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Interaction of p53 with Cellular Proteins

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Summary

Cellular proteins that interact with p53 play a major role in both positive and negative regulation of this tumor suppressor and can fine-tune its response to specific cellular stresses. As a consequence, p53 biology will not be complete until these interacting proteins are fully characterized. This chapter outlines two methods for identifying and characterizing p53-binding proteins: (*i*) glutathione-S-transferase (GST) protein–protein interaction assay; and (*ii*) co-immunoprecipitation (co-IP) assay. These two methods are ideal for any laboratory to perform; the assays are short in duration, do not require specialized expertise to establish in the laboratory, give reliable and reproducible data, and are cost-efficient, because few reagents are needed. This chapter gives a basic description of these two techniques and provides tips that are not found in other protocol manuals on how to achieve the best results.

Key Words

GST pull-down assay, co-immunoprecipitation, protein–protein interaction

1. Introduction

The p53 tumor suppressor protein cannot function alone. Instead, it needs to interact with multiple cellular proteins that serve to both enhance and repress p53 function ***(1,2)*** (*see* **Table 1**). Unlike transcriptional or translational regulation, these protein–protein interactions help provide a mechanism for instant regulation of the p53 molecule by either affecting its transactivation activity and/or protein stability, as discussed below.

Many protein–protein interactions play critical roles in p53 transactivation. For example, p53's amino (N)-terminal transactivation domain directly contacts basal transcription factors, such as TBP ***(3,4)*** and TAFII31 ***(5,6)***, as well as transcriptional co-activators such as p300/CBP ***(7,8)***. Thus, the transcrip-

From: *Methods in Molecular Biology, vol. 234: p53 Protocols*
Edited by: S. Deb and S. P. Deb © Humana Press Inc., Totowa, NJ

Table 1
p53-Interacting Proteins

General transcription factors	*Protein acetylases/deacetylases/ deacetylase adaptors*
TFIID components: hTAFII31 ***(5,6)***	HDAC1 ***(40,41)***
TBP ***(5,6)***	p300/CBP ***(7,8)***
TFIIH components: XPB ***(22–24)***	PCAF ***(42)***
XPD	Sin3a ***(43)***
p62	Sir2α ***(44,45)***
Protein kinases	*Ubiquitination and de-ubiquitination*
Casein kinase 2 ***(25)***	E6-Ap ***(46)***
HIPK2 ***(10,11)***	HAUSP ***(47)***
JNK1 ***(26)***	MDM2 ***(48)***
Redox sensitive proteins	*Replication and repair proteins*
HIF-1α ***(27)***	RP-A ***(49–51)***
Ref-1 ***(9)***	TFIIH components: XPB ***(22–24)***
	XPD
	p62
Miscellaneous p53 activators	*p53 family members*
14-3-3σ ***(28)***	p63 ***(52)***
53BP1 ***(29)***	p73 ***(52)***
53BP2 ***(29)***	
HMG-1 ***(30)***	
Viral proteins	*MDM2 family member*
AdE1B55 kD ***(31,32)***	MDMX ***(53)***
EBV ENBA-5 ***(33)***	
HBV X protein ***(34,35)***	
HPV E6 ***(36,37)***	
SV40 Tag ***(38,39)***	

tional machinery is recruited to promoters to which p53 is bound. Other proteins can further stimulate p53's transcriptional activity in response to certain cell stress stimuli. The redox-sensitive protein Ref-1 binds to p53 and activates its DNA binding and transactivation function in response to redox stress ***(9)***. Several kinases also form stable interactions with p53, resulting in phosphorylation and activation. For example, the homeodomain interacting protein kinase-2 (HIPK2) has been recently shown to bind and phosphorylate p53 at Ser46 after high doses of ultraviolet (UV) light, resulting in induction of p53-dependent apoptosis ***(10,11)***.

Protein stability of p53 is also tightly regulated by protein–protein interactions. Most importantly, the oncoprotein MDM2 binds to the N-terminal domain of p53 ***(12)*** and negatively regulates its stability. MDM2 is a RING-finger containing E3-ubiquitin ligase that ubiquinates lysine residues on p53's carboxy (C) terminus, thus targeting p53 to the proteosome for degradation ***(13,14)***. The functional link between these proteins is clearly shown by knock-out mouse models, in which MDM2 homozygous null mice are embryonic lethal, though double-knock-out MDM2-p53 mice survive ***(15)***. This striking finding indicates that without MDM2 to keep the p53 protein in check, the organism cannot survive p53's growth suppressive effects. Importantly, the MDM2-p53 interaction is also regulated by cell stress stimuli. For example, UV and γ irradiation can activate kinases that phosphorylate p53 on serine residues within the MDM2-binding domain, thereby preventing MDM2 from binding ***(16–21)***.

As suggested by the above examples, p53 protein–protein interactions are often regulated by cellular stress signals. Thus, it is important, when studying a particular p53-interacting protein, to keep in mind that the interaction may be very stress-specific and that optimization of binding conditions is required. This will include testing several cellular stress agents and performing time courses to determine when maximal binding occurs posttreatment.

In this chapter, we describe two commonly used methods for studying p53 protein–protein interactions: (*i*) glutathione-S-transferase (GST) pull-down assays to study interactions in vitro and co-immunoprecipitations (Co-IPs) reactions to study interactions in cells. Briefly, the GST pull-down assay uses recombinant p53 fused to the GST protein, which can then bind to glutathione coupled to agarose beads. Mixing GST-p53 with a recombinant protein of interest to test for binding or with cell lysates to pull out novel p53-interacting proteins is then done. On the other hand, co-IPs use p53-specific antibodies coupled to protein A or G Sepharose to pull-down p53 from cell lysates. If a known protein is being tested for p53 binding activity, then the co-IPs can be visualized by running on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot analysis. Alternatively, to identify novel p53-interacting proteins, the SDS-PAGE gel can be stained with silver solution for example, followed by cutting out the novel protein bands and subjecting them to protein sequencing.

2. Materials

2.1. GST Protein–Protein Association Assay

1. LB broth (Fisher Scientific, Pittsburgh, PA, USA; cat. no. BP1426-2).
2. Isopropyl-β-D-thiogalactoside (IPTG): (Fisher Scientific; cat. no. BP1620-10). Store in aliquots at –20°C.

3. Phosphate-buffered saline (PBS) buffer 20X: 160 g NaCl, 4 g KCl, 23 g $Na_2HPO_2 \cdot 7H_2O$, 4 g KH_2PO_4. Store at room temperature.
4. PBS 1X: Prepare from 20X stock using water and store at 4°C.
5. Glutathione–agarose beads (Sigma, St. Louis, MO, USA; cat. no. G-4510). Swell the lyophilized powder with deionized water at room temperature for at least 30 min (1 g swells to approx 14 mL gel). Wash and store beads in 1X PBS.
6. Protease inhibitors: 1 μg/mL aprotinin, 1 μg/mL leupeptin, 4 μ*M* pepstatin A, 0.2 m*M* phenylmethylsufonyl-fluoride (PMSF) (add fresh just before use).
7. Dithiothreitol (DTT): make 1 *M* stock in water and store in aliquots at –20°C. Add to buffers just before use to make a final concentration of 1 m*M*.
8. GST-lysis buffer: 1X PBS, 0.1% Nonidet® P-40 (NP40), 10% glycerol. Store at 4°C. Add 1 m*M* DTT and protease inhibitors just before use.
9. GST-lysis buffer plus 500 m*M* NaCl: 1X PBS, 0.1% NP40, 10% glycerol, 500 m*M* NaCl. Store at 4°C. Add 1 m*M* DTT and protease inhibitors just before use.
10. SDS protein sample buffer 4X: 200 m*M* Tris-HCl, pH 6.8, 400 m*M* DTT, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol.

2.2. Immunoprecipitation of Cellular Proteins

1. PBS buffer 1X: *see* **Subheading 2.1.**, **item 4** above.
2. Lysis buffer: 50 m*M* Tris-HCl, pH 8.0, 5 m*M* ethylenediamine tetraacetic acid (EDTA), 150 m*M* NaCl, 0.5% NP40. Store at 4°C. Add 1 m*M* DTT and protease inhibitors just before use.
3. SNNTE buffer: 50 m*M* Tris-HCl, pH 7.4, 5 m*M* EDTA, 5% sucrose, 1% NP40, 0.5 *M* NaCl. Store at 4°C. Add 1 m*M* DTT and protease inhibitors just before use.
4. RIPA buffer: 50 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 1% Triton® X-100, 0.1% SDS, 1% (w/v) Na Deoxycholate. Store at 4°C. Add 1 m*M* DTT and protease inhibitors just before use.
5. Protein G Sepharose® (Amersham Pharmacia Biotech, Piscataway, NJ, USA; cat. no. 17-0618-01). Store at 4°C.
6. Anti-MDM2 (Ab-3, monoclonal, clone 4B11) (Calbiochem-Novabiochem, San Diego, CA, USA; cat. no. OP46).
7. Anti-MDM2 (C-18, rabbit polyclonal) (Santa Cruz Biotechnology, Santa Cruz, CA, USA; cat. no. sc-812).
8. Anti-p53 (Ab-1, monoclonal, clone PAb 421) (Calbiochem-Novabiochem; cat. no. OP03).
9. Anti-p53 (Ab-2, monoclonal, clone PAb 1801) (Calbiochem-Novabiochem; cat. no. OP09).
10. Anti-p53 (Ab-4, monoclonal, clone PAb 246) (Calbiochem-Novabiochem; cat. no. OP32).
11. Anti-p53 (FL-393, rabbit polyclonal) (Santa Cruz Biotechnology; cat. no. sc-6243).

3. Methods

3.1. GST Protein–Protein Association Assay

The following protocol is adapted from **refs. *54*** and ***55***, with some modifications (**Fig. 1**).

3.1.1. Purify GST-Fusion Proteins

1. Inoculate 5-mL starter cultures from single colonies of GST-fusion protein and GST-0 (control GST empty vector) constructs. Grow overnight with shaking at 37°C.
2. Add starter cultures to 500 mL of LB broth and incubate with shaking at 37°C until OD_{600} is between 0.6–0.8. Then add 500 μL 0.4 *M* IPTG to the cultures (final is 0.4 m*M*), and incubate for an additional 2–6 h.
3. Centrifuge cultures for 15 min at 5000*g*.
4. Resuspend cell pellets in 25 mL GST-lysis buffer.
5. Lyse cells by passage 2× through a French Press (*see* **Note 1**). Centrifuge lysates for 20 min at 30,000*g* to pellet insoluble fraction.
6. Incubate supernatents with 1 mL glutathione–agarose beads 50% slurry (500 μL bead bed vol) for 5 min at room temperature.
7. Centrifuge for 1 min at 1000*g* in swinging bucket rotor to pellet agarose beads.
8. Wash beads 3× with the following buffers: (*i*) 25 mL GST-lysis buffer; (*ii*) 25 mL GST-lysis plus 500 m*M* NaCl; and (*iii*) 25 mL GST-lysis buffer. During each wash, incubate with rotation at room temperature for 5 min, followed by centrifugation for 1 min at 1000*g*.
9. Check protein expression and purity on SDS-PAGE with Coomassie® brilliant blue staining. Compare the levels of GST-fusion and GST-0 proteins on the stained gel, such that equal amounts are used in the pull-down assay. In addition, these proteins can be run alongside bovine serum albumin (BSA) as a protein standard in order to determine the protein concentration of the GST-fusion protein. Titrate BSA in a range between 100 ng to 2 μg.

3.1.2. In Vitro GST-Fusion Protein Pull-Down Assay

This protocol calls for one protein to be immobilized on glutathione–agarose beads, for example GST-p53, and another protein, for example a candidate p53-binding protein, to be expressed in a soluble form. The soluble protein can be either in vitro translated (Transcription and translation [TNT]-coupled reticulocyte lysate system; Promega, Madison, WI, USA; cat. no. L4610) with ^{35}S methione (NEN® Life Science Products, Boston, MA, USA; cat no. NEG 709-A), purified by one of several established methods (6X histidine-tagged pro-

teins; Qiagen, Valencia, CA, USA; cat. no. 30210, or intein-fusion proteins; New England Biolabs, Beverly, MA, USA; cat. no. E6900S), or can be from cell nuclear extracts to identify novel p53-interacting proteins (*see* **Note 2**).

1. Combine GST-fusion protein (typically 1 μg) immobilized on glutathione–agarose beads with soluble protein (typically 5 μg or an excess of GST-fusion protein) in 30 μL final vol of GST-lysis buffer. As a control sample, add only the GST-fusion protein without adding the soluble protein.
2. Incubate 45 min at room temperature with mixing (*see* **Note 3**).
3. Following the incubation, wash 3× with GST-lysis buffer with light vortex mixing each time (*see* **Note 4**). Pellet agarose beads by centrifugation in microfuge at 5000*g* for 15 s.
4. Finally, remove supernatent and add 4X SDS loading buffer to a final of 1X concentration. Analyze protein interactions on SDS-PAGE and either stain with Coomassie brilliant blue or transfer to polyvinylidene difluoride (PVDF) membrane for Western blotting.

3.2. Immunoprecipitation

When dealing with an uncharacterized p53 binding protein, it is important to test several antibodies for immunoprecipitation (IP) because: (*i*) the antibody may interfere with the protein–protein interaction; and (*ii*) the p53 posttranslational modifications, such as acetylation and phophorylation, may negatively affect the antibody's ability to bind. *See* **Note 5** for information on p53 antibodies. For example, the PAb 421 epitope maps to amino acids 371–380, which is a region that is modified by acetylation, phosphorylation, and ubiquination in cells after various stress-inducing agents. Though this antibody works well for IP, it may only recognize a subset of p53 molecules in the cell that are unmodified in this region. PAb 246 recognizes p53 in a wild-type conformation. This antibody also works well for IP, though it may be possible that some p53-interacting proteins may affect p53 conformation, such that this antibody no longer recognizes it. The epitope for PAb 1801 is in the N terminal of p53, amino acids 46–55. Similar to PAb 421, this region is also posttranslationally modified after p53-activating agents. Refer to **Fig. 1** for the stepwise procedure.

3.2.1. Prepare Cell Lysate

1. Aspirate the medium from the cultured cell plate and wash the cells 2× with cold 1X PBS. Finally, add cold 1X PBS to each plate.
2. Scrape the cells from the plate using a plastic policeman. Transfer the suspensions to 15-mL tubes by pipet, followed by rewashing the plate with cold 1X PBS to both clean the policeman and to collect leftover cells in the plates. Collection tubes should be stuck deeply into the ice during the procedure.

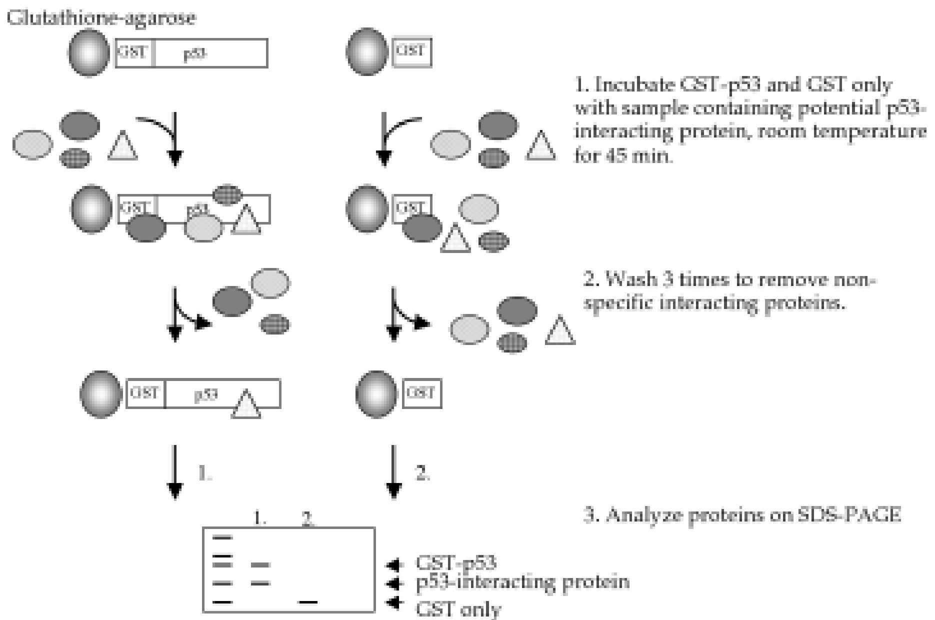

Fig. 1. Schematic diagram showing GST-pull-down assay. IP is performed in a similar manner if GST-beads are replaced with immunoaffinity beads. *See* text for the details on both assays.

3. Centrifuge the cells in a swinging bucket rotor for 3–5 min at 3000*g* and 4°C to pellet the cells. Aspirate the PBS, and then the cells are ready for IP or can be stored at –80°C for at least 1 mo.
4. Add 1.0 mL of cold lysis buffer per 10^7 cells. Resuspend the sample by vortex mixing and leave on ice for 20–30 min with occasional mixing. Transfer the lysate to a 1.5-mL tube and spin in a 4°C microfuge for 5–10 min at maximal speed. Transfer supernatant to a fresh tube and keep on ice.

3.2.2. Preclearing the Lysate

1. Add 30 μL of protein G Sepharose (50%) per 1.0 mL of lysate.
2. Rotate at 4°C for 30–60 min. Spin in a 4°C microfuge for 15 s at 5000*g*.
3. Carefully add supernatant to a new 1.5-mL tube or store at –80°C.

3.2.3. IP Reaction

1. Measure the protein concentration of the precleared cell lysate (*see* **Note 6**).
2. In a 1.5-mL tube, combine at least 300 μg cell lysate with 1 μg antibody of choice and 20 μL protein G Sepharose (50% slurry).
3. Add lysis buffer to bring vol to 500 μL

4. Rotate at 4°C for at least 2 h or overnight.
5. Centrifuge samples in room temperature microfuge for 15 s at 5000*g*.
6. Aspirate supernatent and wash beads with the following buffers (*see* **Note 4**) with centrifugation between each time: (*i*) 1 mL lysis buffer; (*ii*) 1 mL SNNTE buffer; (*iii*) 1 mL SNNTE buffer; and (*iv*) 1 mL lysis buffer.
7. Finally, aspirate supernatent and quickly spin the sample. Use a pipet to carefully remove excess supernatent, leaving approx 20 µL total vol in the tube. Add 4X SDS sample buffer to a final of 1X concentration. The samples are now ready to be boiled and loaded onto SDS-PAGE, followed by Western blotting analysis (*see* **Note 7**).

4. Notes

1. Bacterial cell lysis can be performed several ways, including French Press, sonication, and freeze-thaw, as described in *Current Protocols in Molecular Biology* ***(55)***. We use a Spectronic Unicon French Pressure System with a 40-K manual-fill cell (FA-031) at an internal pressure of 20,000 psi.
2. To identify novel p53-interacting proteins, one can use either whole cell lysates (*see* **Subheading 3.2.1.**) or nuclear extracts. Nuclear extract preparations have been described elsewhere ***(56,57)***. The lysate or extract should be incubated as described in **Subheading 3.1.2.** with beads containing either GST only or GST-p53 fusion protein. Additionally, an unrelated GST-fusion protein can also be used as a control. After running the samples on SDS-PAGE and after Coomassie brilliant blue or silver staining, proteins found only in the GST-p53 lane can be cut out of the gel and sent out for peptide sequencing by mass spectrometry, for example.
3. We perform mixing of GST-fusion proteins immobilized on glutathione–agarose in Eppendorf® tubes using a vortex mixer with a 3-in. rubber platform attachment. We place the Eppendorfs in a Fisherbrand Flat Rack held on by a rubber band while vortex mixing at low speed (Fisher Scientific; cat. no. 05-544-4) or alternatively use an attachable platform head that holds Eppendorf tubes (Fisher Scientific; cat. no. 12-812B).
4. Wash conditions will vary depending on the proteins tested, and so various wash buffers should be tested in order to maximize binding while minimizing background. Many protein–protein interactions are salt-dependent, while other interactions are hydrophobic in nature. Thus, using high ionic-strength washes may be good in some cases, while low ionic-strength washes will be good in others. For GST pull-down assays, the wash buffers we commonly use are: (*i*) GST-lysis buffer; (*ii*) GST-lysis plus 500 m*M* NaCl, to remove background proteins whose interactions are salt-sensitive; (*iii*) 1:3 diluted GST-lysis buffer in water, to remove background proteins whose interactions are hydrophobic; and (*iv*) RIPA buffer, which is a stringent wash good for removing background when using in vitro translated proteins with ^{35}S-methione label. For IPs, we use: (*i*) Lysis buffer, (*ii*) SNNTE (high salt); (*iii*) 1:3 diluted lysis buffer in water (low salt); and (*iv*) RIPA.

5. There are many antibodies to choose from when performing an IP of p53. Four are listed in **Subheading 2.**, including PAb 421, PAb 246, PAb 1801, and anti-p53 polyclonal. We typically use the first three for IPs, though there are others as listed in the Calbiochem-Novabiochem catalog.
6. To determine protein concentrations, we use the 5X protein assay dye from Bio-Rad (Hercules, CA, USA; cat. no. 500-0006) by diluting it in water to 1X concentration. We add 1 mL of 1X protein dye reagent to a 5-mL tube (Sarstedt, Newton, NC, USA; cat. no. 55.476), add 2 μL of cell lysate, vortex mix briefly, leave it at room temperature 5 min, transfer each solution into a 1-mL plastic cuvette, and then measure the color change by spectrophotometer set to wavelength 595 nm. A standard curve should be made using a purified protein of known concentration such as BSA. We have found that as an estimation of protein concentration, the following conversion works well:
   protein concentration = (20 μL/? μL) × OD_{595} = [protein] μg/μL where ? equals the amount of lysate added to the protein assay reagent. For example, if 2 μL of cell lysate were added to the reagent, and the OD_{595} was 0.2, then:
   protein concentration = (20 μL/2μL) × 0.2 = 2 μg/μL.
7. When the IP reactions are run on SDS-PAGE and analyzed by Western blotting, often the immunoglobulin (IgG) heavy chain signal is very prominent. Depending upon the molecular weight of your protein of interest, the IgG signal may overlap. This is especially true for p53, which migrates just above the IgG heavy chain. To avoid this problem, it is beneficial to perform the IP with a monoclonal antibody, e.g., PAb421, followed by Western blot analysis with a polyclonal antibody, e.g., anti-p53 p.c. The opposite is true as well, e.g., using a polyclonal antibody for IP followed by a monoclonal antibody for Western blotting.

Acknowledgments

D. Keller is supported by the National Eye Institute (NEI) predoctoral fellowship. This work is supported partly by National Institutes of Health (NIH) R01 and ACS grants to H. Lu.

References

1. Ko, L. J. and Prives, C. (1996) p53: puzzle and paradigm. *Genes Dev.* **10,** 1054–1072.
2. Vogelstein, B., Lane, D., and Levine, A. J. (2000) Surfing the p53 network. *Nature* **408,** 307–310.
3. Chen, X., Farmer, G., Zhu, H., Prywes, R., and Prives, C. (1993) Cooperative DNA binding of p53 with TFIID (TBP): a possible mechanism for transcriptional activation. *Genes Dev.* **7,** 1837–1849.
4. Horikoshi, N., Usheva, A., Chen, J., Levine, A. J., Weinmann, R., and Shenk, T. (1995) Two domains of p53 interact with the TATA-binding protein, and the adenovirus 13S E1A protein disrupts the association, relieving p53- mediated transcriptional repression. *Mol. Cell. Biol.* **15,** 227–234.

5. Lu, H. and Levine, A. J. (1995) Human TAFII31 protein is a transcriptional coactivator of the p53 protein. *Proc. Natl. Acad. Sci. USA* **92,** 5154–5158.
6. Thut, C. J., Chen, J. L., Klemm, R., and Tjian, R. (1995) p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60. *Science* **267,** 100–104.
7. Gu, W., Shi, X. L., and Roeder, R. G. (1997) Synergistic activation of transcription by CBP and p53. *Nature* **387,** 819–823.
8. Lill, N. L., Grossman, S. R., Ginsberg, D., DeCaprio, J., and Livingston, D. M. (1997) Binding and modulation of p53 by p300/CBP coactivators. *Nature* **387,** 823–827.
9. Jayaraman, L., Murthy, K. G., Zhu, C., Curran, T., Xanthoudakis, S., and Prives, C. (1997) Identification of redox/repair protein Ref-1 as a potent activator of p53. *Genes Dev.* **11,** 558–570.
10. D'Orazi, G., Cecchinelli, B., Bruno, T., et al. (2002) Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. *Nat. Cell Biol.* **4,** 11–19.
11. Hofmann, T. G., Moller, A., Sirma, H., et al. (2002) Regulation of p53 activity by its interaction with homeodomain- interacting protein kinase-2. *Nat. Cell Biol.* **4,** 1–10.
12. Chen, J., Marechal, V., and Levine, A. J. (1993) Mapping of the p53 and mdm-2 interaction domains. *Mol. Cell. Biol.* **21,** 4107–4114.
13. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) Mdm2 promotes the rapid degradation of p53. *Nature* **387,** 296–299.
14. Kubbutat, M. H., Jones, S. N., and Vousden, K. H. (1997) Regulation of p53 stability by Mdm2. *Nature* **387,** 299–303.
15. de Oca Luna, R. M., Tabor, A. D., Eberspaecher, H., et al. (1996) The organization and expression of the mdm2 gene. *Genomics* **33,** 352–357.
16. Bell, D. W., Varley, J. M., Szydlo, T. E., et al. (1999) Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. *Science* **286,** 2528–2531.
17. Canman, C. E., Lim, D. S., Cimprich, K. A., et al. (1998) Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science* **281,** 1677–1679.
18. Chehab, N. H., Malikzay, A., Stavridi, E. S., and Halazonetis, T. D. (1999) Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage. *Proc. Natl. Acad. Sci. USA* **96,** 13,777–13,782.
19. Hirao, A., Kong, Y. Y., Matsuoka, S., et al. (2000) DNA damage-induced activation of p53 by the checkpoint kinase Chk2. *Science* **287,** 1824–1827.
20. Shieh, S. Y., Ikeda, M., Taya, Y., and Prives, C. (1997) DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell* **91** 325–334.
21. Unger, T., Juven-Gershon, T., Moallem, E., et al. (1999) Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2. *EMBO J.* **18,** 1805–1814.
22. Leveillard, T., Andera, L., Bissonnette, N., et al. (1996) Functional interactions between p53 and the TFIIH complex are affected by tumour-associated mutations. *EMBO J.* **15,** 1615–1624.
23. Wang, X. W., Yeh, H., Schaeffer, L., et al. (1995) p53 modulation of TFIIH-associated nucleotide excision repair activity. *Nat. Genet.* **10,** 188–195.

24. Xiao, H., Pearson, A., Coulombe, B., et al. (1994) Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53. *Mol. Cell. Biol.* **14,** 7013–7024.
25. Filhol, O., Baudier, J., Delphin, C., Loue-Mackenbach, P., Chambaz, E. M., and Cochet, C. (1992) Casein kinase II and the tumor suppressor protein P53 associate in a molecular complex that is negatively regulated upon P53 phosphorylation. *J. Biol. Chem.* **267,** 20,577–20,583.
26. Fuchs, S. Y., Adler, V., Buschmann, T., et al. (1998) JNK targets p53 ubiquitination and degradation in nonstressed cells. *Genes Dev.* **12,** 2658–2663.
27. An, W. G., Kanekal, M., Simon, M. C., Maltepe, E., Blagosklonny, M. V., and Neckers, L. M. (1998) Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. *Nature* **392,** 405–408.
28. Waterman, M. J., Stavridi, E. S., Waterman, J. L., and Halazonetis, T. D. (1998) ATM-dependent activation of p53 involves dephosphorylation and association with 14-3-3 proteins. *Nat. Genet.* **19,** 175–178.
29. Iwabuchi, K., Bartel, P. L., Li, B., Marraccino, R., and Fields, S. (1994) Two cellular proteins that bind to wild-type but not mutant p53. *Proc. Natl. Acad. Sci. USA* **91,** 6098–6102.
30. Jayaraman, L., Moorthy, N. C., Murthy, K. G., Manley, J. L., Bustin, M., and Prives, C. (1998) High mobility group protein-1 (HMG-1) is a unique activator of p53. *Genes Dev.* **12,** 462–472.
31. Sarnow, P., Ho, Y. S., Williams, J., and Levine, A. J. (1982) Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. *Cell* **28,** 387–394.
32. Zantema, A., Schrier, P. I., Davis-Olivier, A., van Laar, T., Vaessen, R. T., and van der, E. A. (1985) Adenovirus serotype determines association and localization of the large E1B tumor antigen with cellular tumor antigen p53 in transformed cells. *Mol. Cell. Biol.* **5,** 3084–3091.
33. Szekely, L., Selivanova, G., Magnusson, K. P., Klein, G., and Wiman, K. G. (1993). EBNA-5, an Epstein-Barr virus-encoded nuclear antigen, binds to the retinoblastoma and p53 proteins. *Proc. Natl. Acad. Sci. USA* **90,** 5455–5459.
34. Feitelson, M. A., Zhu, M., Duan, L. X., and London, W. T. (1993) Hepatitis B x antigen and p53 are associated in vitro and in liver tissues from patients with primary hepatocellular carcinoma. *Oncogene* **8,** 1109–1117.
35. Wang, X. W., Forrester, K., Yeh, H., Feitelson, M. A., Gu, J. R., and Harris, C. C. (1994) Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3. *Proc. Natl. Acad. Sci. USA* **91,** 2230–2234.
36. Crook, T., Tidy, J. A., and Vousden, K. H. (1991) Degradation of p53 can be targeted by HPV E6 sequences distinct from those required for p53 binding and trans-activation. *Cell* **67,** 547–556.
37. Werness, B. A., Levine, A. J., and Howley, P. M. (1990) Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* **248,** 76–79.
38. Lane, D. P. and Crawford, L. V. (1979) T antigen is bound to a host protein in SV40-transformed cells. *Nature* **278,** 261–263.

39. Linzer, D. I. and Levine, A. J. (1979) Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell* **17,** 43–52.
40. Luo, J., Su, F., Chen, D., Shiloh, A., and Gu, W. (2000) Deacetylation of p53 modulates its effect on cell growth and apoptosis. *Nature* **408,** 377–381.
41. Murphy, M., Ahn, J., Walker, K. K., et al. (1999) Transcriptional repression by wild-type p53 utilizes histone deacetylases, mediated by interaction with mSin3a. *Genes Dev.* **13,** 2490–2501.
42. Liu, Y., Colosimo, A., Yang, X., and Liao, D. (2000) Adenovirus E1B 55-kilodalton oncoprotein inhibits p53 acetylation by PCAF. *Mol. Cell. Biol.* **20,** 5540–5553.
43. Zilfou, J. T., Hoffman, W. H., Sank, M., George, D. L., and Murphy, M. (2001). The corepressor mSin3a interacts with the proline-rich domain of p53 and protects p53 from proteasome-mediated degradation. *Mol. Cell. Biol.* **21,** 3974–3985.
44. Luo, J., Nikolaev, A., Imai, S., et al. (2001) Negative control of p53 by Sir2 promotes cell survival under stress. *Cell* **107,** 137–148.
45. Vaziri, H., Dessain, S., Ng Eaton, E., et al. (2001) hSIR2SIRT1 functions as an NAD-dependent p53 deacetylase. *Cell* **107,** 149–159.
46. Huibregtse, J. M., Scheffner, M., and Howley, P. M. (1993) Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. *Mol. Cell. Biol.* **13,** 775–784.
47. Li, M., Chen, D., Shiloh, A., et al. (2002) Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. *Nature* **31,** 31.
48. Oliner, J. D., Pietenpol, J. A., Thiagalingam, S., Gyuris, J., Kinzler, K. W., and Vogelstein, B. (1993). Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature* **362,** 857–860.
49. Dutta, A., Ruppert, J. M., Aster, J. C., and Winchester, E. (1993) Inhibition of DNA replication factor RPA by p53. *Nature* **365,** 79–82.
50. He, Z., Brinton, B. T., Greenblatt, J., Hassell, J. A., and Ingles, C. J. (1993) The transactivator proteins VP16 and GAL4 bind replication factor A. *Cell* **73,** 1223–1232.
51. Li, R. and Botchan, M. R. (1993) The acidic transcriptional activation domains of VP16 and p53 bind the cellular replication protein A and stimulate in vitro BPV-1 DNA replication. *Cell* **73,** 1207–1221.
52. Gaiddon, C., Lokshin, M., Ahn, J., Zhang, T., and Prives, C. (2001) A subset of tumor-derived mutant forms of p53 down-regulate p63 and p73 through a direct interaction with the p53 core domain. *Mol. Cell. Biol.* **21,** 1874–1887.
53. Shvarts, A., Steegenga, W. T., Riteco, N., et al. (1996) MDMX: a novel p53-binding protein with some functional properties of MDM2. *EMBO J.* **15,** 5349–5357.
54. GST gene fusion system manual, Pharmacia Biotech, Inc., 3rd ed., revision 3, 18-1123-20.
55. Ausubel, F. M., Brent, R., Kingston, R. E., et al. (eds.) (1998) *Current Protocols in Molecular Biology, Vol. 3, Unit 16*, John Wiley & Sons, New York.

56. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11,** 1475–1489.
57. Ausubel, et al. (eds.) (1998) *Current Protocols in Molecular Biology, Vol. 2, Unit 12*. John Wiley & Sons, New York.