# Behind the scenes: Unravelling the molecular mechanisms of p53 target gene selectivity (Review)

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**Abstract.** The p53 protein is a tumor suppressor that plays a crucial role in cellular growth regulation inducing a plethora of response pathways. p53 post-translational modifications, p53-binding proteins, co-factors and the p53-family members p63 and p73 have all been described to contribute to p53 target gene regulation and hence cellular outcome. However, the molecular mechanisms that discriminate between the different p53-responses towards stress treatments have remained largely elusive. This review focuses on the topic of the molecular mechanisms behind target gene selectivity of the transcription factor p53 and provides insight into the latest era of p53 research.

**Contents**



## 1. Introduction

The p53 protein was first discovered 30 years ago and was found to associate with simian virus 40 Large T protein (SV40LT) while accumulating in the nuclei of cancer cells (1-3). The gene encoding p53 (TP53) was cloned from neoplastic rodent and human cells. Several groups showed at the same time that this form of p53 had oncogenic activity when introduced together with the ras gene in transformation assays (4-6). However, in the late 1980s it turned out that the DNA plasmids used for these original experiments were mutant p53 clones; the wild-type p53 did not transform cells, but rather inhibited oncogene mediated transformation (7-9). Soon, it was discovered that p53 was frequently mutated in a variety of human cancers (10,11) and germ-line p53 mutations were found in patients with Li-Fraumeni syndrome, a rare autosomal dominant hereditary disorder which leads to the occurrence of several types of cancer (12,13). Mice deficient for p53 were shown to be susceptible to spontaneous tumors (14).

One of the first identified functions of the tumor suppressor p53 was its ability to bind to DNA and to function as a transcription factor (15-20). About the same time it was discovered that p53 can control apoptosis (21) and cell cycle arrest (22). In the meanwhile it has become evident that p53 can also act independently from transcriptional regulation to induce apoptosis (23).

In unstressed cells, p53 is maintained at low levels due to a very short protein half-life and it becomes rapidly stabilized upon cellular and genomic stress. A key component in regulating p53 levels is the p53-interacting protein mouse double minute 2 (MDM2) (24-26). MDM2 (HDM2 in humans) is itself a p53 transcriptional target (27,28), and was shown to inhibit the p53 transcriptional activity as well as to decrease p53 protein levels, establishing a negative feedback loop (29). Further studies showed that MDM2 is an E3 ubiquitin ligase and induces ubiquitin mediated proteasomal degradation of p53 thereby regulating p53 stability (30,31). Whereas poly-ubiquitination is important for the p53 protein degradation, monoubiquitination of p53 by MDM2 results in nuclear export of p53 (32). Homozygous deletion of MDM2 is lethal in mice due to p53-mediated apoptosis during early embryogenesis, while deletion of both MDM2 and p53 gives rise to mice that develop normally, demonstrating the importance of MDM2 in regulating p53 activation (33,34). Thus, MDM2 controls the ubiquitination, nuclear export and degradation of p53. Another important regulator of p53 is MDMX (MDM4), a p53-binding protein, homologous to MDM2, but with little E3 ligase activity itself (35,36). MDMX can heterodimerize with MDM2 and this complex appears to be a better E3-ubiquitin ligase than MDM2 alone (37). MDMX knockout mice are also lethal, and similar to MDM2, this lethality is rescued by inactivation of p53 (38,39). Furthermore, MDM2 and MDMX play important and distinct roles in the regulation of the p53 transactivation activity (40). The activity of MDM2

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towards p53 can be regulated by several proteins that bind to MDM2. The p14Arf protein as well as the ribosomal proteins L5, L11, L23, S7, the nucleolar proteins nucleophosmin (NPM) and nucleostemin (NS) impinge on the MDM2-mediated ubiquitination of p53 (41-47). Also other E3-ubiquitin ligases have been reported to ubiquitinate p53, such as Pirh2, COP1 and ARF-BP1 (48-50), but their exact contribution to p53 degradation is unknown. The p53-stability is also regulated via de-ubiquitination, e.g. by the Herpesvirus-associated ubiquitin-specific protease (HAUSP) (51).

One of the first identified p53 downstream target genes is p21 (52). p53 is directly involved in the regulation of cell-cycle and apoptosis through target genes such as p21 and Bax (52,53). Since the first discovery of direct p53 target genes, many downstream p53 target genes have been identified which expanded its biological role to involvement in DNA repair, senescence, aging, differentiation, cell adhesion, cell mobility, membrane functions, metabolism and autophagy (54). The most recently identified function is its involvement in stem-cell biology. Disruption of p53 enhanced the production of so-called induced pluripotent stem (iPS) cells (55-59).

Although p53 has been proven to be involved in many biological pathways, it is still not known how p53 selectively activates or represses a certain set of target genes to evoke a specific cellular response. The more we learn about p53 and its functions, the more complex the p53 response seems to be. This review will focus on the molecular mechanisms behind target gene selectivity of the transcription factor p53.

## 2. Regulation of activity

*p53 regulates cell cycle arrest and apoptosis*. When p53 is activated upon DNA damage it triggers a response to stop the cell cycle to allow the cells to take care of the DNA damage or, if DNA damage is too severe to be repaired, to target the cells for apoptosis to eliminate defective cells, which is crucial to prevent cancer. The molecular mechanisms to selectively evoke a specific cellular response, ranging from cell cycle arrest to apoptosis, remain widely elusive. Upon DNA damage transient alterations in cell cycle permit repair of DNA damage before the cell reinitiates replicative DNA synthesis (G1-arrest) or mitosis (G2-arrest). The G1-arrest is primarily regulated by p21, one of the most intensively studied direct p53 target genes since its discovery in 1993 (52,60). p21 in turn inactivates cyclin E/Cdk2 or cyclinD/Cdk4 resulting in hypophosphorylation of pRB and cell cycle arrest (60,61). p53 also contributes to a G2 arrest by inducing transcription of GADD45, p21 and 14-3-3σ and by repressing cyclin B (61). Besides its involvement in cell cycle arrest, p53 can also induce DNA repair through multiple mechanisms. p53 can directly activate target genes which are involved in DNA repair such as p53R2 (62,63), it can interact with proteins that function in DNA repair pathways such as replication protein A (RPA) (64) or directly with DNA (61).

When DNA damage is too severe to be repaired, p53 can induce apoptosis, the cell death program that is mediated by proteases called caspases. There are two distinct apoptotic signaling pathways; one responding to signals within the cells (the intrinsic pathway) and one responding to outside stress signals (the extrinsic pathway). p53 contributes to both pathways. It can activate the extrinsic pathway through induction of death ligands (65), such as FAS (66) and DR5 (67). The intrinsic pathway induces release of cytochrome C from mitochondria and is largely controlled by Bcl-2 proteins (68), in which p53 can activate pro-apoptotic Bcl-2 proteins such as Bax (69), PUMA (70,71), Noxa (72) and Apaf-1 (73) and it represses anti-apoptotic Bcl-2 proteins such as Bcl-2 itself (74,75) and Bcl-X (76). Both pathways result in the induction of caspase signaling, which induces apoptosis. Crosstalk between the two pathways also occurs. For example, caspase-8 gets activated in the extrinsic pathway and can proteolytically activate Bid, which in turn facilitates cytochrome C release (77). Besides the two p53 transcriptionally-dependent apoptotic pathways, there is an alternative pathway in which p53 acts in a transcriptionally independent manner. In this pathway, p53 translocates to the mitochondria, where it directly acts with anti- and pro-apoptotic multidomain members of the Bcl-2 family to induce mitochondrial outer membrane permeabilization resulting in cell death (23,78).

*p53 can function as a transcription factor*. The protein p53 functions mainly as a transcription factor. p53 interacts with DNA via its DNA binding domain. This domain has been defined by numerous biochemical studies and its interaction with DNA has been visualized by X-ray crystallography, which supported the hypothesis that DNA binding is critical for the biological activity of p53 (79-81). More than 90% of p53 mutations found in human tumors reside in the DNA binding domain. This implies the importance of the transcription factor function of p53 in growth control (82). Not only the DNA binding domain, but also the C-terminal part of p53 has been implicated in DNA binding (83). Besides the DNA binding domain, p53 consists of several other functional domains; transactivation domains, a proline-rich domain, a nuclear localization signaling domain and a tetramerization domain. The two tandem transactivation domains are located at the N-terminal part of p53, which are responsible for activation or repression of target genes. Thus, p53 acts as a transcription factor mostly by sequence-specific binding to the DNA and thereby activating or repressing target genes to control cellular outcome.

*Stabilization and activation of p53 by post-translational modifications*. Numerous post-translational modifications (PTMs) of p53, such as phosphorylation, acetylation, methylation, ribosylation, neddylation, sumoylation and ubiquitination are involved in the stabilization and activation of p53 upon cellular stress (84-86).

One of the best studied post-translational modifications of p53 is its phosphorylation. Phosphorylation of p53 is involved in its stability as well as activity regulation. The phosphorylation sites are mostly clustered within the N-terminal and C-terminal part of p53. p53 can be phosphorylated by several kinases, such as ATM, ATR, p38, Chk1 and Chk2. Phosphorylation close to the N-terminus, the residues to which MDM2 binds, can interfere with the ability of MDM2 to bind to p53, such as phosphorylation of serine 15 (87). Phosphorylation of serine 15 by ATM and ATR can enhance the transcriptional activity of p53 (88). Phosphorylation of the C-terminus has been shown to be important for the activation

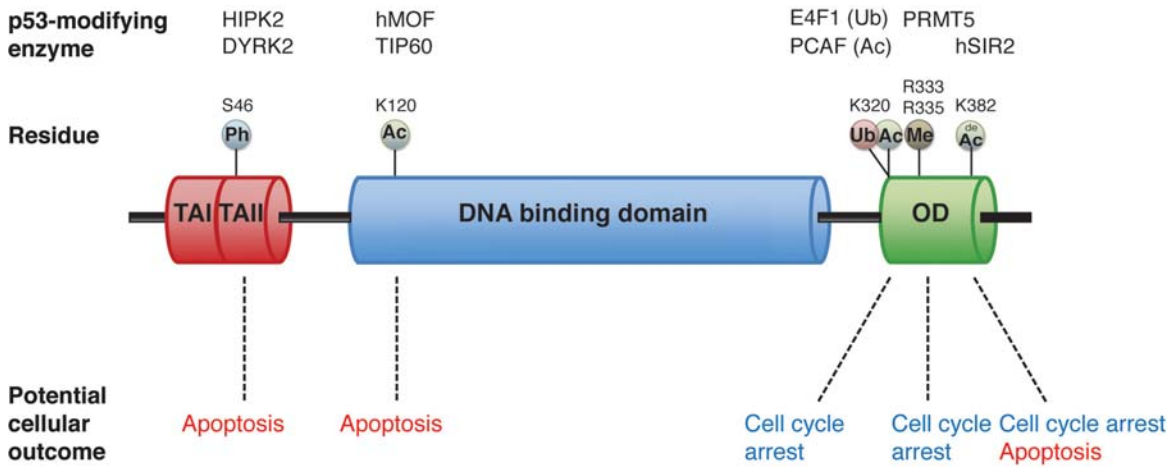


Figure 1. Post-translational modifications which play a role in target gene selection. The transcriptional activation domain I and II (TAI and TAII), DNA-binding domain (DBD) and oligomerization domain (OD) of p53 are indicated. Modified residues which are either phosphorylated (Ph), acetylated (Ac), ubiquitinated (Ub), methylated (Me) or deacetylated (deAc) and play a role in apoptosis or cell cycle arrest are depicted.

of the p53 protein (89). In principle, most phosphorylation events occur upon cellular stress and the patterns and kinetics of the phosphorylation marks can reflect the actual inducing stress signal. UV irradiation, for instance, induces specifically the phosphorylation of serine 392, whereas threonine 18 is mostly phosphorylated upon ionizing irradiation (90-92).

Also, the acetylation levels of p53 are significantly enhanced in response to stress and correlate well with p53 activation and stabilization (85). p53 is acetylated at several lysines by different histone acetyltransferases (HATs), such as p300 and Tip60 (93-95). Acetylation of the C-terminus activates p53's DNA binding. Knock-in studies in which the 6 C-terminal lysine residues were mutated to arginines (K6R), eliminating acetylation of these residues, reveal normal p53 protein stability before and after DNA damage, showing that these lysines are not solely responsible for efficient p53 degradation (96). However, different cells derived from these knock-in mice had an impaired p53-dependent target gene induction upon DNA-damage. Thus the post-translational modifications of these C-terminal residues of p53 are necessary for the proper p53 transactivation function as also seen in *in vitro* studies (97). Conversely, the activity of p53 can be regulated by deacetylation as well. Deacetylation complexes can contain HDAC1 (98), SIRT1 (99) or SIRT2α (100) and can repress p53 transcriptional activation.

Lysine residues of p53 can also be methylated at least at three different sites by Smyd2 and Set8 and 9 (101-103). Furthermore, it was described for the first time that three arginine residues of p53 can be methylated as well (104). The lysine-specific demethylase LSD1 mediates p53 demethylation. This demethylation prevents p53 interaction with its co-activator 53BP1, thereby inhibiting p53 to induce apoptosis (105).

The mechanism to both stabilize and activate p53 is the competition for post-translational modifications on the same residue on p53. The C-terminal lysine residues, which are important for ubiquitination for example, can also be acetylated. Acetylation of these residues blocks p53 degradation and stabilizes p53 (106,107). Crosstalk between the different modifications also exists. Lysine methylation at K372 of p53 for example is important for subsequent acetylation of the C-terminus, which results in stabilization of the protein (108). Furthermore, phosphorylation of p53 at serine 46 induces the acetylation of p53 at lysine 382 and happens upon severe DNA damage that leads to apoptosis (109,110).

In conclusion, the stabilization and activation of p53 is regulated by numerous post-translational modifications. Although it has been shown that these are important for p53 regulation, the exact role of each modifications or the interplay between the modifications has not been shown yet.

## 3. p53 target gene selection

*Post-translational modifications of p53 are important for target gene selection*. Besides stabilization and activation, several post-translational modifications have been described to play a role in target gene selection (Fig. 1) (54,86,111). Phosphorylation of serine 46 (S46) by HIPK2 (109,110) or DYRK2 (112) is proposed to influence the induction of specific apoptotic target genes, such as p53 apoptosis inducing protein 1 (p53AIP1) in response to DNA damage (113-115). Phosphorylation of S46 is differentially regulated by a mechanism involving MDM2 depending on the extent of DNA damage (116). Upon mild DNA damage MDM2 ubiquitinates HIPK2, which is then unable to phosphorylate p53 at serine 46. Upon severe DNA damage, on the other hand, low levels of MDM2 do not ubiquitinate HIPK2 anymore and DYRK2 translocates to the nucleus. This results in phosphorylation of p53 at serine 46 which leads to the induction of specific apoptotic target genes triggering apoptosis (112,116,117). Furthermore, the phosphorylation of serine 46 by HIPK2 has also been reported to be necessary for a subsequent lysine 382 acetylation and both modifications have to be present for p53 transactivation of apoptotic target genes (109,110,118). The serine 46 phosphorylation site can be regulated by several other kinases besides DYRK2 and HIPK2; protein kinase C δ (PKCδ) (119), AMP-activated protein kinase catalytic subunit α (AMPKα) (120) or p38 mitogen-activated protein kinase (p38 MAPK) (121). The fact that several kinases can phosphorylate serine 46 suggests that this is a very important site for the regulation and function of p53 whereas the exact

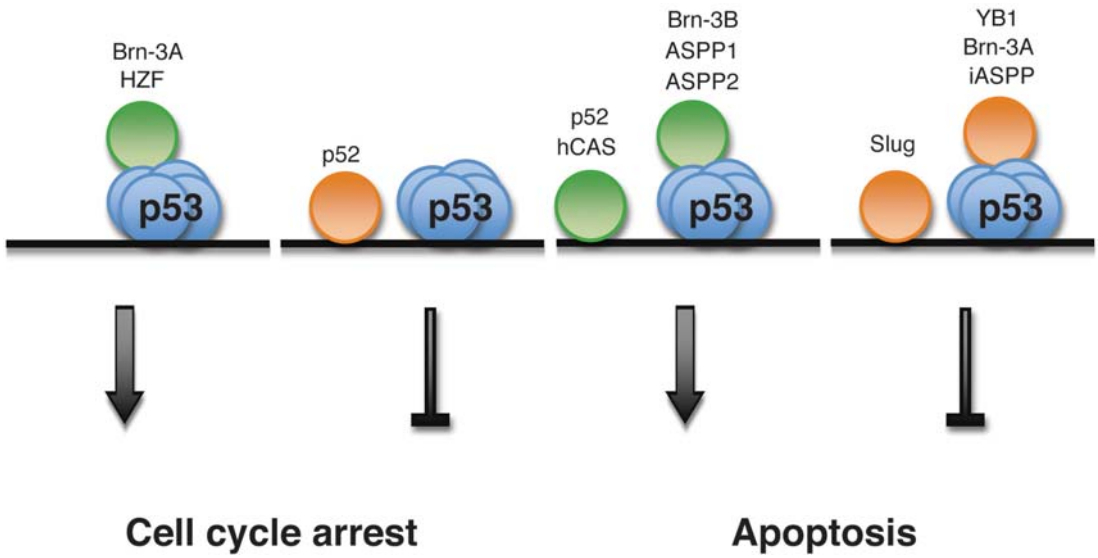


Figure 2. Proteins that influence cellular outcome. Proteins which either bind to p53 directly or act as a co-factor can either enhance (depicted in green) or block (depicted in orange) the p53 stress response resulting in cell cycle arrest or apoptosis.

regulation of this site with respect to the selectivity of the p53-transcriptional program remains to be further elucidated.

Acetylation of lysine 120 (K120) of p53 upon DNA damage by two MYST family histone acetyl transfereases (HATs) hMOF and Tip60 has also been shown to promote apoptosis (95,122). p53 that is acetylated at K120 was reported to accumulate preferentially on the promoters of pro-apoptotic target genes, such as Bax and PUMA, suggesting that this modification can modulate p53 target gene selection (95,122). In human cancers a p53 mutation can occur at K120 in which K120 is mutated to an arginine. This mutated form of p53 can no longer be post-translationally modified at this site. The mutation diminishes the p53-mediated apoptosis without effecting cell cycle arrest, p53 stability or DNA binding (95,122).

Lysine 320 (K320) on the other hand, can be modified independently by both ubiquitination and acetylation to influence promoter selectivity. The p300/CBP-associated factor (PCAF) acetylates p53 at K320 (94, 123). Acetylation of p53 K320 was shown to favor DNA binding to the p21 target gene, promoting cell survival and cell cycle arrest after DNA damage (124). The E3 ligase E4F1 ubiquitinates p53 also at K320 and this increases the activation of cell cycle arrest genes, such as p21 and cyclin G1 as well (125). *In vivo* knock-in mice with a lysine to arginine mutation (p53K317R) corresponding to K320 in human, abolished DNA damage-induced acetylation at position 317, resulting in increased p53-dependent apoptosis. Several apoptotic target genes such as PUMA and Noxa were upregulated in thymocytes from $p53^{K317R}$ mice compared to wild-type mice demonstrating that acetylation of this residue negatively regulates p53 apoptotic activity after DNA damage (126).

Acetylation of p53 by p300 generally induces its pro-apoptotic activity (124). As mentioned before, acetylation appears to depend to some extent on phosphorylation: S46 phosphorylation induces the acetylation of p53 at lysine 382 (110). Furthermore, RNAi mediated knockdown of Chk1 and Chk2, which both induce C-terminal phosphorylation of p53, reduced acetylation greatly (127). Interestingly, acetylation of p53 at carboxy-terminal lysine residues, lysine 320, 373 and 382, have also been reported to be required for its transcription-independent apoptotic function (128). Not only acetylation but also deacetylation of p53 is involved in p53 target gene selection. hSIR2/SIRT1 deacetylates p53 at lysine 382 which can decrease the p53 transcriptional activity at the p21 promoter, whereas in other cellular contexts it can repress p53 function in apoptosis (99,100).

Recently, it was reported that the protein arginine methyltransferase 5 (PRMT5) is responsible for the p53 methylation at arginine residues 333 and 335 (104). PRMT5 appears to be essential for cell proliferation and PRMT5 deficiency triggers G1 cell cycle arrest (129). PRMT5 is also described to be required for p53 expression itself and for the induction of the p53 targets MDM2 and p21 upon DNA damage (129).

While new p53 modifications continue to be uncovered, the impact of single modifications or a combination of all those modifications on p53 outcome and cellular functions remains to be elucidated.

*p53 co-factors and binding proteins involved in p53-mediated transcription*. For the p53-mediated transcription several protein complexes are required. Transcriptional co-factors and p53-binding proteins are essential for the regulation of the transcriptional activities of p53 (54,130). An important general activator of p53 is p300. p300 acts as a p53 co-activator for p53 target genes by acetylating p53 (93,131). The activity of p300 is tightly regulated by p300 co-factors, which enhance or repress the p53 response. Two co-recruited factors, junction-mediating and regulatory protein (JMY) and stress responsive activator of p300 (Strap) are also required for general p53 activation (132,133). A third p300 cofactor, S phase kinase-associated protein 2 (SKP2), on the other hand, has been reported to be involved in the selectivity of the p53 response. SKP2 prevents the interaction between p300 and p53, resulting in suppression of apoptosis (134). Furthermore, several

chromatin modifying proteins are essential for p53 transcriptional regulation. The chromatin-modifying and remodeling proteins BRG1 and hSNF5 enhance p53-dependent gene transcription (135,136). The STAGA complex, a multiprotein complex with the acetyltransferase GCN5L as the catalytic subunit, acts as a coactivator of p53, in which p53 interacts with SAGA subunits GCN5, ADA2b and TAF9 (137). The ATM-related TRAPP protein is recruited by p53 to activate gene transcription (138).

Besides the proteins that are general activators or repressors of p53 there are proteins that can particularly influence p53 target gene selectivity or cellular outcome (Fig. 2). Among the p53-binding proteins that influence the cellular outcome are the apoptosis-stimulating proteins of the ASPP family, which consist of the two pro-apoptotic members ASPP1 and ASPP2 and the anti-apoptotic member iASPP (139,140). ASPP1 and ASPP2 bind to the DNA-binding domain of p53 and specifically modulate p53-induced apoptosis by stimulating p53 binding to the Bax and PIG3 genes, whereas iASPP inhibits p53-mediated apoptosis but not cell cycle arrest, which implies that iASPP competes with ASPP1 and ASPP2 for binding with p53, but the exact mechanism is currently unknown. Downregulation of iASPP enhances the binding of p53 to the Bax promoter, but not to the p21 promoter (141). The hematopoietic zinc finger protein (HZF) is induced by p53 and binds to the DNA-binding domain of p53 (142). It facilitates p53-binding to cell cycle arrest target genes such as p21 and 14-3-3σ resulting in preferential cell cycle arrest.

The Brn3 family of POU domain transcription factors binds to the p53-binding domain (143). While Brn-3a cooperates with p53 to activate p21 and repress Bax expression, Brn-3b has opposing effects. A recently identified p53-binding protein Krüppel-associated box (KRAB)-type zinc-finger Apak is a negative regulator of p53-mediated apoptosis (144). Apak binds directly to p53 in unstressed cells which results in downregulation of pro-apoptotic genes, such as BAX and Puma, probably through recruitment of HDAC1-complexes to p53 and thereby reducing p53 acetylation. In response to stress Apak is phosphorylated and dissociates from p53, resulting in induction of apoptosis (144). The Y-box-binding protein YB1 interacts with p53 and inhibits the ability of p53 to induce apoptosis, but does not interfere with the p53 activation of p21 to induce cell cycle arrest (145). Binding of the co-factor human cellular apoptosis susceptibility protein (hCAS/CSE1L) to a subset of p53 target genes, such as PIG3, has been shown to positively influence the p53-mediated apoptosis (146). The co-repressor CtBP2 reduces p53-dependent transcription of Bax, but not of p21 (147,148). The p52 NF-κB subunit can either cooperate or antagonize with p53 to regulate target genes by regulating coactivator and corepressor recruitment (149). p52 represses p21 expression, but cooperates with p53 to increase PUMA, DR5 and GADD45.

Slug is a transcription factor and a p53 target gene, which plays a role in repressing p53-mediated apoptosis by antagonizing p53-mediated transactivation of PUMA (150). Similarly, the p53 target gene IRF2BP2 increases the threshold for the induction of apoptosis (151). IRF2BP2 inhibits p53 transactivation of p21 and Bax, but upon higher levels of p53, inhibition of Bax transactivation could be overcome. The newly discovered p53 target gene XEDAR on the other hand (152), is a p53 target gene that seems to lower the apoptotic threshold, but the molecular mechanism is not known yet. Besides its involvement in apoptosis-regulation XEDAR is also involved in the anoikis pathway (152).

Since post-translational modifications (PTMs), p53-binding proteins and co-factors are all involved in p53 target regulation, it is likely that crosstalk between PTMs and p53-binding protein or co-factors occurs. An example of this interplay is the p53 binding protein peptidyl-prolyl *cis/trans* isomerase (Pin1). Pin1 regulates conformational changes of certain phosphorylated proteins. Upon DNA damage Pin1 recognizes Serine 46 phosphorylation of p53 and mediates p53 dissociation from iASPP, thereby promoting apoptosis (153). Future research should elucidate how p53 PTMs influence the interaction with p53-binding proteins and co-factors and what result this has on p53 target gene selectivity. Possibly p53-binding proteins or co-factors interactions are regulated by PTMs of p53 and/or interacting proteins promote changes in p53 PTMs to achieve promoter specific transactivation.

Thus, there are various p53-binding proteins and co-factors, which are required for regulation of the transcriptional activities of p53 either as general activators or repressors of p53, or as proteins that are involved in target gene selectivity. Among those, some are also direct p53 target genes. Although they do not necessarily show a direct interaction with p53, they can form regulatory loops, thereby influencing p53-directed cell cycle arrest or apoptosis. Although it is known for many p53-binding proteins and co-factors that they influence p53-directed cellular outcome, most of the time the effect is only tested on selected p53 target genes and therefore the complete molecular mechanism how p53 directs cellular outcome is still elusive.

*Additional layers of p53 gene regulation*. In addition to p53-binding proteins and co-factors that influence the p53 target gene regulation, the p53-family members p63 and p73 can contribute to the p53 cellular outcome (154-156). p63 and p73 were reported to be required for the p53-binding to the p53 response elements of the target genes Perp, Bax and Noxa, but not to those of p21 or MDM2 (155). A 'priming model' was suggested, in which p63 and p73 can bind to a specific chromatin-embedded response element not accessible for p53, and subsequently modify the context of the response element in such a way that it becomes available for p53 binding (111). However, the fact that p63 and p73 are required for p53-dependent apoptosis might not be the case for all cell-types, since studies performed in T-cells showed that p63 and p73 were dispensable for p53-dependent apoptosis of T-cells (157). ChIP-on-chip data showed that the full-length proteins TAp63 and TAp73 can bind to a large amount of p53 binding sites and could thereby play an important role in target gene regulation (158). The ΔN-isoforms of p63 and p73, lacking the transactivation domain, compete with p53 for binding sites and behave as repressors of the p53 target gene response (159). Not only the p53 family members p63 and p73 consist of multiple isoforms, but p53 as well (160). These p53 isoforms can play a role in target gene regulation. The isoform p53ß, for example, binds to p53 binding sites and can enhance p53 target gene expression

(160). ChIP performed with a specific p53ß antibody showed that p53ß binds preferentially to the p21 and Bax promoter but not to the one of MDM2. Thus, also p53 isoforms and its family members p63 and p73 can play an important role in target gene regulation.

Another layer of regulation of target gene expression is likely mediated by p53-regulated miRNAs. The first miRNA family which was identified to be p53 responsive was miR-34 (161,162). miR-34a is a transcriptional target of p53 and induces apoptosis. It inhibits SIRT1, which regulates p53-dependent apoptosis through deacetylation and stabilization of p53 (163). Thus, a positive feedback loop exists in which p53 induces expression of miR-34a, which in turn suppresses SIRT1, resulting in increasing p53 activity (163). Other identified p53 responsive miRNAs are the miR192/194 and miR-194/215 clusters, (164-166). Whereas miR-34a is involved in inducing apoptosis, miR-192 induces cell cycle arrest in a p53-dependent fashion (166). Furthermore, a new function of p53 in regulating cell cycle arrest involving miRNAs has been recently identified. p53 interacts with proteins of the Drosha complex, which promotes processing of a subset of miRNAs with growth-suppressive function, including miR-16-1, miR-143 and miR-145 (167). Thus, by processing of these miRNA cells go into cell cycle arrest, which is a new function how p53 can inhibt cellular growth.

Altogether, many layers of p53 target gene regulation are required to regulate the p53 target response and we still have to learn much more about the cellular decision process.

*Current state-of-the-art techniques to elucidate p53 target gene regulation*. Gene expression is not only regulated by binding of a single transcription factor to a single binding site at a given time point, but by a combination of *cis*-acting regulatory sequences and *trans*-acting factors or post-transcriptional regulation as well (168). Uncovering locations of transcription factor binding sites and regulatory elements is critical for the importance of understanding the genome-wide dynamic view how these factors regulate gene expression. Since the publication of the human genome sequence (169,170), a great effort has been made to elucidate transcriptional circuits that control gene expression in the whole human genome. Chromatin immunoprecipitation (ChIP) is a robust method to identify protein-DNA interactions in chromatin. Essentially, DNA-binding proteins are crosslinked to DNA and cells are lysed and fragmented. Fragments bound by the protein of interest are immunoprecipitated and those enriched DNA fragments can either be checked whether they include a specific gene locus or they can be mapped in a genome-wide manner using different ChIP-based techniques. In 2000, the first study to detect genome-wide binding sites combining ChIP with microarrays (ChIP-on-chip) was reported for gene-specific transcription activators in yeast (171). Technological advances have enabled high throughput sequencing of the immunoprecipitated DNA fragments. Massive parallel sequencing of ChIP-DNA fragments (ChIP-seq) allows identification of binding sites with greater sensitivity and resolution than ever before (172). Massive parallel sequencing has multiple applications. For example, the transcriptional landscape can be studied on the basis of gene expression by direct sequencing of cDNA (RNA-seq). RNA-seq not only provides information about alternative splicing and different promoter usage, but also allows to identify transcripts that are expressed on low levels, something which was not possible with expression microarrays before.

Several studies have identified p53 binding sites using a variety of ChIP-based techniques (158,173-181). Cawley and co-workers were the first to identify p53 binding sites using tiling microarrays representing chromosome 21 and 22 (173). Based on p53 binding to chromosome 21 and 22 they estimated 1600 binding sites for p53 in the whole human genome. In 2006 Wei *et al* (176) identified 542 global high-confidence binding loci by combining ChIP with paired-end ditag (PET) sequencing. The genome-wide set of p53 binding sites was expanded by Smeenk *et al* using ChIP-on-chip identifying 1546 genome-wide high confidence binding sites (158). With the generated data using genome-wide binding techniques new bioinformatic analysis can be performed. In 1992, the p53 binding site has been characterized and described as two copies of the palindromic consensus half-site RRRCWWGYYY, in which R=purine, W=A or T and Y= pyrimidine, with a spacer of 0-13bp (15,182). In 2002, an algorithm (p53MH) was developed based on 37 known p53 binding sites (183). The identification of genome-wide binding sites allowed the development of a more accurate p53 motif finding algorithm of the identified p53 binding sites (p53scan) (158). This showed that the p53 motif is found in ~80% of all binding sites. Both genome-wide ChIP-PET and ChIP-on-ChIP data showed that in most of the p53 binding sites no spacer is present (158,176). Based on the genome-wide data novel aspects of p53 functions could be identified (158,176), and subsequently also clinical relevance was shown by validating target gene response to p53 activation in clinical breast tumors (176). Although a variety of cell lines and treatments were used, many binding sites overlap between the different studies, as up to 69% of the p53 binding sites in the PET5 cluster identified by Wei *et al* (176) were also bound by the set identified by Smeenk *et al* (158). Besides genome-wide binding sites for p53, binding sites for p53 family members p63 and p73 have also been identified (158,177,184). Not only the p53 binding sites overlap to a large extend in different cells and/or treatments, but also within its family members p63 and p73 (158). Whereas microarray data identified many new p53 regulated target genes (185,186), genome-wide p53 binding data combined with expression data can show direct involvement of p53 binding to a target gene and therefore helps to understand the genome-wide dynamic view how p53 regulates gene expression.

## 4. Future outlook

Gene expression regulation is dynamic and thereby very complex. Most likely different cellular outcomes cannot be explained on the basis of selective binding of p53 only. Therefore, it is likely that additional levels of regulation, such as post-translational modifications of p53, co-factors, p53 binding factors, p53 responsive microRNAs (miRNAs), p53 family members or chromatin remodeling factors, mediate target gene expression. Until now, most studies indicating involvement of for example a certain p53 PTM or particular cofactor in target gene expression, have been performed on

the basis of a selected set of target genes. The next step in unraveling the p53 target gene choice will be to combine genome-wide p53 binding data with its PTMs, co-factors, binding proteins, and histone modifications to verify this hypothesis. A combination of for example ChIP-seq with RNA-seq at multiple time-points after multiple treatments in different cells makes it possible to provide insight into the genome-wide dynamic view of gene regulation. Tiling micorarrays containing 30 Mb of the human genome have already started to link p53 binding sites to histone modifications in the human genome (174) and it is possible to do this in a genome-wide approach. Furthermore, by combining ChIP-seq not only with expression data from for example RNA-seq or chromatin modifications, but also with other techniques such as mass spectrometry, target genes affected by specific co-factor interactions could give more insight into p53 target gene selection (146). Thus, this new era of massive parallel sequencing has made it possible to analyze on a global scale how p53, in combination with *cis*-acting regulatory sequences and *trans*-acting factors, can regulate gene expression. Future p53 studies using massive parallel sequencing and profound data and network analysis will provide further insight into the molecular mechanism behind target gene selectivity of the transcription factor p53.

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