The multiple mechanisms that regulate p53 activity and cell fate

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Abstract | The tumour suppressor p53 has a central role in the response to cellular stress. Activated p53 transcriptionally regulates hundreds of genes that are involved in multiple biological processes, including in DNA damage repair, cell cycle arrest, apoptosis and senescence. In the context of DNA damage, p53 is thought to be a decision-making transcription factor that selectively activates genes as part of specific gene expression programmes to determine cellular outcomes. In this Review, we discuss the multiple molecular mechanisms of p53 regulation and how they modulate the induction of apoptosis or cell cycle arrest following DNA damage. Specifically, we discuss how the interaction of p53 with DNA and chromatin affects gene expression, and how p53 post-translational modifications, its temporal expression dynamics and its interactions with chromatin regulators and transcription factors influence cell fate. These multiple layers of regulation enable p53 to execute cellular responses that are appropriate for specific cellular states and environmental conditions.

p53 was initially identified in complex with the simian virus 40 T antigen in transformed rodent cells[1,2] and was first recognized as a tumour suppressor in 1989 (REFS[3,4]); subsequently, p53 has been found to be the most frequently mutated gene in cancer[5,6]. Because of its central role in the DNA damage response (DDR), p53 is often referred to as the 'guardian of the genome'. Although *TP53* is the most studied human gene of all time[7], there are still many open questions concerning the regulation of p53 activity by cellular stresses.

In this Review, we focus on discussing the established function of p53 as a transcription factor. p53 is activated by cellular stresses including DNA damage, hypoxia, oncogene activation and ribosomal stress. In response, p53 can promote cell cycle arrest, DNA damage repair, various pathways of cell death and metabolic changes[8–10]. Activation of p53 in response to stress largely occurs through protein stabilization, thereby enabling rapid (within a few hours) increase in total protein abundance and initiation of the p53 transcriptional response. In nonstressed conditions, p53 levels are kept low by the E3 ubiquitin ligase MDM2 (REFS[11–13]). Upon activation of the DDR, p53 is phosphorylated, rendering it insensitive to MDM2 (REF.[14]) and leading to its accumulation in cells. MDM2 itself is a transcriptional target of p53, thereby forming a regulatory feedback loop[15]. In addition to MDM2, p53 regulates the expression of genes of multiple pathways[16]. The best-studied pathways induced by p53 after DNA damage are cell cycle arrest followed by DNA repair or apoptosis. The choice of pathway activation is based on the extent and type of DNA damage and on cell type. How p53 integrates these and other cues to choose between the competing cell survival and cell death outcomes has been of great interest and is still largely unknown.

In this Review, we discuss the mechanisms regulating p53 that influence cell fate in response to DNA damage. These mechanisms include modulation of DNA binding by p53 through DNA sequence and chromatin structure, post-translational modifications (PTMs) of p53 and interactions with cofactors and temporal expression dynamics of p53. We focus on the choice between cell cycle arrest and apoptosis, which are the most commonly studied p53-dependent cellular outcomes in response to DNA damage.

p53 in the DNA damage response

Following DNA damage, p53 upregulates the expression of genes involved in both cell cycle arrest and DNA repair (leading to cell survival) and apoptosis (leading to cell death). The contexts, timing and extent of pathway induction have important implications for cell fate. The genes induced by p53 activation span multiple biological functions; the most enriched categories in genome-wide analyses (and the best known p53 targets) are the DDR (for example, DNA damage-binding protein 2 (*DDB2*) and *XPC*), cell cycle arrest (cyclin-dependent kinase inhibitor 1 (*CDKN1A*, which encodes p21) and *GADD45A*), apoptosis (*PUMA* (also known as BCL-2-binding component 3) and *BAX*), metabolism (TP53-induced glycolysis and apoptosis regulator (*TIGAR*) and aldehyde dehydrogenase family 1 member A3) and

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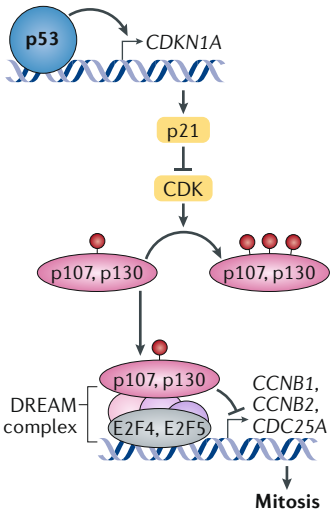
Box 1 | **p53-dependent transcription repression**

Recently published genomic data sets of gene expression and DNA binding showed that high-confidence p53 target genes are almost exclusively upregulated by p53 (REFS[28,36,40]). Similarly, genomic studies showed that p53 binding is not enriched at genes that are repressed following p53 activation[33,86,103–105]. Furthermore, high-throughput enhancer activity assays of p53-bound genomic sites did not find evidence of direct repression by p53 (REFS[40,41]). Nevertheless, multiple studies identified genes that are repressed in a p53-dependent manner, particularly genes involved in cell cycle regulation[36]. Their repression is thought to occur indirectly and to be largely mediated through the canonical p53 target gene *CDKN1A* (encoding p21)[36,203]. Recent genomic analyses attribute a key role for the complex DREAM (dimerization partner, RB-like, E2F, multivulval class B (MuvB)) in p53-mediated transcription repression[36,204] (see the figure). DREAM consists of multiple subunits, including the core MuvB complex proteins, the transcription factor E2F4 or E2F5 and RB-like protein 1 (also known as p107) or RB-like protein 2 (p130; reviewed elsewhere[205]). Inhibition of cyclin-dependent kinase (CDK) mediated by p21 results in hypophosphorylation of p107 and p130 (REF.[206]) and leads to their incorporation into the DREAM complex[207,208] and to repression of mitosis-promoting genes such as *CCNB1* (encoding cyclin B1) and *CCNB2* (encoding cyclin B2) and the phosphatase *CDC25A*[36,207] (other gene targets are compiled elsewhere[205]).



In normal cellular conditions, the interacting MuvB proteins are regulated in a cell-cycle-specific manner, and thus, the transcription-repressing DREAM complex is restricted to G_0 and early G_1 cells. At later cell cycle stages, transcription activators MYB-related protein B[209] and/or forkhead box protein M1 (REF.[210]) replace the E2F and p107 or p130 proteins[208]. In response to DNA damage or other stresses, p53 induction and the resulting p21 activation favour the formation of the suppressive DREAM complex. The transcriptional repressor E2F7, whose gene is another direct p53 target, is thought to function in conjunction with RB-associated protein (pRB) and DREAM to indirectly mediate p53-driven repression of cell-cycle-related genes[31,103,211–213]. Other effectors of p53-mediated transcription repression are noncoding RNAs[37,214,215]. The best known p53-regulated noncoding RNA is the microRNA miR-34a, which regulates cell cycle arrest and anti-apoptotic genes (reviewed elsewhere[31,216]) and long intergenic noncoding lincRNA-p21, which was implicated in p53-dependent transcription repression[215].

post-translational regulators of p53 (*MDM2* and p53-induced phosphatase 1). Genomic and transcriptomic studies have attempted to identify a comprehensive set of p53 target genes from various cell types and DNA damage settings. Transcriptional profiling found a wide range of genes affected by p53, ranging in number from less than 100 to more than 1,500, depending on the context of p53 activation and data processing approach[17–19]; however, these studies could not distinguish between direct and indirect targets of p53, thereby precluding unambiguous identification of direct p53 targets.

Chromatin immunoprecipitation followed by sequencing (ChIP–seq) studies attempted to identify genes that are directly regulated by p53, but many studies found poor correlation between p53 binding and transcriptional effects[20–22]. One major challenge for identifying direct target genes from ChIP–seq data (for p53 as well as for other transcription factors) is connecting binding to the regulation of specific genes. The majority of high-confidence p53 target genes contain a p53 binding site near the transcription start site (TSS)[23–27], either in the promoter region or within the first intron[28–31], making the association and validation of binding site–target gene pairs more straightforward. Generally, a cut-off of maximum distance from the TSS is used to link ChIP signals to specific genes. However, distal binding sites (>10 kb from any TSS) represent a large fraction of p53 binding sites[32,33] and are found not only in enhancers but also in Alu interspersed repeats[23] and other repeat sequences[34]. Although these distal p53 binding sites were generally not considered in p53 target gene analyses, there is evidence that they can regulate transcription. For example, distal binding sites correlated with gene induction[35,36], gave rise to enhancer RNAs (eRNAs)[37,38], which are associated with functional enhancers[39], and induced expression of reporter genes in high-throughput assays[40,41]. Many p53 binding sites, including distal sites as well as those identified as promoters, stimulated transcription in a screen for enhancers[41]. Further confounding genome-wide studies comparing ChIP signals before and after DNA damage induction was the identification of substantial DNA binding of basally expressed p53 (before DNA damage)[20,42–44]. Thus, it is clear that p53 may influence cell fate decisions by a variety of mechanisms, which are discussed below.

In contrast to earlier reports, which suggested that p53 can be both an activator and a repressor of transcription[30,45,46], an emerging theme from multiple large-scale studies is that p53 is a transcriptional activator and some of its targets serve as transcriptional inhibitors and thus lead to p53-dependent but indirect repression (BOX 1). In light of these recent meta-analyses, we focus on the role of p53 as a transcriptional activator.

The p53 domains regulate its function

The p53 protein consists of several well-characterized functional domains. At the amino terminus are two tandem transcription activation domains[47–49] (TADs). Both TADs were shown to be required for proper p53 target gene induction in response to DNA damage and p53 tumour suppressor function in mice[50], although

there were qualitative differences in how mutations in each TAD affected gene expression. In general, a TAD1 mutant showed greater effects on target-gene expression than did a TAD2 mutant; however, the TAD1 mutant maintained expression of some genes, for example, *Bax*, that were lost upon inactivation of both TADs, suggesting a role for the TADs in directing p53 target gene selection[50]. The TADs are followed by a proline-rich domain that contributes to transcription activation and is necessary for restricting cell growth[51]. The following region is the DNA-binding core domain[52], which has been crystallized in complex with DNA[53–55] and is the site of most cancer-associated mutations[52]. p53 binds cooperatively[56] to its target site as a tetramer (dimer of dimers)[57].

Although the core domain can tetramerize on its own when complexed with DNA and forms stable interactions[58], the adjacent oligomerization domain facilitates these interactions[59]. As expected on the basis of crystal structures, mutations in the tetramerization domain impaired p53 DNA binding and led to loss of its transcriptional activity[60–62]. In one study, p53 mutants that generated monomeric, dimeric or tetrameric species activated distinct gene sets, suggesting that oligomerization is critical for cell fate decisions[63]. This idea was supported by findings that p53 tetramerizes in cells following DNA damage[64] and activates gene expression[37] before substantial protein accumulation, suggesting that the initial p53-mediated response may be driven by tetramerization rather than by an increase in protein levels. Using a series of p53 mutants that differed in their extent of cooperative binding to target sites, induction of cell cycle arrest genes was found to be less dependent on cooperative p53 binding than was induction of apoptosis genes[65], and mice expressing cooperativity-deficient mutant p53 proteins developed spontaneous tumours and showed specific deficits in apoptosis[66]. These studies suggest that intermolecular p53 interactions may influence cell fate following DNA damage.

The role of the carboxy-terminal domain (CTD) of p53 has been especially challenging to decipher. It is highly unstructured[67] and post-translationally modified, in particular, serving as the primary site of acetylation[68]. Initial studies suggested the CTD has a negative regulatory role, because recombinant p53 bound weakly to its targets[69] and binding could be enhanced with antibodies against the CTD or by phosphorylation[69] or acetylation[68,70] of the p53 CTD. These studies suggested the existence of allosteric inhibition of p53 DNA binding by the CTD, which could be released by appropriate signals. However, lack of CTD conformational change upon DNA binding[71] and robust p53 DNA binding in vitro in the absence of competing DNA[72] subsequently called this model into question. Nevertheless, the CTD is required for DNA binding and transcription in vitro[73], and roles for the CTD in promoting p53 linear diffusion along DNA[74–76], binding to nonlinear DNA[77,78] and binding to nucleosomes[79] have been proposed (reviewed elsewhere[80] and discussed below). Deletion of the CTD reduced expression of both pro-apoptosis and pro-survival gene candidates[81], obscuring its role in orchestrating a coordinated set of genetic pathways. Expression of p53 lacking 24 amino acids[82] or 31 amino acids[83] of the CTD in mice resulted in reduced viability after birth and a general hyperactivation of p53, leading to deregulation of telomere maintenance[83] or altered expression of distinct subsets of target genes in different tissues[82]. In the latter case, alterations in gene expression resulted either from changes in p53 binding to target sites or from subsequent events[82]. The specific effects of CTD deletion in mice suggest that it may play a role in target-gene selection following DNA damage and not simply dampen p53 function globally.

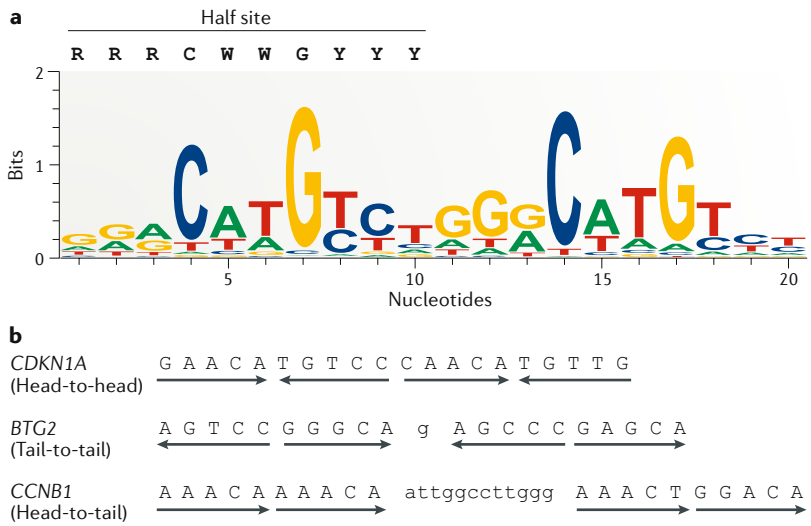


Fig. 1 | **The p53 binding site. a** | The canonical p53 motif in the head-to-head orientation. The IUPAC (International Union of Pure and Applied Chemistry) motif of one p53 half site is shown at the top and the 20 bp position weight matrix derived from p53 ChIP–seq data at the bottom[104]. R represents A or G, W represents A or T and Y represents C or T. **b** | Examples of genomic p53 binding sites. *CDKN1A* (encoding p21) and *BTG2* are activated by p53, whereas *CCNB1* (encoding cyclin B1) is repressed by p53. *CDKN1A*, representing the consensus sequence[84], contains two pairs of quarter sites in head-to-head orientation with no spacer. *BTG2* contains a slightly divergent sequence with two pairs of tail-to-tail quarter sites and a 1 bp spacer[88]. *CCNB1* contains a highly divergent site, with two pairs of head-to-tail quarter sites and a 12 bp spacer[109]; these features are not frequently observed at promoter-proximal p53 binding sites of induced target genes.

The DNA-binding motif of p53

The p53 binding motif was first discovered in the early 1990s[84,85] and consists of two 10 bp half-site sequences, each binding to one p53 dimer and separated by a spacer of variable length, between 0 and 20 nucleotides[25,84,86]. The half-site consensus, which is derived from in vitro selection experiments, is RRRCWWGYYY[84,85] (R represents A or G, W represents A or T and Y represents C or T) (FIG. 1a). Within each 10 bp half site are two 5 bp quarter sites; these are palindromic sequences that can adopt a canonical head-to-head orientation as well as head-to-tail or tail-to-tail arrangements (FIG. 1b). The fact that p53 is tetrameric and its binding site consists of four quarter sites raised the possibility that each monomer recognized one quarter site, which was confirmed by cocrystal structures showing each half site bound by two p53 molecules[54,55]. In vivo determination of p53 binding sites confirmed the binding preference for this consensus site, which also emerged as the most enriched motif in multiple studies using a variety of

genomic approaches[24–26,52,87]. Nevertheless, some strongly p53-induced genes, such as *BTG2* (REF.[88]), have non-canonical binding sites (FIG. 1b). Binding sites with orientations other than head-to-head or containing long spacer sequences[23–25,28,40,89] were not enriched in the genome, suggesting that these may be outliers and/or limited to specific genes.

A range of p53 affinities was observed for different degenerate binding motifs[33,56,57,90], with even single-base substitutions causing up to a half-log change in the dissociation constant of p53 binding to DNA[58]. The degree of cooperativity in p53 binding was influenced by the flexibility of the sequence in the centre of the core motif (WW), with the preferred AT sequence being the most flexible and showing lowest cooperativity, and the weakest-affinity and inflexible TA dinucleotide leading to highly cooperative binding[91]. Single-nucleotide polymorphisms in p53 binding elements that caused core-motif deviation from the consensus resulted in lower transactivation of a reporter gene[92]. On the basis of this information, several algorithms for finding and scoring p53 binding sites have been developed[24,25,93–96]. Scanning the genome with the p53 position weight matrix (representing transcription factor binding preferences) identified ~14,000–21,000 possible p53 binding sites depending on the model and threshold, but <50% were bound by p53 in ChIP–seq experiments[25,32,40,97]. Nevertheless, the position weight matrix of the full p53 binding site was a significant predictor of p53 binding in vivo. Interestingly, analyses of select p53 target promoters revealed that genes involved in cell cycle regulation harboured high-affinity binding sites, whereas those involved in apoptosis displayed a broader range of affinities and/or larger deviations from the consensus site and resulted in reduced p53 binding[56,65,98–100].

The above observations initially led to the 'affinity model', which postulated that p53 concentrations could determine the choice between cell cycle arrest and apoptosis. This model was supported by studies showing that modulating p53 expression resulted in cell cycle arrest at low p53 levels and apoptosis at higher levels[101] and that pro-apoptosis genes required cooperative p53 binding (and by extension, higher p53 levels[102]). However, genes encoding key regulators of apoptosis such as *PUMA* and *NOXA1* have high-affinity binding sites in their promoters[20,56]. Additionally, consistent correlations between p53 levels, binding at promoters of apoptosis genes and induction of apoptosis were not observed[20–22], and even at low p53 levels, the protein was found bound at apoptosis genes[21]. In fact, many p53-bound genes did not display p53-dependent regulation[22,25,65], suggesting that differences in p53 PTMs and expression dynamics and the status of chromatin at target genes may underlie key p53-mediated cell fate decisions.

Although meta-analyses and several genome-wide studies have concluded that p53 functions only as a transcription activator[28,33,36,40,41,86,103–105], many individual studies have reported repressive functions that are intimately linked to the structure of the p53 binding site[22,23,25,65,106]. p53-repressed genes either lack an identifiable binding site or contain weaker binding motifs. For example, the promoters of the p53-repressed survivin (also known as BIRC5)[107] and Alu elements[23] contain 3 bp spacers (in contrast to the 0 or 1 bp spacers identified in p53-activated genes). Half-site orientations other than head-to-head were also reported for some repressed genes[108,109], as in the case of the cyclin B1 (*CCNB1*) promoter[109], which additionally includes a long spacer (FIG. 1b). Furthermore, repressed genes were reported to require highly cooperative binding, suggesting that repression occurred at higher levels of p53 expression[65]. It is possible that p53 functions as a repressor in unique situations (for example, in particular cell types or damage responses or with defined kinetics), thus evading recognition in meta-analyses that compare data sets obtained in disparate conditions.

Chromatin structure tunes p53 function

Following the determination of the p53 DNA-binding site, the ability of p53 to bind chromatin was extensively assessed. An early study[79] found that p53 bound with higher affinity to chromatin than to DNA oligonucleotides, and subsequent studies demonstrated that p53 could bind closed chromatin, thus classifying it as a pioneer transcription factor (transcription factor that can directly bind nucleosomal DNA in chromatin)[110–112]. These studies partially reconciled the discrepancies regarding the role of the CTD in DNA binding, as they revealed that some of the in vitro results were artefacts of binding to short oligonucleotides. In fact, the CTD was found to enhance binding to nucleosomes regardless of its acetylation state (see below)[79]. Some genome-wide analyses documented co-occurrence of other transcription factor binding sites with p53 binding sites[22,106], but a meta-analysis of such studies revealed that p53 binding sites did not cluster with sites for any other transcription factors and that the initial reports were based on low-confidence p53 binding sites[40]. These findings raised the possibility that chromatin structure, rather than binding site affinity, might underlie the genomic binding patterns of p53. However, meta-analyses showed the p53 binding sites were largely conserved across cell types and treatments[40,106], casting doubt on both the affinity model and the idea that chromatin structure has a major regulatory role. In fact, the only parameter determining p53 binding was found to be the presence of the p53 binding site[25,40]. Although chromatin-based selection of target genes was not supported by meta-analyses, at least one p53 target gene — the gene encoding the adaptor 14-3-3-σ — was made unresponsive to p53-dependent activation following DNA methylation[113]. A comparison of p53 binding sites in normal and cancer cells revealed an enrichment for CpG islands and hypomethylated DNA in normal cells but not in cancer cells with wild-type p53 (REF.[26]). This difference likely arises from globally altered chromatin landscapes in cancer cells, which may affect the accessibility of binding sites to p53 either through chromatin structure itself or through binding of other factors such as the transcription factor SP1 (see below)[26].

In support of the role of chromatin in directing p53 binding and function, the chromatin remodeller RSF1 was found to be required for p53 binding, formation of a complex with the histone acetyltransferase p300

and p300-mediated acetylation of p53 target genes in response to DNA damage[114]. RSF1 deficiency reduced cell death following DNA damage and compromised the expression of both cell cycle arrest and apoptosis genes. Although DNA methylation, eRNA production and chromatin remodelling appear to guide p53-binding-site selection, they appear to occur before DNA damage and the choice of which p53-dependent transcriptional programme to activate. Nevertheless, the basal state of chromatin does appear to affect gene expression. Basal gene expression was higher in regions of open chromatin and in regions where p53 binding resulted in chromatin remodelling, leading to higher fold changes in gene expression[33,97]. How the interplay between the chromatin structure and p53, before and after DNA damage, affects cell fate is a fundamental question that is also highly relevant for cancer therapy.

In addition to p53 induction in response to DNA damage or other stimuli, p53 binds to a subset of promoters[42] and enhancers[37,44] in a basal state before gene activation, suggesting that distinct chromatin states at these regions[115,116] help maintain p53 binding[41]. A study using global run-on sequencing (GRO-seq; a method to identify RNAs that are actively transcribed by RNA polymerase II) found that p53-independent production of eRNA correlated with increased p53 binding and the responsiveness of nearby genes[37], raising the possibility that eRNAs may direct p53 target selection. How eRNA-mediated p53 recruitment could lead to gene activation remains unknown, and the enhancer priming model contrasts with studies in mouse embryonic stem cells (ESCs), in which p53 binding at enhancer sites reduced its activation of associated genes[44]. Additional studies are needed to show whether this difference arises from unique properties of stem cells or whether p53 priming and interference occur simultaneously at different sites.

Post-translational modification of p53

More than 300 different PTMs of p53 have been detected by mass spectrometry[117–119]. The role of some PTMs has been well studied and shown to be crucial for regulating p53 levels and activity (FIG. 2a). For example, a key feedback mechanism for controlling p53 expression levels is polyubiquitylation of p53 at the carboxyl terminus by its target, MDM2. Whereas ubiquitylation targets p53 for degradation, phosphorylation of p53 at the amino-terminal serine and threonine residues in response to DNA damage weakens the p53–MDM2 interaction and thus stabilizes p53. PTMs can be specific to the type of DNA damage[120–126] and thus can accordingly direct the p53-mediated response and cell fate.

The p53 CTD is highly regulated by acetylation of several lysine residues, but the full range of consequences of these PTMs and the underlying molecular mechanisms have been difficult to elucidate, in part because the effects of acetylation can depend on both the overall level of acetylation (bulk) and on the specific sites at which it occurs. Bulk p53 acetylation increased following DNA damage, and acetylated p53 was enriched at the promoter of the cell cycle arrest gene *CDKN1A*[70], yet mutating five[70] or six[127] acetylation sites in the p53 CTD resulted in equal or greater effects on apoptosis than on cell cycle arrest[127,128]. The effects of acetylation on DNA binding have been varied: early studies showed that acetylation enhanced specific DNA binding[68] but inhibited DNA binding of the CTD[129]; later studies found no effects of mutating four lysine residues in the CTD to either acetylated or nonacetylated mimics[130] or found effects on gene expression only in specific cell types[127]. A mouse with lysine-to-arginine (KR) substitutions at seven acetylation sites in the CTD showed no grossly abnormal phenotypes, although thymocytes (but not mouse embryonic fibroblasts) showed increased p53 responsiveness to irradiation[131]. In human cells, expression of p53 with eight KR substitutions (six in the CTD and two in the DNA binding domain (DBD)) had no effects on expression of the p53 target MDM2 but did result in reduced expression of *CDKN1A* and multiple pro-apoptotic genes, which was linked to increased interactions between p53 and MDM2 or MDMX[132]. It is likely that MDM2 inhibited p53 function by occluding the transactivation domain[133,134] rather than by ubiquitylation, because the 8KR p53 mutant is expected to have lost most of its ubiquitin acceptor sites. These conflicting reports on the effects of acetylation on p53-mediated gene activation were recently somewhat reconciled by the finding that the CTD enhanced p53 binding preferentially to noncanonical sites and that this function was compromised by acetylation[135], leading the authors to propose that acetylation may turn off the p53 response at these loci. In summary, these studies suggested that p53 acetylation does not have a major regulatory role but could fine-tune p53 function in a context-dependent manner.

Whereas the above studies focused on bulk levels of acetylation, many site-specific PTMs appear to have greater effects on regulation of apoptosis than on cell cycle arrest. These PTMs vary in their domain location, functional consequence and type. For example, combined acetylation of Lys370, Lys372 and Lys373 in the CTD (all substrates of the acetyltransferase p300 (REF.[68])) or of Lys120 in the DBD (a substrate of TIP60)[136–138] promoted apoptosis[136,137,139], whereas acetylation at Lys317 by PCAF suppressed it[140] (FIG. 2a). Lys120 mutations compromised cell cycle arrest as well as affecting apoptosis[136]. By contrast, acetylation of Lys320 showed greater regulation of cell cycle arrest[139]. Although there are no genome-wide measurements of Lys120-acetylated p53, structural studies showed that Lys120 acetylation switches the p53 protein conformation into a state that increases binding to the *BAX* binding site, suggesting that it may promote apoptosis[141,142].

Although acetylation is not prevalent in the amino terminus, phosphorylation is commonly found in this region (FIG. 2a). Notably, phosphorylation of Ser46 by the MDM2-regulated homeodomain-interacting protein kinase 2 (HIPK2)[143–145] was initially shown to regulate apoptosis by inducing a key apoptotic gene, p53-regulated apoptosis-inducing protein 1 (REF.[146]), and subsequently by enhancing binding at promoters of several other apoptosis-inducing genes[22]. By contrast, phosphorylation of Ser15, which is mediated by the major DDR kinases ATM[147–149] and ATR[150], was required for cell cycle arrest. The analogous phosphorylation site in mice (at Ser18) showed similar effects on cell cycle arrest in

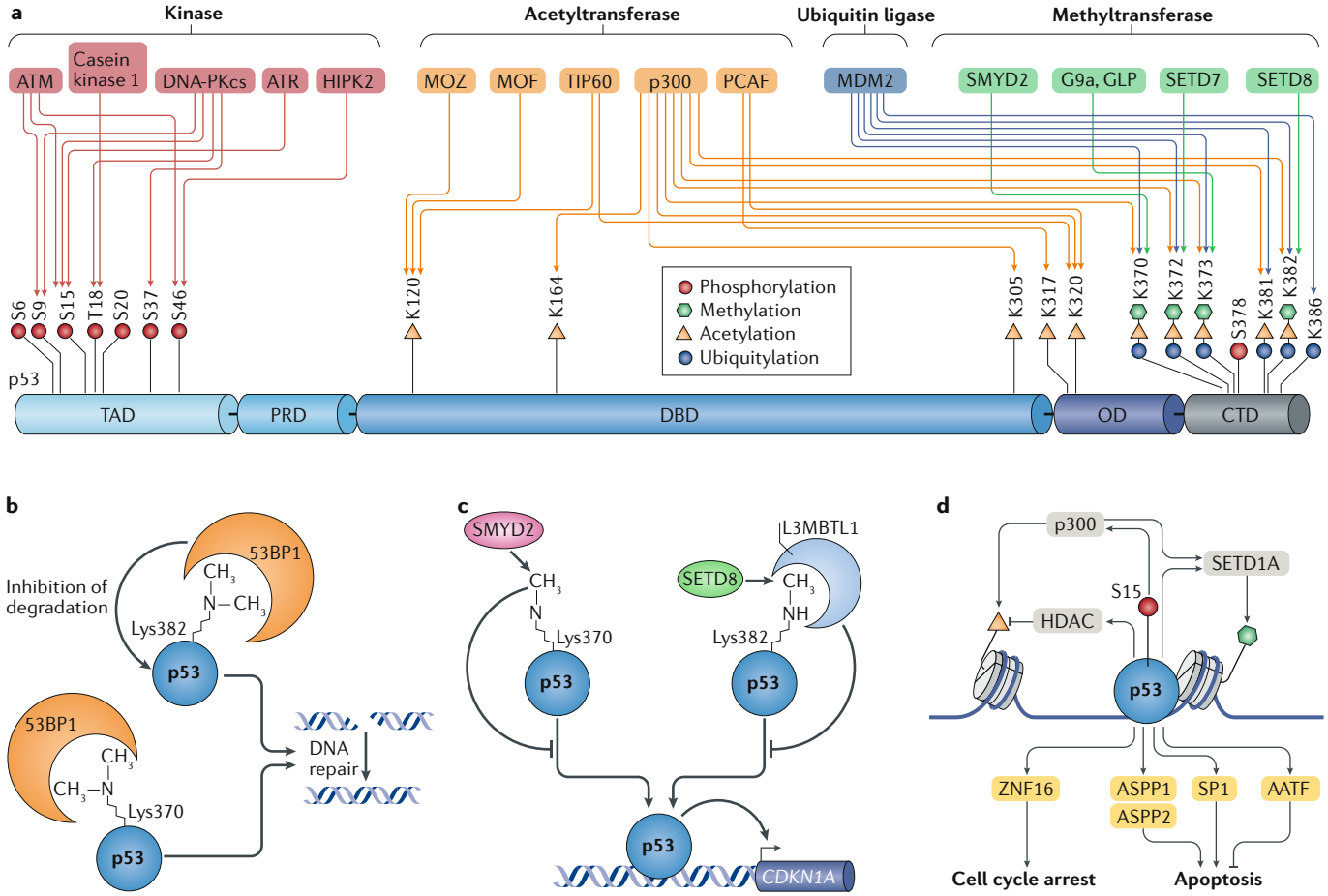


Fig. 2 | **Regulation of p53 target genes by post-translational modification and interactions with chromatin regulators and transcription factors. a** | p53 post-translational modifications and the enzymes responsible for catalysing them. Key experimentally validated modifications known to affect p53 function are shown. Phosphorylation sites are prevalent in the tandem activation domain (TAD), whereas acetylation sites occur in the DNA binding domain (DBD), oligomerization domain (OD) and carboxy-terminal domain (CTD). Methylation sites are enriched in the CTD. The proline-rich domain (PRD) contains few well-studied modifications. A single residue can be modified by multiple enzymes in a mutually exclusive manner, resulting in antagonism between different modifications. **b** | Dimethylation of Lys370 or Lys382 recruits p53-binding protein 1 (53BP1), which promotes DNA damage repair. Specifically, 53BP1 binding to dimethylated Lys382 inhibits p53 degradation. **c** | Monomethylation of Lys370 by SMYD2 or of Lys382 by SETD8 inhibits p53 localization to the promoter of *CDKN1A* (encoding p21). The effect of Lys382 monomethylation is mediated by binding of the transcription repressor L3MBTL1. **d** | Examples of p53 interactions with chromatin-regulating enzymes (grey rectangles) and transcription factors (orange rectangles) and their effects on cellular outcomes. p53 can recruit cofactors independently of its modifications or through specific post-translational modifications. Notably, phosphorylation at Ser15 recruits the histone acetyltransferase p300. Recruitment of chromatin-modifying enzymes results in local changes to chromatin structure, thereby activating or repressing gene expression. Associations with transcription factors direct target-gene choices of p53, thereby influencing cell fate. AATF, apoptosis-antagonizing transcription factor; ASPP1, apoptosis-stimulating of p53 protein 1; DNA-PKc, DNA-dependent protein kinase catalytic subunit; HDAC, histone deacetylase; ZFP16, zinc-finger protein 16.

ESCs[151], but in vivo, the mutation showed greater effects on radiation-induced apoptosis than cell cycle arrest in thymocytes and splenocytes[152]. Several other phosphorylation events differ in their timing and response to different cues. Low levels of gamma irradiation induced phosphorylation of Ser6 and Ser15 very rapidly[126,153] as well as of Ser315, and higher doses induced phosphorylation of Ser20 and Ser37 (REF.[125]). By contrast, phosphorylation in response to ultraviolet light was delayed and prolonged at these sites[125] and even occurred at Ser392, which is not phosphorylated following gamma irradiation[121]. How these phosphorylations mediate the distinct cellular responses to irradiation and ultraviolet light has not been fully elucidated.

Although phosphorylation and acetylation are the best-studied p53 PTMs, in part because of the ease of mimicking modified and unmodified states, additional PTMs also have important roles in p53 regulation. Lysine methylation occurs at multiple residues and can activate or repress gene induction depending on the residue and the extent of methylation (monomethylation, dimethylation or trimethylation). Monomethylation of Lys382 (mediated by the lysine methyltransferase (KMT) SETD8 (also known as KMT5A))[154] or dimethylation of Lys373

by G9a (KMT1C) and GLP (KMT1D)[155] repressed p53 and were accordingly reduced following DNA damage. However, abrogating the methylation of Lys373 (REF.[155]) and Lys382 (REF.[154]) during DNA damage led to premature apoptosis, suggesting the importance of low levels of p53 inhibition by these modifications. By contrast, monomethylation and dimethylation of Lys372 (catalysed by SETD7 (REF.[156])) following DNA damage increased p53 stability, gene activation and apoptosis[157]. Despite these effects, deletion of SET7/9 in mouse embryonic fibroblasts did not impair p53-induced apoptosis, senescence, cell cycle arrest or tumour suppression[158,159], consistent with data showing minimal effects of multiple lysine-to-arginine mutations (including those at the Lys372 site)[127,130,131]. These results suggest that Lys372 methylation may be more important for fine-tuning p53 function than for overall activity. At two lysine residues, Lys370 and Lys382, the degree of methylation showed differing effects on p53 function. At both residues, dimethylation promoted p53 binding to p53-binding protein 1 (53BP1) and DNA repair[160,161] (FIG. 2b). 53BP1 binding at Lys382 mediated this effect by inhibiting p53 degradation[161]. By contrast, monomethylation of Lys370 (mediated by SMYD2 (REF.[156])) or Lys382 (mediated by SETD8 (REF.[154])) inhibited p53 localization to target-gene promoters[156,162] (FIG. 2c). In the case of Lys382 monomethylation, this effect was mediated through specific methyl recognition by the transcription repressor L3MBTL1 (REF.[162]). Lys382 monomethylation inhibited p53 recruitment to both *CDKN1A* and *PUMA* promoters, but it is not known whether monomethylation at either Lys370 or Lys382 affects p53 recruitment equally at all targets. In addition to Lys methylation, methylation of three p53 arginine residues by protein arginine *N*-methyltransferase 5 was found to induce cell cycle arrest genes but not apoptosis genes[163]. Although these examples demonstrate regulation of both cell cycle arrest and apoptosis by p53 PTMs, effects on apoptosis appear to be more prevalent. Because induction of apoptosis is irreversible, additional regulation of this process by p53 PTMs may ensure that it is not selected prematurely.

The different p53 PTMs display considerable crosstalk. Analysis of mutations of amino-terminal phosphorylation sites revealed several PTM interdependencies and a prominent role for Ser15 phosphorylation in inducing the phosphorylation of Ser9, Thr18 and Ser20 (REF.[125]). Phosphorylation of Ser15 (REF.[164]), Thr18, Ser20 (REF.[165]), Ser33 and Ser37 (REF.[126]) promoted binding of the KATs PCAF, p300 and/or CREB-binding protein (CBP) and acetylation of the carboxy terminus. By contrast, Ser378 phosphorylation inhibited PCAF-mediated acetylation of Lys320 (REF.[126]). Crosstalk between methylated residues also occurs, for example, Lys372 methylation inhibits SMYD2-mediated methylation at Lys370 (REF.[156]).

The interplay between acetylation and ubiquitylation is crucial for regulating all functions of p53 by controlling its stability. In general, PTMs inhibit ubiquitylation by decreasing binding of p53 to its E3 ubiquitin ligase MDM2 and/or by occluding the site of ubiquitylation. Mutation of four lysine residues to nonmodifiable alanine residues or p53 CTD acetylation by overexpression of p300 or CBP[166] impaired ubiquitylation despite maintaining efficient p53–MDM2 interactions, suggesting that these residues may serve as ubiquitin acceptors[130] rather than as MDM2 docking sites. A p53 protein bearing mutations of six lysine residues to nonacetylatable arginine residues (6KR) rendered p53-mediated transcriptional activation insensitive to MDM2, even in the absence of DNA damage[167]. Interestingly, in a p53 protein bearing six acetylation-mimic mutations (lysine to glutamine), ubiquitylation was inhibited even more strongly than in the 6KR nonacetylated variant, suggesting that acetylation interfered with ubiquitylation at distal sites[168]. Thus, PTMs regulate MDM2-mediated p53 degradation both by occluding the p53 lysine substrates of MDM2 and by interfering with MDM2 binding. At the amino terminus, phosphorylation of Ser15 and Ser37 by the DNA-dependent protein kinase catalytic subunit increased p53-mediated transcription in vitro by reducing its binding to MDM2, in part by causing a conformational change in p53 (REF.[14]); it is possible that Ser15 phosphorylation additionally stabilizes p53 by promoting carboxy-terminal acetylation[164]. However, the S15A p53 mutant did not have altered stability in vivo[169], and it was later shown that phosphorylation of Ser15 promoted casein kinase 1-dependent phosphorylation of Thr18, which directly inhibited MDM2 binding[170].

In summary, p53 PTMs likely have a role in regulating the strength of DNA binding, target-gene selection, stability and overall p53 function. The varying effects observed in vitro using reporter assays and between cell types highlight the complexity of p53 regulation through PTMs, and future work should elucidate how p53 PTMs act in concert to guide cellular responses.

Cofactors regulate p53 activity

In addition to chromatin context aiding in p53 target gene selection, p53 also cooperates intimately with chromatin regulators to activate its target genes (FIG. 2d). The acetyltransferase p300 was required for p53-dependent activation of *CDKN1A* in vitro and in cells[79,171] and led to an increase in the gene-activating histone H4 acetylation upon p53 activation[42,171]. Another study found that p53-mediated histone acetylation was equal at two different p53-binding sites despite differences in p53 binding[172], suggesting that cofactor recruitment may contribute to the activation of specific gene expression programmes by p53. Histone acetylation was compromised in a nonphosphorylatable S15A p53 mutant[43], consistent with recruitment of p300 by phosphorylation of this residue[164] (FIG. 2d). By contrast, in mouse ESCs, p53 repressed *Nanog* expression by recruiting histone deacetylases to chromatin[173]. Histone methylation also has an important role in target-gene regulation. At the *CDKN1A* promoter, p53 and p300 together recruited the methyltransferase SETD1A, leading to monomethylation and dimethylation of histone H3 Lys4, which are gene-activating PTMs[174].

Unlike most pioneer transcription factors, which co-bind to targets with other transcription factors, p53 binding elements associated with DDR genes were not associated with binding sites for other transcription factors[40,175]. During human ESC differentiation, however, p53 binding sites coincided with the binding motif

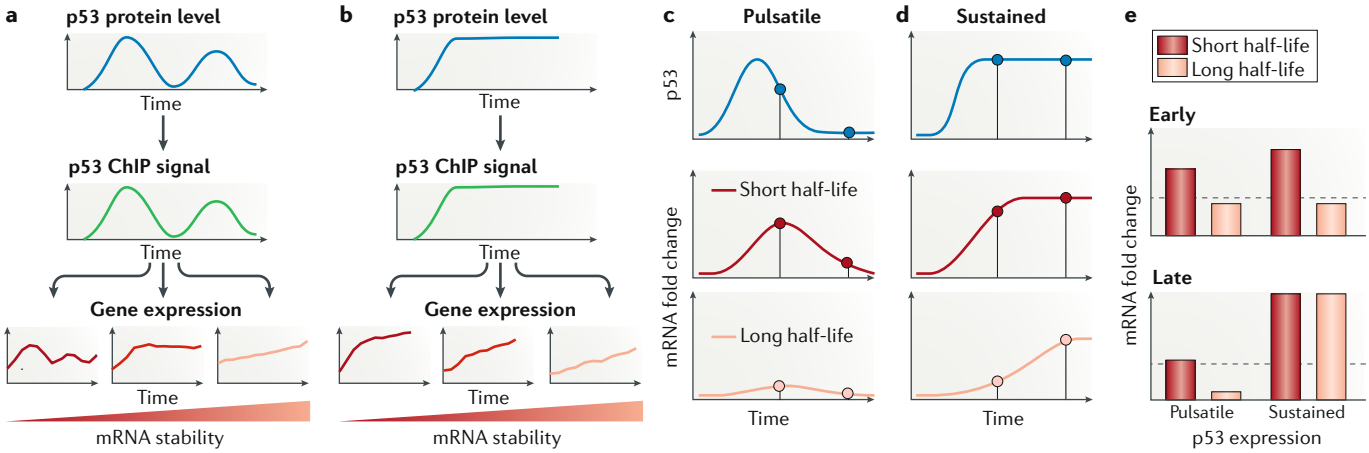


Fig. 3 | **p53 dynamics regulate gene expression.** The dynamics of p53 expression combined with the stability of the mRNA of the target genes influence which gene networks predominate in response to a given stimulus. **a** | Pulsatile p53 protein levels result in pulsatile p53 DNA binding but in heterogeneous gene expression dynamics, which depend on the stability of the mRNAs of p53 target genes. **b** | Sustained p53 expression levels give rise to more uniform mRNA expression dynamics in comparison with pulsatile p53 levels. **c**,**d** | Examples of pulsatile (part **c**) and sustained (part **d**) p53 expression and their effects on the accumulation of stable and unstable target-gene mRNAs. The two points on the graphs mark the early and late measurement time points referenced in part **e**. In the case of pulsatile p53 expression (part **c**), the levels of a short-lived target mRNA will track those of p53 and exhibit pulses of expression. By contrast, a stable target mRNA may not accumulate significantly above the baseline level during the short duration of p53 activity. The maximum levels of both target mRNAs will be detected following the peak of p53 expression, with the lag representing the time required for transcription. In the case of sustained p53 activity (part **d**), unstable transcripts undergo a relatively rapid increase above their basal levels, whereas stable mRNAs require a longer time to achieve elevated expression over their basal levels. **e** | Example of the effects of time of measurement, p53 dynamics and mRNA half-life on the classification of a gene as differentially expressed upon p53 activation. An early measurement, soon after p53 activation, will result in stable mRNAs not being identified as significantly induced by p53 (below the threshold line). By contrast, a later measurement will capture the induction of both stable and unstable mRNAs resulting from sustained p53 expression but can miss or underestimate the induction of stable transcripts after a pulse of p53 expression. ChIP, chromatin immunoprecipitation.

of the pluripotency factors OCT4 and SOX2 (REF.[175]) in several cases, suggesting that p53 might function differently in development than in DDR. Although, generally, other transcription factor motifs are not significantly co-enriched with p53 binding sites, several transcription factors do interact with the p53 DBD to modulate target-gene expression. Apoptosis-stimulating of p53 protein 1 (ASPP1) and ASPP2, which are transcriptional targets of E2F[176,177], interact with the DBD of p53 (REFS[178–180]) to specifically promote p53 binding at and expression of pro-apoptosis genes[181]. Apoptosis-antagonizing transcription factor (AATF) also bound p53 through its DBD[182] at promoters of apoptosis-inducing genes, such as *PUMA*, *BAX* and *BAK1* (REF.[183]). Although AATF can induce p53 transcription, it also reduced p53 occupancy at these apoptosis-inducing gene promoters, thereby inhibiting apoptosis[182]. Thus, although ASPP1, ASPP2 and AATF have similar biochemical interactions with p53 and DNA, they lead to opposite outcomes. p53-mediated apoptosis is further regulated by the pioneer factor SP1 (REF.[184]). Depletion of SP1 protected cells from p53-mediated apoptosis, and its overexpression switched the cellular outcome of p53 activation from cell cycle arrest to apoptosis[185]. Interestingly, global gene expression profiling showed that SP1 depletion did not affect canonical p53 target genes involved in apoptosis (*BAX* and *NOXA1*) or cell cycle arrest (*CDKN1A* and *GADD45A*) but was required for p53-dependent repression of genes in alternative pathways through co-binding with p53 at these genes[106,185]. Finally, the p53 target gene encoding zinc-finger protein 16 binds p53 and increases its binding to and transactivation of the cell cycle arrest genes *CDKN1A* and *SFN* but not apoptotic genes such as *BAX*, *PERP*, *PUMA* or *NOXA1* (REF.[186]). Cofactor binding can also be influenced by p53 PTMs, as observed during *Xenopus laevis* development, in which p53 phosphorylation at Ser6 and Ser9 was required for the recruitment of Smad transcription factors to target genes[187]. As observed for p53 PTMs, cofactors also seem to preferentially regulate apoptotic genes, demonstrating the multiple layers of regulation to which this terminal process is subjected.

The p53 family members p63 and p73 were shown to have tumour suppressive roles and to enhance the p53 apoptotic function[188–190]. In contrast to the above-mentioned cofactors, p63 and p73 have the same DNA recognition sequence as p53; they can bind at the majority of p53 binding sites and are thought to cooperate with p53 in the induction of p53 target genes[24,32,191–193]. Comparison of p63 and p53 ChIP data showed that p63 can also bind to other genomic loci and has an additional and previously unrecognized role in DNA repair[194]. The roles of p53 family members, both together with and independently of p53, are still emerging, and further work is required to fully understand their function.

One difficulty to emerge from these studies is how to reconcile the examples of cofactors that are required for p53 function and specific activity with the observation that p53 binding sites tend not to co-occur with binding

sites for other transcription factors[40]. One possible explanation is that cooperation of some cofactors with p53 is specific to a small subset of genes, and thus, cofactor motifs may not be enriched in genome-wide analyses. The examples above also suggest that rather than having an all-or-nothing effect, cofactors act by modulating the relative strength of p53 binding at different promoters; this situation would be missed in the meta-analysis studies discussed above[28,40].

The role of p53 dynamics

Not only do the absolute levels of p53 protein matter for the choice of cellular outcome in response to DNA damage but the changes in p53 levels over time (p53 dynamics) also affect cell fate[195–197]. For example, ionizing radiation induces pulses of p53 protein levels, which allows cells to repair DNA damage and re-enter the cell cycle. Converting the pulses into sustained p53 activation using a combination treatment of ionizing radiation with MDM2 inhibition led to irreversible cell cycle arrest and senescence[197]. Another striking example is the response to the chemotherapeutic agent cisplatin, in which the rate of p53 accumulation determined whether the cells survived the treatment[196]. Therefore, temporal regulation of p53 protein levels is emerging as an additional modulator of cell fate control by p53.

Owing to the heterogeneity in p53 dynamics at the single-cell level[196,198,199], identifying the molecular mechanisms that connect temporal changes in p53 levels with global gene expression dynamics has been a challenge. Recent studies have focused on the gene expression response to p53 expression pulses induced by ionizing radiation[104,197,200]. In this case, synchrony of the initial p53 pulses between single cells was leveraged to perform population-level measurements of DNA binding and gene expression. A surprising diversity of gene expression dynamics was observed between p53 target genes, which exhibited different timing, levels and patterns of induction. For example, some target genes, such as *CDKN1A*, showed pulses in mRNA levels following p53 protein pulses, whereas others, such as *DDB2*, reached a plateau of expression or, like *RPS27L*, continuously accumulated. Surprisingly, p53 DNA binding dynamics were indistinguishable between the genes that exhibited different mRNA expression dynamics. Mathematical modelling and perturbation experiments showed that mRNA dynamics were largely explained by the mRNA stability of p53 target genes[104,200,201], with unstable transcripts tracking p53 protein dynamics and long-lived mRNAs integrating p53 levels over time (FIG. 3). Moreover, knowledge of p53 protein dynamics and mRNA stability of a target gene were sufficient not only to explain but also to predict target-gene induction dynamics in response to different p53 dynamics[104].

Interestingly, clustering genes on the basis of their dynamic patterns of induction did not distinguish between apoptotic and cell cycle arrest genes, suggesting that the timing of mRNA expression was not sufficient to explain the cellular outcome. Furthermore, the connection between gene expression and cell fate may not be determined by the expression levels of a single gene but by the relative expression levels of multiple genes. Indeed, the ratio of expression between p21 and PUMA was important for the decision between cell cycle arrest and apoptosis[202]. It is indeed more likely that cell fate is coordinated between multiple genes and that relative dynamics and abundance of proteins — which vary with different p53 expression dynamics[104,200] — ultimately determine the cellular outcome.

Finally, we propose that the temporal dynamics of p53 expression should be taken into consideration when defining p53 target genes. We expect that different p53 protein dynamics occurring in response to different types of DNA damage are a source of variability in target-gene identification between studies. Indeed, depending on the type of DNA damage and the time point of measurement, differential expression of target genes may be missed. For example, the induction of a short-lived mRNA will be missed if its expression is measured too long (longer than the mRNA half-life) after p53 levels have decreased. By contrast, a stable mRNA may need more time to reach the fold-change cut-off and thus may be filtered out in early time points (FIG. 3). As an alternative to performing time course experiments to detect target genes, a recent study showed that the confounding effects of mRNA half-life on identification of direct p53 target genes can be circumvented by measuring nascent RNA rapidly following p53 induction (to avoid secondary transcriptional waves) by GRO-seq[37].

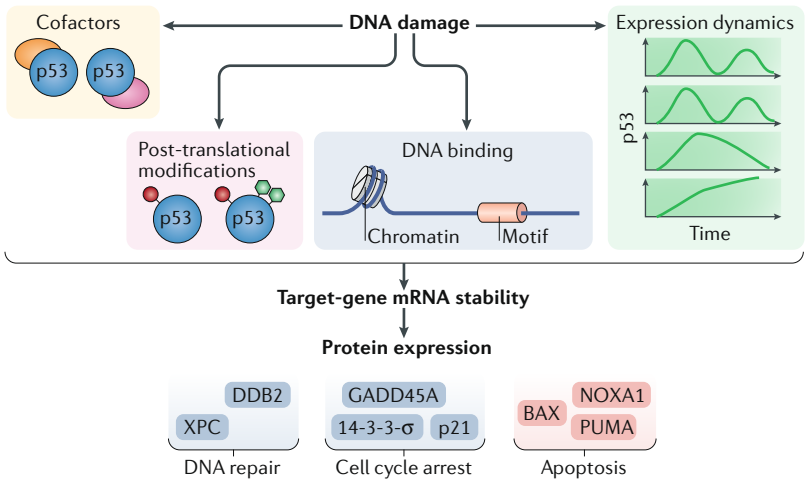


Fig. 4 | **Regulation of p53-mediated cell fate outcomes.** Multiple mechanisms control the activity of p53 in response to DNA damage. The type and extent of damage influence which post-translational modifications occur on p53 and the dynamics of p53 expression. Additionally, the DNA sequence and chromatin structure at each site affect the strength of p53 binding. Promoter-specific association of p53 with different cofactors also influences the extent of gene activation. The magnitude of p53-induced gene activation, combined with the stability of the induced mRNAs, determines protein levels in the cell, which ultimately guide cell fate outcomes towards survival (DNA repair and cell cycle arrest) (blue) or cell death by apoptosis (red). DDB2, DNA damage-binding protein 2.

Conclusion

Following decades of research, the role of p53 in controlling stress-specific responses remains a puzzle. Recent genome-wide analyses showed that p53 robustly binds to a conserved set of genomic loci independently of cell type and treatment. By contrast, studies of specific target genes suggested that the strength of DNA binding and binding to noncanonical sites are tunable

by chromatin structure, p53 PTMs, interaction with cofactors and temporal changes in p53 protein levels (FIG. 4). Additionally, post-transcriptional controls of gene expression, such as mRNA stability, have an important role in the observed heterogeneity in target-gene expression. The large number of p53 regulatory mechanisms and their cooperation in triggering specific expression programmes remain open areas for investigation. Systematic measurements in multiple conditions together with models integrating the multiple layers of regulation on p53 activity will be required to decipher the complexity of p53 function.

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