

## Review Article

# One Function—Multiple Mechanisms: The Manifold Activities of p53 as a Transcriptional Repressor

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Maintenance of genome integrity is a dynamic process involving complex regulation systems. Defects in one or more of these pathways could result in cancer. The most important tumor-suppressor is the transcription factor p53, and its functional inactivation is frequently observed in many tumor types. The tumor suppressive function of p53 is mainly attributed to its ability to regulate numerous target genes at the transcriptional level. While the mechanism of transcriptional induction by p53 is well characterized, p53-dependent repression is not understood in detail. Here, we review the manifold mechanisms of p53 as a transcriptional repressor. We classify two different categories of repressed genes based on the underlying mechanism, and novel mechanisms which involve regulation through noncoding RNAs are discussed. The complete elucidation of p53 functions is important for our understanding of its tumor-suppressor activity and, therefore, represents the key for the development of novel therapeutic approaches.

## 1. Introduction

Complex regulatory systems govern the process of genomic integrity maintenance. Intracellular and extracellular stress signals can lead to induction of central signalling proteins which generate different downstream effects. The transcription factor p53 is the most important known tumor suppressor, and functional inactivation of p53 is frequently associated with loss of genomic stability [1]. Originally thought to be an oncogene due to its binding to the SV40 T-antigen [2, 3], p53 later turned out to be a very potent apoptosis inducer and inhibitor of cell-cycle progression [4]. p53 knockout mice are susceptible to different tumor types [5] and restoration of p53-function leads to the regression of tumors *in vivo* [6, 7]. The tumor suppressive function of p53 is mainly attributed to its ability to regulate numerous target genes at the transcriptional level. Functions both as a transactivator and as a repressor have been described for p53.

The mechanism of transcriptional induction of p53 is well characterized. It involves DNA-binding to the p53

consensus site [8]. Transactivation is achieved by interactions with components of the general transcription factor TFIID like the TATA box binding protein (TBP) [9, 10] and TAFII31 [11]. Numerous target genes upregulated by p53 have been characterized so far. Well-known targets are involved in apoptosis like Bax [12] and in cell-cycle control like the cyclin dependent kinase (CDK) inhibitor p21<sup>CIP1</sup> [13] or the inhibitor of Cyclin B/Cdc2 nuclear import, 14-3-3 $\sigma$  [14]. For all known upregulated target genes DNA-binding of p53 is essential for regulation of gene transcription.

In addition to transcriptional induction, p53 has been shown to repress various target genes, but obviously by different mechanisms [15]. Many of them are involved in cell-cycle control (Figure 1) and contribute to p53-induced cell-cycle arrest. Gene expression analyses showed that p53-dependent repression of target genes is associated with apoptosis after hypoxia treatment, whereas transactivated genes were clearly underrepresented [16]. Therefore, gene repression by p53 contributes to its tumor suppressive activity. In this paper we will summarize the wide diversity

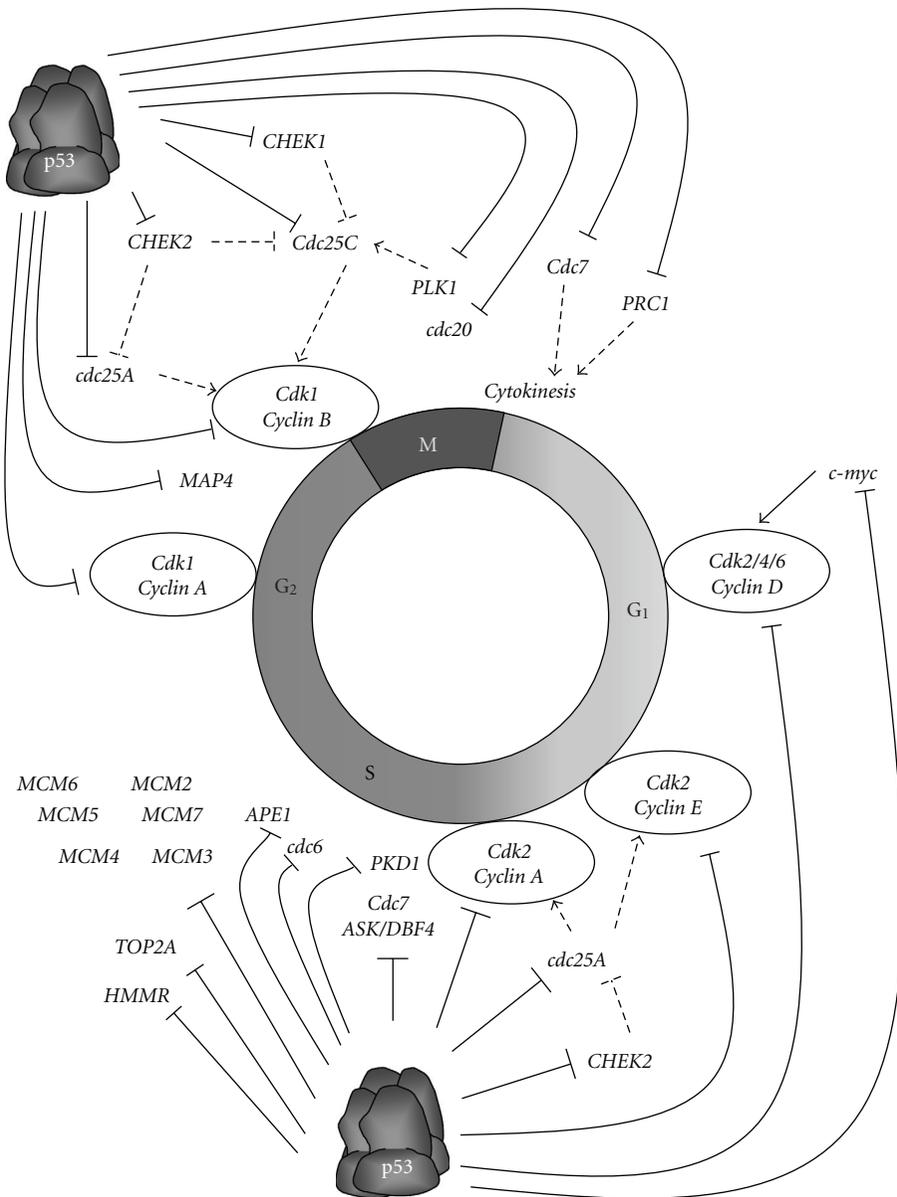


FIGURE 1: The tumour suppressor protein p53 can interfere in multiple ways with cell-cycle progression. All of the cell-cycle regulators shown in this figure are transcriptionally downregulated by p53 (black lines with horizontal bars). Inhibition (dotted lines) and activation (dotted arrows) of a subset of regulation between different p53 target genes is indicated.

of molecular mechanisms of p53-dependent repression described to date. In principle, two categories of p53-repressed target genes can be classified: (I) genes that are regulated by direct interaction of p53 with target gene promoters or bound cofactors, (II) genes that are indirectly regulated by other p53 target genes. But most (if not all) of the mechanisms of transcriptional repression by p53 require its intact DNA-binding domain. Whether the DNA-binding domain is always needed for direct DNA binding to target gene promoters or interaction of p53 with other proteins through this domain can evoke p53-dependent repression, remains to be elucidated.

## 2. Diversity of p53-Binding Sites

The classical consensus site of a p53 binding element consists of two repeats of the palindromic sequence RRRCWW-GYYY (in which R is a purine, Y is a pyrimidine and W is an adenine or thymine) which are separated by a spacer of 0–13 base pairs. p53-binding sites which match this consensus sequence are extensively described in p53-dependent transactivation. Hundreds of p53 target genes (encoding both proteins and noncoding RNAs) have been discovered up to date [17–19] and this number still increases. Among the endogenous human p53 response elements that

have been validated by p53 binding and corresponding gene expression, nearly 95% have mismatches from the consensus sequence [20]. However, these mismatches were not considered significant to result in a change of the transcriptional activation function of p53 [21].

Analogous to transcriptional activation, p53 may repress target genes by binding to a consensus element in the promoter region. In most cases they differ from the canonical consensus sequence. Recently, a specific variation in the central dinucleotide core within the p53-response element was identified to determine whether p53 acts as a transcriptional activator or as a repressor on the target gene [21]. The p53 binding site mediates strong activation of the respective promoters when the central dinucleotide is AT, AA or TT, matching the classical consensus sequence. Core dinucleotide combinations of TG, CA, CC, CG, GG or GC are found in repressive p53 response elements [21]. The function of the remaining dinucleotides depends on the flanking nucleotides of the response element and can either mediate p53-dependent activation or repression whereas the position nearest to the CWWG core motif has the strongest positional effect [21]. In addition to variations within the two decamers of the p53 binding site, the spacer between both half sites also influence the p53 effect. Recent analyses of validated repressor sites demonstrate often longer spacers than activator sites [17].

In addition new p53 binding elements have been found allowing direct interaction of p53 with promoter DNA (Figure 2, panel I). One example is transcriptional repression of the *MDR* promoter [22], where p53 can repress transcription directly by binding to a novel head-to-tail (HT) site within the *MDR1* promoter. A similar element was found in the *CD44* promoter which is efficiently repressed by p53 in a DNA-binding-dependent manner [23]. Work from Marks et al. showed that the rat *Bradykinin B<sub>2</sub>* promoter harbours two binding sites and one of them was responsible for p53-dependent induction while the other mediated transcriptional repression by p53. Intriguingly, the function of the repressive element was strongly dependent upon the promoter context as it turned into an activating element when cloned in front of a TATA-Luc minimal promoter [24]. This mechanism of p53-dependent repression therefore may involve other factors, recruited to the target gene promoters dependent on sequence binding motifs or epigenetic modifications of chromatin.

### 3. Repression by Direct Interactions with Target Gene Promoters or Bound Cofactors

**3.1. DNA-Binding-Dependent Mechanisms.** Different mechanisms which are dependent on a direct DNA binding of p53 have been described (Figure 2). One possibility involves binding of p53 to an element which overlaps binding sites from coactivator molecules (Figure 2, panel II). But direct DNA-binding of p53 to such an element and subsequent interference with the activity of other transcription factors is rarely the exclusive mechanism for p53-dependent repression. Instead, in most cases manifold distinct molec-

ular mechanisms contribute concertedly to p53-dependent response of its repressed target genes. However, in this section we will mention those p53 target genes, whose transcriptional repression is achieved at least in part by direct binding of p53 to their respective promoters. Additional mechanisms described for these p53 target gene will be discussed in detail in later sections.

The expression of the alpha-fetoprotein (AFP) has been shown to be governed by hepatic nuclear factor 3 (HNF3) which binds to a sequence called the developmental repression domain of the promoter. Due to its higher binding affinity, p53 is able to displace HNF3 from the *AFP* promoter. Furthermore, it was demonstrated that p53 can interfere actively with transcription of *AFP* independently of HNF3 most likely through recruitment of a corepressor complex [25]. However, this mechanism occurred exclusively in liver cells [24]. Wilkinson et al. proved that SnoN is the corepressor whose interaction with histone deacetylases (HDACs) is promoted by p53 [25]. Furthermore, they demonstrated additional crosstalk between the TGF- $\beta$  and p53 signalling pathways. Both, p53- and TGF- $\beta$ -activated SMAD transcription factors are able to bind to an intercalating response element in the *AFP* promoter. The interaction of this complex with SnoN and the corepressor mSin3A is essential for repression of *AFP* transcription [26]. mSin3A is a mediator of HDAC recruitment which induce changes in chromatin structure. Therefore, the protein complex needed for the repression of the *AFP* promoter contains several proteins which act on the local chromatin structure. These results indicate that different mechanisms govern repression of the *AFP* promoter by p53 and that tissue-specificity is achieved through recruitment of specific corepressor proteins.

Recently a further p53 target was identified to be regulated by p53 through a similar mechanism. Activity of the human *polycystic kidney disease-1* (*PKD-1*) promoter was shown to be repressed by direct binding of p53 to four p53-response elements (BS1-4) [27]. One of these sites (BS1) dictates active repression in association with an HDAC/mSin3A repressor complex. However, BS1 requires all three other p53-binding sites for full activity, and the authors suggest a model whereby p53 exerts a biphasic control on *PKD1* gene transcription, depending on cellular context and the cognate cis-acting element.

A mechanism involving direct binding of p53 to a promoter element which overlaps a binding site from a coactivator molecule was also shown for the human *AP-endonuclease* (*APE1/Ref-1*) promoter. APE1 is an essential multifunctional protein, and plays a central role in the repair of oxidative base damage via the DNA base excision repair (BER) pathway. A recent study demonstrated that endogenous p53 is bound to the *APE1* promoter region that includes a SP1 site, and that this binding of p53 interferes with SP1 binding to the *APE1* promoter [28].

Such an interference of direct p53 DNA-binding with function of general transcription factor SP1 is described to contribute to p53-dependent repression of several other p53 target genes, including *Cdc25C* and *Cyclin B1*. Repression of both genes were found to be carried out at least in part

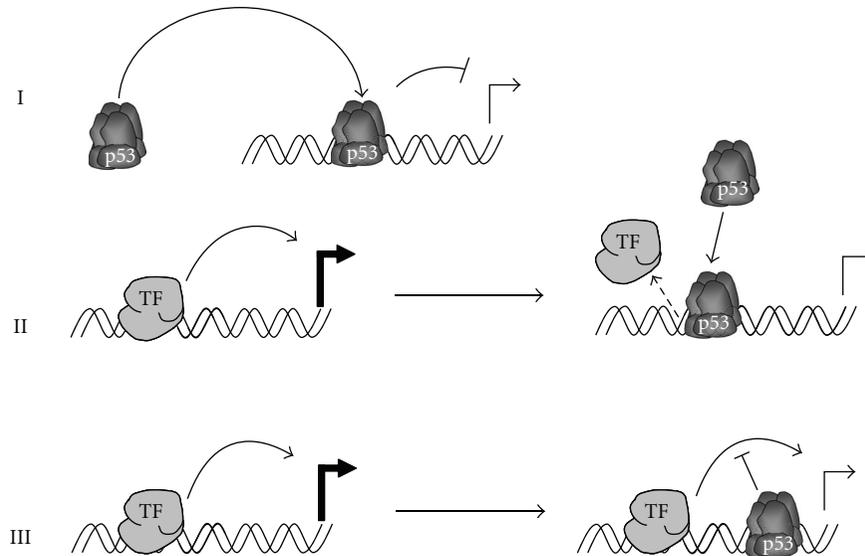


FIGURE 2: DNA-binding dependent mechanisms. Mechanisms of p53-dependent repression which involve direct binding of p53 to the DNA. Binding of p53 to a new p53 binding element leading to direct transcriptional repression by p53 (panel I), displacement of other transcription factors due to overlapping DNA-binding sites (panel II) and concomitant binding of p53 and a second transcription factor with interaction of these two factors leading to repression of the gene (panel III) belong to this group.

by binding of p53 in vicinity to an adjacent SP1-binding site and interaction of these two factors (Figure 2, panel III) [29, 30]. However, several further mechanisms have been described to contribute to p53-dependent repression of both genes. For *Cdc25C*, intact CDE/CHR elements (cell-cycle-dependent element/cell-cycle genes homology region) [30] or CCAAT boxes of the promoter [31–33] are found to be required and/or recruitment of histone- and promoter-modifying enzymes is demonstrated [34, 35]. In addition, for *Cdc25C* indirect mechanism of p53-dependent repression via the target gene  $p21^{CIP1}$  was described [36]. These different types of mechanisms are discussed below.

p53 activity has also been shown to interfere with the transcription of viruses. The HIV-LTR region was shown to be bound by p53 with partial displacement of SP1 leading to transcriptional repression [37]. Another example for overlapping transcription factor binding sites is the *survivin* gene [38]. The mechanism of p53-dependent repression involves direct binding of p53 to the promoter to a sequence which includes an E2F binding site. Hoffman et al. showed that increasing levels of p53 are able to overcome E2F-induced *survivin* expression, suggesting a displacement of E2F from the promoter [38]. Later experimental work by Löhr et al. found that direct binding of p53 is not essential for repression [36]. Instead, an indirect mechanism was proposed involving the p53 target gene  $p21^{CIP1}$  which is discussed in detail below. Raj and coworkers showed that the regulation of *survivin* in melanocytes is carried out by different mechanisms also under nonstress conditions [39]. One of these involves direct p53-binding to the promoter and disruption of this interaction is sufficient to induce basal *survivin* expression. While it has been reported that p53 also affects *survivin* expression via the  $p21^{CIP1}$ -Rb-E2F

pathway mediated by E2F1 and E2F3 [36, 38, 40], Raj and coworkers also demonstrated binding of E2F2 to a newly identified E2F-binding site within the *survivin* promoter [39]. This implicates a novel role for E2F2 rather than E2F1 or E2F3 [41] in the negative regulation of *survivin* expression in human melanocytes. Moreover, in this cell system, p53-dependent repression of the *survivin* promoter appeared to be independent of the retinoblastoma protein Rb [39]. Furthermore, a recent study demonstrated that hypermethylation of the *survivin* promoter in endometrial tumors is correlated with increased *survivin* expression. The authors speculated that DNA-methylation could inhibit the direct binding of p53 to the *survivin* promoter. In addition microarray data were presented, which suggested that de-repression by methylation is a general mechanism of p53 regulation [42]. In conclusion, there seem to be different mechanisms governing p53-dependent regulation of *survivin* expression which might be dependent on the cell type employed for the studies as well as on stress conditions affecting the cells.

In addition to these primary p53 target genes, for a couple of genes direct DNA-binding of p53 to the promoter region *in vivo* was demonstrated for example for the *p202*-[43], *PTTG1*- [44], *PRC1*- [45], *CHEK*- [44], *RAD51*-[46, 47], *hDDA3*- [48], *Hsp90 $\beta$* - [49], and *LASP1*-gene [50]. Here interaction of p53 with its consensus sequence leads to transcriptional repression. However, the underlying mechanisms were not investigated further.

**3.2. DNA-Binding-Independent Mechanisms.** Beside the mechanism relying on direct DNA-binding numerous target genes have been described where p53 regulates

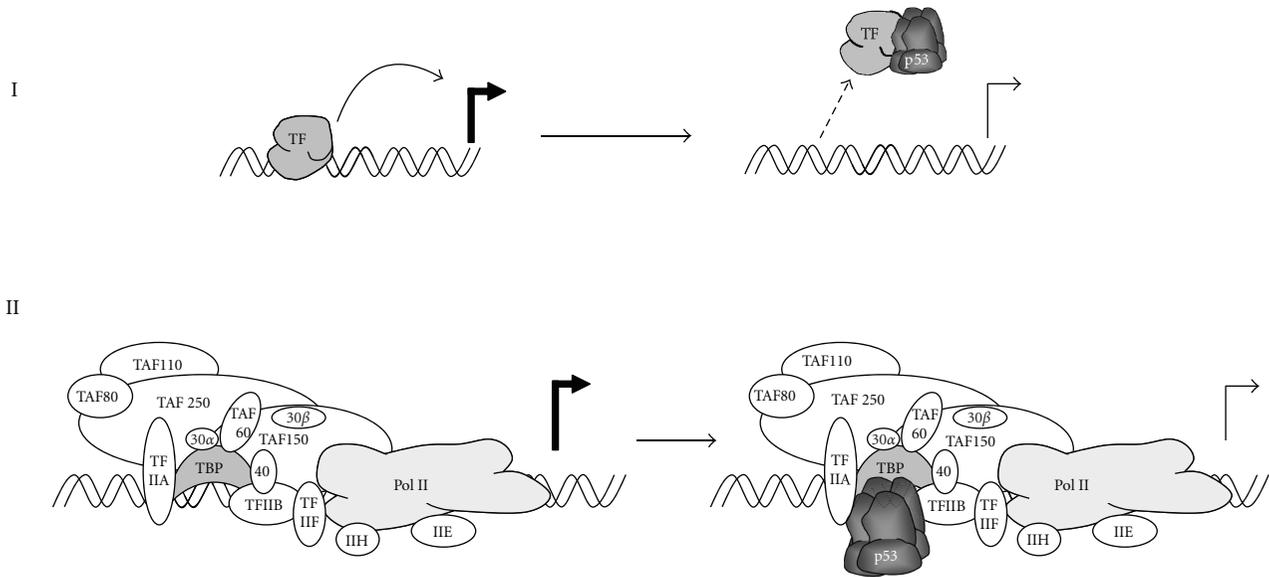


FIGURE 3: DNA-binding independent mechanisms. Mechanisms of p53-dependent repression independent of DNA-binding of p53. We classified these mechanisms roughly into two groups: one comprising interaction with other transcription cofactors (panel I), the second covering interaction of p53 with the basal transcription machinery (panel II). p53 has been shown to interact with TBP, TFIIA, TFIIB and/or diverse TAFs.

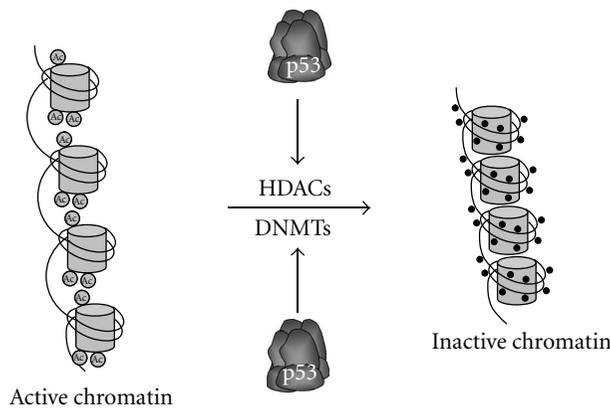


FIGURE 4: Regulation of chromatin structure and promoter methylation. p53 can downregulate target genes by recruitment of histone- and promoter-modifying enzymes or their mediators. A high number of genes were shown to be regulated, at least in part, by altering chromatin structure rendering the promoter regions inaccessible for the transcription machinery and cofactors.

promoter activity but direct DNA-binding is not required. We classified these mechanisms roughly into two groups: one comprises interaction with other transcription cofactors (Figure 3, panel I) or interaction with the basal transcription machinery (Figure 3, Panel II), the second covers regulation of chromatin structure and promoter methylation (Figure 4).

Different evidences show that p53 is able to bind to a variety of coactivators and interferes with their transactivator function. Promoter regulation of the *hTERT*- [51], *insulin receptor*- [52], *IGF-I receptor*- [53], *VEGF*- [54] and *hGR1*-genes [55] depends on binding of p53 to SP1. This protein-protein interaction impairs binding of SP1 to promoter DNA leading to decreased promoter activity. Binding of p53 to the promoter was not detected for these target genes.

It has been known for some time, that matrix metalloproteases are repressed by p53 [56] and mechanistic investigations had been carried out showing that p53 interacts with promoter-bound AP-1. Although p53 did not modulate AP-1 binding to its consensus sequence, the repressive effect was completely abrogated after deletion of the AP-1 binding site [57]. Thus, this repression mechanism seems to depend rather on the modulation of AP-1 activity than on competitive promoter-binding.

The CCAAT-Box binding factor NF-Y has been implicated in the repression of several cell-cycle genes. In addition to the *Cyclin B1* gene mentioned before, repression of its homologue, *Cyclin B2* [58], has been attributed to NF-Y function, too [31]. p53 binds to the NF-YC subunit of NF-Y which is recruited to cell-cycle promoters of the *Cyclin*

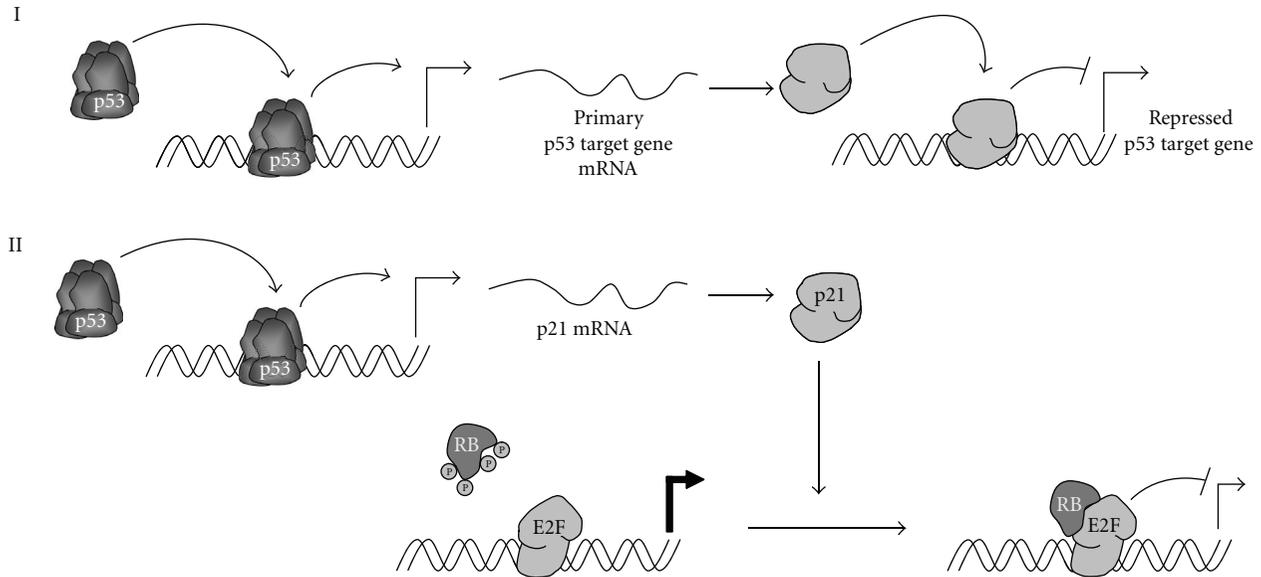


FIGURE 5: Indirect repression through regulation of other target genes. Indirect repression mechanisms are characterised by complete absence of p53 at the target gene promoters. It binds neither at the promoter itself nor at the periphery through protein-protein interactions. p53 regulates expression of numerous transcription factors and changes in their expression should have an impact on their target genes, too (panel I). Furthermore, the CDK-Inhibitor p21<sup>CIP1</sup> has been implicated in the repression mechanism of several genes (panel II). Increased expression of p21<sup>CIP1</sup> protein due to p53-dependent transactivation leads to inhibition of several CDKs. Consequently the retinoblastoma protein remains hypophosphorylated. Hence, instead of releasing E2F which can act as a transcriptional activator, hypophosphorylated RB remains complexed with E2F and functions as a repressor.

*B2*, *Cdc25C* and *Cdc2* genes. Following DNA-damage, p53 is acetylated and induces changes in chromatin structure through recruitment of histone deacetylases HDAC1, 4 and 5 [31, 34]. The NF-Y binding sites are essential for both, p53-dependent repression and HDAC-recruitment. These results indicate that repression of at least some cell-cycle promoters is dependent on intact binding of NF-Y to CCAAT-Boxes and in this context NF-Y bound p53 is part of a large chromatin remodelling repressor complex. Other experiments showed, however, reduced basal activity after mutation of the CCAAT-Boxes of the *Cyclin B2* promoter. Repression by p53, however, was only moderately impaired [58], which implies that there are different mechanisms leading to *Cyclin B2* repression. Another group showed that NF-Y binding to the *Cyclin B1* and *Cdc2* promoters itself is impaired after overexpression of p53 [59]. In addition, the transcriptional repression of the *Cdc2*-gene whose gene product interacts with Cyclin B to form the mitosis promoting factor (MPF) [60], depends essentially on NF-Y [61], too. Also the Checkpoint kinase *Chk2* [62] and the *securin* gene [63] are repressed by p53 in a NF-Y dependent manner. This mode of repression has also been demonstrated for the *topoisomerase II $\alpha$*  gene [64, 65].

Further transcription factors bound by p53 to induce transcriptional repression are c-myc [66], CBF [67], STAT5 [68], HIF1 $\alpha$ , [69], Estrogen Receptor [70] and the hepatic nuclear factors (HNF) 4 $\alpha$ 1 and 6 $\alpha$  [71, 72]. Transcriptional repression of target genes is mostly achieved through preventing the activating transcription factors from binding to the respective promoter. It applies to *HNF4 $\alpha$ 1*, that p53

also binds directly to the *HNF4 $\alpha$ 1* promoter and represses its activity which was in part relieved by inhibition of HDACs. In addition, p53 binds to the ligand binding domain of HNF4 $\alpha$ 1 and inhibits its transactivating function. Therefore, the regulation of the *HNF4 $\alpha$*  promoter involves different mechanisms. This illustrates a well-known feature of p53-function: p53 represses its targets often by interfering with their expression on multiple ways. In conclusion, the prevention of transactivators from binding to promoters by p53, either through protein-protein interaction or through competition for DNA binding, is a widespread mechanism which has implications on the expression of numerous genes.

Apart from the interaction with coactivators, different studies have shown that p53 interacts with the basal transcription machinery and thereby represses promoter activity [73–76] (Figure 3, panel II). For some target genes this mechanism was assumed, such as *MCL-1* [77], *Bcl-2* [78] and *Cox-2* [79]. Binding of p53 to TBP was shown to counteract its association to other components of the basal transcription machinery like TFIIA [80] and TFIIB [81]. The functional significance of this interaction of p53 and TBP was elucidated by Crighton et al. [81]. They found that p53 specifically binds to TBP and disrupts TBP-TFIIB interactions. This prevents TFIIB from binding to TFIIC2 which is a critical step for the assembly of transcription machinery on tRNA gene promoters. Additionally, the interaction between TFIIB and RNA polymerase III is also impaired. Finally, the amount of tRNA is markedly decreased after expression of p53 which is not bound to tRNA promoters thereby raising the question of how specific this repression mechanism is. The authors also

showed that TBP promoter occupancy after p53 expression is indeed lower at tRNA promoters but increased on the p21<sup>CIP1</sup> promoter region. So, p53-TBP interaction is involved in repression and transactivation as well. Another study, however, showed that p53-TBP interaction is not sufficient for repression of stimulated promoters. p53 was able to repress promoter activity stimulated by different coactivators in *Drosophila* Schneider cells, but overexpression of TBP did not rescue the inhibition of activated promoters [82]. Therefore, it is likely that there are other, maybe basal, transcription cofactors which are involved in the repression by transcription machinery interference. One proof of this principle was demonstrated by Gridasova and Henry who investigated repression of small nuclear RNAs U1 and U6 by p53 [83]. Intriguingly, they found that p53 is in fact recruited to the snRNA promoters but their repression is not dependent on that interaction. p53 binds to TBP and to snRNA-activating protein complex (SNAP<sub>C</sub>), a basal transcription factor specific for snRNA genes which binds to proximal sequence elements [84]. Therefore, SNAP<sub>C</sub> might guide p53 to snRNA promoters and cooperate in the repression mechanism.

**3.3. Regulation of Chromatin Structure and Promoter Methylation.** Regulation of epigenetic modifications on histones or promoters is a common mechanism for transcriptional control. p53 downregulates target genes by recruitment of histone- and promoter-modifying enzymes or mediators of these. A high number of genes were shown to be regulated, at least in part, by altering chromatin structure rendering the promoter regions inaccessible for the transcription machinery and cofactors (Figure 5). The *survivin* [85], *PSA* [86], *c-myc* [87], *AFP* [88–91] and *HNF4α* genes [71, 72] are regulated in part by recruitment of histone deacetylases (HDACs). In addition numerous cell-cycle genes like *Cdc2* [31], *Cyclin B2* [31, 34], *Microtubule associated protein 4 (Map4)* [92, 93], *Snk/Plk-akin kinase (SAK)* [94], *Mitosis Arrest Deficiency 1 (MAD1)* [95] and *Cdc25C* [31] are downregulated by this mechanism. *Cdc2* and *Cyclin B* are important for G<sub>2</sub>/M progression and their expression levels depend on NF-Y. As described above, p53 was shown to interact with promoter-bound NF-Y. After DNA-damage p53 becomes acetylated at the C-terminal domain and recruits HDAC1, 4 and 5 followed by decreased histone acetylation and promoter repression [31, 34]. In addition to chromatin modification, also promoter methylation by DNMT1 is involved into the repression of the *Cdc2* and *Cdc25C* gene [96]. Chromatin immunoprecipitations of Doxorubicin treated cells showed that the repressor complex which mediates downregulation of promoter activity contains p53, HDAC1, DNMT1 and correlated with dimethylated histone H3 (H3K9me2) but no NF-Y was detected. Furthermore, these promoters displayed internal CpG methylation in the vicinity of the p53 binding sites after DNA-damage and this methylation was dependent on p53 [35].

Depending on the target gene, the repressor protein complexes assembled may show different components. The repression of the *Map4* and *MAD1* genes [93, 95], for

example, is carried out by a complex including p53, HDACs and the corepressor mSin3a, which was shown to mediate the interaction between p53 and HDAC [92]. mSin3A also plays a pivotal role in the p53-dependent repression of other genes like *AFP* [26] and *c-myc* [87]. Ho et al. [87] observed that p53-induced G1-arrest is dependent on functional *c-myc* repression and this regulation is sensitive to the HDAC-inhibitor trichostatin A (TSA). Accordingly, the corepressor mSin3a is recruited on the *c-myc* promoter possibly for mediating the interaction with HDACs. In conclusion the repressor complexes which control chromatin structure of repressed target genes contain different components depending on the gene to be silenced. p53 is often directly associated with the promoter but may also be bound indirectly through interactions with other promoter-binding proteins.

#### 4. Indirect Repression through Regulation of Other Target Genes

For indirect repression, p53 binding to the promoter or to cofactors is not required (Figure 5). Indeed, p53 regulates expression of numerous transcription factors like TCF4 [97], ATF3 [98] or *c-fos* [99], and changes in their expression influence transcription of their target genes, too (Figure 5, panel I). One example of indirect p53-dependent repression is the regulation of the *Cyclin D1* gene by crosstalk with the NF-κB pathway [100]. A NF-κB p52/Bcl3-complex controls expression from the *Cyclin D1* gene and p53 inhibits expression of the Bcl-3 coactivator unit. Interestingly, the p52-subunit was required for *Cyclin D1* repression, suggesting that it acts as a repressor when Bcl-3 is absent. The p52-subunit is able to recruit HDAC1 to the *Cyclin D1* promoter and interactions of these proteins increase significantly after p53 induction [100]. So, this repression mechanism ultimately culminates in changes in chromatin structure which leads to transcriptional repression.

Special importance to gene repression by p53 has the target gene p21<sup>CIP1</sup>. This CDK inhibitor protein has been implicated in the repression mechanism of several genes like *CHK1* [101], *survivin* [36, 39], *topoisomerase IIα* [102, 103], *Cyclin B1* [36, 103–105], *Cyclin A2* [36, 103], *Cdc2* [36, 103, 104], *Cdc25A* [106, 107], *Cdc25C* [36], *hTERT* [108], *PLK1* [109], *Rad51* [103], the *MCM genes* [110] and *BRCA1* [36]. The p21<sup>CIP1</sup> protein was shown to inhibit several CDKs which are essential for cell-cycle progression. Usually, the retinoblastoma protein gets hyperphosphorylated by CDKs in G1/S-Phase of the cell-cycle which leads to release of bound E2F transcription factor complexes mediating transcriptional regulation of numerous genes during the cell-cycle. p21<sup>CIP1</sup> causes decreased CDK-activity leading to hypophosphorylated Rb which in turn remains complexed with E2F [111, 112] (Figure 5, panel II). Expression of E2F-dependent genes is therefore decreased as a consequence of p21<sup>CIP1</sup> expression. When p21<sup>CIP1</sup> is involved in the molecular mechanism, p53-dependent repression at the promoter level should depend on E2F-binding sites, which in fact was demonstrated for *survivin* [39], *hTERT* [108], *Cdc2*

[103, 104, 113], *Cyclin A* [114] and also suggested for *Cyclin B1* [103, 104].

Analyses of the *cdc2* promoter showed that its repression is dependent on both p21<sup>CIP1</sup> and the retinoblastoma proteins (Rb, p130 and p107). p21<sup>CIP1</sup> activity induces a p130/E2F4 complex which is recruited to the CDE/CHR elements of the *cdc2* promoter. Mutation of these elements impairs p53-dependent repression [113]. The remaining potential of p53 to repress the promoter independent of the CDE/CHR elements may be attributed to other repression mechanisms, for example the recruitment of chromatin remodelling enzymes with subsequent alterations in the chromatin structure. So *cdc2* is another example of a p53-target gene, where many different molecular mechanisms act together for mediation of p53-dependent repression.

Beside *cdc2*, p53-dependent repression of several genes is considered to rely on the presence of CDE/CHR tandem elements within the respective promoters [115]. *Plk1* and *topoisomerase IIa* genes were shown to be downregulated through CDECHR sites upon p21<sup>CIP1</sup> overexpression [103]. The CDE/CHR tandem element and p21<sup>CIP1</sup> are also found to be responsible for mediation of p53-dependent repression of the *Cdc20* promoter [116]. However, these results have been challenged by a recent study, demonstrating that under physiological levels of p53, neither p21<sup>CIP1</sup> nor the CDECHR elements are required for p53-dependent repression. Instead, the authors identified a p53-binding element further upstream in the *Cdc20* promoter as the major regulatory site [117].

Nevertheless, consistent with the findings of Kidokoro et al. [116] is the mechanism of transcriptional repression of G<sub>2</sub>/M genes after DNA damage postulated by Mannefeld et al., which require functional p21<sup>CIP1</sup> [105]. As described above, p21<sup>CIP1</sup> inhibits activity of CDK complexes, thereby triggering hypophosphorylation of retinoblastoma proteins and formation of repressive pocket protein-E2F complexes on E2F-dependent promoters. Mannefeld and coworkers found that in response to DNA damage binding of E2F4 and p130 to the recently identified E2F-pocket protein complex LINC/DREAM is induced and displaces transcription factor B-Myb from this complex. It is postulated, that the switch of LINC/DREAM associated proteins depends on the phosphorylation level of p130 and thereby on p21<sup>CIP1</sup> activity. The G<sub>2</sub>/M genes *Cyclin B1*, *CDC2*, *BIRC5* and *UBCH10* were shown to be regulated by this mechanism [105]. In addition to these G<sub>2</sub>/M genes participation of E2Fs and pocket proteins, especially p130, in DNA damage-induced inhibition of several other cell-cycle regulators is described [109, 113, 118, 119] suggesting that this mechanism is another widespread mode of p53-dependent repression.

A further example for p21<sup>CIP1</sup>-dependent downregulation is the *telomerase (hTERT)* gene. Mutational analyses of the promoter showed that an atypical E2F site in the 5'UTR region is responsible for the repression of this promoter. Cotransfection experiments with a dominant-negative E2F mutant showed that p53 is not able to repress *hTERT* promoter after inhibition of E2F activity. Furthermore, the knockdown of the Rb, p130 and p107 proteins significantly reduced the p53-mediated repression [108]. Therefore, the

p21<sup>CIP1</sup>-Rb-E2F axis controls repression of the *telomerase* gene after p53 expression. As the Rb-proteins were shown to recruit HDACs to target gene promoters which is critical for specific gene repression [120], the *hTERT* downregulation was investigated after trichostatin treatment. The inhibition of HDACs attenuated the repression effect of p53, indicating that histone deacetylation is involved in the repression of *hTERT* promoter [108]. As described above, the repression of *hTERT* was also demonstrated to be dependent on an interaction of p53 with the SP1 coactivator [51]. Again, there are different mechanisms which may act together to repress *hTERT* gene expression.

The expression of the cell-cycle phosphatase *Cdc25A* is also repressed by p53 [106]. We observed that *Cdc25A* repression after DNA-damage is abrogated after p21<sup>CIP1</sup>-knockdown. In consistence with that we and others found that translation is essential for transcriptional repression. Inhibition of translation via cycloheximide abrogates repression of *Cdc25A* and other target genes [121] indicating that other proteins have to be synthesised during p53 expression to take part in the repression mechanism (unpublished results). This suggests contribution of the p21<sup>CIP1</sup>-Rb-E2F pathway in the p53-dependent repression of *Cdc25A*. It was also suggested that p21<sup>CIP1</sup> directly binds as a transcriptional regulator to *Cdc25A* promoter DNA [107]. Its presence on DNA is associated with an inhibition of the recruitment of the p300 histone acetylase and with a downregulation of histone H4 acetylation [107].

In conclusion, gene regulation by indirect mechanisms is a common feature of p53 activity. In particular, p21<sup>CIP1</sup> is an important mediator of p53-dependent repression of many target genes, mainly by inhibition of cyclin dependent kinases provoking subsequent interference with the Rb-E2F-pathway.

## 5. p53-Dependent Repression by Regulation of Noncoding RNAs

In recent years, nonprotein coding RNAs (ncRNA) have emerged as novel regulators of different signalling pathways. Notably, ncRNAs regulate the p53 pathway upstream and downstream of p53. A transcript located antisense to the *p53* gene (*Wrap53*) is induced after DNA damage and directly interacts and stabilizes the *p53* mRNA [122]. Interestingly, the *p53* mRNA itself acts as a ncRNA by interaction with E3 Ubiquitin Ligase MDM2 [123]. This interaction contributes to inactivation of MDM2-mediated p53 degradation after DNA damage. Together, ncRNAs are an integral component of the p53 signalling network.

Regulation of ncRNAs by p53 has been investigated in detail for a class of small RNAs called miRNAs. Different groups showed that p53 upregulates genes of the miRNA-34 family [124–128]. MicroRNAs are small 21–25 nucleotide long noncoding RNAs which can regulate gene expression posttranscriptionally by interference with mRNA-translation or by induction of mRNA-decay [129, 130]. By proteomic studies it was demonstrated that one miRNA may have hundreds of targets [131, 132]. The p53 mediated repression

of gene expression by miRNAs is not carried out at the promoter level but leads to changes in mRNA- and protein-levels. Only recently, it was demonstrated that miRNAs primarily act through induction of mRNA decay and not through translation inhibition alone as anticipated earlier [133]. Interestingly, miRNA-34 induction promotes apoptosis and leads to repression of diverse genes involved in cell-cycle progression, apoptosis, DNA repair, and angiogenesis [127]. The regulation of miRNA-192 and -215 by p53 also influences cell-cycle progression [134, 135]. This function has been attributed, in part, to the miRNA-mediated induction of p21<sup>CIP1</sup> in cells after overexpression of miRNA-192 and -215. On the other hand, miRNA-mediated repression of cell-cycle genes may also contribute to cell-cycle arrest. miRNA-107 is transcriptionally induced by p53 and represses CDK6 and the Rb Related 2 (p130) [136]. Overexpression of this miRNA has been shown to induce a G<sub>1</sub>/S cell-cycle arrest [137]. An interesting aspect of miRNA-107 regulation is that this miRNA also influences the angiogenesis pathway by interfering with HIF1 $\beta$  expression [138]. This shows that p53-dependent regulation of one miRNA may have an impact on different signaling pathways. The miRNA-25, -93, -106b cluster, which is intronic to the *MCM7* gene, was shown to be repressed by p53. This repression is mediated by inhibition of E2F1 activity, which controls expression of the host gene *MCM7*. Overexpression of these miRNAs promotes cell proliferation and delays senescence [139]. Another example is the proto-oncogene *c-myc* which is repressed by different p53-dependent mechanisms. In one of these pathways p53 transcriptionally induces miRNA-145 which then represses *c-myc* expression [140]. In addition, *c-myc* is regulated by interference of p53 with components of the basal transcription machinery, as described above [80]. miRNA genes are also downregulated by this mechanism. The miR-17-92 cluster is transcriptionally downregulated under hypoxic conditions. p53 binds at a response element in the proximal promoter region in which this site overlaps with the TBP binding site. Thus, p53 displaces TBP at the miR-17-92 cluster promoter to repress transcription. Lower expression of these miRNAs assists in hypoxia mediated p53-dependent apoptosis induction [141].

In contrast to miRNAs, long noncoding RNAs are a heterogeneous class of transcripts which have diverse functions in different processes. Genome wide investigation of RNA expression and chromatin modifications has shown that there are over a thousand long intergenic ncRNAs (lincRNAs) in the human genome [142, 143]. A subset of these lincRNAs is regulated by p53. Only recently, lincRNA-p21 was discovered as a global regulator of p53-dependent repression in mouse. This RNA interacts with the heterogeneous nuclear ribonucleoprotein K (hnRNP-K) and is needed for recruitment of this complex to specific genomic sites of repressed genes [144]. Knock down of lincRNA-p21 prevented p53-dependent repression of many genes and also attenuated induction of apoptosis. These results show that regulated noncoding RNAs also have a great impact on gene repression by p53. It can be assumed that in the near future a multitude of other ncRNAs, which are involved in the p53-pathway, will be discovered.

## 6. Conclusion

Several genome wide expression analyses show that repressed genes make up a large proportion of p53-regulated targets [16, 121]. For example, 5-FU treatment of HCT116 cells results in p53-dependent regulation of 230 of 19,000 genes screened, only 41 of them were activated but 189 were repressed [44]. Results of another study suggest, that p53-induced repression plays a decisive role in cell-cycle control, as nearly half of repressed p53 targets are cell-cycle related [121]. However, genes affected by p53-mediated repression cover a wide range of cellular functions [145]. In addition to cell-cycle control and induction of apoptosis, metabolism, infection and immune response, adhesion, DNA replication and repair, cytoskeleton organization and cell signalling are affected [145]. However, the observed spectrum of target genes depends on the stimulus used [16].

As demonstrated here, the mechanisms leading to p53-dependent transcriptional repression are diverse. Generally, in contrast to activation there are many ways of p53-dependent repression. Direct mechanisms of target gene repression comprise DNA-binding-dependent mechanisms and DNA-binding independent mechanisms. In these cases, p53 acts directly on the target gene promoter by displacement of coactivators or general transcription factors. p53 can also be bound to the promoter through interaction with other DNA-binding proteins. A lot of target gene promoters are downregulated by changes in chromatin structure which leads to inaccessibility of the promoter region. The second large class of repression mechanisms comprises the indirect modes, that is, downregulation of promoter activity where p53-interactions at the target gene promoters are completely absent. Particularly, the relevance of the p53 target gene p21<sup>CIP1</sup> for this mechanism is often published. p21<sup>CIP1</sup> is thought to contribute to the p53-dependent repression mainly by inhibition of cyclin dependent kinases provoking subsequent interference with the Rb-E2F-pathway. In many cases there is more than one repression mechanism influencing the promoter activity of a target gene. It is likely that different mechanisms work synergistically together and that they may also compensate for each other.

This is an intriguing difference to p53-dependent trans-activation which is carried out in most cases by a similar mechanism. Why are there such diverse repression mechanisms? First of all, this diversity may contribute to a higher flexibility in terms of cell type and tissue-dependent regulation of target genes after cellular stress. p53 up- and downregulates different target genes in different tissues which leads to different physiological outcomes [146]. These tissue-specific adjustments of the p53 signalling pathway are achieved through modulation or binding to tissue-specific coactivators (e.g., liver specific downregulation of the *AFP* gene). Another reason for such a diversity of repression mechanisms may be the enhanced robustness in the signalling network in response to cellular stress. Aberrant changes in expression or mutation of single components of these pathways might have only a minor impact on the

stability of the whole system. However, mutation of p53 is widely observed in different tumors and some mutants may have even oncogenic functions. In conclusion, care has to be taken in respect to the different cell systems used for investigations, as this may have influences on the repression mechanisms. The heterogeneity and the mechanistic diversity of transcriptional repression is an interesting feature of p53 function making it impossible to assign one mechanism to one target gene. However, up to date there are a lot of other candidate genes that are repressed by p53, including *SDF-1* [147], *Interleukin-2* and *Interleukin-4* [148], *Interleukin-6* [149], *stathmin* and *FKBP25* [150], *PCNA* [151], *MGMT* [152], *Ets1* and *Ets2* [153], *Cks1* [154], *Cks2* [155], *BRCA1* [156, 157], *PIK3CA* [158], *hGRI* [55], *p202* [43], *RECQ4* [159], *Clusterin* [160], *PIQ* [161], *TCF4* [97], and *HMMR (RHAMM)* [162], but the detailed mechanisms of their regulation are not yet clear. As expression of p53 leads to cell-cycle changes it is difficult to determine for some genes whether their regulation is the cause or the consequence of cell-cycle arrest. As outlined above, several cell-cycle genes are efficiently repressed by p53 and their expression pattern might additionally be influenced by cell-cycle arrest.

In addition to proteins, noncoding RNAs have evolved as important components of the p53 network. Many miRNAs are regulated by p53 and may have an impact on hundreds of other proteins in the cell. The mechanisms leading to transcriptional repression mediated by long noncoding RNAs are only beginning to emerge. Many of them interact with chromatin modifying complexes and target them to specific sites in the genome. p53-induced lincRNA-p21 recruits hnRNP-K to genes which are repressed in a p53-dependent manner and this pathway is important for apoptosis induction. To date, the underlying mechanisms of this regulation pathway for specific targets are not clear and more research is needed in order to elucidate the contribution of many other ncRNAs to the p53-dependent gene regulation.

The knowledge of repressed target genes and the complexity of the signalling pathways leading to transcriptional downregulation is rapidly expanding due to new data provided by genome-wide transcriptome profiling and ChIP-Seq methods. More insights into the different mechanisms may enable the researchers in the future to predict repression mechanisms on the basis of promoter sequences. The elucidation of the complete p53-“regulome” is important for our understanding of its tumor suppressor function and understanding its mechanisms of gene regulation represent also the key for therapeutic approaches like the reconstitution of wild-type functions in tumors where p53 was mutated.

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