Aldrich, St. Louis, MO). Store stock at –20°C. Use 2 mL per mL of lysis buffer.

    (c) 1.5 M 4-Nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich, St. Louis, MO). Store stock at –20°C. Use 1 μL per mL of lysis buffer.

    Protease inhibitors

    (a) 50 mM Phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO). Store stock at –20°C. Use 20 μL per mL of lysis buffer.

    (b) Aprotinin, from bovine pancreas (Sigma-Aldrich, St. Louis, MO). Store at 4°C. Use 30 μL per mL of lysis buffer.

    (c) 2 mg/mL leupeptin (Sigma-Aldrich, St. Louis, MO). Store stock at –20°C. Use 1 μL per mL of lysis buffer.
15. 3× Protein sample buffer, prepared with 3 mL glycerol, 1.5 mL β-mercaptoethanol, 0.9 g SDS, 3.75 mL 0.5 M TRIS PO_4 pH

6.8 (all available through FisherScientific, Fair Lawn, NJ), adjusted to 10 mL with water. 1.0 mL Aliquots should be stored at –20°C.

16. 1.7 mL Microcentrifuge tubes (VWR Scientific, Buffalo Grove, IL).
17. Anti-actin rabbit polyclonal antibody (Sigma-Aldrich, St. Louis, MO), diluted 1:1,000 in 1×PBS/0.1% TWEEN®20.
18. Broad range protein standards (Fermentas, Glen Burnie, MD).
19. HRP-conjugated goat anti-rabbit secondary antibody (Millipore, Billerica, MA), diluted 1:1,000 in blocking solution.
20. Enhanced chemiluminescence (ECL) substrates for HRP detection (Thermo Scientific, Rockford, IL).
21. Autoradiography film and cassettes (MidSci, St. Louis, MO).

2.4. Annexin V Detection by Flow Cytometry

1. FITC-conjugated anti-Annexin V antibody (BD Pharmingen, San Diego, CA).
2. Annexin V binding buffer (AVBB) stock reagents:
   (a) 1 M HEPES pH 7.5 (FisherScientific, Fair Lawn, NJ).
   (b) 5 M NaCl (FisherScientific, Fair Lawn, NJ).
   (c) 1 M $MgCl_2$ (FisherScientific, Fair Lawn, NJ).
   (d) 1 M $CaCl_2$ (FisherScientific, Fair Lawn, NJ).

Prepare AVBB with final concentrations of reagents as follows: 10 mM HEPES pH 7.5, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$. AVBB should be stored at 4°C for up to 1 month.

2.5. Equipment

1. Laminar flow hood for sterile cell culture work.
2. CO_2 incubator, 37°C.
3. Refrigerator, 4°C.
4. Freezers, –20°C and –80°C.
5. Microcentrifuge.
6. Low speed centrifuge with swinging bucket rotor capable of accommodating 15 mL tubes (see Subheading 2.2, above).
7. FACS brand flow cytometer (BD BioSciences, San Jose, CA).
8. Appropriate apparatuses for SDS-acrylamide gel casting, SDS-PAGE, gel transfer, and subsequent immunoblotting (available through Bio-Rad Laboratories, Rockaway, NJ).
9. Dark room with film developer capable of processing autoradiographs.

3. Methods

3.1. Staining with Propidium Iodide for Cell Cycle Analysis

p53 and Cell Cycle Effects after DNA Damage Chapter 4, "Determine the effect of p53 on cell cycle," for the relevant protocol.

3.2. Detection of Serine 10-Phosphorylated Histone H3 by Immunoblotting

It is recommended that SDS-PAGE gels be cast ahead of time. Gels may be stored at 4°C for up to 5 days:

12.0% Resolving gel: Combine 5.9 mL 12% gel mix, 4.0 mL 30% Acrylamide/Bis solution, 37.5:1, 100 μL 10% APS, and 10 μL TEMED. Mix thoroughly and add 7.5 mL to an 8 cm × 10 cm × 1.5 mm gel cassette. Overlay with 70% EtOH. Incubate 15 min at room temperature. Dispose of ethanol overlay and rinse gently five to six times with distilled water.

5.0% Stacking gel: Combine 8.3 mL 5.0% gel mix, 1.7 mL, 4.0 mL 30% Acrylamide/Bis solution, 37.5:1, 100 μL 10% APS, and 10 μL TEMED. Mixed thoroughly and add a volume sufficient to fill casting apparatus. Insert a 10- or 15-well comb (depending on the number of samples), taking care not to introduce air bubbles. Incubate 15 min at room temperature.

1. Cells are harvested by trypsinization at relevant points in time following treatment (e.g., every 24 h). Cells should be spun down at 2,000*g* × 5 min at 4°C, rinsed once with 1× PBS, and spun again. Pellets may be frozen for lysis at a later time, provided that all supernatants are aspirated and pellets are kept on dry ice for 5–10 min prior to storage at −80°C.
2. Cell pellets are lysed in an appropriate amount of Triton-X-100-based lysis buffer, prepared with the addition of protease and phosphatase inhibitors as detailed in Subheading 2. Cells should be lysed on ice for 10–15 min.
3. Whole cell lysates (WCLs) are spun at 16,000*g* × 5 min at 4°C. The supernatant is transferred to a new 1.7 mL microcentrifuge tube. The pellet is discarded.
4. The protein content of each WCL is determined by Bradford assay.
5. Samples are prepared using a sufficient volume of WCL for 50 μg of protein in a 1.7 mL microcentrifuge tube. Volume across samples should be equalized using Triton-X-100 lysis buffer, as prepared above. Add an appropriate volume of 3× protein sample buffer to each sample.
6. Samples are vortexed, spun down, and incubated at 95°C for 3 min to denature proteins. To avoid having tubes pop open during this incubation, a syringe needle may be used to produce a hole in the lid of each tube.

7. Samples are again vortexed and spun down after incubation.
8. Samples are loaded into the wells of a pre-cast 12.0% resolving/5.0% stacking gel. 10 μL Protein standards in an appropriate amount of 3× protein sample buffer should be loaded into one lane at either end of the gel.
9. Samples are run at 130 V for approximately 1.5 h, until sample buffer has run out of the gel and into the running buffer.
10. After electrophoresis, the gel is transferred to a nitrocellulose membrane at 110 V for 60 min. Gel transfer should be carried out at 4°C.
11. The nitrocellulose membrane is cut horizontally at the ~35 kDa marker. The top half of the membrane can be used for actin immunodetection (~43 kDa). The bottom half is to be used for phosho-$H3^{Ser10}$ immunodetection (~17 kDa).
12. The nitrocellulose membrane sections are blocked in blocking solution for 1 h. The upper half is incubated in an appropriate volume and dilution of anti-actin antibody, the lower half in anti-phosho-$H3^{Ser10}$, overnight with gentle agitation.
13. The membrane sections are washed three times, 10 min each, in blocking solution.
14. The membrane sections are incubated in an appropriate volume and dilution of HRP-conjugated anti-rabbit secondary for 1 h at room temperature with gentle agitation.
15. The membrane sections are washed four times, 10 min each, in blocking solution. Two final washes in 1× PBS/0.1% TWEEN®20, 10 min each, are performed to wash out any residual milk prior to developing.
16. The membranes are coated in ECL reagent for 1 min. The ECL is blotted off on a paper towel. The membranes are wrapped in transparent film and fixed to the inside of an autoradiography cassette with clear tape.
17. In a dark room, membranes are exposed against autoradiography film for a range of different exposure lengths (e.g., 5, 10, 30, and 60 s). Films are developed and labeled appropriately.

3.3. Assessment of Apoptosis by Annexin V Staining and Detection by Flow Cytometry

1. Unlike PI staining for flow cytometry analysis of DNA content, cells harvested for Annexin V staining are not fixed in EtOH and cannot be stored at −20°C for later analysis. All samples must be harvested and analyzed at the same time. For assessment of up to 72 h of treatment, for example, cells should be split evenly 4 days prior to analysis and treated on days 3, 2, and 1 prior to harvest. An untreated plate of cells may be harvested on the day of analysis as a control sample.
2. Cells are harvested by trypsinization. Cell culture medium should be retained in a 15 mL conical-bottom Falcon tube, as

this is likely to contain dead cells that will be relevant for the analysis. This medium can be used to neutralize the trypsinization reaction and collect adherent cells into the same tube. Add 2–3 mL of 1× PBS and collect into relevant tube of cells to ensure maximum recovery.

3. Cell suspensions are spun at $800g \times 5$ min at room temperature.
4. Cell supernatant is aspirated. All visible traces of trypsin should be removed, as residual enzyme may cleave exposed annexin V.
5. The FITC-conjugated annexin V antibody is diluted 1:250 in AVBB.
6. Cells are resuspended in 500 μL to 1.0 mL of FITC-annexin V dilution and transferred to round-bottom 12×75 mm Falcon tubes.
7. Cells are kept in the dark at 4°C for 30 min. Cells are resuspended every 5–10 min to ensure adequate antibody binding.
8. Samples are analyzed for annexin V expression on a BD BioSciences FACScalibur flow cytometer. The FL1 laser is used to detect the FITC-annexin V antibody. CellQuest software is used to generate both acquisition and analysis plots of 2,000 cells. The percent of cells that are annexin V positive is used as a surrogate measure for cells that have undergone apoptosis.

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Chapter 10

Measurement of Chemosensitivity and Growth Rate in p53 Expressing Cells

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Abstract

Chemoresistance and increased growth rate are two gain-of-function functions that mutant p53 is thought to possess. Here, we describe two methods for measuring the sensitiveness of cells to chemotherapeutic drugs and the rate of cell growth. Both of which can be used with a wide range of cell types. The clonogenic assay can be used with many different chemotoxic drugs and the growth assay described here presents an alternative to the MTT assay and allows for a long-term measurement of cell growth. These protocols are both easy, flexible, require relatively little effort, and are inexpensive to carry out.

Key words: Chemotoxic, Gain of function, Clonogenic assay, Growth assay

1. Introduction

Clonogenic assays are considered the ‘gold standards’ for the measurement of cellular sensitivity. As described by Puck and Marcus, the clonogenic assay relies on the ability of individual mammalian cells to replicate and form colonies (1). At least 50 cells are required to define a colony. The first clonogenic experiments were carried out to measure the ability of HeLa cells to survive radiation (2).

Subsequently, the assay has been used to measure the sensitivity of a large variety of cells including stem cells to a wide range

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of agents, with the ‘surviving’ fraction of cells retaining the ability to form colonies. Further, the assay has been widely used to test the ability of particular gene(s) to confer resistance or sensitivity to treatment with agents. The expression of wild type p53 is generally correlated with sensitivity to radiation and treatment with various chemotherapeutic agents, where p53 is activated when cells are subject to stress and triggers cell death or senescence. The clonogenic assay has been used over the years to determine the contribution of p53 towards resistance or sensitivity to various chemotherapeutic agents, radiation and other stresses (3–7).

Cell proliferation assays have been performed to study the roles of various microRNAs (8), the effect of treatment with growth factors (9), and with inhibitors (10). Most of the cell proliferation assays used now are based off of the MTT assay (11) or colony formation assays (11, 12). Counting cells can also be accomplished through the use of trypan blue staining, but that method is slow and time consuming when a large number of population of cells need to be counted.

The MTT assay is a colorimetric assay where the reduction of salts is used to produce a color that is measured using a spectrophotometer. Others have used the CyQUANT assay (13) which measures the amount of DNA present in cells to calculate proliferation. The growth assay as described here is especially useful in studying the gain of function phenotype of mutant p53 and has been used extensively in cells that have had p53 knocked down. This assay may be used after any treatment to cells such as transient transfection, siRNA mediated knock-down, or drug or steroid treatment.

Cells are counted and plated at a specific density and then harvested either every other day or every day depending on how fast the cell line grows. A coulter counter is used to count cells and the total number calculated. This method is easy to perform and quick to complete.

2. Materials

2.1. Clonogenic assay

1. Petri plates- 10cm or 6cm; Alternatively 6 well plates can be used.
2. Pipettes.
3. Sterile tubes for serial dilution.
4. Coulter counter or Haemocytometer.
5. Microscope.
6. Colony counting pen.
7. Light Box.

8. Appropriate cell culture medium. Most cells require serum to be added.
9. Phosphate Buffered Saline (PBS) or Hanks Balanced Salt Solution (HBSS).
10. Trypsin with EDTA.
11. Isotonic solution for counting cells.
12. Chemotherapeutic drug(s) to be tested. At least 3 different concentrations to be tested.
13. Vehicle for diluting drugs and also for control plates.
14. Methanol for fixation of colonies.
15. Methylene Blue or Crystal Violet Dye for staining colonies.

2.2. Plating Cells

1. Adherent cultured cells.
2. Cell culture incubator.
3. 60 mm Culture dishes.
4. Cell counter or hemacytometer.
5. HBSS or PBS.
6. 10× Trypsin in EDTA.
7. 15 mL Tube.

2.3. Harvesting Cells

1. HBSS or PBS.
2. 10× Trypsin in EDTA.
3. 1.5 mL Tubes.

2.4. Counting Cells

1. 1× PBS.
2. Coulter Counter or haemacytometer.
3. Trypan blue if using a haemacytometer.

2.5. Plotting Data

1. Excel or other suitable software.

3. Methods

3.1. Clonogenic Assay

3.1.1. Plating cells

1. Before starting the experiment, ensure all the paraphernalia that will be used is kept inside a sterile tissue culture hood including the tubes in which the dilutions will be carried out. Also label the plates (along with the lid) that will be used for the assay appropriately. This ensures that the plates are not switched when multiple assays are carried out together. Make sure the label is resistant to methanol, used for fixation.
2. Remove the media from the cells that will be used to plate for the assay (see Note 1). Wash the plate two times with either

PBS or HBSS and aspirate the wash solution. Trypsinize the cells and collect the cells in 10 mls media in a tube.

3. Count the cells using either a haemocytometer or using a coulter counter.
4. Dilute the cell suspension serially to obtain desired seeding concentrations (see Note 2). It is advisable to use at least 1 ml of cell suspension for dilution and also for the final seeding. This ensures minimum error.
5. Allow the cells to plate before the treatment. Generally, 24 hours is a good time period for this purpose.

3.1.2. Treating cells

1. Perform the treatment (see Note 3). Some treatments call for at least two washes with PBS and a change of media immediately after treatment to remove the drug from the plates after exposure. Others vary depending on the agent used.
2. The plates are then kept back in the incubator.
3. Change media on the plates every 4-5 days, as this allows fresh supply of nutrients. If required, leave back some of the old media (for conditioning). Constantly monitor the cells for the formation of colonies-every two days.

3.1.3. Staining

1. Once the colonies form, remove the media and was the plates with PBS.
2. Remove the PBS and fix the colonies with ice cold methanol. Let the methanol sit on the plates for at least 20 minutes.
3. Rinse the plates with distilled water. Add either Methylene Blue or Crystal violet stain and leave the plates overnight.
4. Remove the stain and wash the plates with water and leave them to dry. The colonies should be visible and are now ready to count.

3.1.4. Counting colonies

1. Use either an automatic counting colony counter or manually count the colonies using an alcohol resistant pen to mark individual colonies. Determine the plating efficiency of control cells, that is, the fraction of colonies from cells exposed to the vehicle of no treatment.

3.1.5. Analysis of Results

Plating efficiency is calculated as below:
P.E.= number of colonies formed/ number of cells seeded.
Plot a graph of percent survival (y axis) against drug concentration (x axis) to get the survival curve.

3.2. Growth Assay

3.2.1. Plating Cells

1. Take the cells to be used for the growth assay out of the incubator and remove media. Wash once with 10 mL HBSS or PBS and remove.

2. Add 1.8 mL HBSS and 0.2 mL 10× Trypsin to the cells and incubate at 37°C for 2–3 min or until the cells release from the culture dish.
3. Add 3 mL of complete medium to the trypsinized cells and pipette up and down to completely suspend cells and transfer to a 15 mL tube.
4. Count cells using a hemacytometer and serially dilute the cell solution to get at least 15 mL of a 5×10^4 cells/mL concentration (see Note 4).
5. Plate 1 mL of the cell solution in fifteen 60 mm dishes (see Note 5).

3.2.2. Plating Cells

1. Remove each 60 mm culture dish of cells that were plated the day before from the incubator and remove media.
2. Wash each plate in 2 mL HBSS or PBS and remove.
3. Harvest the cells by trypsinization in 0.9 mL HBSS and 0.1 mL 10× trypsin and incubate at 37°C for 2–3 min. Add 1 mL of complete medium and pipette up and down to completely suspend cells.
4. Put 1 mL of the cell mixture into a 1.5 mL tube for counting (see Note 6).
5. Harvest the cells either every day or every other day for five timepoints (see Note 7).

3.2.3. Counting Cells

1. Add 9 mL of PBS to the 1 mL of cells and count in a Coulter Counter (see Note 8). Each plate of cells will be counted three times.

3.2.4. Plotting the Data

1. Average the three counts for each plate and then average the three total plate counts to give an average of all three plates (per condition). Multiply the average by two (see Note 9) to give the total amount of cells for that timepoint.
2. Calculate the standard deviation from all nine counts for each condition.
3. Plot the total counts in a line graph and add standard error bars.

4. Notes

1. Depending on the type of treatment, cells are either plated before the treatment (using chemotherapeutic drugs) or the treatment of the cells occurs prior to their plating onto

subsequent dishes (gamma irradiation). It is advisable to save the medium in which the cells have been growing prior to them split into treatment plates and adding this medium to the plates. This ‘conditions’ the media with growth factors generated when the cells were grown at a higher density.

2. It is important to remember to make enough cell suspension to allow plating for at least a minimum of three different concentrations of the treatment with allowance for triplicates for each concentration. Plating for the non treated control plates should come from either the stock used for plating for the various treatment or should be a dilution of the stock with the dilution factor accounted for in the final analysis. The accuracy is important as this accounts for the plating efficiency of the cells.

3. As previously mentioned, it is important to have at least three concentrations of the treatment. This allows plotting for a survival curve. If the assay is done with untested agents or untried strategies, it is recommended to test the agent in a series of concentrations increasing logarithmically and the nailing on a range of three concentrations to be tested before the actual experiment. It is imperative to identify the vehicle to be used for dissolving chemical agents and use the vehicle to treat the control plates (vehicle control).

4. 7.5×10^5 Total cells are needed.

5. At least three plates are needed for each cell type, condition the cells have been treated, and for each time point that will be used (Ex. three plates of H1299 siRNA treated for at least five timepoints; giving a total of 15 plates).

6. Make sure that all cells have been collected from the culture dish. If the trypsinization has been inefficient the cell counts will be inaccurate.

7. Depending on how fast the cell line grows, the times at which to harvest the cells will vary. For example, H1299 cells grow quickly and are harvested every day while slower growing cells are harvested every other day.

8. Cell counting can also be accomplished with a haemacytometer, but using a Coulter Counter can save time and be more efficient.

9. The average number is multiplied by two since the cells were harvested in a total amount of 2 mL and only half was used for counting.

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Chapter 11

Mutant p53 in Cell Adhesion and Motility

W. Andrew Yeudall, Katharine H. Wrighton, and Sumitra Deb

Abstract

Pro-oncogenic properties of mutant p53 were investigated with the aid of migration assays, adhesion assays, and soft agar growth assays using cells stably expressing gain-of-function p53 mutants. To determine cell migration, "wound-healing" (scratch) assays and haptotactic (chamber) assays were used. H1299 cells expressing mutant p53 were found to migrate more rapidly than cells transfected with empty vector alone. Results from both types of migration assay were broadly similar. Migratory ability differed for different p53 mutants, suggesting allele-specific effects. Cells expressing p53 mutants also showed enhanced adhesion to extracellular matrix compare to controls. Furthermore, stable transfection of mutant p53-H179L into NIH3T3 fibroblasts was sufficient to allow anchorage-independent growth in soft agar.

Key words: Adhesion, Motility, Migration, Gain-of-function, Chemotaxis, Extracellular matrix, Anchorage independence

1. Introduction

Although wild-type p53 is well recognized as a gatekeeper of normal cellular homeostasis (1), the functions attributable to mutant p53 have been less well studied. While much earlier research focused on dominant-negative inactivation of wild-type p53, it is becoming increasingly clear that "gain-of-function" (GOF) mutations may endow p53 with additional properties, making it oncogenic in its own right (2–13). It is important to identify these aberrant proteins, not only as a means to understand mechanisms of tumor progression but, also, because such mutants will likely be good targets for therapeutic intervention if their oncogenic properties can be neutralized (14).

During tumor progression, cancer cells gain many properties that may give them growth and/or survival advantages and include

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altered interactions with the extracellular matrix (ECM), increased motility, and, in some cases, anchorage-independent growth. These properties are straightforward to measure using a range of assays. Thus, the contribution of GOF p53 proteins to the aforementioned aspects of tumor progression can be readily determined using appropriate cell lines. Adhesion assays, in which multi-well tissue culture plates are coated with the ECM protein of choice (routinely we use fibronectin, vitronectin, laminin, and collagen IV), can be used to investigate altered cell–ECM interactions. Enhanced cell motility can be determined by wound-closure or "scratch" assays, which measures the time taken for cells to migrate across a denuded area of a previously confluent cell monolayer. Alternatively, cells may be plated in the upper chamber of porous supports, and the number of cells migrating to the opposite side of the porous membrane in a given time is then determined after staining and counting. Further, inoculation of cells into suspension culture ("soft agar") measures growth in the absence of solid support and can be a good determinant of the aggressive nature of a cell line.

We used H1299 lung cancer cells stably transfected with GOF p53 mutants: R175H, H179L, R273H, D281G; and NIH3T3 fibroblasts (which have endogenous wild-type p53) stably transfected with p53-H179L, as well as appropriate vector-transfected controls (10, 11, 13). We found that H1299 cells expressing p53 mutants at similar levels showed increased adhesion to fibronectin (FN)-coated plates and enhanced migration in scratch and haptotactic migration assays. Furthermore, fibroblasts expressing p53-H179L formed colonies in soft agar, consistent with the aggressive phenotype of these cells as reported previously by our laboratory.

2. Materials

2.1. Adhesion Assays

1. 24-Well tissue culture plates (Cellstar 662160; Greiner Bio-One, Monroe, NC, USA [or equivalent]).
2. Fibronectin (F1141; Sigma Aldrich, St. Louis, MO, USA) [or vitronectin (V8379), laminin (L2020), collagen IV (C0543)—all from Sigma Aldrich].
3. Bovine serum albumin (BSA; A9418; Sigma Aldrich, St. Louis, MO, USA).
4. Calcium- and magnesium-free (CMF) phosphate-buffered saline (Dulbecco's PBS, DPBS, pH 7.4; 55-031-PB; Mediatech Inc., Manassas, VA, USA). Reconstitute in tissue culture grade water and sterilize by filtration.

5. Growth medium (e.g., Dulbecco's modification of Eagle's medium, DMEM). We recommend purchasing DMEM in powdered form (DMEM/High Glucose; HyClone SH30003.03; Thermo Scientific HyClone, Logan, UT, USA [or equivalent]), reconstituting in tissue culture grade water together with $NaHCO_3$ (Cellgro 61-065-RO; Mediatech Inc., Manassas, VA, USA) and sterilizing by filtration. This allows preparation of 2× concentrated medium, as required for soft agar assays (see Subheading 2.4). Be sure to supplement with sodium pyruvate (and antibiotics, if desired).
6. Crystal violet (C3886; Sigma Aldrich, St. Louis, MO, USA), prepared as a 0.1% solution in DPBS.
7. Ethanol (absolute, 200 proof; 459844, Sigma Aldrich, St. Louis, MO, USA).

2.2. Wound-Closure Migration Assays

1. 12-Well tissue culture plates (Costar 3513; Corning Inc., Corning, NY, USA [or equivalent]).
2. DMEM (see Subheading 2.1) supplemented with 10% (v/v) fetal bovine serum (HyClone, Thermo Scientific HyClone, Logan, UT, USA [or equivalent]).
3. Light microscope with camera and imaging software (e.g., Axiocam and Axiovision, Carl Zeiss Inc., Thornwood, NY, USA).

2.3. Haptotactic Migration Assays

1. Transwell tissue culture inserts (6.5 mm) with polycarbonate membranes of pore-size 8 μm (Costar 3422, Corning Inc., Corning, NY, USA).
2. Cellstripper (Cellgro 25-056-CI; Mediatech Inc., Manassas, VA, USA).
3. DPBS and DMEM (see Subheading 2.1).
4. Glutaraldehyde (G5882; Sigma Aldrich, St. Louis, MO, USA) prepared as a 0.025% (v/v) solution in DPBS.
5. Hematoxylin solution (H-3401; Vector Laboratories, Burlingame, CA, USA [or equivalent]).
6. Scalpel blades (#11).

2.4. Soft Agar Assays

1. 35 mm Diameter tissue culture dishes (Cellstar 627170) or 6-well plates (Cellstar 657185; Greiner Bio-One, Monroe, NC, USA [or equivalent]).
2. Low melting point (LMP) agarose (V3841; Promega Corp., Madison, WI, USA [or equivalent]).
3. Tissue culture grade water.
4. DMEM (see Subheading 2.1), reconstituted to 2× working concentration and filter-sterilized.

5. Methylene blue stain (M9140; Sigma Aldrich, St. Louis, MO, USA), prepared as a 0.15% (w/v) solution in distilled water.

3. Methods

The methods outlined below describe assays for (a) adhesion; (b) wound-closure; (c) haptotactic migration; and (d) anchorage-independent cell growth.

3.1. Adhesion Assay

For this assay, H1299 cells stably expressing mutant p53 proteins, or transfected with the empty plasmid vector as control, were cultured under standard conditions (humidified incubator, 37°C, 90% air/10% CO_2) in complete growth medium (DMEM containing 10% FBS) until 80% confluent. Cells were washed twice in DPBS and then released from the tissue culture plate using 0.25% trypsin/2.21 mM EDTA in HBSS, pre-warmed to 37°C. Cells were washed in fresh medium twice by centrifugation, counted in a hemocytometer, and resuspended in DMEM/0.1% (w/v) BSA at a concentration of 1×10^5 cells/ml. DMEM/BSA is prepared by adding the required amount of BSA into serum-free DMEM, stirring gently until dissolved, and then filter-sterilizing the medium through a standard 0.22 μm filter. The assays were then carried out as follows:

1. Coat a 24-well plate with ECM protein (fibronectin, 1 μg/cm^2) for 2 h at 37°C. Alternatively, coat wells with vitronectin (0.1 μg/cm^2), laminin (1 μg/cm^2), or collagen IV (6 μg/cm^2).
2. Wash wells with DPBS three times for 5 min each, using a minimum of 1 ml per well.
3. Block the remaining binding surfaces by incubation in DMEM/3% BSA for 1 h at 37°C.
4. Wash wells with DPBS three times for 5 min each, using a minimum of 1 ml per well.
5. Plate cells (1×10^5) in DMEM/0.1% BSA. Set up at least four replicate wells for each sample.
6. Incubate for 1 h at 37°C in a humidified incubator, then remove medium by aspiration.
7. Wash wells with DPBS once for 5 min, using a minimum of 1 ml per well.
8. Add 0.1% crystal violet solution for 5 min to stain attached cells.
9. Wash wells carefully, but thoroughly, four times in DPBS.
10. Aspirate all remaining DPBS, and then add 1 ml of 70% ethanol to each well to extract the crystal violet dye. Quantify

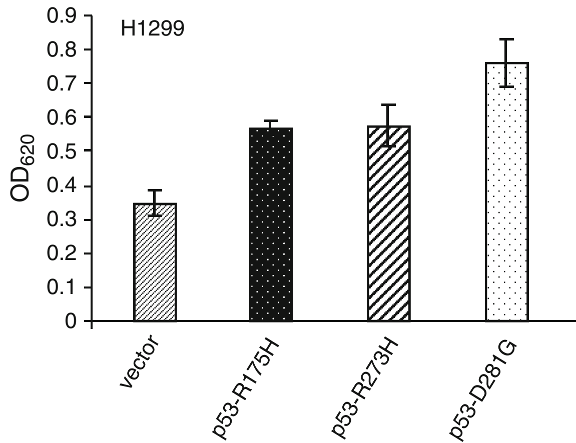

Fig. 1. H1299 lung cancer cells, stably transfected with empty vector or plasmids encoding the indicated p53 mutants, were tested for adhesion to fibronectin substrate, as described in the text. After crystal violet staining, dye was extracted and quantified by spectrophotometry. Values are means of quadruplicate wells and representative of three independent experiments. Bar = s.d.

by reading the absorbance at 620 nm in a standard visible light spectrophotometer. Be sure to include blank wells without cells as control. Absorbance is then plotted after subtracting the value for the blank. The results of a typical assay are shown in Fig. 1, with mutant p53-expressing H1299 cells showing enhanced adhesion to fibronectin-coated plates compared to control-transfected cells.

3.2. Wound-Closure Migration Assay

In this assay, we measured the speed with which cells expressing mutant p53 proteins are able to migrate across a denuded area ("wound" or "scratch") of a tissue culture plate. Cells were grown in complete medium until 80% confluent, then released from the culture plate using 0.1% trypsin–2.21 mM EDTA (Mediatech, Herndon, VA), counted in a hemocytometer, and resuspended in complete medium at a concentration of 1×10^5 cells/ml.

1. Mark the undersurface of each well of 12-well culture plates with three parallel straight lines using an indelible pen. This will allow for orientation of plates for measurement.
2. Plate 2×10^5 cells in triplicate wells of these culture plates and incubate at 37°C until cells are completely confluent.
3. Using a sterilized disposable pipette tip ("yellow tip"), make a scratch across the surface of the plate to remove the complete layer of cells within the scratch area. This should be at 90° to the orientation lines drawn in step 1. Following cell removal, wash each well twice with DPBS and then replace with complete growth medium.

4. Immediately, measure the width of the scratch either side of where it intersects with the three orientation lines. This is carried out under a low power objective using a light microscope with camera and suitable analysis software. We use an Axiocam MRm and Axiovision software. You should have 18 measurements for each cell line being analyzed—triplicate wells and six points per well.
5. Return cells to the 37°C incubator for 10 h, after which time the scratch width is measured at exactly the same positions as at the starting time. Subtract final distance from starting distance and then divide by time to give migration rate (μm/h). As shown in Fig. 2, expression of mutant p53 proteins enhances cell motility compared to vector-transfected control H1299 cells. Furthermore, different mutant p53 alleles increase motility by varying amounts, suggesting allele-specific effects.

3.3. Haptotactic Migration Assay

Migratory ability can also be measured readily with the aid of Transwell tissue culture inserts, which contain a porous membrane that separates an upper chamber from the remainder of the culture well. For migration assays, we find that the inserts with 8 μm diameter pores are suitable for most of the cancer cells with which we work.

1. Coat the underside of 8 μm pore size Transwell inserts with fibronectin at a density of 1 $\mu g/cm^2$ and incubate for 2 h at 37°C in a humidified incubator.
2. Rinse the fibronectin-coated inserts once in DPBS, and then place the inserts in the wells of the 24-well plate containing 0.5 ml of DMEM/0.5% BSA per well. Return plates/inserts to the incubator. Note: DMEM used here does not contain serum.
3. Culture H1299 cells expressing mutant p53 or vector-transfected control cells to 80% confluence. The cultures are then washed twice in DPBS, and cells detached from the tissue culture plate using Cellstripper (*Note*: avoid enzymatic dissociation).
4. Wash the cells twice in DMEM/0.5% BSA by centrifugation, count the cells in a hemocytometer, and then resuspend in DMEM/0.5% BSA at a density of 1×10^6 cells/ml. Add 5×10^4 cells to the upper chamber of the Transwell insert (use triplicates at a minimum), return to the incubator and allow the cells to migrate for 6 h.
5. Fix the cells using 0.025% glutaraldehyde in DPBS for 5 min at ambient temperature.
6. Stain in Gill's hematoxylin for 20 min at ambient temp, and then destain thoroughly in tap water, until no more stain is seen in the rinse.

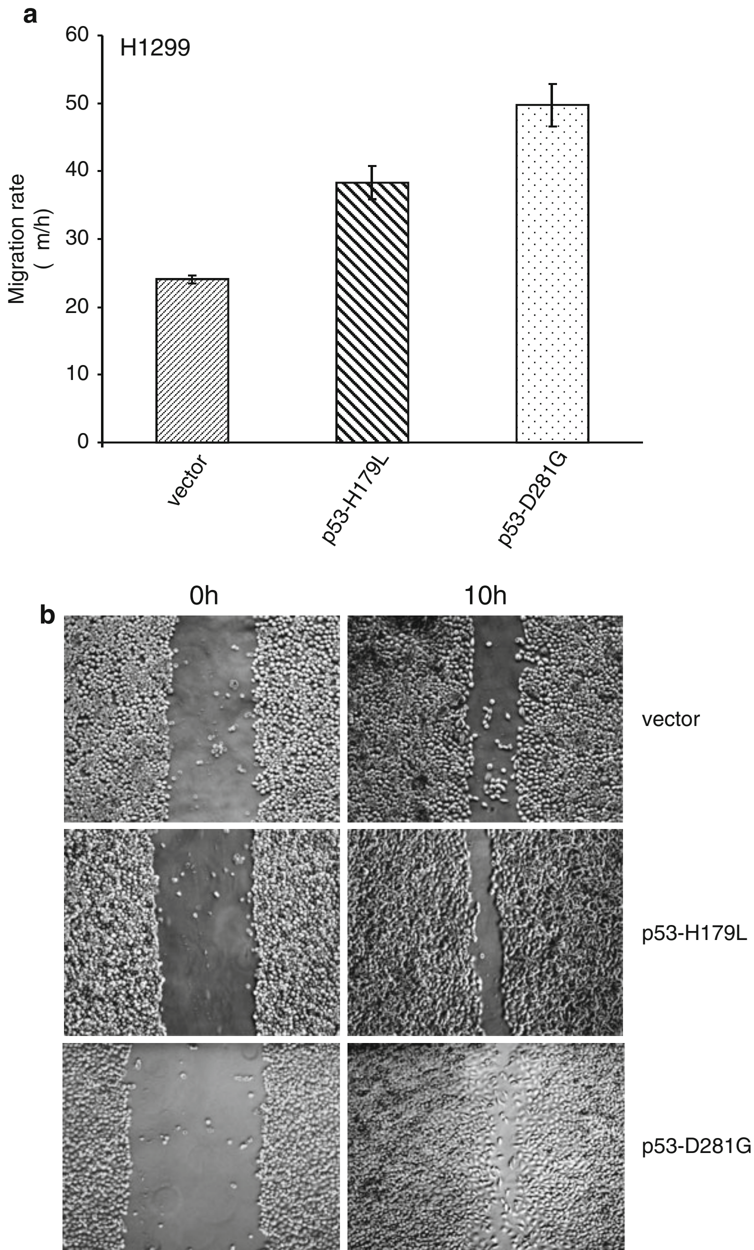

Fig. 2. H1299 lung cancer cells, stably transfected with empty vector or plasmids encoding the indicated p53 mutants, were cultured to confluence and the monolayer denuded, as described in the text, and the distance across the denuded area was measured at 0 h and again after 10 h. Migration rate is shown graphically (**a**) and pictorially (**b**). Data are means ± s.e.m. of 18 measurements per sample and representative of multiple independent experiments. Original magnification ×50.

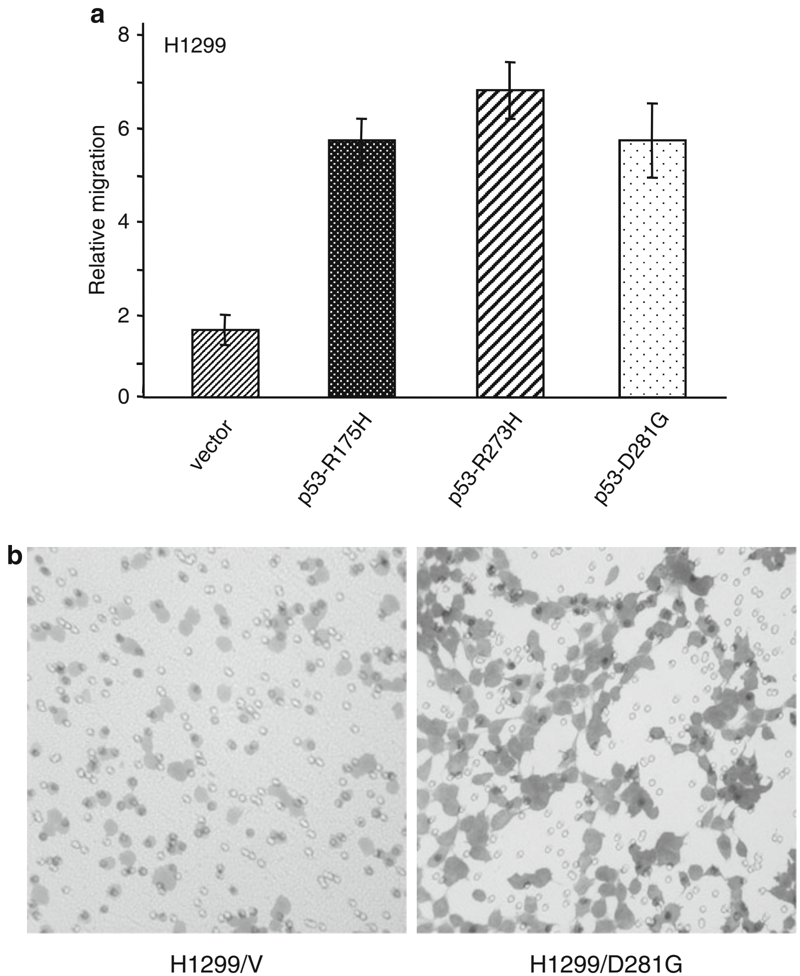

Fig. 3. H1299 lung cancer cells, stably transfected with empty vector or plasmids encoding the indicated p53 mutants, were assayed for migration using Transwell chambers, as described in the text. Number of cells in 20 random high power fields was determined. Relative migration is shown as mean ± s.e.m. of triplicate samples for each cell line and representative of multiple independent biological experiments (**a**). Of note, H1299 cells expressing p53-D281G showed a spindle-like morphology compared to the more rounded vector-transfected controls (**b**). Original magnification ×200.

7. Any non-migrating cells present on the upper surface of the porous membrane can be removed with a Q-tip. Membranes are then excised from the Transwell insert using a sharp #11 scalpel blade and mounted on microscope slides. Add a drop of permanent mounting medium and a coverslip.
8. Using a high power objective (e.g., 20×), count the number of cells present in 20 random, representative fields of view.

Figure 3 indicates enhanced motility of H1299 cells expressing p53-D281G relative to vector-transfected control cells. Of note,

the mutant p53-expressing cells show altered morphology on fibronectin, with a more stellate appearance compared to control cells.

3.4. Growth in Soft Agar

The ability to grow in suspension culture (anchorage-independent growth) is a characteristic of more aggressive tumor cells. Thus, it is an additional parameter that we can use to determine the contribution of GOF mutant p53 proteins to tumor progression.

1. Prepare a 1% solution of LMP agarose in tissue culture grade water, autoclave it, and then store in a water bath at 42°C to prevent solidification.
2. Mix equal volumes of 1% agarose and 2× complete growth medium to form a solid underlayer. Add 1.5 ml of agarose/DMEM solution to each 35 mm tissue culture dish (or to each well of a 6-well plate) and allow to solidify for 1 h at ambient temperature. This gives a final underlayer concentration of 0.5% agarose in 1× complete growth medium.
3. While the agarose underlayer is solidifying, trypsinize and count the tumor cells. Wash the cells by centrifugation and then resuspend them at a density of 7.2×10^4 cells per 1.2 ml of complete growth medium.
4. Add 1.2 ml of 2× complete growth medium and 1.2 ml of 1% agarose to the cell suspension, and mix quickly by pipetting.
5. Add 1 ml of the cell suspension per 35 mm dish or well of a 6-well plate. This gives 2×10^4 cells/well in a suspension of 0.33% agarose and 1× complete growth medium. Set up three plates/wells per cell line, and allow the cell suspension to gel at ambient temperature for 30 min.
6. Incubate cultures at 37°C under standard conditions for 2 weeks. Add 100 μl of complete growth medium (containing serum) to each plate/well every 2 days. Inclusion of a positive control consisting of a cell line known to form colonies in suspension allows monitoring of growth.
7. After 2 weeks, photograph and measure the colonies under a light microscope, and then fix and stain colonies for 2 h at ambient temperature in a solution of 0.15% (v/v) methylene blue prepared in 10% EtOH. Destain by gentle washing under running tap water for 4 h. Photograph under a low power (e.g., 5×) objective lens, and quantify by counting the number of colonies in 20 random fields of vision.

As can be seen in Fig. 4, NIH3T3 fibroblasts stably transfected with p53-H179L form large colonies in soft agar (even larger than those formed by the positive control WT2 chondrogenic tumor cell line), whereas vector-transfected NIH3T3 cells and HN4 (negative control) do not grow under these conditions.

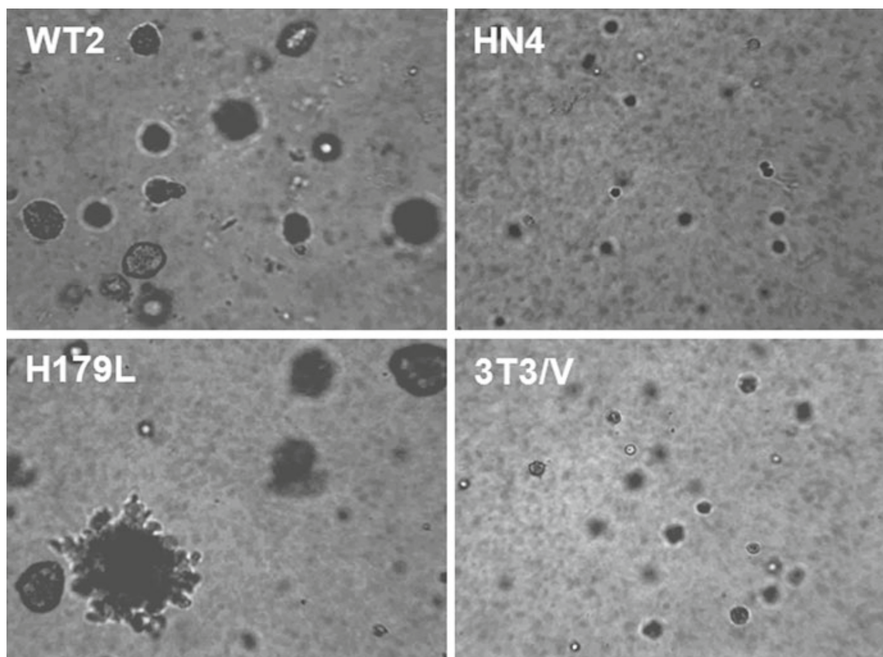

Fig. 4. NIH3T3 fibroblasts stably expressing p53-H179L or empty vector (3T3/V) were grown in soft agar culture as described in the text, with WT2 and HN4 cells as positive and negative controls for colony formation, respectively. Cells were stained with methylene blue and photographed. Original magnification ×50. Similar results can be obtained with H1299 cells expressing p53 mutants.

4. Notes

1. *Adhesion assay*: For all assays that involve the use of ECM proteins, it is important to dilute the proteins in cold DMEM/ BSA and maintain them on ice until wells or membranes have been coated. While we used fibronectin in the assays described here, it may be more appropriate for other cell lines to use alternative ECM substrates depending on their integrin expression profile.
2. *Wound-closure assay*: To denude cell monolayers, we routinely use a sterile pipette tip. This takes practice, as well as attention to angle of applying the tip and the pressure used, in order to produce wounds of equivalent size and the edges of which are visible under low power of the microscope being used. An alternative is to use sterile blocks machined to a precise width in each well and allow the cells to grow until they are in contact with the block. The blocks can then be removed and the time taken for cells to migrate across the cell-free area measured. For different cell lines, it will be necessary to determine appropriate times over which to carry out these assays empirically.

The same is true for chamber-type (haptotactic) migration assays.

3. *Haptotactic migration assay*: Prior to setting up these assays, cells are best removed from culture plates without the use of trypsin. Commercial non-enzymatic agents, as described above, or EDTA (versene) can be used. This is especially important if chemohaptotactic assays (for example, migration towards a growth factor gradient) are being performed, as trypsin can digest growth factor receptors on the cell surface very efficiently. There are many alternatives for staining cells in these assays, such as crystal violet and Diff-Quik (Baxter). The choice is largely down to personal preference. Also, some protocols suggest extracting dye from stained cells and measuring by spectrophotometry, much as we have described for adhesion assays (above). However, it should be cautioned that sometimes stain can be retained in the pores of the membrane if washing has not been thorough, resulting in an overestimation of the number of migrated cells.

Acknowledgments

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Chapter 12

Use of the DNA Fiber Spreading Technique to Detect the Effects of Mutant p53 on DNA Replication

Rebecca A. Frum, Sumitra Deb, and Swati Palit Deb

Abstract

DNA replication involves a coordinated progression through S phase, and disruption of these regulated steps may cause gene abnormalities, which may lead to cancer. Different stages of DNA replication can be detected immunofluorescently that would indicate how replication is progressing in a cell population or under specific conditions. We describe a method for labeling replicating DNA with two nucleotide analogs, and then detecting the sequential patterns of incorporation using fluorescently labeled antibodies on DNA spread onto a glass slide. Quantification of the different types of replication patterns produced by this method reveals how replication is achieved under different conditions by the predominance and lengths of elongating replication forks progressing from single or clustered origins, as well as the sites of termination from two converging forks.

Key words: DNA replication, Fiber spreading, Origins, Clusters, Fork elongations and terminating forks

1. Introduction

The replication of DNA has been studied on the level of individual fibers for almost five decades, and the techniques for studying it have developed over the years to detect and address different aspects of the replication process. Early replication studies used autoradiography to show that replication proceeds in distinct patterns and made use of a pulse-chase with tritiated thymidine to show the direction of fork movement from origins as indicated by the different intensities of the incorporated radioactivity (1–3). In the 1990s, several groups were able to use fluorescence in situ hybridization (FISH) to map decondensed DNA in nuclei or to

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detect single fibers of DNA extended onto slides (4–7). Other groups have subsequently combined the incorporation of single (8) or double (9) nucleotide analogs such as iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU) and their detection by fluorescently labeled antibodies to show different patterns of replication by color instead of radioactivity intensity. The double labeling method has been used for analyzing replication dynamics in subsequent studies (10–12).

The use of more than one marker such as different intensities of incorporation of radioactivity, or the use of more than one nucleotide analog sequentially incorporated into replicating DNA, enables the detection of different types of replication events occurring in the cell based on the directionality of fork movement. In the method we describe here, asynchronous cells are first pulsed with IdU for 10 min, which is then washed out and is followed by a pulse with CldU for 20 min. The IdU and CldU are incorporated into replicating DNA sequentially in the direction of fork movement. The cells are then collected, and a defined small number of cells is streaked across the top of a silane-coated slide and is then lysed. The slides are then tilted to a 45° angle, and as the droplet of lysis buffer is pulled down the slide by gravity, one end of the DNA sticks to the slide and the fiber is straightened by the force of the movement of the buffer down the slide. The slides are then air-dried and fixed, and the incorporated IdU and CldU are detected with fluorescent-coupled antibodies that identify IdU in red and CldU in green to reveal the direction of fork movement.

There are four readily distinguishable patterns of red and green that can be produced by this labeling strategy that indicate sites where replication is initiating at single or clustered origins bidirectionally, elongating or extending at forks, or where replication is merging between two adjacent replicons (terminations). As shown in Fig. 1a, a single red track at the center of two green tracks indicates a single bidirectional origin, with the origin of replication initiating during the first pulse with IdU (detected in red) and extending in both directions from the point of initiation as determined by the subsequent bidirectional incorporation with CldU (detected in green). Clusters of replicons are defined by the merging of two or more red and two or more green tracks that indicate two or more adjacent replicons that complete replication within the defined total pulse time (in this case, 30 min). If a red track within a cluster is surrounded by a green track on both sides, this center red track likely contains an origin. Elongating forks are represented by red and green tracks, while a red track flanked by a green track indicates fork movement from a different replicon towards the other origin in the cluster. Merging of two forks from two adjacent replicons indicates the site of termination by the pattern of a green track at the center of two red tracks, where replication forks are extending in the direction from red to green, since

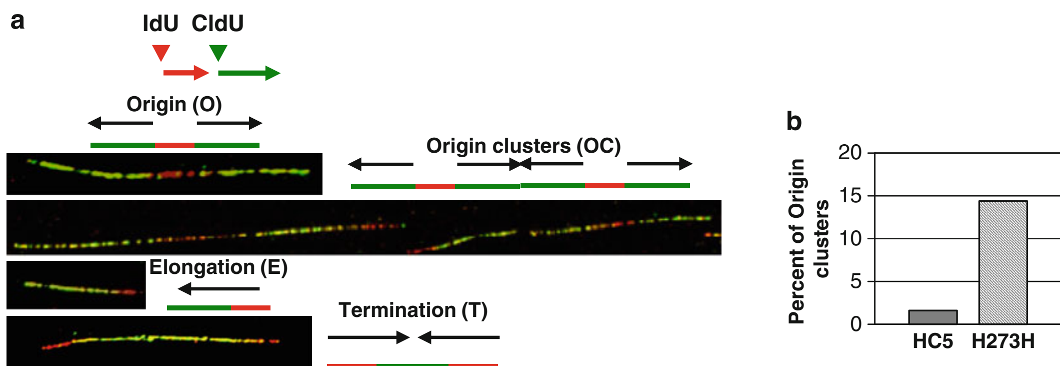

Fig. 1. The different types of replication patterns produced by the fluorescent detection of the sequential incorporation of IdU followed by CldU into replicating DNA are shown in (**a**). Since cells are labeled first with IdU (detected in *red*) followed by CldU (detected in *green*), the direction of fork movement can be determined in merged *red-green* tracks to reveal bidirectional replication from origins, elongating forks, as well as merged tracks of two or more adjacent replicons (*clusters*) and sites where two converging unidirectional forks from adjacent replicons merge (*terminations*). The bar graph in (**b**) shows higher percentage of origin clusters in H1299 cells expressing a tumor-derived mutant of p53 (273H).

IdU (red) was incorporated first followed by CldU (green). Single tracks of red or green can also be detected in this type of analysis as well, with a red track likely indicating a site where replication terminated or stalled before the nucleotide from the second pulse could be incorporated, while a single green track is likely indicative of a site where replication initiated after the first pulse with IdU had already been washed out. Asynchronous or synchronized cells at different hours of S phase could be used to study replication (11). Also, different pulse times with nucleotide analogs can be employed to reveal how replication extends from newly fired origins (12). In addition to these studies, analyses of replication on individual DNA fibers have been used to show the rate of fork elongation (11, 13), origin interference (14), the use of latent origins caused by a slower rate of replication (15), and differences in replication between normal and cancer cells (11, 12, 16).

Here, we describe a method for fluorescently detecting the different patterns of replication using DNA from H1299 cells either expressing mutant p53 or stably transfected with vector. The method involves the incorporation of IdU and CldU into replicating DNA, the creation of DNA fiber spreads by extending DNA linearly onto glass slides, and the immunofluorescent detection of the incorporated nucleotide analogs by confocal microscopy. We also describe strategies for quantifying and analyzing the replication patterns after images are obtained and discuss the different types of information that can be obtained from this type of analysis.

2. Materials

2.1. Nucleotide Analogs (Sigma)

1. Iododeoxyuridine (IdU, 100 mM).
2. Chlorodeoxyuridine (CldU, 100 mM).

2.2. Reagents

1. Hanks balanced salt solution (HBSS), phosphate-buffered saline (PBS), or Dulbecco's phosphate-buffered saline (DPBS).
2. DNA lysis buffer: 0.5% SDS, 200 mM Tris–HCl pH 7.4, 50 mM EDTA.
3. Methanol/Acetic acid (3:1).
4. 2.5 N HCl.
5. PBS/0.1% Tween 20.
6. 2% BSA. Make fresh. Dilute in PBS.
7. Rat anti-Bromodeoxyuridine.
8. Mouse anti-Bromodeoxyuridine.
9. Stringency buffer: 10 mM Tris–HCl pH 7.4, 400 mM NaCl, 0.2% Nonidet P40 (NP40).
10. Alexafluor 488-conjugated chicken anti-rat (Molecular Probes) (1:250).
11. Alexafluor 594-conjugated rabbit anti-mouse (Molecular Probes) (1:333).
12. 2% Normal goat serum (NGS). Make fresh. Dilute in PBS.
13. Alexafluor 488-conjugated goat anti-chicken (Molecular Probes) (1:250).
14. Alexafluor 594-conjugated goat anti-rabbit (Molecular Probes) (1:333).
15. Antifade without DAPI (Invitrogen).

2.3. DNA Fiber Spreading

1. Silane-coated slides (Sigma).
2. Coplin jars.
3. Plastic strips cut into the size of the glass slide surface.
4. Nail polish.
5. Confocal microscope.
6. Image J: This software can be downloaded from the NIH Web site.

2.4. Optional Reagents for Synchronization or S Phase Checkpoint Analysis

1. Aphidicolin.
2. Caffeine (50 mM).

3. Methods

3.1. Labeling of Replicating DNA

1. Add 2 μl of 100 mM IdU to 4 ml of media in the plate. Incubate for 10 min.
2. Wash off IdU two times with HBSS. Work quickly since cells are still actively replicating to ensure continuity of labeled tracks.
3. Add 4 μl of 100 mM CldU to 4 ml of media in the plate. Incubate for 20 min.
4. Wash two times with HBSS and trypsinized cells. Collect into a tube.
5. Spin cells at 500 × *g* for 6 min.
6. Wash with 4 ml of DPBS.
7. Repeat steps 5 and 6 once.
8. Count the number of cells present in the sample. Add DPBS to the sample so that the cells are suspended at 200–300 cells per μl.

3.2. Creating Spreads of DNA Fibers

1. Label slides in pencil. Make enough slide duplicates so that enough fibers will be obtained for analysis, which is usually three to ten depending on the experiment.
2. Vortex cells. Immediately streak 2 μl of the cell suspension across the glass near the top of the slide.
3. Allow most of the liquid to evaporate but do not let the streak dry.
4. Add 9 μl of DNA lysis buffer across the initial streak of cells. Allow cells to lyse for 10 min.
5. Tilt the slide to an approximately 45° angle to allow the buffer to run down the slide. Add 2 μl of extra buffer to the droplet if it starts to dry out.
6. Leave slides at the same tilted angle and allow to air-dry for 2 h.
7. Fix in 3:1 methanol/acetic acid. Dip in slide fixing chamber for 2 min. Place slides on an absorbent surface and allow them to dry overnight in a fume hood.
8. The next day, place the cells in a freezer at −20°C for at least 24 h.

3.3. Immunostaining

1. Remove slides from freezer and allow them to return to room temperature.
2. Fill Coplin jar(s) with 50 ml of 2.5 N HCl to cover the slides. Place slides into the jar and mix up and down a few times. Incubate for 30 min.

3. Using additional Coplin jars, wash once in PBS/0.1% Tween 20, followed by two washes in PBS for 3 min each.
4. Fill unused pipette tip boxes with hot water almost to the top to create a humid chamber. Remove the slides from the last wash and dry the back of the slide with a paper towel. Place onto dry surface of the tip box filled with hot water. Add 2 ml of 2% BSA onto the glass surface of the slide to block nonspecific binding of the antibodies. Spread 2% BSA solution evenly over the surface of the slide by creating surface tension between the solution on the slide and the wide opening of a 1 ml pipette tip. Incubate 2 h.
5. Discard the blocking solution on the slides into a beaker. Dry the back of the slide and add 100 μl of the primary antibody solution (1:250 rat anti-bromodeoxyuridine (detects CldU) plus 1:250 mouse anti-bromodeoxyuridine (detects IdU) in 0.2% BSA in PBS). Tilt the slide to make sure the antibody solution covers the entire glass surface and cover with a piece of plastic. Place onto dried surface of humid chamber and incubate for 1 h.
6. Wash slides for 10 min in stringency buffer in Coplin jar.
7. Wash slides two times in PBS in Coplin jar.
8. Dry the back surface of the slides and add 100 μl of the secondary antibody solution to the slide (1:250 Alexafluor 488-conjugated chicken anti-rat plus 1:333 Alexafluor 594-conjugated chicken anti-rat in 0.2% BSA in PBS). Tilt the slide to make sure the antibody solution covers the entire glass surface and cover with a piece of plastic. Place onto dried surface of humid chamber and incubate for 30 min in the dark.
9. Wash slides once in PBS/0.1% Tween 20, and then twice in PBS for 3 min each in a Coplin jar.
10. Dry the back surface of the slide and replace slide onto dried surface of the humid chamber. Add 1–2 ml of 2% NGS and spread the solution evenly over the slide as in step 3. Incubate for 15 min.
11. Discard the NGS blocking solution into a beaker and dry the back surface of the slide. Add 100 μl of the third antibody solution to the slide (1:250 Alexafluor 388-conjugated goat anti-chicken plus 1:333 Alexafluor 594-conjugated goat anti-rabbit in 0.2% NGS in PBS). Tilt the slide to make sure the antibody solution covers the entire glass surface and cover with a piece of plastic. Place onto dried surface of humid chamber and incubate for 30 min in the dark.
12. Wash slides once in PBS/0.1% BSA, then twice in PBS for 3 min each in Coplin jar.
13. Mount coverslips onto slides using antifade mounting solution. Seal coverslips onto slide with nail polish if necessary.

14. Collect images using a confocal microscope. Analyze images using Image J software program.

3.4. Optional Treatments

1. Normal cells can be synchronized by confluence arrest. Change the media as required by the cell line.
2. Replate cells at 374,000 cells on a 60 mm plate the day before labeling. Optionally, aphidicolin can be added to the plate for 24 h at a final concentration of 2 μg/ml for synchronization of cells.
3. To knockdown the intra-S phase checkpoint, treat cells with 2 mM caffeine for 30 min prior to labeling. During labeling with IdU and CldU, keep the same concentration of caffeine in the media to ensure that the checkpoint remains inactivated.

4. Notes

1. IdU and CldU are light sensitive, so it is recommended that labeling is performed without the light on in the hood.
2. Plating 374,000 cells per 60 mm plate is recommended to ensure proper availability of nucleotide analogs during pulse labeling.
3. Aphidicolin can be used to increase the number of cells synchronously entering S phase after 24 h; however, this may result in checkpoint activation. Confluence arrest alone generally creates a sufficient population of cells entering S phase synchronously. It generally takes about 13–14 h for confluence-arrested cells to reach early S phase following replating and approximately 8–10 h for cells to progress through S phase.
4. To save time during labeling, mixtures of IdU/media or CldU/media can be made in advance on the day of labeling and added directly to plates to save time with multiple additions.
5. While creating DNA fiber spreads, keeping the cell sample at room temperature just before streaking the cells onto the slide creates an even spread of cells and prevents the cell sample from forming droplets when streaked across the slide.
6. After streaking the cell sample on the slide, the streak appears rounded on the surface. Add the lysis buffer when the surface of the cell sample is flat to the surface of the slide but before it is dry.
7. After tilting the slide to create the fiber spreads, use a p20 pipette tip to create a small “point” on the edge of the droplet.

This encourages the drop to proceed down the slide at a good rate (usually 5–10 min).

8. To save time, the antibody mixtures can be made up during the initial blocking step in BSA and stored in dark at 4°C.
9. Bubbles generated when placing the plastic strips over the slides during antibody addition steps can be removed by sliding the plastic a little off the slide and then returning it.
10. When mounting coverslips with antifade, bubbles can also be generated. To prevent bubbles, place the glass coverslip on the bench and place three drops of antifade (about 100 μl total per slide) onto the coverslip. Dry the back surface of the slide and place it face down onto the antifade drops. Usually, keeping a hold of the slide while allowing it to just barely touch the surface of the drops at the same time creates an even spreading of the antifade with minimal bubbles. When bubbles are present, they can be removed by tapping one side of the bubble with tweezers.
11. When focusing the confocal microscope to find the fibers, they are on the same plane as the surface of the slide. Focusing on the surface of the slide first (tiny dots of fluorescence or any label on the slide) will enable the detection of the DNA fibers.
12. Images can be analyzed using the Image J software program. To ensure that all fibers in an image are scored, create an RGB image and after measuring the track length or counting the number of fibers, erase the scored fiber in the RGB image using the eraser tool. Since there are always fibers present in the images that are unscorable (overlapping fibers, overly stretched fibers, undistinguishable labeled tracks), this helps ensure that all scorable fibers are counted in the image.
13. Slides can usually be analyzed for up to a month after staining.
14. This type of analysis can give both quantitative (length of fibers) and qualitative (percent of type of replication patterns) present in the samples. If checking for both parameters, it is recommended that the length of the fibers be measured and the number of these scored fiber lengths be used to generate the percent of each replication pattern out of the total number of scored patterns. This ensures that the same fibers are used for both analyses.

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Chapter 13

Generation of p53-Deficient Induced Pluripotent Stem Cells from Mouse Embryo Fibroblasts

Evguenia M. Alexandrova and Ute M. Moll

Abstract

Here we describe a method for generating induced pluripotent stem (iPS) cells from mouse embryonic fibroblasts (MEFs). Recombinant retroviruses carrying human transcription factors for Klf4, Oct3/4, Sox2, with or without c-Myc, are used to transduce early passage MEFs several times. Based on morphologic criteria, the resulting iPS colonies are picked manually at first, and then propagated and expanded by standard methods. iPS cells can then be differentiated into virtually any cell type or lineage, thus allowing for discoveries of new functions of p53 and mutant p53.

Key words: iPS, MEF, Retrovirus, Reprogramming

1. Introduction

Induced pluripotent stem (iPS) cells are now widely used as a more accessible surrogate for embryonic stem (ES) cells. Similarly to ES cells, iPS cells can be differentiated *in vitro* and *in vivo* into virtually any cell type (1). Reprogramming somatic cells to pluripotency was first successfully achieved by Yamanaka and colleagues in 2006, by means of retroviral transduction of mouse fibroblasts with four human transcription factors involved in pluripotency maintenance: Klf4, c-Myc, Oct3/4, and Sox2 (2). Here we describe a method for reprogramming mouse embryonic fibroblasts (MEFs) into iPS cells with either four or three of Yamanaka's factors (3). The reprogramming factors are cloned into the retroviral genome and packaged into retroviruses in Phoenix E cells (4). The retroviral

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supernatant is then used to transduce MEFs. In the course of a few weeks, ES-like colonies appear and are manually picked for further propagation by standard methods. If starting with p53−/− MEFs or MEFs that carry mutant p53, possibly in combination with another gene deletion/mutation, one can use the resulting iPS cells to analyze the role of p53 in: (1) differentiation into a certain cell type, (2) a cell signaling pathway involved, (3) in the specification to certain lineages, or (4) in the process of reprogramming itself.

2. Materials

1. Accutase solution (Sigma).
2. Antibiotic–antimycotic, 100× (Gibco).
3. DPBS: Dulbecco's phosphate-buffered saline, 1×.
4. ES medium: DMEM/F12+GlutaMAX-I (Gibco), 10% KSR (knockout serum replacement, Gibco), 2 mMl-Glutamine (Gibco), 1× MEM nonessential amino acids (Gibco), 55 μm 2-mercaptoethanol (Gibco), 1× penicillin–streptomycin, 1,000 U/ml ESGRO mLIF (Millipore).
5. ES medium/FBS: same as above, except that 10% FBS (Hyclone) is used instead of KSR.
6. Freezing medium: 50% FBS (Hyclone), 40% ES medium/FBS, 10% DMSO Hybri-MAX (Sigma).
7. Gelatin, 0.1% in ultrapure water (Millipore).
8. Lipofectamine reagent (Invitrogen).
9. MEFs: mouse embryonic fibroblasts obtained by standard methods from day E12-14 embryos (5).
10. MEF medium: 1× high glucose DMEM (Gibco), 10% heat-inactivated fetal bovine serum, 1× penicillin–streptomycin.
11. Mouse feeders: mytomycin-treated or gamma-irradiated MEFs (GlobalStem).
12. Phoenix E cells (4).
13. pRebna-hKlf4, pRebna-hOct3/4, pRebna-hSox2 (optional: pRebna-h-c-Myc and pRebna-GFP) plasmids (6) from midi- or maxi-prep (see Note 1). Alternatively, the same human genes in pMXs vector (Addgene).
14. Puromycin, 1 mg/ml (500×).
15. Trypsin–EDTA, 0.05% (Gibco).

3. Methods

3.1. Production of Retroviral Supernatants

1. Apply 4 ml gelatin to a 10 cm tissue culture dish, wait 20 min, remove gelatin, let dry 1–2 min, seed Phoenix E cells in MEF medium (see Note 2).
2. In a 10 cm dish, obtain actively growing Phoenix E cells at 60–80% confluency (see Note 3).
3. Transfect Phoenix E cells for 12 h or overnight with a mixture of four (5 μg each) or three (6.7 μg each) DNAs encoding Yamanaka's factors in pRebna or pMXs vector, using Lipofectamine reagent according to the manufacturer's directions (see Note 4). At the end of transfection, add 10 ml MEF medium.
4. 24 h after transfection, puromycin selection can be started if reprogramming factors were in puromycin-selectable pRebna vector. Select cells for 2–3 days (see Note 5).
5. After selection is completed, add MEF medium for at least 12 h. Collect the virus-containing supernatants every 12–24 h, filter through a 0.45 μm filter to get rid of Phoenix E cells, and use immediately for steps in Subheading 3.2 or freeze in −80°C, since viral particles are unstable at RT or +4°C (see Note 6).

3.2. Retroviral Transduction of MEFs

1. Treat the desired number of 6 cm plates with 2 ml gelatin as in step 1 of Subheading 3.1. Add 2 ml fresh or just thawed retroviral supernatant per plate and put the plates into 37°C incubator.
2. Trypsinize and spin down actively growing, early passage (passage 0–3, fresh or frozen) MEFs in two tubes, approximately 0.1–0.5 million cells per tube for one reprogramming experiment (see Note 7). Remove the supernatant.
3. Add 1 ml filtered retroviral supernatant to one tube of cells, resuspend, and count cells. Add approximately 250,000 MEFs to the warm plate prepared in step 1 in Subheading 3.2. If there are not enough cells in one tube, add the viral supernatant to the other tube, resuspend the cell pellet, and add the required number of cells to the same plate (see Notes 8 and 9).
4. Collect, filter, and add fresh retroviral supernatants to MEFs every 12–24 h for a total of three to five times. If at the time of last transduction MEFs appear overgrown, split them 1:2 or 1:3 directly in the retroviral supernatant, as in steps 1–3 of Subheading 3.2. For the rest of the experiment, the MEFs will not be split.
5. 24 h after the last transduction, change the medium to ES medium. Add fresh ES medium every other day and observe

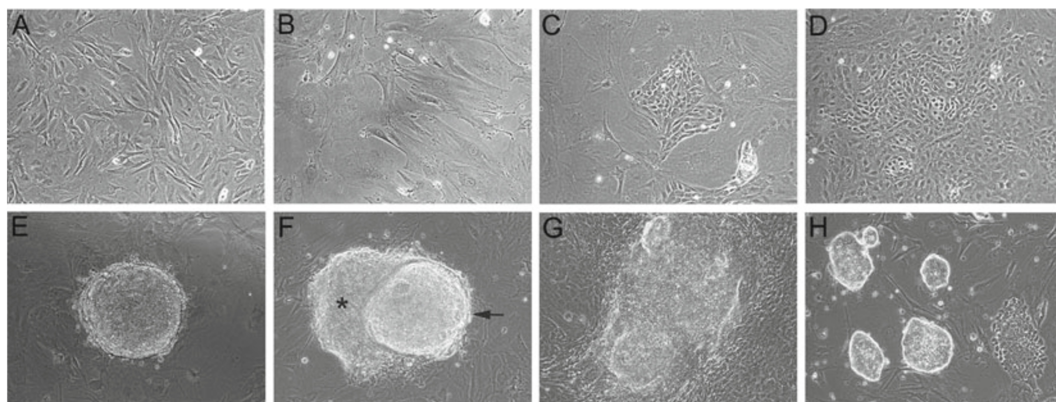

Fig. 1. Morphologies of MEF cells and iPS colonies during different stages of reprogramming. (**a**, **b**) MEFs from two different embryos were seeded (230,000 cells per a 6 cm dish) and retrovirally transduced with 3F supernatants for five times once a day. At that point images were taken. Cells in (**a**) proliferate nicely and show a healthy compact morphology. These cells likely will yield a high efficiency of iPS colony formation. Cells in (**b**) show a large, flat, spread-out morphology and low cell density, indicative of poor MEF quality with poor proliferation. They will likely result in low reprogramming efficiency and delayed iPS formation. In such a case it is best to repeat the transduction with better cells of lower passage and/or higher cell density, while keeping the original dish. In this particular case MEFs in (**a**) were p53–/– and MEFs in (**b**) were wild type. (**c**, **d**) 1–3 days after the last retroviral transduction, epithelial patches (**c**) become visible among the sea of MEFs with typical spindly mesenchymal morphology. These epithelial cells are compact, polygonal, tightly attached to each other and often have enlarged nuclei and shiny cell borders. The epithelial patches increase in size over time and may eventually occupy almost the entire plate (**d**). (**e**) A high quality *un*differentiated iPS colony has a "tight" morphology, i.e., smooth shiny borders and usually a round shape. The colony is elevated above the rest of the cells. This is the one to pick. (**f**) A partially differentiated iPS colony shows a flat sheet of cells (*asterisk*) with a rough, somewhat irregular border growing out from an iPS colony (*arrow*) either on one side, as shown here, or all around (not shown). Do not pick such colonies. (**g**) A completely differentiated iPS colony is flat and has poorly defined borders. Do not pick such colonies. (**h**) When primary iPS colonies are passaged, they are often not perfectly round, but still should have well-defined shiny borders and compact cells. In contrast, a differentiated colony (shown on the right) has poorly defined borders and flat spread-out cells. (**c**–**h**) Note, the morphologies of these stages are identical between wild-type and p53–/– genotypes.

cells (see Notes 10 and 11). The cell morphology should change to epithelial-like by day 5–7 (Fig. 1c, d) and iPS colonies should appear by day 15–20 from the beginning of reprogramming (Fig. 1e–f).

3.3. Manual Collection of iPS Colonies

1. Carefully observe your plates at low magnification every day and mark appearing colonies with a marker on the bottom of the dish. The colonies should be picked 2–4 days after their first appearance. Avoid picking big and partially or completely differentiated colonies (Fig. 1e, h) (see Note 12).
2. One day in advance, estimate the number of colonies to be picked (typically 10–30 per plate) and seed mouse feeders in MEF medium on the desired number of gelatin-coated wells (see step 1 in Subheading 3.1), plus some additional wells, in 24-well plates (see Note 13).

3. On the next day, wash the feeders twice for at least 30 min at 37°C with ES medium/FBS without LIF. Change the medium to ES medium/FBS with LIF and return plates to the 37°C incubator.
4. In a 96-well plate, add 40 μl accutase solution to the number of wells corresponding to the number of colonies to be picked, plus several additional ones. Keep it in the tissue culture hood. Spray the microscope to be used for picking colonies with 70% EtOH, wipe dry, and put under the hood.
5. In the hood, put one dish with iPS colonies under the microscope at low magnification and open it (see Note 14). Locate the first colony to be picked, separate it from the surrounding MEFs with a plastic 10 μl pipette tip (without the pipetman) so that it floats above the rest of the cells. With a pipetman set for 10 μl and a new pipette tip, suck in the floating colony and transfer it to one well of the 96-well plate with accutase, pipetting up and down gently about 20 times. Leave the pipette tip in the well until about eight colonies are picked (one column in a 96-well plate).
6. When eight colonies are picked, pipette each again up and down about 20 times and examine the cells under the microscope. There should be single cells floating in the wells but no big clumps (see Note 15). If necessary, destroy the remaining cell clumps by additional pipetting, possibly with a pipetman set for 40 μl. Bring one 24-well plate prepared in step 3 of Subheading 3.3 from the incubator, transfer iPS cells onto feeders, and return the plate to incubator. Proceed the same way with the remaining colonies.
7. Approximately 5 h later (or after overnight), change the medium to ES medium.
8. Multiple colonies should appear 2–4 days later. To further expand them, prepare feeders in 6 cm plates as in steps 2 and 3 of Subheading 3.3 and transfer all or part of the cells from each well of the 24-well plates (see Note 16) into one 6 cm plate by trypsinization (Subheading 3.4).

3.4. Maintenance and Passage of Mouse iPS Cells

iPS cells can be passaged onto mouse feeders by standard trypsinization (7). It is recommended to seed 100,000–300,000 cells per a 6 cm plate or 500,000 cells per a 10 cm plate (see Notes 17 and 18).

3.5. Cryopreservation and Thawing of Mouse iPS Cells (7)

1. To freeze iPS cells, label and pre-cool cryovials in a styrofoam box at −80°C (see Note 19). Prepare the freezing medium and keep it at 4°C.
2. Trypsinize, spin down, and remove the supernatant from the cells to be frozen. Keep cryovials in the styrofoam box (this way, they are cold throughout the procedure). Add freezing

solution to the cells, resuspend them, and distribute to cryovials (see Note 20). It is recommended to freeze 0.5–1 million iPS cells per ml. Close the cryovials, cover them with another styrofoam box, and put at −80°C (the styrofoam box will make the freezing process gradual). After 1 day, the cryovials can be put in liquid nitrogen for long-term storage.

3. To thaw iPS cells from a frozen stock, prepare plates with feeders in advance as in steps 2 and 3 of Subheading 3.3. Let the frozen cells half-melt in a 37°C water bath. Promptly add the cells drop-wise to a tube of pre-warmed ES medium/FBS, spin down to remove DMSO, resuspend in fresh ES medium/FBS, and plate. Add ES medium after 5 h to overnight.

4. Notes

1. The human reprogramming factors in pRebna vector were generated by Dr. Olexi Petrenko in our laboratory. They have not been previously published and are available upon request.
2. For Phoenix E cells, always use gelatinized dishes and add the medium gently along the dish wall, as these cells easily detach. Ideally, have a separate bottle of MEF medium designated for phoenix E cells and be careful not to contaminate any other cells, including MEFs to be reprogrammed, with phoenix E cells, as they are highly proliferative and can make false ES-like colonies.
3. For best transfection efficiency, Phoenix E cells should be single, not in patches. If you have the desired density but cells in patches, trypsinize, resuspend, and re-seed them several hours before transfection.
4. It may be beneficial in certain cases to use only three factors without c-Myc, since the latter confers tumorigenicity onto iPS cells (1). Moreover, we also noticed that reprogramming efficiency is higher with three factors without c-Myc compared to four factors.
5. To see transfection efficiency and the progress of puromycin selection, one can use pRebna-GFP in a parallel plate of cells. It is not recommended to add pRebna-GFP to the mixture of reprogramming factors, as it will reduce both cell viability and the percentage of cells that produce recombinant retroviruses.
6. If during puromycin selection or viral production Phoenix E cells detach from the plate or overgrow, which is evident by a high cell density and yellow color of the medium, they can be replated with or without splitting onto gelatinized dishes. After plating, always wait for the cells to attach well before adding

puromycin, for at least several hours. Transfected Phoenix E cells will produce retroviruses for about a month. Additional puromycin selection can be done during this period.

7. p53−/− MEFs can also be used at a later passage, since they proliferate better and are reprogrammed easier than wild-type MEFs, which senesce after about 10–12 passages (8, 9).
8. Alternatively, MEFs can be plated in advance in MEF medium. After the cells are attached, remove the MEF medium and add 3–4 ml of retroviral supernatant.
9. If MEFs in the starting culture do not look healthy and do not actively proliferate, e.g., appear too flat and spread (Fig. 1a, b), they will not be effectively transduced with the viruses and produce few or no iPS colonies. To overcome this problem, either use earlier passage cells if possible, or begin with more cells, e.g., 400,000–500,000.
10. If you are concerned that the preparation of primary MEFs might be contaminated, antibiotic–antimycotic can be added to the ES medium.
11. If control MEFs were transduced with the GFP virus, fluorescence should be visible 2–4 days after the last transduction. Transduction efficiency can be assessed at this time by either microscopy or FACS analysis.
12. If you get too few colonies, you might try to collect and propagate the not-perfect looking ones as well. Since they probably contain undifferentiated iPS cells, those can outgrow differentiated cells and still make a good iPS culture.
13. At this time, extra feeders can be seeded onto gelatin-coated 6 cm plates to be used in step 8 of Subheading 3.3, to expand iPS clones. Seeded mouse feeders can be used for 1–2 weeks, provided that fresh MEF medium is added every 3 days.
14. In order not to contaminate your iPS cells, frequently spray gloves with EtOH during the procedure.
15. When working with iPS cells, always make sure to passage single cells, since clumps will promote cell differentiation.
16. In the 24-well plate (passage 0), not all clones will be established. For expansion, select the wells that have at least several colonies and also make sure that the majority of the colonies are undifferentiated (Fig. 1d).
17. Care should be taken: (1) not to let the colonies overgrow (which would promote differentiation), (2) to propagate single cells (cell suspensions can be passed through a sterile 45 μm mesh to remove cell clumps, prior to seeding), (3) to treat cells with warm or room temperature trypsin at 37°C just until they lift off the plate (usually about 2 min), since overtreated cells will attach poorly, (4) to plate the cells into warm ES

medium/FBS (FBS will promote their attachment better than KSR), and (5) to change the medium to KSR-containing ES medium no later than after one night.

18. If, during early passages, too many iPS colonies appear differentiated, it is possible to repeat the manual selection of undifferentiated colonies as in Subheading 3.3.
19. We typically use the styrofoam racks that come with the 15 ml conical tubes to put cryovials into and cover them with.
20. When freezing iPS cells, work promptly to reduce the amount of time the cells are in contact with DMSO, which is toxic for them.

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Chapter 14

p53 Actions on MicroRNA Expression and Maturation Pathway

Hiroshi I. Suzuki and Kohei Miyazono

Abstract

The tumor suppressor p53 orchestrates multiple cellular pathways as a central node of anti-oncogenic programs in response to DNA damage, oncogene activation, and several stresses. In addition to the principal role as a transcription factor that transactivates many target genes involved in apoptosis and cell cycle control, p53 has been shown to exert various transactivation-independent effects both in the nucleus and in the cytoplasm. Diversity of p53 activities is further emphasized by the recent studies revealing the close interaction between the p53 and microRNA (miRNA) world. We recently demonstrated that p53 promotes the processing of several primary miRNA transcripts through association with Drosha, a central RNase III in miRNA biogenesis, under DNA damage-inducing conditions. In contrast to wild-type p53, cancer-derived p53 mutants attenuate miRNA maturation. These findings reveal a novel aspect of p53 activities and suggest complex crosstalks between miRNA biogenesis and intracellular signaling pathways. In this chapter, we describe the methods for evaluation of the effects of p53 on miRNA expression, an interaction between pri-miRNA and Drosha complex, and pri-miRNA processing activity of the Drosha complex.

Key words: microRNA, p53, p68, Drosha, Dicer, Processing

1. Introduction

p53 tumor suppressor is at the center of a complex web composed of numerous signaling pathways triggered by a wide range of cellular stresses including DNA damage, oncogene activation, and hypoxia (1). Loss of p53 function occurs in most human tumors by either mutation of the p53 gene itself, or by inactivation of the p53

Potential conflicts of interest: none.

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signal transduction pathway (2). p53 is well characterized as a sequence-specific transcription factor that transactivates many target genes such as p21, 14-3-3σ, GADD45, and PUMA upon activation by several stresses. The target genes of p53 are involved in various cellular pathways such as apoptosis, cell cycle arrest, and metabolism. In addition to the role as a transcriptional activator, p53 has been shown to exert several transcriptional-independent and/or cytoplasmic activities (3). In the nucleus, p53 regulates DNA replication and homologous recombination apart from the transactivation-dependent effects. It has also been demonstrated that p53 promotes apoptosis through induction of mitochondrial outer membrane potential at the mitochondria and regulates autophagy at the cytoplasm. Furthermore, recent studies revealed a close relationship between the p53 pathway and microRNA (miRNA) network (4).

miRNAs are emerging regulators of post-transcriptional gene regulation through RNA-induced gene silencing (5). miRNAs are short 20–23-nucleotide single-stranded RNA molecules derived from miRNA genes and introns of various mRNAs. Mature miRNAs form RNA-induced silencing complex (RISC) with Argonaute (Ago) proteins and guide RISC to target mRNAs through nucleotide complementarity between miRNAs and target mRNAs, leading to degradation, destabilization, or translational inhibition of target mRNAs. One miRNA species targets multiple target mRNAs and modulates diverse cellular functions, taking advantage of the flexible target recognition based on partial complementarity. Intense researches have disclosed that miRNA expression alters in several cellular contexts and that various miRNAs are involved in diverse physiological and pathological processes (6).

miRNAs originate from primary miRNA transcripts containing one or more stem-loop structures, which are mainly transcribed by RNA polymerase II from miRNA genes. These primary miRNAs are subjected to two-step RNase III-mediated cleavage to generate mature miRNAs. Drosha, an RNase III enzyme, cleaves out the stem-loop portion (precursor miRNA, pre-miRNA) from pri-miRNAs in the nucleus (7–9). The Drosha complex accompanies several cofactors such as DGCR8 (DiGeorge critical region 8), certain heterogeneous nuclear ribonucleoproteins (hnRNPs), p68 (DDX5), and p72 (DDX17) DEAD-box helicases (7). p68 and p72 are required for adequate processing of a subset of, but not all, miRNAs (10). The resultant pre-miRNAs are exported to the cytoplasm and further processed to double-stranded miRNA duplexes by Dicer, another central RNase III in miRNA biogenesis (11, 12). One strand of miRNA duplex is finally loaded onto the RISC and executes RNA silencing. Recent advances disclosed that miRNA biogenesis is dynamically regulated at multiple steps of miRNA maturation (13).

p53 modulates the expression of various miRNAs through several mechanisms. As a transcriptional activator, p53 activates the transcription of the miR-34 family (miR-34a, miR-34b, and miR-34c) and elevates these miRNAs (14–17). miR-34s regulate targets involved in the cell cycle and apoptosis, such as cyclin-dependent kinase 4 (cdk4), cyclin E2, and Bcl-2. miR-192, miR-194, and miR-215 are additional miRNAs that are transcriptionally activated by p53 (18, 19). p53 has been also shown to suppress miRNA expression including miR-17-92 cluster through transcriptional repression. Under hypoxia, the expression of miR-17-92 cluster is repressed through a competitive block of the recruitment of TATA-binding protein (TBP) to the miR-17-92 promoter by p53 binding to the promoter (20).

We have recently demonstrated that p53 modulates the miRNA system in a more intimate manner (21). In addition to miR-34 induction by p53, DNA damage induction resulted in upregulation of several miRNAs, including miR-143 and miR-16, in a p53-dependent fashion (21, 22). This upregulation of mature miRNA expression was accompanied with the upregulation of pre-miRNAs, but not pri-miRNAs, suggesting the enhancement of pri-miRNA processing activity. In consistent with the previous report of an interaction between p53 and p68/p72 (23), it was confirmed that p53 was associated with the Drosha complex through the interaction with p68/p72. RNA immunoprecipitation (RIP) analysis showed that p53 enhanced the association of Drosha and p68 with the target pri-miRNAs. In vitro pri-miRNA processing analysis further revealed that the pri-miRNA processing activity of Drosha for pri-miR-143/-16 increased in a p53-dependent manner under DNA damage-inducing conditions. In contrast to the enhancement of miRNA processing by wild-type p53, transcriptionally inactive tumor-derived p53 mutants have been shown to hinder the miRNA processing of certain miRNAs including miR-143 and miR-16. These findings indicate that p53 modulates miRNA biogenesis through the interaction with the Drosha complex and suggest that transcription-independent regulation of miRNA biogenesis is embedded in an anti-oncogenic program governed by p53.

In addition, another line of evidence showed the close connection between the p53 network and miRNA system (4). Several miRNAs such as miR-29 family, miR-125b, miR-122, and miR-372/373 have been shown to regulate the p53 activity and the p53 downstream pathway (4, 24–27). It was also reported that a loss of mature miRNAs results in increased DNA damage and p53 activity (28), revealing a reciprocal connection between the p53 and miRNA pathways. Most recent study has identified Dicer and miR-130b as transcriptional targets of p63, a p53 family member, but not p53, underscoring the crosstalk between p53 family members

and miRNAome (29). Further investigations would provide insights about miRNA involvement in the p53 network.

In this chapter, we detail the general methods for evaluation of the effects of p53 on miRNA expression and biogenesis; (1) miRNA expression analysis (qRT-PCR analysis and northern blot analysis); (2) RIP assay examining the interaction between pri-miRNA and target proteins; and (3) in vitro pri-miRNA processing analysis assessing the activity of Drosha complex.

2. Materials

2.1. Isolation of RNA and miRNA Expression Analysis

2.1.1. RNA Preparation

1. Cells of interest.
2. Trizol (Invitrogen).
3. Chloroform, molecular biology grade.
4. Isopropyl alcohol, molecular biology grade.
5. Nuclease-free water.
6. Ethanol: 100%, 75%.
7. Glycogen (5 mg/ml, Applied Biosystems, Cat No. AM9510): optional use.
8. mirVana miRNA isolation kit (Applied Biosystems, Cat. no. AM1560).

   *Other equivalent kits for preparation of <200 nt small RNA (e.g., miRNeasy Mini kit (Qiagen)) can be used.

2.1.2. miRNA RT-PCR Analysis

1. RNA samples from Subheading 3.1.1.
2. Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System.

Analysis of Mature miRNA

1. Taqman microRNA Reverse Transcription kit (Applied Biosystems).
2. Taqman microRNA assays (for the target miRNA) (Applied Biosystems).
3. Taqman 2× Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems).

   *Other alternative kits for miRNA detection (e.g., miScript PCR System (Qiagen)) can be used.

Analysis of Pri-miRNA and Pre-miRNA

1. Quantitect Reverse Transcription kit (Qiagen).
2. FastStart Universal SYBR Green Master (Roche).

   *Items 1–2: other equivalent kits can be used.
3. RT primers for amplification of pri-miRNA and pre-miRNA.

2.1.3. Northern Blot Analysis for miRNA

Probe Generation

1. Probe: DNA oligonucleotide complementary to the microRNA of interest.
2. T4 Polynucleotide kinase (TAKARA).
3. [γ- ^{32}P]ATP (PerkinElmer).
4. ProbeQuant G-50 Micro Columns (GE Healthcare).

Preparation of Denaturing Urea Polyacrylamide Gel

1. Gel cast.
2. Gel solution: Urea 7.2 g, 30% Acrylamide solution 7.5 ml, and 10× TBE 1.5 ml.
3. 20% APS solution.
4. TEMED.

Gel Electrophoresis and Blotting

1. RNA samples from Subheading 3.1.1.
2. RNA loading buffer.
3. Gel from Subheading "Preparation of Denaturing Urea Polyacrylamide Gel."
4. Running Buffer: Dissolve 48 g Urea, 50 ml 10× TBE, and DEPC 1 ml in water and autoclave to make 1 L.
5. RNA size marker (Ambion Decade RNA Marker (Applied Biosystems) or DynaMarker Prestain Marker for Small RNA (Plus)).
6. 0.5× TBE (pre-cold).
7. Nylon Membranes, positively charged (Roche).
8. 3 M Paper.
9. Semi-Dry Transfer Unit.
10. UV crosslinker.

Hybridization

1. Membrane from Subheading "Gel Electrophoresis and Blotting."
2. 20× SSC.
3. ExpressHyb Hybridization Solution (Clontech).
4. 10% SDS solution.
5. Washing solution I: 2× SSC, 0.05% SDS.
6. Washing solution II: 0.1× SSC, 0.1% SDS.
7. Autoradiography system (e.g., Fujifim BAS-2500).

2.2. RIP Analysis

2.2.1. Preparation of Whole Cell Lysates

1. Cells of interest.
2. Phosphate-buffered saline (PBS).
3. RNase inhibitor.
4. Formaldehyde solution (37%).
5. Glycine solution (1–1.5 M).
6. SDS-Lysis buffer: 1% SDS, 10 mM EDTA, 50 mM Tris–HCl pH 8.0, 1% protease inhibitor mixture, and 0.5 U/μl RNase inhibitor.

7. Bioruptor sonicator or equivalent.
8. ChIP dilution buffer: 1% Triton X-100, 50 mM Tris–HCl pH 8.0, 165 mM NaCl, 0.11% sodium deoxycholate, 1% protease inhibitor mixture, and 0.5 U/μl RNase inhibitor.

2.2.2. Immunoprecipitation and Wash

1. Cell lysate samples from Subheading 3.2.1.
2. Protein G (A) agarose beads (Sigma).
3. Antibody to the protein of interest (e.g., p53 DO-1 (sc-126) or FL-393 (sc-6243) (Santa Cruz); p68 05-850 (Upstate); Drosha 07-717 (Upstate)).
4. 1× RIPA buffer/150 mM NaCl: 0.1% SDS, 1% Triton X-100, 1 mM EDTA, 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% sodium deoxycholate, 1% protease inhibitor mixture and 0.5 U/μl RNase inhibitor.
5. 1× RIPA buffer/500 mM NaCl: 0.1% SDS, 1% Triton X-100, 1 mM EDTA, 50 mM Tris–HCl pH 8.0, 500 mM NaCl, 0.1% sodium deoxycholate, 1% protease inhibitor mixture and 0.5 U/μl RNase inhibitor.
6. LiCl wash buffer: 250 mM LiCl, 1% Nonidet P40, 1 mM EDTA, 10 mM Tris–HCl pH 8.0, 0.5% sodium deoxycholate, 1% protease inhibitor mixture and 0.5 U/μl RNase inhibitor.
7. TE pH 8.0.

2.2.3. Elution, Reversal of Crosslinks, and RNA Purification

1. Immunoprecipitated complex on beads from Subheading 3.2.2.
2. ChIP elution buffer: 0.5% SDS, 5 mM EDTA, 10 mM Tris–HCl pH 8.0, 300 mM NaCl, and 0.5 U/μl RNase inhibitor.
3. Proteinase K (10 mg/ml).
4. Trizol, glycogen, and reagents for RNA isolation (see Subheading 2.1.1).

2.2.4. RT-PCR Analysis

1. DNase I.
2. Quantitect Reverse Transcription kit (Qiagen).
3. FastStart Universal SYBR Green Master (Roche).

   *Items 2 and 3: Other equivalent kits can be used.
4. Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System.

2.3. In Vitro Pri-miRNA Processing Analysis

2.3.1. Preparation of Pri-miRNA Template and (Radiolabeled) Pri-miRNA

1. Linearized DNA template containing T7 promoter and a target pri-miRNA fragment, or equivalent.
2. DIG RNA Labeling Kit (SP6/T7) (Roche).
3. ATP, CTP, GTP, and UTP (Roche).
4. [α-^{32}P]UTP (PerkinElmer).

5. RNase inhibitor.
6. RNase-free water.
7. ProbeQuant G-50 Micro Columns (GE Healthcare).

2.3.2. Preparation of Drosha Complex

1. Cells of interest, which produce FLAG-Drosha, and FLAG-Drosha expression vector.
2. PBS.
3. Lysis buffer: 1% Nonidet P40, 20 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, and 1% protease inhibitor mixture.
4. Anti-FLAG M2 Affinity Gel (Sigma).

2.3.3. In Vitro Processing Reaction and Analysis

1. Radiolabeled pri-miRNA substrates.
2. Immunoprecipitated FLAG-Drosha complex on beads.
3. Processing buffer: 20 mM Tris–HCl pH 7.9, 0.1 M KCl, 10% Glycerol, 5 mM dithiothreitol, and 0.2 mM PMSF in water.
4. Energy solution buffer: 32 mM $MgCl_2$, 10 mM ATP, and 200 mM creatine phosphate in water.
5. RNase inhibitor.
6. Nuclease-free water.
7. Trizol, glycogen, and reagents for RNA isolation (see Subheading 2.1.1).
8. TBE-urea 15% acrylamide precast gels.
9. Running Buffer: Dissolve 48 g Urea, 50 ml 10× TBE, and DEPC 1 ml in water and autoclave to make 1 L.
10. Small RNA marker (e.g., Ambion Decade RNA Marker (Applied Biosystems) or DynaMarker Prestain Marker for Small RNA (Plus)).
11. Autoradiography system (e.g., Fujifilm BAS-2500).

3. Methods

3.1. Isolation of RNA and miRNA Expression Analysis

The following methods for miRNA expression analysis are composed of (1) preparation of RNA samples, (2) qRT-PCR analysis, and (3) northern blot analysis. Stem-loop reverse transcription (RT)-based TaqMan® MicroRNA assays are most widely used for the evaluation of mature miRNA expression by RT-PCR analysis (30).

3.1.1. RNA Preparation

1. Prepare the cells of interest. Before RNA preparation, cells would be transfected with siRNAs of interest (e.g., p53, p68,

and so on), infected with adenovirus carrying wild-type p53 or p53 mutants and/or treated with various DNA damage-inducing agents.

2. Remove the media and lyse cells by directly adding 1 ml of Trizol to a 35–100-mm dish and pipetting the cell lysate several times (see Notes 1 and 2).
3. Incubate the homogenized samples for 5 min at room temperature.
4. Add 0.2 ml of chloroform per 1 ml of Trizol reagent. Shake tubes vigorously for 15 s.
5. Incubate for 2–3 min at room temperature.
6. Centrifuge at 12,000 × *g* at 4°C for 15 min.
7. Transfer the upper aqueous phase containing RNA to a new tube and add 0.5 ml of isopropyl alcohol per 1 ml of Trizol reagent initially used. During isopropanol precipitation, glycogen can be added as a career.
8. Incubate for 10 min at room temperature.
9. Centrifuge at 12,000 × *g* at 4°C for 10 min.
10. Remove the supernatant. Add 1 ml of 75% ethanol and vortex.
11. Centrifuge at 7,500 × *g* at 4°C for 5 min.
12. Remove the supernatant and dry the RNA pellet.
13. Dissolve RNA in nuclease-free water by pipetting several times and incubating at 55–60°C for 10 min.
14. (Optional) For small RNA (<200-nt) purification, total RNA purified by Trizol reagent can be subjected to further fractionation using mirVana miRNA isolation kit (Applied Biosystems) or miRNeasy Mini kit (Qiagen), according to the manufacturer's instructions. Cells can be directly subjected to the RNA preparation protocol using these kits (see Note 3).

3.1.2. miRNA RT-PCR Analysis

Analysis of Mature miRNA

1. The RNA purified from the previous step is used as a template to synthesize cDNA using Taqman microRNA Reverse Transcription kit (Applied Biosystems) and miRNA-specific RT primers (Applied Biosystems), according to the manufacturer's instructions.
2. Quantitative real-time RT-PCR analysis is performed using Taqman 2× Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems), miR-specific RT-PCR primers (Applied Biosystems), and the 7500 Fast Real-Time PCR System (Applied Biosystems), according to the manufacturer's instructions. Data analysis is done by the comparative C_T method. Results are normalized to U6 snRNA or RNU44 snoRNA for human samples.

Analysis of Pri-miRNA and Pre-miRNA

1. The RNA purified from the previous step is used as a template to synthesize cDNA using Quantitect Reverse Transcription kit (Qiagen) and random hexamers, according to the manufacturer's instructions. For analysis of pre-miRNA expression, small RNA fraction is subjected to cDNA synthesis.
2. Quantitative real-time RT-PCR analysis is performed using FastStart Universal SYBR Green Master (Roche) and the 7500 Fast Real-Time PCR System (Applied Biosystems). Results are normalized to GAPDH for pri-miRNA detection and U6 snRNA for pre-miRNA detection.

3.1.3. Northern Blot Analysis for miRNA

Probe Generation

1. Combine the following in a total volume of 50 μl:
   (a) 10–50 pmol of DNA oligonucleotide probe.
   (b) 5 ml of 10× T4 PNK buffer.
   (c) 1 ml of T4 PNK.
   (d) 5 μl of [γ-^{32}P]ATP (20 μCi/μl, 800 mCi/mmol).
   (e) Nuclease-free water.
2. Incubate at 37°C for 1 h.
3. Incubate at 90°C for 2 min to inactivate T4 PNK and transfer the tube on ice.
4. Gel-filter with a ProbeQuant G-50 Micro Column according to the manufacturer's instructions, to remove unincorporated radioisotope.

Preparation of Denaturing Urea Polyacrylamide Gel

1. Assemble a gel cast after washing.
2. Mix Gel solution (Urea 7.2 g, 30% Acrylamide solution 7.5 ml, and 10× TBE 1.5 ml) and shake in the dark for 1 h at room temperature.
3. Mix Gel solution, 60 ml of 20% APS, and 16 ml of TEMED. Pour gel solution into a gel cast immediately.

Gel Electrophoresis and Blotting

1. Set the gel cast to the gel box and add Running buffer to the reservoirs.
2. Rinse out wells with a syringe and 23 G needle.
3. Pre-run the gel at 120 V for 30 min.
4. During pre-running, prepare RNA samples.
   (a) Mix equivalent volumes of sample and DNA loading buffer.
   (b) Incubate at 65°C for 10 min, transfer to ice.
5. After pre-running, rinse out wells with a syringe and 23 G needle and load samples.
6. Run gel at 240 V for approximately 1–2 h or until BPB reaches about 2 cm above the bottom of the gel.

7. Disassemble the gel cast and transfer the gel into pre-cold 0.5× TBE buffer.
8. Add a few drops of EtBr to the 0.5× TBE and stain the gel for 10 min using a shaker.
9. Wash the gel in 0.5× TBE for 10 min. Check the gel under UV for RNA quality.
10. Soak Nylon Membranes and six pieces of 3M papers in 0.5× TBE.
11. Assemble the Semi-Dry Transfer Unit: three pieces of 3M paper, gel, membrane, and three pieces of 3M paper.
12. Roll out bubbles and transfer at 250 mA for 1 h at room temperature.
13. After dissembling, UV crosslink at 1,200 mJ by UV crosslinker.
14. Bake the membrane between 3M papers at 80°C for 30 min.

Hybridization

1. Soak the membrane with 2× SSC.
2. Put the membrane into the hybridization bottle.
3. Add 5 ml of ExpressHyb Hybridization Solution (Clontech) (pre-warmed to 37°C) into the bottle.
4. Incubate at 37°C for 30 min for pre-hybridization in hybridization oven.
5. Replace the solution with 5 ml of fresh hybridization solution.
6. Add appropriate volume of the radiolabeled probe and incubate at 37°C for 12–24 h in hybridization oven.
7. Wash the membrane in the bottle with 10 ml of Washing solution I.
8. Transfer the membrane to a glass tray filled with 200 ml of Washing solution I.
9. Rinse for 30 min at room temperature.
10. Repeat washing with Washing solution I.
11. Rinse with Washing solution II for 15 min, twice.
12. Remove the residual liquid, wrap the membrane in plastic wrap, and analyze by Autoradiography system (e.g., Fujifim BAS-2500).

3.2. RIP Analysis

The potential interaction of various RNA species including miRNA precursors and miRNAs and target proteins can be evaluated by a modified version of the chromatin immunoprecipitation (ChIP) assay widely used for DNA–protein interactions. This procedure is named RNA immunoprecipitation (RIP) assay or RNA-ChIP and has been used for investigation of various RNA–protein interactions (10, 31–33). The interactions between pri-miRNAs and p53,

p68, or Drosha are investigated according to these methods, with some minor modifications (21). In RIP assay, the interactions between RNAs and proteins are reversibly fixed through chemical crosslinking with formaldehyde. RNA interacting with target protein(s) is immunoprecipitated with antibodies against the candidate protein and detected by RT-PCR analysis. RIP assay is performed throughout the procedures using the buffers containing RNase inhibitor.

3.2.1. Preparation of Whole Cell Lysates

1. Prepare the cells in one to three 100-mm dishes, which are transfected with siRNAs of interest (e.g., p53, p68, and so on) and/or treated with various DNA damage-inducing agents (see Notes 4–6).
2. Wash cells twice with PBS.
3. Add formaldehyde to a final concentration of 1% in PBS and perform crosslinking by shaking for 10 min at room temperature.
4. Add glycine to a final concentration of 125 mM to quench crosslinking and shake for 5 min at room temperature.
5. Wash twice with cold PBS.
6. Add 1 ml of ice-cold PBS containing 1% protease inhibitor mixture, collect the cells by scrapping, and transfer to a tube.
7. Centrifuge at 800 × *g* at 4°C for 5 min.
8. Add 150–200 ml of SDS-Lysis buffer to the pellets, mix well, and incubate on ice for 10–20 min.
9. Sonicate lysates on crushed ice using Bioruptor (power high, on 30 s/off 1 min for 5–6 cycles) (see Note 7)
10. Centrifuge at maximum speed (20,400 × *g*) at 4°C for 10 min.
11. Transfer the supernatant to a new tube and dilute the sonicate tenfold into ChIP dilution buffer to a final volume of 1 ml per immunoprecipitation assay. A 1–10% aliquot is preserved as an input sample and preserved at –80°C until the reverse cross-linking step.

3.2.2. Immunoprecipitation and Wash

1. Add 40 ml of protein G agarose beads and preclean the sonicate from steps 1 to 11 of Subheading 3.2.1 by rotating at 4°C for 1–2 h.
2. Centrifuge at 9,000 × *g* at 4°C for 10 s.
3. Transfer the supernatant to a new tube, add antibodies of interest (0.5–5 mg/ml) or normal control IgG, and rotate at 4°C overnight.
4. Add 50 ml of protein G agarose beads and rotate at 4°C for 2 h.

5. Centrifuge at 1,500 × *g* at 4°C for 1–2 min to collect immune complexes. The supernatant would be preserved as an unbound fraction.
6. Wash the beads five times using the following buffer. After each wash, beads are collected by gentle centrifugation (1,500 × *g*, 1–2 min).
   (a) 1× RIPA buffer/150 mM NaCl
   (b) 1× RIPA buffer/500 mM NaCl
   (c) LiCl wash buffer
   (d) TE pH 8.0
   (e) TE pH 8.0

3.2.3. Elution, Reversal of Crosslinks, and RNA Purification

1. Add 200 ml of ChIP elution buffer to the beads from the previous step, vortex briefly following gentle centrifugation. From this step, input sample and/or unbound fraction would be subjected to the same procedures.
2. Reverse crosslinks by incubating at 65°C for 1–2 h.
3. Add 1 ml of 10 mg/ml Proteinase K and incubate at 42°C for 45 min.
4. Purify RNA using Trizol and glycogen, according to the manufacturer's instructions.

3.2.4. RT-PCR Analysis

1. DNA from the samples is removed with DNase I treatment (buffer composition: 70 μl nuclease-free water with 1 μl of 40 U/μl RNase inhibitor, 5 μl of 1 M Tris–HCl pH 7.5, 20 μl of 50 mM $MgCl_2$, and 4 μl of 10 U/μl DNase I) for 30 min at 37°C, followed by Trizol purification (dissolved in 30 ml of nuclease-free water) or removal of the DNase I using DNase-inactivating reagent (Ambion Inc.).
2. The RNA purified from the previous step is used as a template to synthesize cDNA using Quantitect Reverse Transcription kit (Qiagen) and random hexamers, according to the manufacturer's instructions. 29 ml of RNA samples was usually used for a 60 ml cDNA synthesis reaction.
3. Quantitative real-time RT-PCR analysis is performed using FastStart Universal SYBR Green Master (Roche) and the 7500 Fast Real-Time PCR System (Applied Biosystems).

3.3. In Vitro Pri-miRNA Processing Analysis

In mammalian miRNA biogenesis, long pri-miRNA transcripts are sequentially processed to mature miRNA in the nucleus and cytoplasm. Two members of the RNase III, Drosha and Dicer in the nucleus and cytoplasm, respectively, are responsible for this two-step processing of miRNA precursors (13). Drosha-mediated processing of pri-miRNAs to pre-miRNAs can be reconstituted in in vitro system using immunoprecipitated Drosha complex and

radiolabeled pri-miRNA substrates, as demonstrated by several reports (7, 10, 34, 35). The effects of p53 on pri-miRNA processing by Drosha were previously examined according to these methods, with some modifications (21). The methods in in vitro pri-miRNA processing analysis are composed of (1) preparation of pri-miRNA template, (2) preparation of radiolabeled pri-miRNA, (3) preparation of Drosha complex, and (4) in vitro processing reaction and analysis using the products of (2) and (3).

3.3.1. Preparation of Pri-miRNA Template

To make pri-miRNA templates of interest, short fragments of pri-miRNAs containing pre-miRNA and flanking sequence on both sides of pre-miRNA are PCR-amplified and cloned into pcDNA3 expression vector or equivalent expression vector containing the T7 promoter. The resultant expression vector carrying the T7 promoter and pri-miRNA is linearized at the 3′-end of pri-miRNA by an available restriction enzyme and purified by ethanol precipitation. Restriction enzymes with activities that leave 3′ overhanging ends should not be used. SP6 promoter also can be used.

3.3.2. Preparation of (Radiolabeled) Pri-miRNA

Radiolabeled ([α-^{32}P]UTP internally labeled) pri-miRNAs are generated by in vitro transcription according to the manufacturer's instructions (DIG RNA Labeling Kit, Roche) with some minor modifications, as described below.

1. Mix the in vitro transcription reaction mixture in a total volume of 20 μl:
   (a) 2 μl of 10× transcription buffer.
   (b) 1 μl of RNase inhibitor.
   (c) 0.77 μl each of 100 mM ATP, CTP, and GTP and 0.07 μl of 100 mM UTP.
   (d) 1 μl of [α-^{32}P]UTP (20 μCi/μl, 800 mCi/mmol).
   (e) About 0.2–1 μg of template DNA.
   (f) 1 μl of T7 RNA polymerase.
   (g) Nuclease-free water.
2. Incubate at 37°C for 3 h.
3. Add 1 μl of DNase I and incubate at 37°C for 30 min.
4. Gel-filter with a ProbeQuant G-50 Micro Column according to the manufacturer's instructions, to remove unincorporated radioisotope.
5. As an option, radiolabeled pri-miRNA can be further purified by gel purification (34).

3.3.3. Preparation of Drosha Complex

1. Prepare the cells transfected with the FLAG-Drosha expression vector. In combination, cells would be co-transfected with siRNA and treated with various DNA damage-inducing agents.

Cells stably expressing FLAG-Drosha by lentivirus gene transfer could be similarly used.

2. Use cells in one (to three) 100 mm dish at near confluency for one in vitro reaction.
3. Remove the media and wash the cells with ice-cold PBS.
4. Add 1 ml of ice-cold PBS, collect the cells by scrapping, and transfer to a tube.
5. Centrifuge at 800 × *g* at 4°C for 5 min.
6. Remove the PBS and suspend the cell pellet in 500–1,000 ml of lysis buffer.
7. Disrupt the cells by passing the pellet 5–10 times into a 23–27 G needle with a syringe. Cells can be disrupted by sonicating ten times (for 5 s each) with short intervals at 30% amplitude (see Note 8).
8. Centrifuge at 13,000 × *g* at 4°C for 25 min.
9. Transfer the supernatant into a new tube.
10. Add 25 μl of Anti-FLAG M2 Affinity Gel and rotate the tube at 4°C for 3 h or overnight (see Note 9).
11. Wash the beads five times with 1 ml of lysis buffer.
12. Wash the beads with 500 ml of processing buffer before the final in vitro processing reaction. Change the tube during the last washing. Carefully remove the residual buffer and keep the tube on ice.

3.3.4. In Vitro Processing Reaction and Analysis

1. Combine the following (see Note 10):
   (a) FLAG-Drosha on beads (option: with/without p53 on beads).
   (b) 3 ml of energy solution buffer.
   (c) 2.4 ml of RNase inhibitor (4–8 U/ml).
   (d) 15 ml of processing buffer.
   (e) 1–10 ml of radiolabeled pri-miRNA.
2. Incubate at 37°C for 90 min or various time periods. Shake the tube sometimes to float the beads.
3. Add 1 ml of Proteinase K and incubate at 37°C for 10 min. This step could be omitted.
4. Add 100 ml of Trizol to the reaction mixture.
5. Purify the RNA using Trizol and glycogen, according to the manufacturer's instructions (see Subheading 3.1.1).
6. Prepare RNA samples for electrophoresis and autoradiography.
   (a) Mix appropriate volumes of sample and DNA loading buffer.
   (b) Incubate at 65°C for 10 min, transfer to ice.

7. After pre-running of urea polyacrylamide denaturing gel, load samples to electrophoresis, as described in Subheading "Gel Electrophoresis and Blotting."
8. After electrophoresis, dry the gel with a gel dryer, according to the manufacturer's instruction, and analyze by Autoradiography system (e.g., Fujifim BAS-2500).

4. Notes

1. Follow normal precautions, including the use of filter tips, while handling RNA samples, to avoid the contamination of RNase.
2. Wear gloves and check other usual safety precautions, in particular, while handling chloroform, acrylamide, and the radiolabeled samples.
3. The mirVana™ miRNA Isolation Kit or miRNeasy Mini kit can be used to extract both total RNAs or small RNAs, as an alternative to Trizol.
4. All the buffers used in RIP analysis contain 0.5 U/µl RNase inhibitor.
5. To avoid protein degradation, the procedures dealing with intact proteins should be performed on ice or at 4°C (pre-cool the centrifuge rotor).
6. Several reagents such as PMSF, DTT, and protease inhibitors should be added immediately before using.
7. In some protocols, the step of fragmentation of crosslinked RNA samples can be omitted to examine whether a protein potentially interacts with a target RNA, while the fragmentation step is important for the precise mapping of the protein binding region (31).
8. Intensive sonication might tend to result in a loss of activity for pri-miRNA processing because of heating.
9. Use wide-bore pipette tips for transfer of Protein G agarose or FLAG agarose beads.
10. We usually add Mg^{2+} to buffers at a final concentration of 3–6 mM. RNase III family proteins need Mg^{2+} for miRNA processing. The optimal Mg^{2+} concentration for Drosha is 6.4 mM. The processing efficiency is not significantly reduced down to 3.2 mM, but the processing efficiency decreases gradually below 3.2 mM.

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Chapter 15

Isolation and Characterization of Murine Multipotent Lung Stem Cells

Venkat S. Gadepalli, Catherine Vaughan, and Raj R. Rao

Abstract

Cancer stem cells possess the ability to self-renew and differentiate into specific cells found in tumor types, a characteristic feature of normal multipotent stem cells. These cells harbor within the bulk of tumors and if the tumor suppressor p53 is mutated in these cells, can be more likely to cause relapse and metastasis by giving rise to new tumors. This new paradigm of oncogenesis has been observed in various cancers, including lung cancer. Determining the interaction of critical cellular pathways in the ontogeny of lung tumors is expected to lead to identification of molecular targets for effective therapeutic strategies. To achieve this, it is important to characterize and dissect the differences between the cancer cells with aberrant stem cell like properties and normal multipotent stem cells that contribute to regeneration. This could be accomplished by using cell surface markers unique for certain cell types by employing techniques such as flow cytometry and magnetic bead isolation. This chapter summarizes the isolation process of the resident stem cell Sca1 (+ve), CD-45 (−ve), and CD-31 (−ve) populations for its potential use in assessing correlations between specific p53 gain of function phenotypes in different murine lung cancer models.

Key words: Lung stem cells, Flow cytometry, MACS

1. Introduction

Stem cells possess distinct characteristic features such as self-renewal and differentiation potential. However, depending on the source of resident stem cells, these characteristics differ; e.g., embryonic stem cells are pluripotent (ability to differentiate into any cell type in a body), while somatic stem cells are multipotent (ability to differentiate into cells of specific lineage). Isolation, characterization, and propagation of these multipotent stem cell lines have been a challenge for researchers. In the context of lung regeneration,

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the main purpose of the multipotent lung stem cells is to generate new cells and maintain structure and function of the tissue throughout the life of a multicellular organism. Scientists have characterized multipotent stem cells from different tissues and have inferred that different organs use different strategies to renew themselves with results suggesting adoption of a diverse and flexible renewal process by respective tissue and organs (1). The adult lung is a vital and complex organ in multicellular organisms and exhibits a slower regeneration process than rapidly regenerating hematopoietic and epithelial tissues. There is growing evidence of resident multipotent stem cell populations in different anatomical regions of murine lung tissue that posses different differentiating capacities (2, 3).

Recent studies on phenotypical and functional properties of human lung samples have shown the evidence of resident stem cell population that participate in tissue homeostasis and regeneration in animal models (4). The existence of cancer stem cells in different tumor types has set a challenge in identifying and characterizing lung cancer stem cells, which could serve as relevant targets in understanding and treating human lung cancer. The tumor suppressor p53 is mutated in a high number of lung cancers. Certain gain of function phenotypes result from a mutated p53 protein such as increased oncogenecity and tumorigenecity.

Results obtained from the studies of animal models of lung injury and carcinogenesis have led to the idea that several potential stem cell compartments are located along the respiratory tract and in the lung parenchyma (5). Identification of these potential stem cells is primarily based on expression of specific surface markers (2, 3). Based on different studies, it is hypothesized that Sca1 (+ve), CD-45 (−ve), and CD-31 (−ve) cell population harbors one or more endogenous stem cell populations (3). In order to characterize the difference between cancer stem cells and normal stem cells it is important to develop reproducible methods to identify, isolate, and propagate these stem cells. This chapter details cell sorting techniques such as magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS), which take advantage of specific cell surface marker expression in the isolation, sorting, and characterization of resident stem cell populations in the lung. This will enable researchers to study the role mutant p53 may play in cancer stem cells and gain of function effects in those cells throughout the lungs.

2. Materials

2.1. Equipment

1. Sterile Laminar Air flow hood.
2. MACS (Milteny Biotec, Auburn, CA, USA).
3. Fluorescence-activated cell sorter (BD FACSAria™ II High-Speed Cell Sorter, BD Biosciences).

4. Desktop centrifuge.
5. Flow cytometer (BD Accuri® C6 Flow Cytometer, Accuri, Ann Arbor, MI, USA).

2.2. Anesthesia Preparation

1. *Stock solution*: Add 5 ml T-amyl alcohol to 5 g tribromoethanol (tbe) (Aldrich St. Louis, MO, USA) in a dark environment.
2. *Working solution*: Mix 0.1 ml stock solution with approximately 7.9 ml normal saline, (or PBS), in a glass vessel wrapped in foil or a dark bottle. Dosage is about 0.2 ml/10 g of body weight.

2.3. Dissection Kit

1. Sterile stainless steel narrow blade scalpel, sharp/blunt operating scissors, straight iris scissors, thumb forceps, fine-point forceps, straight teasing needle, surgical thread, syringe, 18, 21, 30 gauge needle, 0.40 µm mesh filter, 1 ml SubQ Syringe.

2.4. Digestion Media

1. Dispase solution, 0.6–2.4 U/ml working concentration, prepared in sterile Phosphate buffer saline (PBS) (Ca^{2+} and Mg^{2+} free). Alternatively more efficient dissociation of tissue is obtained by mixing the Dispase at 0.3–0.6 U/ml and collagenase (60–100 U/ml) (Invitrogen, Carlsbad, CA, USA) (see Note 1).
2. 0.001% Dnase solution (Invitrogen, Carlsbad, CA, USA), to hydrolyze DNA from damaged cells.

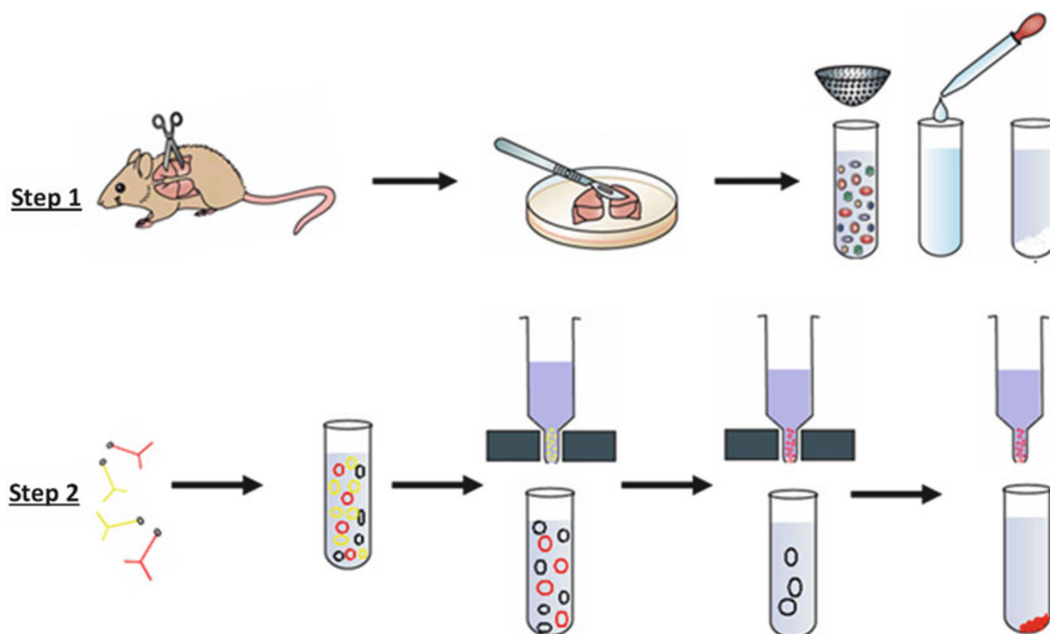

Fig. 1. Schematic detailing the steps involved in isolation of multipotent lung stem cells. *Step 1*: Lung isolation, digestion, sieving tissue debris, Red Blood cell (RBC) lysis, and pelleting by centrifugation. *Step 2*: Separation of Sca1 (+ve), CD-31 (–ve), CD-45 (–ve) cells by magnetic-activated cell sorter (MACS). *Note*: Sca1-APC (*red*), CD-31–PE and CD-45–PE (*yellow*).

2.5. Sorting Medium

1. Phosphate buffer saline (PBS^{++} with Ca^{2+} and Mg^{2+} ions) with 10% fetal bovine serum (FBS).

2.6. Antibodies, Micro-beads, and Fluorescent Stains

1. Anti-mouse CD-31–PE conjugate (R&D systems, Minneapolis, MN, USA).
2. Anti-mouse CD-45–PE conjugate (R&D systems, Minneapolis, MN, USA).
3. Anti-PE and Anti-APC Micro-beads (Milteny Biotec, Auburn, CA, USA).

2.7. Multipotent Lung Stem Cell Medium

1. DMEM high glucose supplemented with sodium pyruvate and glutamine (Invitrogen, Carlsbad, CA, USA).
2. 1% Penicillin and streptomycin.
3. 1% Nonessential amino acids.
4. 10% FBS (Fig. 1).

3. Methods

3.1. Lung Isolation

The experimental mice used in this study are maintained in our animal facility in pathogen-free conditions according to the guidelines of IACUC and Virginia Commonwealth University animal care facility.

1. Select three 6–8 weeks old mice for dissection.
2. Place the mice on a cage lid and grasp the loose skin behind the ears with your thumb and forefinger. As soon as the mouse's head is restrained pick up the tail and secure it within your ring finger and little finger. Make sure movement of mice is restrained completely.
3. Anesthetize mice by Intra-peritoneal injection method. The injection site should be in the lower left quadrant of the abdomen. Disinfect the site by 70% alcohol and carefully penetrate the tip of the needle into mice abdomen wall at a 45° angle.
4. It will take about 5 min for the mouse to become fully anesthetized (evidenced by lack of response to toe or tail pinch). An additional 0.05–0.1 ml can be given if any sense of pain is observed. Once completely anesthetized, sacrifice the mice by cervical dislocation.
5. Using sterile pointed scissors make a small incision near the posterior end of abdomen. Excise towards the anterior end cutting the rib cage to expose internal organs. Care should be taken to near the throat region to avoid any damage to trachea. Dissect away the platysma and anterior tracheal muscles in order to visualize and access tracheal rings.

6. Slightly make a cut on trachea to insert syringe tip. Inject 1 ml of dispase (2 U/ml) into the trachea using a 1 ml subcutaneous (SubQ) syringe with appropriate needle. The lung inflation is observed during injection. Carefully remove the syringe and tie a surgical thread at cut end of trachea, to prevent draining of dispase.
7. Pulling up trachea with thumb forceps, carefully excise and separate other internal organs to reach lungs. Pull out lungs along with heart, trachea (referred as "pluck"), and place them on a sterile Petri dish. Separate trachea, heart, and other connective tissues to collect lung lobes.
8. Place the lung lobes in cold PBS^{--} (Ca^{2+} and Mg^{2+} free) supplemented with Penicillin and streptomycin, for about 5 min to drain out blood.
9. Discard the mice according to IACUC protocol.

3.2. Lung Digestion

1. In a tissue culture hood remove lung lobes from PBS^{--} and place them on a 100 mm Petri dish. Using a sharp scalpel, mince lung lobes finely into small pieces. Add 10 ml of digestion medium. Incubate for 30 min at 37°C on a rotator. Observe it every 10 min to make sure that all the pieces are immersed in digestion medium.
2. After 30 min, digested lung slurry should be easily pipetted up and down using a 10 ml pipette. This is an indication of good digestion. Repeat this few times (see Note 2).
3. Collect the slurry in a 10 ml syringe and pass through 18 and 21 gauge syringes. Repeat this step few times, until clear slurry is obtained.
4. Filter the slurry using a sterile 0.40 μm mesh filter into a 50 ml conical tube.
5. Centrifuge the filtered solution using desktop centrifuge at 300 × *g* for 5 min and aspirate out the supernatant.
6. Add sterile RBC lysis solution and vortex gently. Incubate it for 2–3 min. Again gently vortex few times and centrifuge at 300 × *g* for 10 min. A white pellet should be observed following centrifugation. Repeat RBC lysis if necessary.
7. Re-suspend the pellet in 1 ml of fresh sorting medium and count the cells using a hemocytometer.

3.3. Depleting CD-31 and CD-45 Positive Cells

1. Re-suspend the pellet in 100 μl of sorting media (PBS and 10% FBS).
2. Incubate the cell suspension with 10 μl (for up to 10^7 cells) CD-45–PE conjugate antibody for 15 min at 4°C in dark. After incubation add 1–2 ml of sorting media and wash unbound antibody. Centrifuge at 300 × *g* for 10 min. Aspirate the supernatant and re-suspend in 100 μl of sorting media.

3. Incubate the cell suspension with 20 µl (for up to 10^7 cells) of Anti-PE micro-beads for 15 min at 4°C in dark. After incubation add 1–2 ml of sorting media and wash unbound antibody. Centrifuge at 300×*g* for 10 min. Finally re-suspend the pellet in 500 µl of sorting media.
4. Prepare the MACS column by adding 500 µl of sorting media. Now add 500 µl of cell suspension and allow cell suspension to flow through the magnetic columns by gravity. Collect the solution in a 2 ml centrifuge. Wash the column two to three times with 500 µl sorting media (see Note 3).
5. Repeat the steps 2–4 using CD-31–PE Antibody and Anti-PE micro-beads (see Note 4).

3.4. Collecting Scal Positive Cells

1. After depletion of CD-31 and CD-45 positive cells, re-suspend the pellet in 100 µl of sorting media (PBS and 10% FBS).
2. Incubate the cell suspension with SCA1-APC conjugate antibody for 15 min at 4°C in dark. After incubation add 1–2 ml of sorting media and wash unbound antibody. Centrifuging at 300×*g* for 10 min. Aspirate the supernatant and re-suspend in 100 µl of sorting media.
3. Incubate the cell suspension with 20 µl (for up to 10^7 cells) Anti-APC micro-beads for 15 min at 4°C in dark. After incubation add 1–2 ml of sorting media and wash unbound antibody. Centrifuge at 300×*g* for 10 min. Aspirate the supernatant and re-suspend in 500 µl of sorting media.
4. Prepare the MACS column by adding 500 µl of sorting media. Add 500 µl of cell suspension and allow the cell suspension to flow through the magnetic columns by gravity. Remove the MACS column from magnet holder and add 1 ml of sorting media to the column. Using a plunger, force the collected Sca1 positive cells in column into a 1.5 ml centrifuge tube (see Note 5).
5. Centrifuge the cell suspension, re-suspend the pellet in fresh multipotent lung stem cell (MLSC) media, and count the cells using a hemocytometer.

3.5. Plating Scal Positive Cells

1. Plate the Sca1 positive lung stem cells on 3-day-old inactivated mouse embryonic fibroblasts (iMEF) dishes. Change the MLSC media daily. Primary colonies should be observed in 7–15 (Fig. 2) (see Note 6).
2. Alternatively, Sca1 positive cells can also be plated on normal tissue culture dishes. Primary colonies may be observed in 20–25 days on tissue culture dishes.
3. Characterization studies could be conducted by flow cytometry (Fig. 3) or immuno-staining (see Note 7).

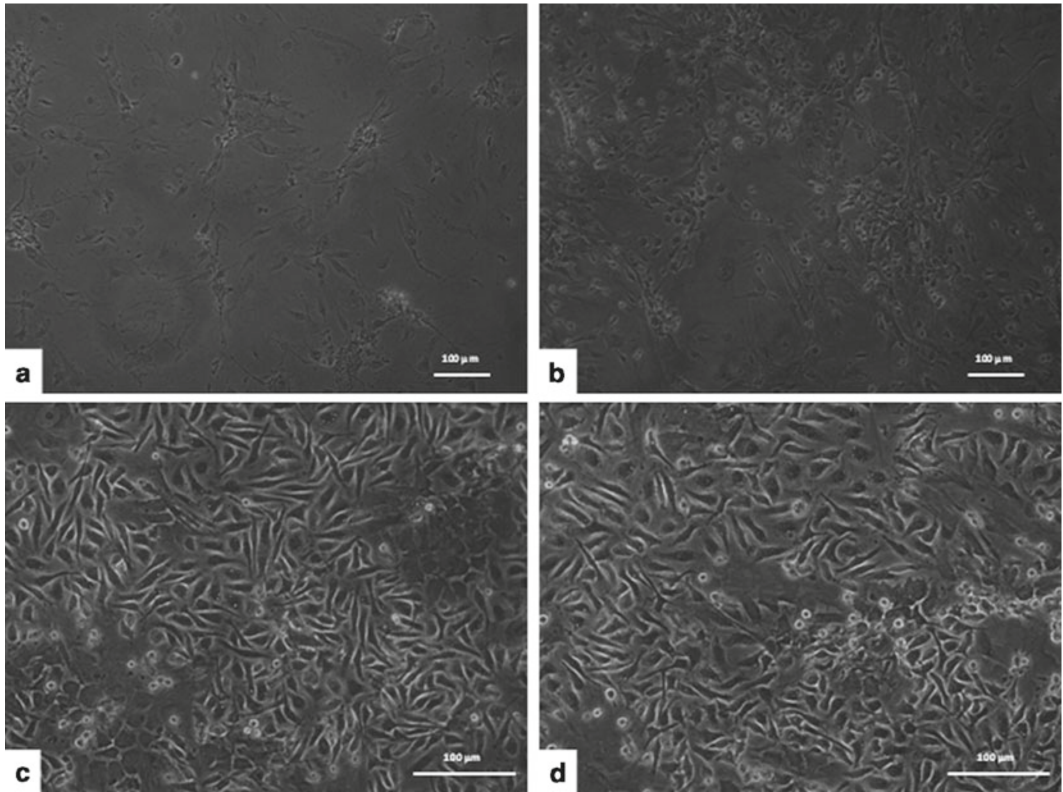

Fig. 2. Multipotent lung stem cells propagated on inactivated MEFs. Spindle-shaped cells show up on (**a**) day 5 and (**b**) day 8; while they start exhibiting stem cell morphologies of high nuclear–cytoplasmic ratios on (**c**) day 13 and (**d**) day 15.

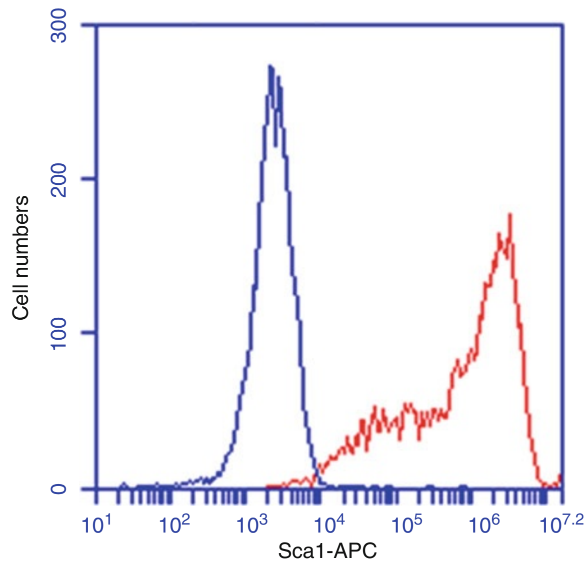

Fig. 3. Flow cytometry analysis of Sca1-APC stained (*red*) and unstained (*blue*) isolated multipotent lung stem cells.

4. Notes

1. It is important to dissolve lyophilized dispase enzyme in PBS (Ca^{2+}/Mg^{2+} free), because these ions reduce the activity of the enzyme. Concentrations higher than 2.4 U/ml are not recommended.
2. In general, 30 min incubation at 37°C is required for soft tissue digestion. If incomplete digestion is obtained, increase the reaction time accordingly with addition of fresh digestion medium.
3. It is important to allow the flow of cell suspension through the column by gravity. Do not flush the column with force. Please review the use of antibody concentration according to the manufacturer's protocol.
4. Mouse lung suspension cells have higher number of CD-45 positive cells. Hence it is important to deplete these cells first. Based on the cell numbers obtained from three lungs, depletion of CD-45 and CD-31 could be performed in a single step. If there are more than 10^7 cells obtained from three mice lungs, it is important to perform depletion steps independently, to avoid the clogging of the column. Please review manufacturer's column sizes and cell number recommendations (Milteny Biotec, Auburn, CA, USA).
5. The efficiency of MACS separation is about 70–80%. To further achieve a more homogenous population of Sca1 positive, and CD-45 and CD-31 negative cells, it is important to perform FACS sorting (BD FACSAria™ II High-Speed Cell Sorter). To reduce the stress on cells, propagate MACS sorted cells at in vitro conditions for two passages and then further sort them on FACS Aria.
6. In co-culture with iMEFs, we observe compact spindle-shaped cells (Fig. 2). Once the primary colonies are established these cells can be propagated directly on tissue culture plates without the need of iMEF. Enzymatic passage using 0.25% Trypsin is recommended.
7. Additional characterization studies are important to establish these isolated cells as multipotent lung stem cells. Immunostaining, flow cytometry, limiting dilution assays to assess colony forming ability, and differentiation into specific lineage helps us to categorize them as stem cells. Flow cytometry analysis on these isolated lung stem cells is shown in Fig. 3.

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Chapter 16

Generation of p53 Knock-down Cell Lines

Catherine Vaughan, Swati Palit Deb, and Sumitra Deb

Abstract

In order to study the functions of a cell's endogenous mutant p53, the p53 protein levels must be knocked-down. Transient transfection of small interfering RNAs is one way to accomplish this. Another is the stable expression of short hairpin RNAs. This chapter presents a method by which a short hairpin RNA (shRNA) targeting p53 is inserted into the genome of a cell via lentivirus infection. These p53 knock-down cell lines are stable and may be grown long term for use in a wide range of applications.

Key words: p53 knock-down, shRNA, Lentivirus, Gain of function

1. Introduction

There are thousands of different p53 gene mutations. According to the International Agency for Research on Cancer (IARC) TP53 database there are 27,580 somatic and 597 germline mutations alone (1). One way of studying the functions of a particular mutation is to overexpress that particular cDNA clone into a p53-null cell line, such as H1299. However, if the functions of a cell line's endogenous mutant p53 were to be studied, the protein levels need to be reduced. Transient transfections are a quick and easy method by which this can be done and can be accomplished using several different reagents. The problem with transient knock-down is that if long-term functions, such as cell growth or tumorigenicity, are to be studied, there is no way of preventing the protein levels from coming back.

Stable expression of a shRNA against a particular protein, and the generation of a cell line expressing this, can be used in any type of functional assay without the worry of short-term problems.

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shRNA-mediated knock-down of proteins has been used successfully by many different groups (2–4). Generating cell lines with p53 knocked-down is accomplished using a short hairpin RNA targeting p53 cloned inside a lentiviral vector that is always expressed. Cells are transfected with the shRNA using a lentivirus system to fully integrate it into the cell genome. After integration, transfected cells are selected for with the addition of an antibiotic. A puromycin resistance gene is located within the shRNA vector allowing only the cells that have been transfected to survive. Cells that continue to grow are cloned and p53 protein levels are checked by Western blot analysis. Many successfully knocked-down cell clones may be created following this protocol.

2. Materials

2.1. Preparation of shRNA Against p53

1. TRCN0000003756 (supplied as a bacterial culture, Open Biosystems).
2. STE: 0.1 M NaCl, 10 mM Tris–Cl pH 8.0, 1 mM EDTA pH 8.0.
3. Solution I: 50 mM Glucose, 25 mM Tris–Cl pH 8.0, 10 mM EDTA pH 8.0.
4. Lysozyme.
5. Solution II: 0.1% NaOH, 1% SDS.
6. Solution III: 3 M Potassium Acetate, 11.5% Glacial Acetic Acid.
7. 1× TE.
8. Cesium chloride.
9. 10 mg/mL Ethidium bromide.
10. Dialysis tubing.
11. Dialysis tubing clips.

2.2. Generation of shp53 Lentivirus

1. Purified shp53 lentiviral DNA.
2. Packaging plasmid.
3. Packaging envelope.
4. pmax GFP.
5. Fugene 6 Transfection Reagent (Roche).
6. HEK293T cells in culture.
7. DMEM.
8. Disposable pipettes.
9. 1.5 mL tubes.

2.3. Lentivirus Infection

1. Adherent cells in culture.
2. 10 cm Culture dishes.
3. shp53 lentivirus.
4. HBSS.
5. Complete growth medium.

2.4. Clone Selection

1. Lentivirus-infected cells.
2. Complete growth medium.
3. 1 mg/mL Puromycin.
4. Cloning cylinders.
5. 2× Trypsin in EDTA.
6. 24-Well plates.
7. 12-Well plates.
8. 6-Well plates.
9. 10 cm Culture dishes.

2.5. Western Blotting

1. Cold 1× PBS.
2. Rubber policemen.
3. 1.5 mL Tubes.
4. Refrigerated centrifuge.
5. 5× Reporter Lysis Buffer (diluted to 1×).
6. Dry ice.
7. Protein Assay Dye Reagent Concentrate (BioRad).
8. Spectrophotometer.
9. 4XLLB with β-mercaptoethanol.
10. Western blotting apparatus.
11. Electrode buffer: 3.03 g Tris Base, 5% Glycerol, 14.42 g Glycine, 1% SDS for 1 L.
12. Transfer apparatus.
13. Transfer buffer: 5.8 g Tris Base, 2.9 g Glycine, 0.37 g SDS, 20% Methanol for 1 L.
14. TBST: 10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20 for 1 L.
15. Nonfat dry milk.
16. pAb 1801 p53 primary antibody.
17. Anti-mouse HRP-linked secondary antibody.
18. ECL.

3. Methods

3.1. Preparation of shRNA Against p53

1. Large-scale preparation of the shRNA against p53 is accomplished using the protocol found in the Molecular Cloning laboratory manual (see Notes 1 and 5).

3.2. Generation of shp53 Lentivirus

3.2.1. Transfection

1. Split a confluent plate of HEK293T cells so that the new plates will be 60–70% confluent on the day of transfection.
2. The day of transfection, change media on the plates to be transfected in the morning.
3. Combine 2.5 μg packaging plasmid, 2.5 μg packaging envelope, and 5 μg shRNA against p53 or positive control DNA (see Note 2) in a 1.5 mL tube and make the volume up to 100 μL with DMEM.
4. Put 100 μL DMEM in a separate 1.5 mL tube and add 25 μL of the Fugene 6 solution directly to the DMEM slowly. Flick tube to mix, *do not vortex*, and spin down.
5. Add the DNA–DMEM mix to the Fugene 6–DMEM mix slowly. Flick tube to mix, *do not vortex*, and spin down.
6. Incubate the solution (now 225 μL) in the cell culture hood for 15 min and add dropwise to the plate of 293T cells. Swirl plate gently and keep in incubator for 2 days.

3.2.2. Harvesting the Lentivirus

1. Before harvesting the virus, view the positive control plate (pmax GFP) under fluorescent light to make sure the transfection worked.
2. Using a disposable pipette, collect the entire amount of media from the plate and put it into a 50 mL tube, this is the virus. Mix gently.
3. Aliquot the virus in 1 mL aliquots and flash freeze in liquid nitrogen for 5 min. Store at –70°C until needed (see Note 3).

3.3. Lentivirus Infection

1. The day before virus infection, split the cells to be infected into a 10 cm dish so they will be 20% confluent on the day of transfection.
2. Remove the culture medium from the cells and wash with 10 mL HBSS or PBS, and then remove.
3. Add 1 mL lentivirus to the plate and rock to coat all the cells. Put the dish in the incubator.
4. Rock the plate every 15 min for 2 h to ensure even distribution of the virus and to make sure the cells don't dry out.
5. After 2 h, add 10 mL complete medium back to the cells.

3.4. Clone Selection

1. Two days after infection add puromycin to the cells to begin selection and change the media plus puromycin for every 3–4 days until colonies are visible (see Note 4).
2. When nicely separated colonies are visible, remove the culture medium and wash the plate with 10 mL HBSS or PBS, and remove.
3. Put one cloning cylinder around each colony and add 100 μL 2× trypsin inside. Incubate at room temperature for 2–3 min.
4. Pipette up and down using a micropipettor and put each colony into one well of a 24-well plate containing 0.5 mL complete medium plus puromycin.
5. When the clones become confluent in their wells, move them to the next well size higher (e.g., 24-well to a 12-well).
6. When the clones become confluent in a 6-well dish, split them so that 80% of the cells are transferred to a 10 cm dish and 20% remain in the 6-well dish (see Note 5).
7. Upon Western blotting verification, split the cells to a 10 cm dish and utilize for various experiments.

3.5. Western Blotting Verification of p53 Knock-down

3.5.1. Harvesting and Preparing Extract

1. Take the 10 cm dish for each clone for Western when it is about 80–90% confluent.
2. Aspirate the media and wash the cells with 10 mL ice-cold PBS. Aspirate.
3. Add 1 mL ice-cold PBS to the cells and scrape off the culture dish with a rubber policeman. Pipette the cell/PBS mix into a 1.5 mL tube.
4. Pellet the cells by centrifugation at 4,000 rpm (1,700 rcf) for 10 min at 4°C and remove the supernatant.
5. Resuspend the cell pellet in 1× Reporter Lysis Buffer and perform a freeze–thaw cycle by putting the tube in a dry ice/ethanol bath for 5 min. Thaw the extracts and pellet the cell debris by centrifugation at 10,000 rpm (1,700 rcf) for 10 min at 4°C.
6. Transfer the supernatant into a new 1.5 mL tube and perform a protein assay (see Subheading 3.5.2).
7. Add one-third the volume of the protein extract of 4XLLB with β-mercaptoethanol and mix.
8. Boil the extracts for 5 min and load in a polyacrylamide gel.

3.5.2. Protein Assay

1. Combine 960 μL sterile water, 240 μL Protein Assay Reagent, and 3 μL extract or water for blank into a 1.5 mL tube. Mix and take an optical density reading using a spectrophotometer at 595 nm.

2. Normalize the amount of extract to use for each sample in the polyacrylamide gel.

3.5.3. Running and Transferring the Gel

1. Pour a 10% polyacrylamide gel with spacers of 1.5 mm.
2. Load a maximum volume of 20 μL per extract and a protein ladder for band identification (see Note 6).
3. Run the gel at 30 mA until the dye band almost runs out of the gel.
4. Transfer at 350 mA for 2 h using a 0.45 μm membrane.

3.5.4. Developing the Blot

1. Block nonspecific antibody binding by washing the blot in a 3% milk-TBST solution for 45 min at room temperature.
2. Wash the blot twice with 10 mL TBST for 10 min.
3. Incubate in pAb 1801 for 1 h.
4. Repeat step 2.
5. Incubate the blot in anti-mouse HRP-linked secondary diluted 1:1,000 in TBST for 30 min at room temperature.
6. Repeat step 2.
7. Develop the blot for p53 and keep all clones that show an absent p53 band (see Note 7).

4. Notes

1. Preparation of the lentivirus DNA may be done outside of a biosafety cabinet, the DNA will be dialyzed to clean it. All steps from this point onward should be carried out inside a sterile biosafety cabinet with the exception of Western blotting.
2. Use pmax GFP as a positive control for transfection. Two days later view the cells under fluorescent light; the cells should express GFP and provide support to the success of the transfection.
3. Take care to only use disposable pipettes and treat everything the virus touches with bleach for at least 24 h. The lentivirus is replication deficient but can still infect tissue it comes into contact with.
4. It is useful to add puromycin at different concentrations starting at 1 μg to the original cell line to determine which concentration would be the best for clonal selection. After selection, cells need to continue to be grown in media containing puromycin to ensure the knock-down effect is not lost.
5. The 10 cm dish will be used for Western analysis while the remaining cells grow in the 6-well dish until p53 knock-down verification.

6. Use either the original cell line or cells infected with shGFP as a positive p53 band control.
7. Make sure to develop the blot for a loading control such as Actin, GAPDH, or Erk2.

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Chapter 17

ChIP for Identification of p53 Responsive DNA Promoters

Jun-Ming Liao and Hua Lu

Abstract

Chromatin immunoprecipitation assay (ChIP) has been frequently used to determine whether a transcriptional regulator can bind to a specific DNA element in the chromatin content of cells. Here, we describe a detailed protocol for this assay with hands-on tips based on our own experience in working on the transcriptional regulator and tumor suppressor p53.

Key words: Chromatin immunoprecipitation, p53 responsive DNA element, Transcription

1. Introduction

Although p53 can induce apoptosis in a transcription-independent fashion (1), it has been well established that p53 functions as a tumor suppressor primarily through its transcriptional regulation of a number of target genes, which encode proteins and miRNAs important for executing the p53 response to a variety of stress signals (2–5). This transcriptional activity is exerted via the direct binding of p53 to the canonical p53 responsive DNA elements of its target promoters. Thus, monitoring the binding of p53 to its target gene promoters becomes one critical measurement of p53 transcriptional functions for specific target genes in cells. Chromatin immunoprecipitation (ChIP) was initially developed to define the Polycomb-repressed chromatin domains in cells (6) and later on often applied for the detection of the binding of a number of transcriptional factors to their responsive DNA elements in the chromatin content in cells (7). This method has also been successfully used not only to detect the binding of p53 to its known responsive DNA elements in vivo (8), but also to identify new p53 target promoters, including some of our own studies in the laboratory (9–11). Hence, ChIP has become one of the frequently used

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and important methods in the field of p53 to analyze in vivo transcriptional functions of this tumor suppressor.

In 1991, p53 was identified as a transcription factor that was able to bind to a sequence-specific DNA promoter (12, 13), and the consensus sequence of p53 response elements was resolved by in vitro assays 1 year later (14). By using a global ChIP-sequencing assay, Wei et al. have recently refined the consensus sequence with the pattern 5′-RRRC(A/T)(A/T)GYYY-3′ (15). Although several studies showed that p53 can also bind to some DNAs without the consensus sequence (16, 17), the identification of p53 as a sequence-specific DNA-binding transcription factor is clearly a landmark in the p53 field, since most tumor-associated p53 mutations occur within the DNA-binding domain (18) and more than 150 p53 target genes including those encoding miRNAs have been reported thus far since the *p21* and *mdm2* genes were identified as the first batch of the p53 target genes (19–23). In spite of the possibility that p53-DNA binding may be affected by other factors, like DNA structure and topology, defining the sequence-specific DNA elements that specifically interact with p53 in cells is critical for verifying bona fide target genes for this transcription factor.

It has been debated whether binding of p53 to chromatin DNA in cells is stress-induced or not. While the binding of p53 to the promoter sequences of its target genes was previously believed to require stress induction, later studies showed that p53 is able to bind the promoter DNA element of *p21*, *mdm2*, or *pig3* genes in unstressed cells, though to a lesser extent, when compared to stressed cells (24, 25). These findings reveal that although the inactive form of p53 in unstressed cells could bind to its target promoters, it is not sufficient to induce transcription of target genes. Considering that the procedures of cell culture and cell isolation could cause cellular stress, the basal level of the binding of inactive p53 to its promoter might be due to this type of artificial stress. Thus, to achieve a conclusive result for the definition of a new p53 responsive (p53RE) DNA element in vivo, it is recommended to induce p53 in cells with a specific stressor, such as treatment with 5-FU or actinomycin D, prior to conducting a ChIP assay as detailed below.

In this chapter, we describe a hands-on protocol for conducting a ChIP assay with some tips gained from our own direct experience in studying the binding of p53 to its target promoters in cells. Although we use p53 as a working model here, this protocol can be used for studying other p53 family members, such as p73 or p63, and other transcription factors as well. This protocol could also be used to identify new p53 target DNA elements. In the latter case, putative p53 responsive DNA elements in the promoter region of a putative target gene could be readily identified through bioinformatic analysis of human genome sequences. Two pairs of primers should be designed based on the sequence information, including one that covers the putative p53RE DNA element and another pair of control primers derived from downstream or upstream sequences irrelevant to this

DNA element. In principle, p53-containing cells can be treated with anticancer drugs, such as actinomycin D or 5-FU, for several hours before being harvested to ensure that p53 is induced. Harvested cells are then subjected to cross-linking and sonication prior to being used for immunoprecipitation with anti-p53 antibodies. Immunoprecipitated lysates are then used for Polymerase Chain Reaction (PCR) to amplify the DNA element that may associate with p53. The detailed procedure with all necessary materials is described below.

2. Materials

1. Formaldehyde (Fisher Scientific, F75P1GAL).
2. 1.25 M Glycine (Fisher Scientific, G45-212). Store at 4°C.
3. 20× Phosphate-buffered-saline (PBS) buffer: 160 g NaCl, 4 g KCl, 23 g Na_2HPO_2-$7H_2O$, and 4 g KH_2PO_4. Store at room temperature.
4. 1×PBS: Prepare from 20× stock using water and store at 4°C.
5. Protease inhibitors: 1 μg/mL Aprotinin, 1 μg/mL Leupeptin, 4 μM Pepstatin A, and 0.2 mM Phenylmethylsufonyl-fluoride (PMSF). (Add fresh just before use).
6. Lysis buffer: 150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS, 50 mM Tris, and 5 mM EDTA, pH 8.0. Store at 4°C. Add protease inhibitors just before use.
7. ProteinA/GPLUS-Agarose(SANTACRUZBIOTECHNOLOGY, sc-2003).
8. Pre-sheared salmon sperm DNA (Sigma-Aldrich, S3126).
9. Wash buffer: 100 mM Tris, 500 mM LiCl, 1% Np-40, and 1% deoxycholic acid, pH 8.5.
10. 500 mM $NaHCO_3$.
11. 10% SDS (Sigma-Aldrich, 71725).
12. 5 mg/mL RNAse A (Sigma-Aldrich, R4642).
13. 5 M NaCl.
14. Yeast tRNA (Ambion, AM7118).
15. Ethanol.
16. 5× PK buffer: 50 mM Tris, 25 mM EDTA, and 1.25% SDS, pH 7.5.
17. 20 g/L Proteinase K (New England BioLabs, P8102S).
18. 70% ethanol.
19. Agarose gel.
20. Ethidium bromide.

3. Methods

The following protocol is based on the previously described method (26) with some modifications according to our own experience (Fig. 1).

3.1. Chromatin Preparation

1. Culture sufficient cells (at least 10^7 cells per IP) with desired treatments (see Note 1).
2. Add 37% formaldehyde (30 μL/mL media) directly into the plate with media. Gently shake the plates at RT for 10 min (see Note 2).

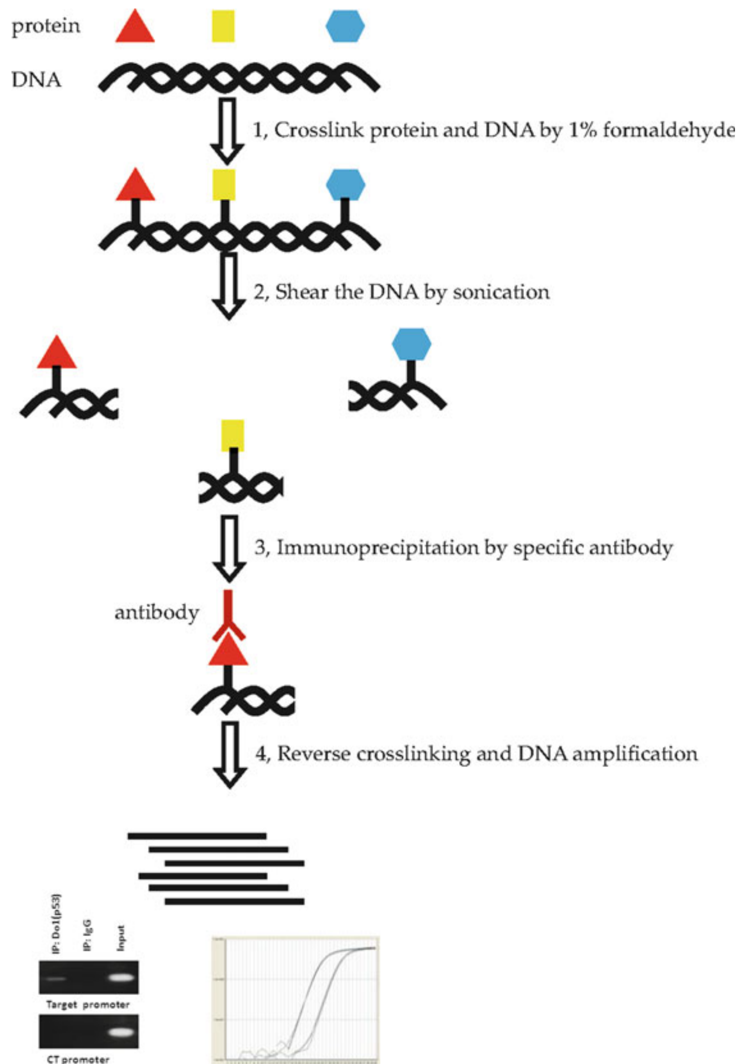

Fig. 1. Schematic diagram showing ChIP.

3. Add glycine to a final concentration of 0.125 M to stop the cross-linking reaction. Incubate at room temperature (RT) for 5 min.
4. Wash cells twice with PBS and scrape 10^7 cells in 1.5 mL PBS with PMSF. Transfer the cells to 1.5 mL tube.
5. Pellet cells for 5 min at 1,000×*g* at 4°C and aspirate off the supernatant. *The cells can be used for ChIP directly or frozen in liquid N2 and stored at −80°C for months. From this step, keep your sample and reagents at 4°C or in an ice bucket.*
6. Add protease inhibitors into the RIPA buffer (prepare 5 mL per IP).
7. Resuspend cell (do not vortex) pellets with 1 mL RIPA with protease inhibitors (see Note 3).
8. Incubate the mixture in ice for 30 min by reversing the tube occasionally to avoid precipitation.
9. Shear DNA to yield fragments of 300–1,000 bp by sonication (Fig. 2), 10 s, 8 times. We use Fisher Scientific Model 100 Sonic Dismembrator and set up the output power to 14 W (see Note 4).
10. After sonication, spin the lysates for 10 min at 16,000×*g* and collect the supernatant into a new 1.5 mL tube. *The chromatin can be stored at −80°C at this step.*

3.2. Immunoprecipitation

For the first ChIP experiment, we strongly recommend that you set up both positive and negative controls for both antibodies and primer sets.

1. Take out 50 μL for the input (see Note 5).
2. Prepare DNA–protein A/G slurry (1:1 mixture of 10 g/L presheared salmon sperm DNA and protein A/G).
3. The prepared chromatin was precleared with 50 μL DNA–protein A/G slurry on rotating platform for 30 min at 4°C.
4. Spin at 1,500×*g* 4°C for 2 min, collect the supernatants to a new 1.5 mL tube (see Note 6).
5. Take 250 μL of mixtures to a new tube for each antibody, add appropriate antibody (1–5 μg) to each sample, and immunoprecipitation was performed on a rotating platform at 4°C overnight (see Note 7).

End of day 1

6. Add 50 μL Protein A/G sepharose beads and incubate for two more hours at 4°C on the rotating platform.
7. Wash the immunocomplexes twice with 1 mL RIPA buffer, four times with IP wash buffer, and twice with RIPA buffer. Between washes, samples are rotated for 5 min and spun at 1,000×*g* 1 min (see Note 8).

3.3. Elution and Reverse Cross-links

1. Freshly make the IP elution buffer (300 μL/IP).
2. Add 150 μL IP elution buffer, put on a rotating platform at RT for 15 min, spin at 2,000 × *g*, 1 min RT, and transfer supernatants to a new tube. Repeat with another 150 μL IP elution buffer. Combine supernatants and spin at 16,000 × *g* for 5 min to remove any traces of beads.
3. Add 250 μL IP elution buffer into the input sample taken out from step 1 in section 3.2.
4. Add 2 μL 5 g/L RNAse A and 12 μL 5 M NaCl. And incubate at 65°C for overnight. The sample could be stored at −20°C (see Note 9).

End of day 2

5. Add 10 μg tRNA and 650 μL 100% ethanol. Mix and store at −20°C more than 3 h or overnight.

End of day 3

6. Centrifuge at top speed for 20 min. Discard ethanol and dry pellet at RT for 10 min.
7. Resuspend DNA in 100 μL TE buffer and add 25 μL 5× PK buffer. Vortex the sample and add 30 μg proteinase K. Mix well and incubate at 45°C for 2 h (see Note 10).
8. Add 175 μL TE buffer to final volume of 300 μL and add 300 μL 25:24:1 phenol–chloroform–isoamyl alcohol. Vortex vigorously for 1 min. Transfer the top phase to a new tube and extract with 300 μL chloroform and transfer the top phase to a fresh tube.
9. Add 30 μL 5 M NaCl, 10 μg yeast tRNA, and 750 μL 100% ethanol. Mix well and precipitate at −20°C more than 3 h or overnight.

End of day 4

10. Spin samples at top speed for 20 min and wash DNA with 70% ethanol.
11. Dissolve pellets in 100 μL water. The DNA can be stored at −20°C for months.
12. Analyze the pulled down DNA by either a Real-Time PCR assay or regular PCR reaction with relevant or control primers (see Note 11).
13. For Real-Time PCR, use 2 μL DNA as a template and follow the standard qPCR protocol (10). If possible, always do one positive control with the following p21 primers, forward: 5¢-GCTCCCTCATGGGCAAACTCACT reverse: 5¢-TGGC

TGGTCTACCTGGCTCCTCT, with annealing temperature: 60°C and data collecting temperature: 75°C.

14. Or, run regular PCR products on a 2% agarose gel with ethidium bromide staining (Fig. 1) (see Note 12).

4. Notes

1. Start with three confluent 10-cm dishes. A sufficient number of cells are vital to the success of the ChIP assay. Fewer cells may result in weak signal and high background. The required number of cells may vary depending on cell type. Therefore, we suggest measuring the DNA concentration for each cell line and start with 25 μg DNA per IP.
2. The time and formaldehyde concentration may be optimized. However, do not cross-link the proteins to DNA longer than 30 min, for it would affect the antibody binding and the efficiency of sonication or cause cell aggregation.
3. Optional steps to purify the nuclei may reduce the nonspecific binding of primary antibodies. Before adding RIPA buffer, add 1 mL Swelling buffer (5 mM PIPES, 80 mM KCl, and 1% NP-40, pH 8.0) with protease inhibitors to 5×10^7 cells, incubate on ice for 30 min, reverse the tube during the incubation to avoid the precipitation. Centrifuge at $2{,}300 \times g$ at 4°C for 5 min, dump the supernatants, and go to step 7 in this section.
4. Make sure to keep cell lysates cold. Optimizing the conditions for the sonication is crucial, for sonication efficiency varies depending on cell type. Test the pulse duration, intensity, and time course to establish optimal conditions. Make sure to carry on the reverse cross-linking after sonication and apply the DNA fragments to 2% agarose gel as shown in Fig. 2. Do not shear or sonicate DNA for too long, for long time sonication may result in disruption of nucleosome-DNA binding. The fragments shorter than 250 bp could be an indication for too long sonication. Choose the lowest power output that gives ideal fragmentation to avoid overheat.
5. For the first ChIP assay, it is recommended to measure the DNA concentration after reverse cross-linking. Briefly, take the input DNA at step 1 in section 3.2, go to step 3 in section 3.3 directly, and measure the concentration after step 11 in section 3.3. The DNA concentration should be about half of the concentration measured after step 11 in section 3.3.
6. The preclear step is important for reducing the background. Leave 100 μL supernatant in the original tube to avoid any

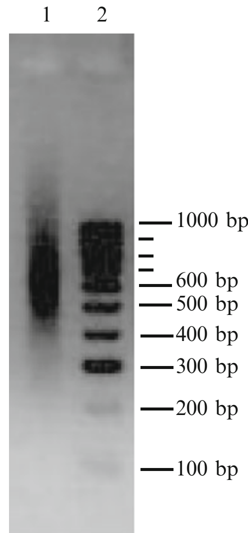

Fig. 2. The sheared DNA fragments from H1299 cells. *Lane 1*, sheared DNA fragments. *Lane 2*, DNA markers.

contamination. The remaining salmon sperm DNA also plays a role as blocking reagent for the immunoprecipitation.

7. Use an equal volume of about 25 μg prepared chromatin for each immunoprecipitation with each antibody. The amount of antibodies may vary, and trial experiments may be needed to determine the amount of antibodies suitable for each immunoprecipitation and PCR. Start with 2 μg antibodies for 25 μg prepared chromatin. If this is the first ChIP assay, do not forget to set up proper positive and negative controls, like anti-pol II and IgG.
8. Additional washes may be needed if the signal–noise ratio is low. The first two washes could be done by inversing the tube several times; but it is recommended to put the tube on a rotating platform at 4°C for 5 min for each wash.
9. Removing RNA by RNAse A treatment will increase the efficiency for the DNA purification, especially when DNA is purified using a PCR purification kit, for the columns could be saturated by a high level of RNA. After this step, the DNA can be purified by using a PCR purification kit, or a miniprep column. For Qiagen QIAprep Spin Miniprep Kit, go through the following steps. Mix 700 μL of buffer PB with 300 μL of elutions and pass through a miniprep column; wash with 750 μL buffer PE; then elute the DNA with 50 μL buffer EB or H_2O and go to step 12 in section 3.3.
10. The predominant site of proteinase K cleavage is the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids with blocked alpha amino groups. The proteinase

K treatment disrupts the binding of protein to DNA and increases the efficiency of DNA purification.

11. The primers could be designed based on the p53RE DNA element within a target gene. The p53RE DNA element could be bioinformatically predicted by p53MH program (27). A good negative control could be the primers encompassing the 3 UTR-coding region of a target gene.

12. It is necessary to optimize the PCR cycle number, for too many cycles will amplify the nonspecific DNA as immunoprecipitated with IgG; however, fewer cycles could not show the DNA pulled down by the p53 antibody used. A titration of immunoprecipitated DNA is recommended to figure out the proper cycle number for a specific PCR reaction.

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Chapter 18

ChIP-on-chip to Identify Mutant p53 Targets

Frauke Goeman, Giulia Fontemaggi, and Giovanni Blandino

Abstract

Chromatin immunoprecipitation (ChIP) followed by microarray hybridization (on-chip) is a technique well suited for a comprehensive analysis of transcription factor binding sites, histone modification patterns, and nucleosome occupancy. It can be restricted to a subset of genes or regions but also expanded up to a genome-wide range yielding insight into the functional elements of gene regulatory networks. Mutant p53 proteins have lost their capacity to bind to its cognate binding sites, but it is well established that it has retained the ability to bind indirectly to DNA via other transcription factors and therefore change the expression of several target genes. The identification of those transcription factors and binding regions sheds light on how mutant p53 is able to exert oncogenic functions.

Key words: Mutant p53, ChIP, ChIP-on-chip, Microarray, Binding sites, ChIP-chip, Chromatin, Target genes, Transcription factor, Transcriptional signature

1. Introduction

"ChIP-on-chip" (also called ChIP-chip) is a technique that allows anyone to perform a large-scale identification of genomic regions occupied by DNA-binding proteins under physiologically relevant, in vivo conditions (1, 2). This method combines the already well-established procedure of chromatin immunoprecipitation with DNA-microarrays (Fig. 1). The probes on the microarray can be designed in several ways, by using libraries of CpG islands, cloned PCR fragments of promoter regions or oligonucleotides which nowadays constitute the most frequently used probe set (3, 4). The oligonucleotides can be spotted in house or bought by several companies that offer fixed catalogue arrays but also the possibility to arrange custom-designed arrays, providing in addition, all the necessary software for the probe design. The first crucial step in

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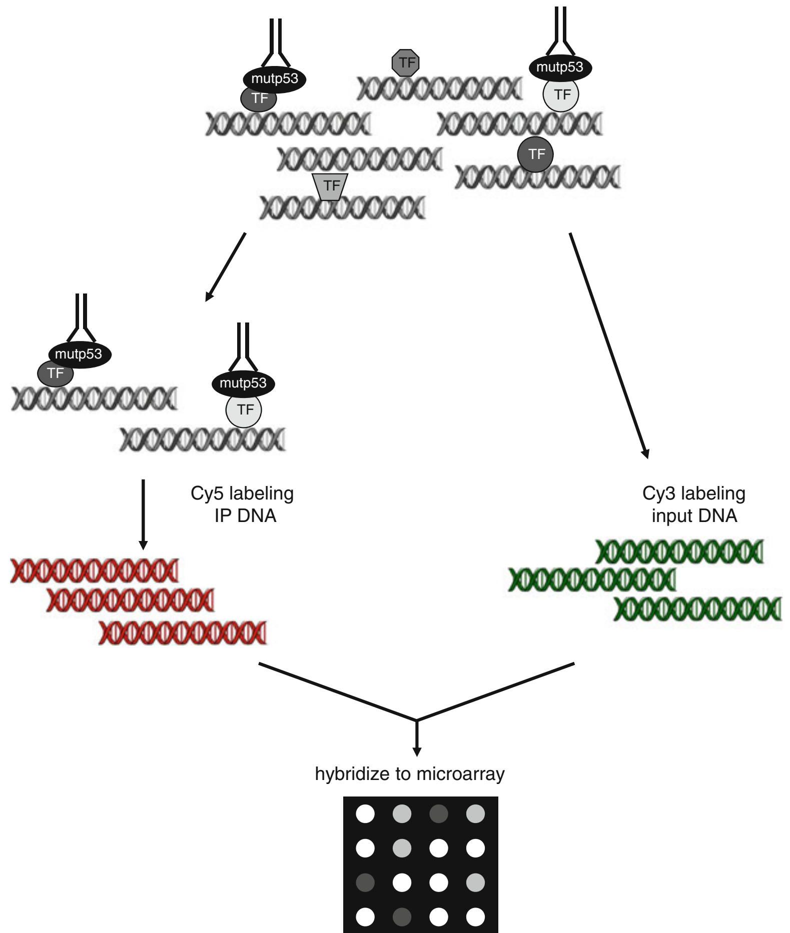

Fig. 1. Schematic overview of the ChIP-on-chip procedure.

performing a successful ChIP-on-chip is to carry out an efficient chromatin immunoprecipitation and therefore enrich the target regions of the transcription factor of interest. Consequently, a highly specific antibody that is able to deliver a specific enrichment of at least eight to tenfold over the background is essential. The initial immunoprecipitated DNA amount is very low and therefore has to be amplified to ensure enough material for the subsequent fluorescent labeling and hybridization onto the microarray. The starting material before the amplification should be in a certain range, around 10 ng, to minimize the risk of an introduction of a bias during the amplification process. In this protocol the amplification is achieved via ligation-mediated PCR. The chromatin is blunted and then ligated to a unidirectional double-stranded oligonucleotide linker that permits the subsequent PCR amplification. The quality of the immunoprecipitated chromatin should be tested before and after the amplification process via PCR with known target regions to ensure a sufficient enrichment before

and after the amplicon generation that should not change during the amplification. The actual enrichment is measured by comparing it to the input and a PCR using primers against regions that should not be enriched. The resulting DNA population is labeled by Cy5 and hybridized to the microarray. To determine the enrichment of the immunoprecipitated material, it has to be co-hybridized with a reference DNA, which is the starting chromatin fraction (input) that should undergo the same treatments like the IP material and is labeled with Cy3. To exclude enrichments that are not due to the antibody used for the ChIP, we suggest to also include chromatin that was immunoprecipitated with either no antibody or with an IgG control and co-hybridize it with the input amplicon on a parallel array.

2. Materials

1. 250 mM PIPES [piperazine-N,N′-bis(ethanesulfonic acid)]: Add 18.9 g of solid PIPES to 200 mL of ultrapure water. Adjust the pH with 1 M NaOH to pH 8 and make up to 250 mL with ultrapure water.
2. 1 M KCl: Solve 7.45 g of KCl in 70 mL of ultrapure water. After the salt has dissolved, make up to 100 mL with ultrapure water and filter it with a 0.2 μm cellulose acetate filter.
3. 10% SDS: Add 10 g SDS to 90 mL of ultrapure water.
4. 0.5 M EDTA pH8: Add 93.06 g disodium ethylenediaminetetraacetate (EDTA) to 450 mL ultrapure water. Adjust pH with 10 M NaOH which will render the EDTA solute. Finally, the EDTA solution has to be filled up with ultrapure water to 500 mL and autoclaved.
5. 1 M Tris–HCl pH 8: Weigh 60.57 g Tris [Tris (Hydroxymethyl) Aminomethane] into 450 mL of ultrapure water. Adjust the pH with HCl, make up to 500 mL with ultrapure water and filter through a 0.2 μM cellulose acetate filter.
6. 5 M NaCl: Weigh 5.75 g into finally 50 mL of ultrapure water. Filter the solution with a 0.2 μm cellulose acetate filter.
7. 1 M $NaHCO_3$: Weigh 840 mg of $NaHCO_3$ and dissolve it into finally 10 mL of ultrapure water.
8. Lysis Buffer: 5 mM PIPES, 85 mM KCl, and 0.5% NP-40 substitute. Add 1 mL of the 250 mM PIPES and 4.25 mL of the 1 M KCl stock solution together with 0.25 mL of NP-40 substitute, fill it up with ultrapure water up to 50 mL. Store at 4°C.
9. Sonication buffer: 1% SDS, 10 mM EDTA, and 50 mM Tris–HCl pH 8. Mix 5 mL of 10% SDS solution with 1 mL of 0.5 M

EDTA pH 8, 2.5 mL 1 M Tris–HCl pH 8, and 41.5 mL of ultrapure water. Store at room temperature to avoid a precipitation of the SDS under low temperatures.

10. Dilution buffer: 0.01% SDS, 1.2 mM EDTA pH 8, 16.7 mM Tris–HCL pH 8, 1.1% Triton-X100, and 167 mM NaCl. Add 0.05 mL of 10% SDS, 120 μL of 0.5 M EDTA pH 8, 0.835 mL of 1 M Tris–HCl pH 8, 5.5 mL of Triton-X100, and 1.67 mL of 5 M NaCl solution to 41.83 mL ultrapure water.
11. Elution buffer: 100 mM $NaHCO_3$ and 1% SDS. Add 1 mL of 10% SDS solution and 1 mL of 1 M $NaHCO_3$ to 8 mL of ultrapure water. The buffer has to be prepared fresh and used within 2 weeks!
12. Wash buffer A: 0.1% SDS, 2 mM EDTA pH 8, 20 mM Tris-HCl pH 8, 1% Triton X-100, and 150 mM NaCl. Add 2.5 mL of 10% SDS, 1 mL of 0.5 M EDTA pH 8, 5 mL of 1 M Tris–HCl pH 8, 2.5 mL of Triton X-100, and 7.5 mL of 5 M NaCl stock solution to 231.5 mL of ultrapure water (final volume 250 mL).
13. Wash Buffer B: 0.1% SDS, 2 mM EDTA pH 8, 20 mM Tris–HCl pH 8, 1% Triton-X-100, and 500 mM NaCl. Add 2.5 mL of 10% SDS solution, 1 mL of 0.5 M EDTA pH 8, 5 mL of 1 M Tris–HCl pH 8 solution, 2.5 mL of Triton-X-100, and 25 mL of the 5 M NaCl stock to 214 mL of ultrapure water (final volume 250 mL).
14. Wash Buffer TE: 10 mM Tris–HCl pH 8, 1 mM EDTA pH 8. Dilute 0.5 mL of 1 M Tris–HCl pH 8, and 0.1 mL of 0.5 M EDTA pH 8 into finally 50 mL of ultrapure water.
15. BSA (100 μg/μL) solution: Weigh 1 g of BSA fraction V into finally 10 mL of ultrapure water. Then, filter the solution with a 0.2 μm cellulose acetate filter.
16. 0.125 M Glycine solution: Weigh 1.88 g glycine into 200 mL of PBS buffer. For cells in suspension: Prepare a 10× concentrated glycine solution by weighing 4.7 g glycine into finally 50 mL 1× PBS.
17. 100 mM PMSF: Weigh 174.2 mg and dissolve it into 10 mL of isopropanol.
18. p53 specific antibody: sheep polyclonal Ab-7 (Calbiochem/Merck).
19. PBS/BSA wash buffer: 1× PBS plus 5 mg/mL BSA. Weigh 250 mg of BSA fraction V into 50 mL of 1× PBS. Filter with 0.2 μm cellulose acetate filters.
20. Magnetic beads: Use Dynabeads® Protein G for Immunoprecipitation (Invitrogen).
21. 1 M Tris–HCl pH 6.5: Weigh 60.57 g Tris [Tris (Hydroxymethyl) Aminomethane] into 450 mL of ultrapure water. Adjust the pH with HCl to pH 6.5. Make up to 500 mL with ultrapure water and filter through a 0.2 μM cellulose acetate filter.

22. 3 M NaAc pH 5.2: Weigh 61.52 g of sodium acetate into 220 mL of ultrapure water and adjust the pH with glacial acid to pH 5.2. Fill it up to 250 mL with ultrapure water and filter through a 0.2 μm cellulose acetate filter.
23. 70% ethanol: Combine 70 mL of ethanol with 30 mL of ultrapure water.
24. Primers used for qPCR:

    ID4-C-F: GGCGCACGGCTCTATAAATAC

    ID4-C-R: CAAAAGCACCGGAAGAAAAGTA

    HIST1H2BA_F: ACTCTCCTTACGGGTCCTCTTG

    HIST1H2BA_R: AGTGCTGTGTAACCCTGGAAAA.
25. PicoGreen® or Qubit dsDNA HS Assay (Invitrogen).
26. 0.1 M $NaHCO_3$: Weigh 840 mg of $NaHCO_3$ and dissolve it in 100 mL of ultrapure water.
27. 0.1 M Na_2CO_3: Weigh 1.06 g of anhydrous Na_2CO_3 and dissolve it in finally 100 mL of ultrapure water. The solution must always be prepared *fresh*.
28. 0.1 M $NaHCO_3$ solution pH 9: Always prepare it *fresh*. Adjust the pH by mixing the 0.1 M $NaHCO_3$ solution with the 0.1 M Na_2CO_3 solution until it reaches pH 9.
29. Cyanine dyes: Amersham CyDye Post-Labeling Reactive Dye Packs (GE Healthcare).
30. High concentrated Klenow: BioPrime® DNA Labeling System (Invitrogen).
31. Hybridization buffer: 2×SDS based hybridization buffer (Genisphere).
32. Human Cot-1 DNA (Invitrogen)
33. 1× SSC with 1% SDS: Dilute 10 mL of a 20× SSC stock solution and 20 mL of 10% SDS with 170 mL of ultrapure water.
34. 0.2× SSC: Mix 1 mL of 20× SSC with 99 mL of ultrapure water.
35. NanoDrop® spectrophotometer.
36. Microarray scanner.

3. Methods

3.1. Chromatin Immunoprecipitation

The ChIP-on-chip assay begins with a chromatin immunoprecipitation similar to the one described in Chapter 17. Two important changes have to be considered: In order to obtain the immunoprecipitated chromatin amount of around 10 ng, we usually start with a higher quantity of cells/chromatin for the IP and with a higher

amount of μg antibodies compared to the classical ChIP. The second important step is to use protein G Dynabeads® instead of agarose beads. These kinds of beads tend to have a lower unspecific binding and do not need to be blocked with salmon sperm DNA, which should be avoided using for instance commercially available oligonucleotide microarrays. For one immunoprecipitation use 1×10^7 cells (ca. 80 μg chromatin) employing 7 μL of the p53 specific Ab-7 antibody. Perform in parallel an immunoprecipitation with no antibody or IgG as negative control.

3.1.1. Cross-linking and Cell Harvesting

1. To freeze the transcription factors that are bound in certain regions on the DNA, add 1% of formaldehyde (final concentration) to the live cells. The formaldehyde generates protein–protein and protein–DNA cross-links in this way fixing the proteins in proximity to the DNA. The formaldehyde should be incubated EXACTLY 10 min at room temperature (22–24°C) under a hood (see Note 1).
2. To terminate the cross-linking, the adherent cells should be washed quickly with cold 1× PBS and then incubated at room temperature for 5 min with 1× PBS supplemented with 0.125 M glycine. The glycine quenches the formaldehyde and therefore stops the cross-linking (if you use cells in suspension you can add glycine directly to a final concentration of 0.125 mM after the cross-linking. In this case, prepare a 10× concentrated solution and add 1/10 of the overall volume to the cells).
3. After the 5 min incubation period, rinse the cells again with cold 1× PBS and collect them into a 50 mL Falcon by scraping. Centrifuge the cells for 5 min at 1,000 rpm, 4°C, remove the supernatant, and wash the cell pellet with cold 10 mL 1× PBS supplemented with 0.5 mM PMSF. Centrifuge again at 1,000 rpm and remove the supernatant. The resultant cross-linked cell pellet is ready for storing at −80°C or immediate cell lysis.

3.1.2. Cell Lysis and Nuclei Isolation

The cells are lysed by adding 5 cell pellet volumes of a hypotonic buffer, the cell lysis buffer, and incubation for 30 min on ice (see Note 2). Transfer the solution into a 1.5 mL microfuge tube and isolate the nuclei of the cells by rigorously pipetting up and down the solution at the end of the incubation time and subsequent centrifugation at 3,000 rpm in a table centrifuge. The nuclei of 2×10^7 cells (for one p53 IP and one IgG IP) are resuspended in 250 μL of sonication buffer, supplemented with protease inhibitors.

3.1.3. Sonication

Sonicate the lysate to shear the DNA to a size range of 100–600 bp (after reverse cross-linking). The sonication power and time will depend on your type of sonicator and cell type, which has to be determined empirically (see Note 3). Control 1 μL of the cross-linked chromatin over the time-course of sonication on a 0.8% agarose gel

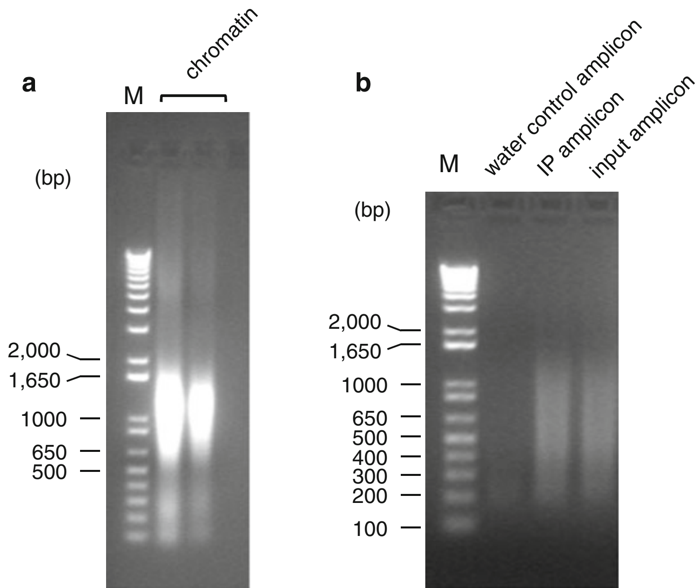

Fig. 2. (**a**) Size distribution of the sonicated chromatin before reverse cross-linking; M marker. The length of the DNA marker fragments is indicated. (**b**) Size distribution of the immunoprecipitated DNA after Ligation mediated PCR (LM-PCR).

that should run like shown in Fig. 2a. Centrifuge the lysate for 20 s at 14,000 rpm in a table centrifuge to pellet the cell debris and transfer the supernatant into a new 1.5 mL microfuge tube (see Note 4). The lysate can be frozen at −80°C.

3.1.4. DNA Concentration Determination

1. To quantify the chromatin and to control its actual size after reverse cross-linking, remove 8 μL of the nuclei lysate. Fill up the volume by adding 192 μL dilution buffer and 0.5 μL RNase A (20 μg/μL) plus 8 μL of the 5 M NaCl solution (200 μM final NaCl concentration) and reverse the cross-linking by incubating the samples for 5 h or overnight at 65°C in a thermo mixer with shaking (see Note 5).
2. Afterwards, add 4.2 μL of 5 M EDTA pH 8, 8.4 μL of 1 M Tris–HCl pH 6.5, and 1 μL of proteinase K (20 μg/μL) and incubate the samples for 2 h at 42°C (see Note 6). The final concentration of EDTA and Tris–HCl pH 6.5 are therefore, 10 mM and 40 mM, respectively.
3. The DNA is recovered by phenol–chloroform purification and ethanol precipitation. Therefore, 200 μL of phenol–chloroform–isoamyl alcohol is added and vigorously mixed for 30 s.
4. A centrifugation step at 11,000 rpm in a table centrifuge at room temperature separates the DNA that remains in the aqueous phase from the proteins that are in the organic phase and interphase (see Note 7).

5. The DNA is precipitated by adding 20 μL of 3 M NaAc pH 5.2, 0.5 μL of glycogen (20 μg/μL), and 440 μL of 100% ethanol to the samples (see Note 8). Leave the samples overnight at −20°C and centrifuge them the next day for 30 min at full speed (13,000 rpm) in a table centrifuge at 4°C. Remove the supernatant and wash the pellet once with 70% ethanol. Centrifuge again for 5 min at 4°C, 13,000 rpm and remove the supernatant again. Let the DNA pellets dry and resuspend them subsequently in 15 μL of ultrapure water.
6. Use a NanoDrop® spectrophotometer to determine the DNA concentration and run ca. 1 μg DNA on a 1.5% gel to control the correct size range of the chromatin which should be around 100–600 bp (see Note 9). The remaining DNA can be used as input which will serve as reference control.

3.1.5. Immunoprecipitation

1. For each immunoprecipitation use 50 μL of Dynabeads® and resuspend them in a 1.5 mL microfuge tube on ice containing 1 mL PBS/BSA. Vortex briefly to mix well. To wash the beads place them on the magnet and remove the supernatant. Resuspend the beads in 1 mL PBS/BSA and repeat the step before, using the magnet. Wash the beads four times in total with PBS/BSA.
2. Resuspend the beads again in 1 mL of PBS/BSA and add 7 μL of the p53 specific Ab-7 antibody or control IgG. Incubate the beads with the antibodies rotating overnight at 4°C (see Note 10).
3. Wash the beads again three times with 1 mL of PBS/BSA the next day and resuspend them finally in 100 μL PBS/BSA.
4. Dilute the chromatin obtained in Subheading 3.1.3 with the dilution buffer 1:10, adding 2.25 mL of dilution buffer, supplemented with protease inhibitors, to the 250 μL of cell nuclei lysate. Finally, add the chromatin solution to the bead-antibody mix and incubate them together overnight at 4°C. For each immunoprecipitation use 1,100 μL of chromatin solution.
5. Collect the beads containing immuno-bound chromatin by placing the microfuge tube on the magnet. Discard the supernatant and initiate the washing procedure by washing them five times with 700 μL of wash buffer A, then five times with wash buffer B and finally, two times with TE (see Note 11).

3.1.6. Elution

Discard all the liquid and elute the immuno-bound chromatin by adding 200 μL of elution buffer to the beads. Incubate them on a rotator at room temperature for 15 min and collect the supernatant after placing the tubes on the magnet. Repeat this elution process by adding 200 μL of elution buffer to the beads once more

and incubate them for 15 min rotating at room temperature. Collect the supernatant by placing the samples on a magnet and combine it with the first eluate.

3.1.7. Reverse Cross-links and DNA Purification

1. To reverse the cross-links, add 16 μL 5 M NaCl (final concentration 200 mM) and 0.5 μL RNase A (20 μg/μL) and incubate them with continuous shaking for at least 5 h or overnight at 65°C.
2. Afterwards, add 8.33 μL 0.5 M EDTA pH 8, 16.66 μL 1 M Tris–HCL pH 6.5, and 1 μL proteinase K (20 μg/μL) to the samples and incubate them for 2 h at 42°C.
3. Next, add 440 μL phenol–chloroform–isoamyl alcohol to the samples, mix rigorously for 30 s and divide the two phases by centrifugation at room temperature for 10 min at 11,000 rpm.
4. Collect the upper aqueous phase that contains the DNA and place it into a new microfuge tube (see Note 7). Add 44 μL 3 M NaAc pH 6.5, 0.5 μL glycogen (20 μg/μL), and 880 μL ethanol to precipitate the DNA. Leave the samples overnight at −20°C and centrifuge them the following day for 30 min at 13,000 rpm, 4°C (see Note 12). Remove the supernatant, add 700 μL of 70% ethanol and centrifuge the samples again for 5 min. Remove the supernatant and allow the samples to dry (see Note 13). Resuspend the samples in 50 μL of ultrapure water.

3.1.8. Quality Control of ChIP Material

It is crucial to control the quality and the quantity of the immunoprecipitated DNA. The quantity is determined by employing PicoGreen® or the Qubit dsDNA HS Assay. We immunoprecipitated approximately 2–3 ng per single ChIP. Instead, the quality is controlled by PCR or qPCR using primers for targets that should be enriched but also primers for regions that should not be enriched to estimate the fold enrichment over the background. Use 2 μL of each ChIPed DNA and 10 and 25 ng of input. If you carry out a qPCR, prepare a standard curve by serial dilutions of your input. Your ChIP should display an eight to tenfold enrichment and contain 10 ng of immunoprecipitated chromatin to go on with the on-chip part. If necessary, pool several ChIPs. For mutant p53 we used the ID4 promoter as a positive and the HIST1H2BA promoter as a negative control in SKBr3 and MDA-MB-231 cells (5) (Fig. 3).

3.2. Amplification of the Immunoprecipitated Chromatin

To obtain sufficient material for microarray hybridization the immunoprecipitated DNA has to be amplified, which is usually done by ligating a unidirectional linker to the DNA followed by PCR amplification. Sonication generates overhanging ends. Therefore, to allow an efficient ligation of the linker the chromatin has to be blunted first.

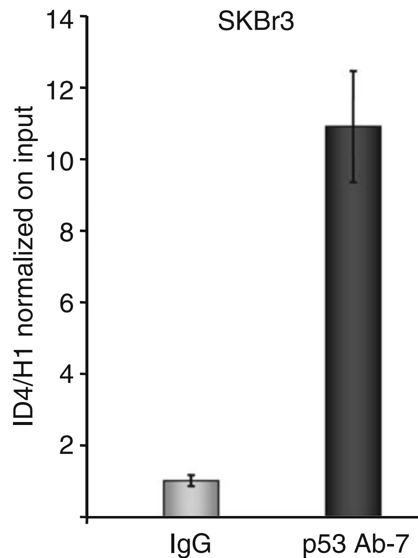

Fig. 3. Quality control for the mutant p53 chromatin immunoprecipitation. The ChIP was performed on cross-linked extracts from proliferating SKBr3 cells using either the p53 specific antibody Ab-7 or IgG as control. The efficiency of the ChIP was analyzed by qPCR. The fold enrichment was calculated by comparing the quantity between the positive control promoter ID4 to the negative control promoter of HIST1H2BA (H1), normalized to the input.

3.2.1. Blunting of the Immunoprecipitated DNA

The end-repair of the DNA is accomplished by T4 DNA polymerase, mixing 1 μL of the enzyme (5 U/μL) with 0.5 μL BSA (10 mg/mL), 1.6 μL dNTPs (20 mM total), 10 ng of input or pooled ChIP samples (mutant p53 IP or IgG IP), and 22 μL T4 DNA polymerase buffer (5×), filled up until 110 μL with ultrapure water. Let the samples incubate in a thermo mixer for 1 h at 37°C (see Note 14). Purify the DNA with QIAquick PCR purification columns following the manufacturer's instructions and elute in 30 μL elution buffer (EB).

3.2.2. Annealing of the Primers to Form the Unidirectional Linker

The oligonucleotides JW102 (5′ gcggtgacccgggagatctgaattc 3′) and JW103 (5′ gaattcagatc 3′) (2) have to be annealed by combining 6.7 μL of each oligonucleotide (100 μM) with 86.6 μL ultrapure water and boiling them for 5 min in a water bath. It is important to let them cool down very slowly within the water bath until they reach room temperature. The linkers can be stored infinitely at –20°C. Thawing should be done slowly on ice.

3.2.3. Ligation of the Linkers

To ensure efficient ligation, use a high concentrated T4 DNA ligase (400 U/μL). Combine 10.3 μL of ultrapure water with 5 μL ligase buffer (10×), 6.7 μL linker, 27 μL blunted chromatin and 1 μL of the T4 DNA ligase. Let the samples incubate overnight at 16°C. Purify them again with QIAquick PCR purification columns following the manufacturer's instructions and elute them in 30 μL of elution buffer (EB).

3.2.4. Ligation Mediated PCR

1. After the ligation of the linker, the chromatin can be amplified by PCR. The PCR reactions contain 27 μL of the chromatin ligated to the linkers, 4.66 μL of ultrapure water, 5 μL PCR buffer (5×, 25 mM $MgCl_2$), 2.24 μL dNTPs (20 mM each), 10 μL betaine (5 M), 0.5 μL JW102 primer (100 μM), and 0.6 μL HotMaster Taq (5 U/μL, Eppendorf).

   Perform the PCR with the following program:

55°C	2 min	1 cycle
72°C	5 min	
95°C	2 min	
95°C	30 s	15 cycles
55°C	30 s	
72°C	1 min	
72°C	4 min	
4°C	□	

   Purify the DNA again with QIAquick PCR purification columns following the manufacturer's instructions and elute it in 50 μL of elution buffer (EB).

2. Repeat this PCR step. Use 25 μL for the second PCR reaction and purify the samples again with QIAquick PCR purification columns but elute them two times with 50 μL ultrapure water. Quantify the DNA with NanoDrop®.

3. A third PCR amplification round might be necessary to obtain sufficient DNA for microarray hybridization. Use 50 ng for the third PCR and control the resulting DNA amount after six cycles by loading 10% of the PCR reaction on a 1% agarose gel. Afterwards, you may continue the PCR with some more cycles or perform the purification procedure with PCR purification columns, eluting each sample two times with 50 μL ultrapure water.

3.2.5. Quality Control of the Amplicons

PCR amplification might introduce a bias compared to the original chromatin population. Therefore, it is strongly recommended to perform PCR or qPCR using the same primers as before to validate the ChIP, using 10 ng of the mutant p53 IP, the IgG or no antibody IP and the input DNA. The fold enrichment of known target regions of mutant p53 compared to the input should remain unchanged. Furthermore, control the size distribution of the amplicons by running 10 μL on a 1% agarose gel. The size distribution should be within the range of 200–800 bp (Fig. 2b).

3.3. Labeling of the Amplicons

The amplicons are labeled with two color fluorescence, Cy5 for the IP and Cy3 for the input chromatin. In this way, a microarray can be co-hybridized with the IP and the input amplicon, which serves as

an internal reference control. Enrichment in the IP chromatin can be easily detected by the ratio generated between the two colors. In general, there are two ways of labeling, direct vs. indirect. Since direct labeling in one step—where the fluorescent dye is already conjugated to a nucleotide analogue—can lead to different incorporation rates (due to their bulky character) and thus leads to a labeling bias of the Cy5 and Cy3 conjugates, we accomplished labeling of the amplicons by indirect means. In the first step the aminoallyl-dUTPs are incorporated, which happens equally because of their small size. In the second step the cyanine dyes in the form of NHS-ester are covalently bound to the aminoallyl of the nucleotide analogue (2).

1. Dry 400 ng of each amplicon in a speed vac with heat (60°C). Resuspend the pellet in 39.95 μL of ultrapure water, add 30 μL of random primer buffer (2.5×, BioPrime® DNA Labeling System, Invitrogen) and incubate the samples for 5 min at 95°C. Place them immediately on ice and incubate them for 5 min.
2. Incorporating aminoallyl-dUTPs is mediated by incubating the amplicon with 1.8 μL aminoallyl-dUTP (10 mM), 0.75 μL dNTP 100× (20 mM dATP/dCTP/dGTP, 3.5 mM dTTP), and 2.5 μL of highly concentrated Klenow (40 U/μL) for 4 h at 37°C. Purify it with QIAquick PCR purification columns following the manufacturer's instructions and elute it in 2 × 50 μL ultrapure water (see Notes 15 and 16).
3. Subsequently, dry the samples in a speed vac with heat (60°C). To couple the NHS-ester cyanine dye to the chromatin, resuspend the DNA in 4.5 μL of ultrapure water. Resuspend each cap of Cy dye (= 40 nmol) with 4.5 μL 0.1 M $NaHCO_3$ (pH 9) (see Note 17). Take care to dissolve the dye well by vortexing and spinning down the liquid several times. Combine the IP chromatin solution with the Cy5 dye, the input DNA with the Cy3 dye. Mix them well and incubate them in the dark for 1.5 h. Resuspend them every 15 min.
4. The unincorporated dye is removed by column purification. To allow for efficient binding of the DNA to the column, the pH has to be lowered by adding 35 μL of 3 M NaAc pH 5.2 and 56 μL of ultrapure water to the samples. Purify the labeled DNA with QIAquick PCR purification columns like before, following the manufacturer's instructions and elute it in 2 × 50 μL of ultrapure water.
5. Control the successful labeling by using a microarray specific program of the NanoDrop® spectrophotometer. It controls absorbance at 650 nm for Cy5 and 550 nm for Cy3, together with absorbance at 260 nm in order to determine the quantity of the DNA simultaneously (see Note 18). Dry the samples in a speed vac with heat (60°C) (see Note 19).

3.4. Hybridization of the Array

The total amount of DNA that should be hybridized onto the array depends on the kind and size of the array. Nowadays, there are different formats commercially available, which allow hybridizing various samples also on one slide depending on the number of probes requested, etc. For one entire slide, we recommend to use between 1 and 5 μg of labeled DNA each, IP and input that will be co-hybridized on one slide. You might want to use one additional array to hybridize the IgG or no antibody control together with the input amplicon. In this way, unspecific enrichments that were not due to the p53 antibody can be excluded.

1. Dissolve the desired amount of DNA (which should be equal for IP and input) in 105 μL of ultrapure water. Next, combine the Cy5 and Cy3 labeled DNA, each one dissolved in 105 μL ultrapure water, add 50 μL Cot-1 DNA (1 mg/mL) and finally, add 260 μL of the hybridization buffer (Genisphere, 2×SDS based, C600V600 S25) (see Note 20). The mixture is denatured at 95°C for 5 min and then immediately transferred to a thermo mixer at 37°C and incubated for 30 min (see Note 21).
2. Spin at 13,000 rpm at room temperature for 1 min. If you use the Agilent SureHyb system dispense 490 μL of the hybridization mix onto the gasket slides, which should already be loaded into the Agilent SureHyb chamber base, and carefully place the microarray slide on top. Close the chamber and incubate the slides rotating in an oven at 60°C for 24 h.

3.5. Washing of the DNA Microarray

The washing procedure includes several buffers, differing in SSC and SDS composition. Use glass dishes, slide racks, and magnetic stir plates. Let the buffers swirl during the incubation of the slides by using a little magnetic stir bar.

1. Remove the gasket slides carefully in 1× SSC 0.1% SDS, preheated to 50°C. Place the microarray slide into a glass rack that already resides in a new glass dish, filled up with 1× SSC 0.1% SDS, preheated to 50°C (see Note 22) and incubate it for 5 min with a magnetic stir bar by swirling (medium speed).
2. Next, transfer the slides with the glass rack into a new glass dish containing 1× SSC, 0.1% SDS and let them incubate with magnetic stirring at room temperature for 5 min.
3. The last wash is performed by using 0.2× SSC for 5 min at room temperature. The last buffer will remove any residual SDS that could otherwise create a huge background. The microarrays are dried by spinning them in a 50 mL Falcon tube for 5 min at 600 rpm and immediately scanned in a microarray scanner.

4. Notes

1. In this step it is very important to follow exactly the 10 min incubation to avoid cross-linking that is too strong or too low. Exceeding the 10 min could result in excessive cross-linking, which reduces antigen accessibility and sonication efficiency. Too low cross-linking instead does not allow efficient freezing/cross-linking of the transcription factors on the DNA.
2. Henceforth, keep the samples on ice and always add protease inhibitors (for instance protease inhibitor cocktail from Roche).
3. It is essential to keep the samples during the sonication on ice and strictly prevent the samples from warming. Avoid foaming of the samples.
4. In this step it is important that the samples are not kept cold for a very short moment in order to avoid the SDS from precipitating. Centrifuge shortly at room temperature. The pellet should be very small, otherwise it is an indication that SDS precipitated.
5. The RNA will be efficiently removed by the RNase treatment. This step is also strongly advised for a classical ChIP, if you use a column (PCR purification kit) for the subsequent DNA purification because the RNA can dramatically reduce the DNA yield as the column becomes saturated.
6. Proteinase K has broad substrate specificity and therefore, digests and removes proteins from DNA. Furthermore, it eliminates nucleases from the purified DNA which prevents degradation.
7. It is critical not to carry over any phenol which would precipitate later on together with the DNA and hinder subsequent enzymatic reactions. One could either use a PCR purification kit instead of the phenol–chloroform–ethanol precipitation step or a Phase Lock® gel tube (Eppendorf) that separates reliably the aqueous phase from the organic phase without any carryover.
8. Glycogen is an inert carrier, which significantly increases the recovery of nucleic acids by alcohol precipitation. It is especially useful for precipitating low amounts of DNA like in the samples of the final chromatin IP.
9. The DNA determination is especially important to control the total DNA amount used for each immunoprecipitation, if you wish to compare DNA-binding events under different treatments that may have influenced the cell growth and therefore, the cell number cross-linked.
10. Dynabeads® have the big advantage to bind less DNA in an unspecific manner compared to agarose beads. Furthermore,

salmon sperm DNA for blocking is not necessary which can create a background in several kinds of oligonucleotide based arrays. Please note that the antibody affinity of these beads is lower and requires an overnight incubation with the antibody for efficient binding.

11. When you remove the unbound chromatin make sure to use filter tips and to eliminate the waste to prevent any contamination of the samples later on. Take care not to contaminate your gloves with chromatin.
12. The DNA pellet should be visible but not too big or fuzzy. If the pellet is huge or fuzzy it is an indication that your sample contains phenol precipitates which will disturb subsequent enzymatic reactions. If you use PhaseLock® gel tubes you can be sure that you will never carry over any phenol traces.
13. The ethanol must be completely removed to avoid any disturbance of subsequent enzymatic reactions.
14. You might perform all the following steps with one additional sample using only water instead of DNA as a control.
15. Avoid using elution buffer because the amine groups of the Tris can react with the monofunctional NHS-ester of the cyanine dyes.
16. Control the quantity of the purified DNA with the NanoDrop®. The amount should have been increased significantly (at least three to eightfold).
17. The cyanine dyes are light sensitive. Perform all steps under minimal light exposure.
18. The minimum amount of incorporated dye should be 100 picomol.
19. At this point, you could store the samples at –20°C in the dark overnight. The cyanine dyes are sensitive to light but also to air humidity, therefore, it is important to store them dry if necessary.
20. The amount of hybridization buffer depends on the hybridization chamber used. We recommend employing the gasket slides, SureHyb hybridization chambers and oven from Agilent that ensure a good and even hybridization. The most important thing to remember is that the slides should never become dry during the hybridization process which would otherwise result in a huge background due to precipitated SDS.
21. This step allows the Cot-1 DNA to hybridize with the repetitive DNA sequences to reduce the background in the subsequent microarray hybridization.
22. Do not allow the slide to dry during the washing steps! Scan the slides immediately at the end of the washing procedure. Cy5, especially, degrades rapidly if exposed to ozone.

Acknowledgments

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Chapter 19

ChIP Sequencing to Identify p53 Targets

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Abstract

Mutant p53 may activate target genes through the interaction of transcription factors or through histone modifications. Chromatin immunoprecipitation (ChIP) is a method commonly used to study these types of protein interactions. In order to generate a list of target genes that may be activated through this mechanism, ChIP sequencing may be used. ChIP sequencing involves the mass parallel sequencing of ChIP DNA fragments. We describe a method by which to prepare chromatin immunoprecipitation sequencing libraries and how to analyze sequencing data. In this procedure, prepared libraries have been sent to a core facility. The results have been verified using quantitative PCR.

Key words: p53, Chromatin immunoprecipitation, ChIP sequencing, Histone modification

1. Introduction

Mutant p53 targeted gene transcription may be accomplished in a variety of ways. The promoter region of genes contains regulatory elements which bring an RNA polymerase to the promoter and starts the process of transcription. Histone modifications may occur which unwind the chromatin and make it accessible for transcription. In order to study how genes become activated in the presence of mutant p53, chromatin immunoprecipitation, or ChIP, can be used.

Cross-linked ChIP, the method used here, was first developed in the 1980s by Varshavsky's group and is continuing to be updated (1, 2). ChIP is a useful tool in understanding the relationship between proteins and genomic DNA, and can be used with a wide variety of antibodies. ChIP DNA can now be sequenced to determine the exact location within the genome of where a particular protein binds (3). Used on cells expressing mutant p53, and using

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antibodies against different transcription factors or histones, a profile of potential target genes may be derived (4).

In the basic ChIP procedure, chromatin-associated proteins are cross-linked to the DNA using formaldehyde. The genomic DNA is then sheared, the proteins are released from the DNA and digested, and the DNA purified. ChIP sequencing then utilizes that DNA for large scale parallel sequencing to the genome. The DNA libraries are prepared by blunt-ending the DNA fragments generated by ChIP and attaching an “A” base to the 3′ ends. Adapter sequences are then ligated to the DNA and amplified using polymerase chain reaction (PCR) (5). The analysis is performed by aligning sequencing reads to the genome. Once the sequencing of these libraries is analyzed, quantitative PCR is performed to validate the data.

2. Materials

2.1. Chromatin Immunoprecipitation

1. H1299 cells.
2. Trypan blue.
3. Hemocytometer.
4. 10 cm cell culture dishes.
5. 37% Formaldehyde.
6. 1 M Glycine.
7. 1× PBS.
8. Lysis Buffer: 150 mM NaCl, 25 mM Tris–HCl pH 7.5, 5 mM EDTA pH 8.0, 1% Triton X-100, 0.1% SDS, and 0.5% NaDoc.
9. Protease Inhibitor Cocktail mix.
10. 1 mL syringes.
11. 27 G1/2 needles.
12. 5 mL snap-cap tubes.
13. Misonix Sonicator.
14. Refrigerated centrifuge.
15. RIPA Buffer: 150 mM NaCl, 50 mM Tris–HCl, pH 8.0, 0.1% SDS, 0.5% NaDoc, and 1% NP-40.
16. High Salt Buffer: 500 mM NaCl, 50 mM Tris–HCl pH 8.0, 0.1% SDS, and 1% NP-40.
17. LiCl Buffer: 250 mM LiCl, 50 mM Tris–HCl pH 8.0, 0.5% NaDoc, and 1% NP-40.
18. 1× TE Buffer.
19. 5 M NaCl.
20. Absolute EtOH.

21. 10 mg/mL RNase A.
22. 10 mg/mL Proteinase K.
23. Phenol.
24. Chloroform–Isoamyl alcohol 24:1.
25. 7.5 M Ammonium Acetate.
26. Glycogen.

2.2. Chromatin Immunoprecipitation Sequencing

1. 20–60 ng ChIP DNA.
2. End-It DNA End-Repair Kit (Epicentre Biotechnologies).
3. Klenow Fragment ($3' \rightarrow 5'$ exonuclease-) (New England Biolabs).
4. 1 mM dATP.
5. T4 DNA Ligase (New England Biolabs).
6. Illumina Adapter Oligo Mix (1:10).
7. QIAquick PCR Purification Kit (Qiagen).
8. QIAquick MinElute PCR Purification Kit (Qiagen).
9. QIAquick Gel Extraction Kit (Qiagen).
10. 2× Phusion Master Mix with HF Buffer (Finnzymes).
11. Illumina PCR Primer 1.1.
12. Illumina PCR Primer 2.1
13. Sterile water.
14. Low-melt agarose.
15. 1× TAE.
16. ChIP Loading Buffer: 50 mM Tris–HCl pH 8.0, 40 mM EDTA, and 40% (w/v) sucrose.

3. Methods

3.1. Chromatin Immunoprecipitation

1. ChIP H1299 cells expressing an empty vector or a p53 mutant, such as R273H (H273H). Plate the cells at 3×10^6 cells per 10-cm culture dish (see Note 1). Cross-link the cells the following day using 540 μL of 37% formaldehyde at 37°C. Add glycine to a final concentration of 0.125 M to stop the cross-linking reaction and harvest the cells in 1× PBS.
2. Sonicate cross-linked cells to shear DNA to a size of 0.2–1 kb and remove cellular debris via centrifugation. Immunoprecipitate extracts using the antibody of interest and suitable control antibody (see Note 2) overnight at 4°C after preclearing the extracts with Protein A agarose saturated with BSA and sonicated salmon sperm.

3. In the morning add Protein A agarose beads to the IPs for 1 h at 4°C, then collect protein-DNA complexes by centrifugation. Keep an aliquot of the supernatant and discard the rest and wash the beads with RIPA once, High Salt Buffer once, LiCl Buffer Twice, and 1× TE pH 8.0 twice. Elute protein-DNA complexes from the Protein A agarose beads in a total volume of 400 μL of fresh elution buffer and incubate at 65°C for 6 h-overnight with 16 μL of 5 M NaCl. After reverse cross-linking put the samples to ethanol precipitation for at least 2 h at −70°C.
4. Spin down and dry the samples and put to RNase A digestion for 30 min then Proteinase K digestion for 1 h. Phenol–chloroform extract the DNA and do a final ethanol precipitation.
5. Validate the efficiency of the ChIP by performing quantitative Polymerase Chain Reaction on the ChIP samples for selected target genes, and then proceed to the sequencing preparation.

3.2. Chromatin Immunoprecipitation Sequencing Library Preparation

3.2.1. End Repair

1. End repair 20–60 ng of each ChIP DNA and positive control (see Note 3) using the End-It DNA End-Repair Kit by Epicentre Biotechnologies following the manufacturer's standard reaction. Incubate each reaction at 25°C for 30 min followed by 10 min at 70°C to stop the enzyme reaction.
2. Purify the blunt-ended DNA using the QIAquick PCR purification kit and elute in 34 μL of elution buffer.

3.2.2. A-Tailing

1. A-tail the total amount of end-repaired DNA from the previous step by incubating with 5 μL of NEB Buffer 2, 10 μL of 1 mM dATP, and 1 μL of Klenow fragment (3′–5′ exonuclease minus) at 37°C for 30 min.
2. Purify A-tailed DNA using the QIAquick MinElute PCR purification kit and elute in 17 μL of elution buffer.

3.2.3. Adapter Ligation

1. Ligate the total amount of A-tailed DNA to genomic DNA adapters from Illumina by incubating with 2 μL of 10× T4 DNA Ligase Buffer, 1 μL of Illumina adapter mix (diluted 1:10), and 0.5 μL of T4 DNA Ligase at 16°C for 18 h.
2. Purify ligated DNA using the QIAquick MinElute PCR purification kit and elute in 20 μL of elution buffer.

3.2.4. Polymerase Chain Reaction

1. Perform the polymerase chain reaction by combining the following components: 25 μL of 2× Phusion Master Mix, 1 μL each of 25 μM Primers 1.1 and 2.1 from Illumina, the 20 μL of ligated ChIP DNA or positive control from the previous step, and sterile water to 50 μL (see Note 4).
2. PCR at 98°C for 30 s; then 20 cycles of 98°C for 10 s, 65°C for 30 s, and 72°C for 30 s; 72°C for 5 min; and finally hold at 4°C.

3.2.5. Gel Analysis of ChIP Sequencing Sample Preparation

1. Pour a 2% low-melt agarose TAE gel and set with an 8-well comb.
2. Add 16 μL of ChIP Loading Buffer to each PCR product and mix.
3. Load the entire amount of PCR product/loading buffer into the gel along with appropriate controls and using 100 bp DNA ladder (NEB) as a marker.
4. Run at constant 50 V until a good separation of the ladder can be seen.
5. Excise a region of the gel where the most signal is seen after viewing under short wave UV light (250–500 bp, see Note 5).

3.2.6. Agarose Gel Extraction

1. Extract DNA from the agarose using the QIAquick Gel Extraction Kit and elute in 30 μL of elution buffer (see Note 6).

3.3. Library Sequencing

Samples are sent to a facility for sequencing, and aligned sequence data is sent back for analysis. For the example of analysis presented in this chapter, the Illumina GA II Genome Analyzer was used, though other systems may also be used.

3.4. Analysis of ChIP Sequencing Data

3.4.1. p53 ChIP-Seq: The Principles

ChIP of p53 generates a collection of DNA fragments, purified because they either directly bind to p53 or because they bind other proteins that interact with DNA and chromatin that are complexed with p53. Thus, the DNA isolated is a reflection of direct and indirect DNA and chromatin interactions.

Once the fragments of DNA are isolated, the goal is to determine from where in the genome these fragments come. There should be substantial representation of fragments in promoter regions, but other specific locations, and random locations from nonspecific interactions are possible. The nonspecific interactions can be from p53 or the Ab used in precipitation, or just inefficient purification. The genomic locations represented by the fragments can be determined by sequencing the ends of the fragments. Let us say 2 million fragments are sequenced. Enough of each fragment is sequenced (35–200 bases) so that the sequence can be effectively aligned to the known genome sequence. Usually one or more possible mismatches are allowed so as not to miss mapping fragments with small errors in the read, or miss regions with polymorphisms. Later, if desired, one can analyze only perfect matched data. Not all sequences will align using the desired criteria, and alignment efficiency may be as little as 50%, so one could end up with 1 million fragments mapped in this example. Once the chromosome and base position of fragments are known, the density of these aligned sequences along the genome represents the relative amount of p53 binding, reflecting the binding site properties.

ChIP-Seq can be also used to map changes in histone modifications throughout the genome affected by p53. Instead of

using an Ab to p53 in the ChIP, an Ab to a modified histone is used, such as to acetylated histone H3. An increase in acetylated histones in promoter regions corresponds to increased transcription for the associated genes. ChIP studies of histone modification changes mediated by p53 are consistent with associations of p53 with histone acetyl transferases on promoters.

3.4.2. Data Interpretation

There is a significant distinction between mapping p53 binding sites and mapping histone modification changes. For the modified histone mapping, there are sites of histone modification in the control that change levels of modification when p53 is altered, though most sites do not change. The mapping of p53 binding sites merely locates sites of binding with signals significantly above the noise of the assay.

Interpretation of the data for binding site properties of p53 is a matter for discussion that goes beyond this chapter. While locating the peaks is reasonable, quantifying the peaks for comparison between samples without a viable normalization strategy is problematic. The challenges in analyzing ChIP-Seq data include identifying peaks, normalization of the data, quantifying peaks within replicates, comparing those peak signals between samples, and statistical analysis. An approach that avoids many of these issues and challenges is to quantify binding signal within defined regions of the genome, such as defined gene promoter regions. We use this approach in the analysis described in this chapter.

3.4.3. Bioinformatics of ChIP-Seq Data

The basic information needed for analysis is (1) the sequence, (2) the location (chromosome and base position) of the aligned sequence, (3) the strand of alignment, (4) how the sequence matched the location (perfect match or allowable mismatches), and (5) a numerical indicator of how well the sequence matched the location. This data should be provided in a text format, such as BED, though any text format and arrangement of fields of data is usable. The data may include both aligned and unaligned sequences.

The unaligned sequences will be filtered out leaving the aligned sequences, the tags or hits. The number of hits to expect can range from 1×10^6 to 100×10^6. The more hits to be analyzed, the greater the sensitivity and reliability, but also the greater the time for analysis. The desired minimum number of hits can depend on the results expected. A protein with high specificity for binding and few binding sites will not require a large number of hits, as few as 1×10^6, while proteins that bind throughout the genome, e.g., modified histones, will generate a large number of sites and require a larger number of hits to quantify those sites. Any study with a high hit background throughout the genome would need a larger number of hits.

3.4.4. Analysis of Histone H3 Acetylation Changes Mediated by Mutant p53

The analysis starts with aligned sequences. The first step in analyzing the data is to place it where it can be accessed. While Microsoft Excel does a good job handling data for manipulation and calculations, it can't hold the number of records for the sequences generated by most ChIP-Seq experiments. We use the database software Filemaker Pro because it has no problem holding the amount of data generated by ChIP-Seq and because it runs on both Windows and Macintosh operating systems.

The next step in analysis is to summarize the data. The summary may still be quite large and extensive but it is more manageable. The different types of summaries include binning the data so that the hits are summed over fixed intervals such as 100 bases. Another summary includes locations of peaks found. The summary used in this chapter determines the number of hits within a defined promoter region for each of the known genes.

We use the genes and positions as determined by NCBI RefSeq, with 21,518 genes (6), and transcript start sites as mapped to HG18. This data was collected from the UCSC Genome Browser ((7); http://hgdownload.cse.ucsc.edu/goldenPath/hg18/database/refGene.txt.gz).

The transcription start site for each gene is determined and a defined region of each gene's promoter is determined; 1,000 bases upstream and 1,500 bases downstream of this site is one possible condition. Too narrow a region could result in not collecting data for the entire peak. Also, some peaks can exceed the size of a 2,500 base window or span further into gene coding regions, so initial visual inspection of various promoter regions can help in deciding the size of the promoter window.

Figure 1 shows the distribution of H3 acetylation sequence hits near the promoters for three example genes; the arrows show the transcription start sites and direction of transcription on a 1 Kb increment scale.

The number of hits for each gene promoter region is determined from unbinned data. The hits per promoter are normalized for the average number of hits for the sample run. The average from replicates is calculated and a student *t*-test is used to determine statistical significance between the controls and the p53-manipulated cells. Because of the greater than 21,000 tests performed, the Benjamini–Hochberg correction for multiple testing (8) is used to help identify genes with significant changes based on a desired false discovery rate.

This analysis provides a list of genes whose promoters are affected in H3 acetylation levels. This gene list can be further analyzed just as a list of genes with altered gene expression can be analyzed, such as analysis of over-represented genes from GO biological processes or determining the correlation with known transcription factor binding sites.

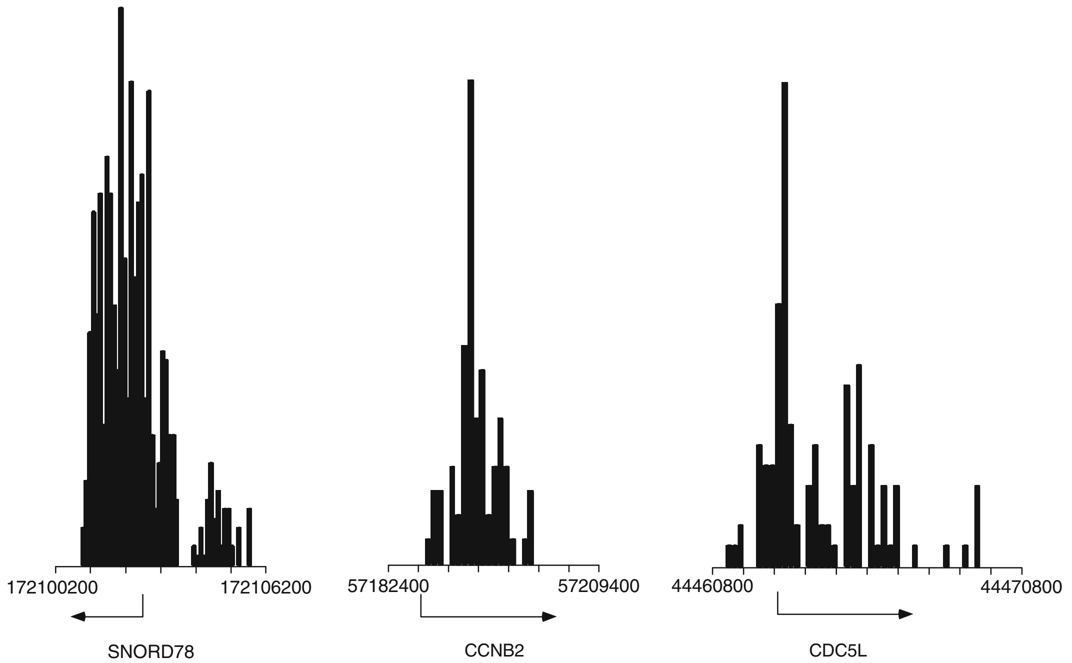

Fig. 1. Peaks of H3 acetylation near promoters.

A visual inspection of peaks can be performed by exporting all or a subset of data from the database for generating density plots using the graphing software of choice.

3.4.5. Caveats and Considerations

When aligning the sequences to the genome, a version of the genome must be selected. The latest human genome version is HG19, but it is not annotated to as great an extent as the previous version, HG18. Therefore, as of this date, to relate the ChIP-Seq data to the greatest amount of data within the Genome Browser database, alignment to HG18 may be preferred.

The results for mapping hits to gene promoters will depend on how a promoter is defined in size and position. If the hits are low, then using a wide window may be necessary to establish enough hits per promoter for analysis. However, the wider the window, the greater the background. The position of this window around the promoter region or the transcription start site can make a significant difference. Peaks of histone modifications are not necessarily within promoters; they can be downstream past the transcription start site and well into the coding region of genes. The peaks can also be narrow or broad, spanning many kilobases (9).

One can see from the UCSC Genome Browser data that the transcription start site for all genes (Tx) is lower in position than the end site regardless of which strand codes the genes. The start site is not always the actual start site. For genes coded on the plus

strand, the start site (lower value) is the transcription start site and for genes coded on the minus strand, the end site (higher value) is the transcription start site.

3.5. Quantitative PCR Verification of ChIP Sequencing Analysis

Perform quantitative PCR on the leftover DNA to validate the sequencing analysis. Ensure all primer sets are specific for the gene of interest and that the PCR will amplify a region of DNA about 500 bp before the promoter start site.

4. Notes

1. The optimal number of cells plated for cross-linking should be determined empirically to achieve the best results later on.
2. Make sure the antibody used for immunoprecipitation is good for ChIP before proceeding with the ChIP Sequencing procedure.
3. Take the aliquot of the immunoprecipitation supernatant and carry it through the rest of the ChIP procedure and run it in a 2% agarose TBE gel. Cut out a region of the gel around 200 bp and extract out the DNA. This DNA can be used as a positive control for ChIP Sequencing preparation. One of the bands in the PhiX HaeIII DNA Ladder (New England Biolabs) such as the 234 bp band may be used as well but does not need to be end repaired.
4. To ensure the PCR works, PCR half of the positive control sample as normal and take the other half of the positive control sample and PCR without the enzyme. This will show the difference between the ligated material and unused primers. Always perform a negative for PCR, where water is used instead of DNA.
5. Do not view the gel under long wave UV light, this will damage the DNA and seriously compromise both sequencing and analysis.
6. Make sure to perform quantitative PCR on the samples after the ChIP Sequencing preparation to ensure sample integrity.

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ERRATUM TO

Chapter 4
P53 and Cell Cycle Effects After DNA Damage

Emir Senturk and James J. Manfredi

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The publisher regrets that the coauthors of Chapter 4 were incorrect. The coauthors of Chapter 4 should be Lois Resnick-Silverman and James J. Manfredi and not Emir Senturk and James J. Manfredi.

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