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Review

Reverting p53 activation after recovery of cellular stress to resume with cell cycle progression

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ABSTRACT

The activation of p53 in response to different types of cellular stress induces several protective reactions including cell cycle arrest, senescence or cell death. These protective effects are a consequence of the activation of p53 by specific phosphorylation performed by several kinases. The reversion of the cell cycle arrest, induced by p53, is a consequence of the phosphorylated and activated p53, which triggers its own downregulation and that of its positive regulators. The different down-regulatory processes have a sequential and temporal order of events. The mechanisms implicated in p53 down-regulation include phosphatases, deacetylases, and protein degradation by the proteasome or autophagy, which also affect different p53 protein targets and functions. The necessary first step is the dephosphorylation of p53 to make it available for interaction with mdm2 ubiquitin-ligase, which requires the activation of phosphatases targeting both p53 and p53-activating kinases. In addition, deacetylation of p53 is required to make lysine residues accessible to ubiquitin ligases. The combined action of these downregulatory mechanisms brings p53 protein back to its basal levels, and cell cycle progression can resume if cells have overcome the stress or damage situation. The specific targeting of these down-regulatory mechanisms can be exploited for therapeutic purposes in cancers harbouring wild-type p53.

Contents

Abbreviation: AMPK, AMP - activate kinase alpha 1; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related protein; CHK2, checkpoint kinase 2; CHK1, checkpoint kinase 1; CDK, cyclin-dependent kinase; CDKN2A, cyclin dependent kinase inhibitor 2A; DNA-PK, DNA-activated protein kinase; DRAM, damage-regulated autophagy modulator; DUSP4, dual specificity phosphatase 4 (MKP2); DUSP6, dual specificity phosphatase 6 (MKP3); DYRK2, dual specificity tyrosine phosphorylation regulated kinase 2; ERK2, extracellular signal-regulated kinase 2 (MAPK1); GRWD1, glutamate rich WD repeat containing 1; HDAC, histone deacetylase; Hdm2, human double-minute 2; HIPK2, homeodomain interacting protein kinase 2; JNK2, c-jun n-terminal kinase 2 (MAPK9); LSD1, lysine demethylase 1 (KDM1A); MAPK1, mitogen-activated protein kinase 1; Mdm2, murine double-minute 2; MKP2, mitogen-activated protein kinase phosphatase 2 (DUSP4); PKCδ, protein kinase C δ; PP, phosphatase; PPM1D, protein phosphatase, Mg2+/Mn2+ Dependent 1D (WIP1); RPL11, ribosomal protein L11; RUNX2, runt related transcription factor 2; SIRT1, sirtuin 1; TBK, TANK binding kinase 1; VRK1, vaccinia-related kinase 1; WIP1, wild-type p53-induced phosphatase (PPM1D).

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1. Introduction

Cells undergoing any type of stress, including DNA damage, have to react individually, and independently, of cell cycle progression and these reactions are mediated by p53 that functions as an integrator of multiple cellular responses to cellular stresses [1,2]. The cellular responses mediated by p53 can have an effect at both the cellular and organism levels. First, each individual cell within a tissue has to react on its own to the specific stress to which it is exposed. The initial protective response consists in the arrest of the cell cycle to allow specific repair processes to solve the problem. In those cells that cannot cope with cellular stress or damage, the p53 response triggers cell death and in that way protects the organism from the expansion of a damaged cell population, which is an important component in cancer development. Thus, mutations or deletions of *TP53* can facilitate tumour development [3]. The p53 protein has a regulatory role in all biological processes that contribute to cancer hallmarks [4], including cell proliferation, genomic stability, cell death, senescence, hypoxia, angiogenesis, and tumour metabolism in which it plays different roles [5]. The complexity of these roles requires very fine regulatory mechanism controlling p53 protein levels and functions. The activated p53 protein channels distress signals towards the selection of the appropriate response pathway in order to initiate specific cellular reactions, aiming either to protect either the individual cell or the organism. Therefore, the p53 protein is an intermediate switch, which is activated by multiple signalling pathways and mediates selection of specific cellular responses, which represent their biological outputs [6]. The change in p53 protein level and its pattern of posttranslational modifications regulate and determine the specific biological responses mediated by the activation of p53. These p53 changes determine the selection of interacting proteins, transcriptional cofactors and gene promoters [7]. In normal growth conditions, the level of the p53 protein undergoes temporal fluctuations that may be important for specific roles within a tissue, normal or tumoral, and it can also vary among cells forming the affected tissue, which reflects the individual cell situation [8,9]. In response to cellular stress, the level of the p53 protein raises immediately because of its stabilization by phosphorylation. This increased in p53 level triggers its biological effects, mostly as a consequence of p53-dependent activation of gene transcription [10,11], so that cells are able to start an immediate and specific response [12–14]. These fluctuations are underlined by a complex network of autoregulatory loops [15].

The 53 activation, if sustained in time, will cause a stable accumulation of p53 protein that is incompatible with cell life. When the cellular protective response to stress is completed, p53 has to return to its basal protein level so that cells can resume with their normal functions. Therefore, once the protective actions have been successfully performed, cells need to have active mechanisms that will revert the accumulation of p53. These p53 down regulatory mechanisms are late events in the cellular response to stress, and are a direct consequence of an activated p53. Because of these roles, the accumulation of p53 protein has to be necessarily transient, so that cell viability can be maintained. In this context, mechanisms that participate in p53 downregulation have not received as much attention as its activation, but they play a fundamental role in the cycling behaviour of p53 and form a complex network of regulatory mechanisms.

2. P53 activation mechanisms

2.1. Importance of p53 levels to trigger different biological effects

Any response to stress has an immediate initial phase that cannot depend on de novo gene transcription and translation, because this will require several hours, and by that time the consequences of cell damage will accumulate, and might become irreversible. However, all cells in order to initiate an immediate response to any type of stress must have a basal p53 level. In the basal situation of non-stressed cells, the level of p53 protein is very low, and in this readiness state p53 is forming a stable basal complex with VRK1 [14], the most abundant nuclear kinase [16]. The level of p53, its posttranslational modifications and protein-protein interactions determine the specificity of the cellular response to stress. Within a tissue, the individual cell situations are different, but they will have to react to a common type of stress. In a tissue there are dividing and non-dividing cells might react differently, and cells differ in their local microenvironment and cellular interactions that can be homotypic or heterotypic or stroma. There are also known variations in the context of cell cycle phases [17]. Therefore, even in a tissue exposed to a common stress signal, for example in skin exposure to UV damage, each cell has to respond individually. The different mechanisms contributing to p53 regulation are summarized in Table 1.

2.2. Structural bases of p53 activation and its selection of transcriptional cofactors

The p53 protein has two different binding modes whose roles are determined by the phosphorylation state of the N-terminal trans-

Table 1
Mechanisms involved in p53 regulation.

Effect	Consequence	Mediator
Direct regulation of p53 by covalent modification		
Phosphorylation of p53 by kinases	Stabilization of p53 by preventing interaction with mdm2/Hdm2	Kinases: ATM-CHK2 ATR-CHK1 VRK1
	Contributes to specificity of the selection of transcriptional coactivators	DNA-PK HIPK2
	Partial exit of p53 to cytosol	
Acetylation of p53	Required for transcriptional activity of p53	Acetylases: PCAF P300
Sumoylation		
Ubiquitylation	Binding to ubiquitin ligases and proteasomal degradation of p53	Ubiquitin ligases: mdm2/hdm2
Indirect regulation of p53: control of genes and proteins that affect p53		
Expression of ubiquitin ligase genes	Targeting p53 or its activating cofactors	Mdm2/hdm2
Deactivation of p53 activating-kinases	Targeting VRK1 and ATM	Phosphatases Ubiquitin ligases
Gene expression of p53 negative regulators	Regulation of phosphatase and deacetylase genes	Phosphatase genes Deacetylase genes
Proteolytic removal of activating-kinases	VRK1	Autophagy genes lysosome

activation domain of p53 (Fig. 1). The dephosphorylated state of p53 is required for its interaction with ubiquitin ligases, such as mdm2/hdm2, that will tag dephosphorylated p53 molecules with ubiquitin and is followed by proteasome mediated degradation and down-regulation [8,18]. The phosphorylated state of p53 determines the binding and selection of transcriptional cofactors [19] that will result in expression of one gene or another depending on the particular context of the cell and the type of stress [20].

Binding to ubiquitin ligases only occurs with a p53 molecule dephosphorylated in its N-terminal transactivation domain, so that its α-helix has the adequate folding to present a hydrophobic side required for interaction with mdm2 [21]. The alteration of this α-helix mediates the switch from a non-binding mode to a binding mode to facilitate specific interactions and selection of transcriptional factors that will also regulate specific genes. The binding to transcription factors requires a phosphorylated p53 in several N-terminal amino acids that affect the selection of binding partners [22,23]. Transcriptional activation of p53 is mediated by phosphorylation of its N-terminal transactivation domain mediated by several kinases that target specific p53 amino acids

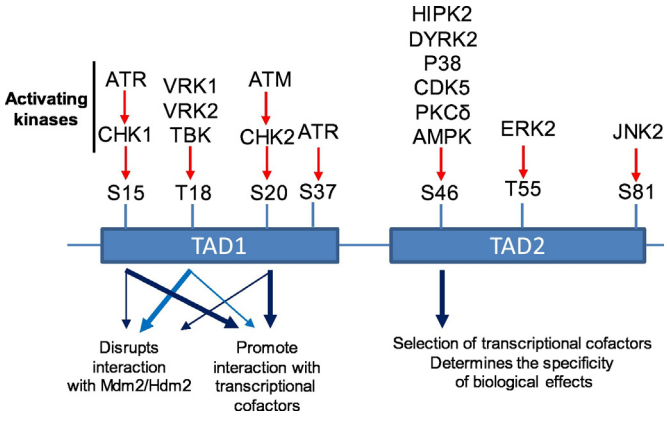

Fig. 1. Structure of the transactivation domain of p53 with the residues phosphorylated. The kinases identified phosphorylating specific p53 residues are indicated at the top.

[7,24] (Fig. 1) participating in different signalling pathways [7]. P53 is phosphorylated in multiple residues within its N-terminal domain, but the combinatorial patter of phosphorylation differs depending on the type of stimuli [25]. The phosphorylation profile of p53 is a determinant of its specific effect, and depending on the residue phosphorylated, the functional implications are different [26].

The initial switch from binding to mdm2 or to transcriptional cofactor is mainly mediated by a unique and specific phosphorylation of p53 in Thr18, which is required to maintain a p53 α-helix with a hydrophobic side that fits within a hydrophobic pocket in mdm2 [21]. A hydrogen bond between Thr18 and Asp21 maintains the stability of this α-helix [21], which is disrupted by phosphorylation. This Thr18 is the critical residue for switching between binding mdm2 or to binding transcriptional cofactors [13,19]. The phosphorylation of p53 in Thr18 mediated by the VRK1 Ser-Thr kinase [12,27]. VRK1 is a chromatin kinase [28–30] that VRK1 is activated by serum [28,31] and is also involved in DNA damage responses to different types of DNA damage [14,28]. *VRK1* is cell cycle regulated kinase and behaves as an early gene, like *MYC* and *FOS*. VRK1 also regulates several transcription factors including p53 [12,27,32], c-Jun [33], ATF2 [34], and CREB1 [35]. Thus, the early activation of p53 by VRK1 is very suitable to make the initial decision, either inducing cell cycle arrest or cell death, depending on the individual cell situation. Also VRK1 is activated by similar types of stress as p53, including DNA damage by ionizing radiation, chemotherapy [28] or ultraviolet light [36]. In this context, VRK1 is a suitable candidate for this early role in p53 reactions and it forms a stable basal complex with in non-stressed cells in which VRK1 interacts with the DNA binding domain of p53 and that is phosphorylated immediately in response to DNA-damage [14], and which is independent of the type of DNA damage [28].

Additional p53 phosphorylation in residues Ser15 and Ser20, mainly by the ATM-CHK2, DNA-PK and ATR-CHK1 pathways, regulate the selection of specific transcriptional cofactors, including acetyl transferases [19], which have been extensively studied and reviewed [7,24,37].These additional residues have different kinetics of phosphorylation depending on the type of DNA damage [25].

3. Steps for reversion of p53 activation and accumulation

After a successful completion of the repair responses mediated by p53, it is necessary the reversion of the activated state of p53 in order to be able to resume with cell cycle progression and normal cell functions, otherwise cells will enter into an irreversible cell cycle arrest or die. In this context, the activated p53 molecule is a key regulator and target of its own downregulatory mechanisms. The activated of p53 has two different types of activating covalent modifications, phosphorylation and acetylation and both modifications have to be removed before p53 protein can become a substrate for downregulatory mechanisms. The role of activating kinases is the best known process [7]. In addition, different regulatory posttranslational modifications occur at the C-terminal p53 domain, including acetylation and ubiquitylation that have functionally opposite roles. While acetylation is associated to activation of transcription factors such as p53 [38], ubiquitylation is a signal for downregulation of p53 [39]. Thus, in order to down-regulate p53 its dephosphorylation and deacetylation have to occur before its degradation by ubiquitylation can take place. Reversion of p53 activation requires the participation of several and different regulatory mechanisms that affect both genes and proteins that directly regulate p53, and indirectly by acting on its additional regulatory proteins (Table 1).

The p53 downregulatory mechanisms require a sequential order for p53 inactivation. The order is dephosphorylation of p53 and of its activating kinases, followed by deacetylation and finally ubiquitylation of p53. Initially, dephosphorylation and deacetylation of active p53 have to occur before p53 C-terminal lysine residues can ubiquitylated. In this context, there are three main levels of action: a) p53 activation of gene expression of phosphatases, deacetylases and ubiquitin ligases;

b) Enzyme activation of phosphatases, ubiquitin ligases and proteases; c) Targets of phosphatases, ubiquitin ligases and deacetylases, which include p53 or p53-activating kinases.

4. The role of phosphatases: activated p53 induces gene expression of phosphatases that directly or indirectly dephosphorylate p53

The first requirement in the reversion of an activated p53 is the need for dephosphorylation of p53 in those residues located in its transactivation domain that interfere with its binding to hdm2 and thus making it accessible for ubiquitylation. Moreover, the inactivation of p53 activating kinases is also necessary to prevent p53 rephosphorylation.

The p53 protein is only accessible to the mdm2/hdm2 ubiquitin ligase when it is in a dephosphorylated state, which is necessary for its degradation in the proteasome after ubiquitylation by the Hdm2 ubiquitin ligase [40]. Active and phosphorylated p53 has altered the structure of its N-terminal alpha helix required for its interaction with hdm2 [21,41]. Temporally, this dephosphorylation has to occur sometime after p53 phosphorylation in order to permit p53-dependent activation of transcription and its specific biological effects. The p53 deactivation by dephosphorylation requires acting at two different levels; one is by direct dephosphorylation of the phosphorylated p53 protein or alternatively it is and indirect effect that is performed by the inactivation of the kinases that target either p53 or p53-activating kinases, such as ATM and VRK1, in order to prevent a continuous p53 rephosphorylation. Thus, in this downregulatory situation, dephosphorylation is delayed and the participating phosphatases might be regulated by p53 itself, and in that way the sequential action can be achieved and two different time windows for p53 effects are synchronized. There are ninety phosphatases either Ser-Thr phosphatases or dual phosphatases targeting Tyr, Ser and Thr residues [42]. Ser-Thr phosphatases belong to three main groups. Type 1 phosphatases belong to the PP family (PP1), which have two subunits that directly interact and form a heterodimer with a highly conserved catalytic subunit and a regulatory subunit that determines the substrate specificity. The PP type 2 family, which includes PP2A, PP4 and PP6, are phosphatases in which the interaction between the catalytic and regulatory subunits is indirect and mediated by a scaffold protein. There is a third PPM separate family of phosphatases, which does not require a regulatory subunit, and only has the catalytic subunit that requires magnesium. The representative member is Wip1 (PPM1D). There is an additional and separate group of dual phosphatases (DUSP) phosphatases, composed of twenty-five members, and which dephosphorylate Tyr or Ser-Thr residues [43,44]. All these four types of phosphatases are involved in the regulation of p53 or its activating kinases. Activated p53 control the expression of phosphatase genes and in that way, the immediate effect of p53 phosphorylation and the later expression of phosphatase genes generate a time window in which p53 exerts its protective functions followed by the reversal of its effect after solving the stress situation.

4.1. P53 regulates gene expression of phosphatases that target p53 protein or p53-activating kinases

The phosphorylated p53 protein activates the transcription of several phosphatase genes as part of the stress response. However, phosphorylated p53 is also a protein target of these phosphatases. The role of these phosphatases is described below in connection with p53. There are two levels of regulation; one is phosphatase gene regulation at the start of the stress response mediated by p53. The other occurs later as part of the stress signal downregulation, which comprises the phosphorylated targets of the phosphatase protein, which includes both p53 and deactivation of kinases involved in activation of p53.

Within cells, p53 is always in the presence of very high levels of VRK1, one of the most abundant nuclear kinases [16] and that is activated by DNA damage [28,29,45]. Therefore, even if p53 is dephosphorylated the kinase can rephosphorylate it, and contribute to the maintenance of a p53 basal pool ready that is ready for reacting to any stress situation [14]. However, after the response to stress activation and solution of the problem, it is also necessary the down-regulation of kinases phosphorylating p53 that is mediated by p53-dependent mechanisms. The p53-activating kinases can be downregulated by several different mechanisms, which overlap with those dephosphorylating p53. Some are immediate by direct dephosphorylation of specific residues by phosphatases, or indirectly by degradation of the protein kinase by either the proteasome or autophagosome. The evidence for the contribution of specific phosphatases to downregulation of p53 is very heterogeneous.

4.2. PP family

4.2.1. PP1 and p53

Protein phosphatase 1 (PP-1) plays an important role in cell survival, which is partly mediated by its contribution to dephosphorylation of p53, particularly of residues Ser15 and Ser37, and in that way negatively regulates p53 dependent pathways [46].

4.2.2. PP2A, mdm2 and ATM

PP2A has a dual role by regulating p53 and making it accessible for degradation, or alternatively by inactivation of its negative regulator mdm2 contributes to p53 stabilization. PP2A dephosphorylates at least six different p53 phosphopeptides [47] in cells that were transformed with the SV40 large T antigen. The activity of PP2A is regulated by the interaction between its B subunit and cyclin G, a complex formed after induction of p53 activation [48]. In addition, PP2A can also interact with hdm2 and induces its dephosphorylation in T216 and of S166 [49]. Phosphorylated mdm2 is unable to degrade p53. Consequently, PP2A can also indirectly regulate p53 by facilitating the effect of mdm2/hdm2. Furthermore, PP2A [50], and PP5 [51], also dephosphorylate and inactivate ATM, a p53 activating kinase, as part of DNA damage checkpoints.

4.2.3. PP4 and cell cycle

PP4 has an essential role for reentry in the G1 phase of the cell cycle [17]. This effect of PP4 is indirect and mediated by dephosphorylation of S473 in Kruppel-associated box domain-associated protein 1 that represses p53-dependent transcriptional activation of p21 [17]. This step is negatively controlled by cell-cycle checkpoints that are regulated by activated p53 and some of its transcriptional targets, such as p21 or p16. Thus, dephosphorylation of p53 will result in downregulation of cell cycle inhibitors and permit progression to G1/S or G2/M.

4.3. PPM family of phosphatases

4.3.1. Wip1(PPM1D) on p53, ATM, ATR/CHK1 and p38/CDKN2

Different types of DNA damage including ionizing radiation, ultraviolet light, oxidative stress by hydrogen peroxide and some DNA damaging drugs activate p53, which induces *PPM1D* gene expression that codes for the Wip1 serine-threonine phosphatase [52]. The Wip1protein has significant homology to type 2C protein phosphatases, and is magnesium dependent but insensitive to inhibition by okadaic acid [52]. Wip1 is needed for re-entry in G2 [17]. Wip1 targets both p53 and p53-activating kinases. Wip1 dephosphorylates Ser33 and Ser46 in p53, which are induced by UV light [53], and Ser15 [54]. Wip1 also dephosphorylates Thr380 in p38, a p53 activator, and thus permitting a return of p53 to its basal state [53,55]. Wip1/PPM1D also dephosphorylates and deactivates ATM and ATR serine/threonine kinases [56–58]. Moreover, accumulation of Wip1 also deactivates Chk1 by dephosphorylation of Ser345 [54]. These dephosphorylations reduce the S and G2/M checkpoints in response to DNA damage that activates p53 [17,54]. Cells return to their normal homeostasis, in situations of replicative stress, partly by this mechanism. In ovarian cancer, Wip1 confers resistance to *cis-*

platinum treatment by this reduction in p53 and Chk1 phosphorylations, which makes cells less sensitive to induction of apoptosis, which is manifested as resistance to treatment [56]. Thus, high levels of Wip1 are oncogenic because of the down-regulatory role of p53 protective responses, and thus there is a failure in induction of cell cycle arrest and apoptosis. But in cells lacking p53, the overexpression of Wip1 confers sensitivity to cell death by dephosphorylation of RUNX2 phospho-S432, which resulted in increased expression of Bax [59]. The loss of Wip1 sensitizes cells to stress and DNA damage induced apoptosis by lowering its threshold [60]. Furthermore, a reduction of Wip1 results in an increased activity of p38, which activates the expression of *CDKN2A* tumour suppressor gene (p16 and 19). This effect reduces the formation of tumours in murine mammary tumourigenesis [61]. In addition, the removal of the Wip1 phosphatase reduces the activation threshold of p53 in APC deficient mice [62], a consequence of accumulating an active p53 that can induce apoptosis leading to a reduction of the stem cell pool and consequently to a reduction in polyp formation [62]. Therefore, there is a complex regulatory interaction between WIP1 and p53 than cab have different outcomes depending on the particular situation of the cell.

4.3.2. WIP1 on HIPK2

The kinase HIPK2 phosphorylates p53 in Ser46, a phosphorylation that has been associated to the induction of apoptosis and a reduced cell viability [63]. In this context, downregulation of HIPK2 causes a resistance to cisplatin-resistant in bladder carcinomas, both in vitro and in vivo [64]. This phosphorylation facilitates the exit of p53 to cytosol, where it can also induce apoptosis independent of gene transcription [65]. Overexpression of HIPK2 overcomes resistance and sensitizes cells to treatment in two ways; one is by direct phosphorylation and activation of p53, and the other by phosphorylation of WIP1 that facilitates its accessibility to proteasomal degradation and thus cannot dephosphorylate p53 and other activating kinases. In this way HIPK2 overexpression potentiates the defensive role played by activated p53 [64]. This loop is also regulated in a temporal order by the phosphorylation of HIPK2 by ATM that results in the inactivation of HIPK2, and permits the reactivation of WIP1 that will down-regulate ATM and p53 [66].

4.4. DUSP family of dual phosphatases

4.4.1. DUSP4/MKP2 on p53 and VRK1

The promoter of the *DUSP4* gene (MKP2) has a novel palindromic p53-response motif [67] is activated by phosphorylated p53 [67]. The expression of *DUSP4* is important for the induction of apoptosis, but it has no effect on the p53 role in cell cycle [67]. These results indicated that p53 induces a different set of genes depending on the pathway triggered. Furthermore, overexpression of *DUSP4* is able to facilitate apoptosis in a p53 independent manner [67]. However, the phosphorylated proteins targeted by DUSP4/MKP2 are not well known. One of them is the VRK1 kinase that interacts with MKP2 [68] and inhibits the kinase activity of VRK1 [68]. Once MKP2 is present in the cell it can down-regulate VRK1 [68], probably by its dephosphorylation, since VRK1 is extensively phosphorylated in its active state [27,28]. The down-regulation of VRK1 activity by MKP2o can indirectly prevent further activation of p53. However, no phosphatase has been identified or tested on p53-Thr18 phosphorylation [7]. The loss of p53 phosphorylation will facilitate its interaction with mdm2. Alternatively, MKP2 might alter the pattern of phosphorylated residues in p53 and in part contribute to induction of apoptosis, but not to cell cycle arrest, because of differential selection of transcriptional cofactors. The different functions of p53 have different thresholds and are a consequence of different phosphorylation patterns and protein levels [13].

4.4.2. DUSP6 loss affect ATK-CHK2 and p38

The activation of the ATM/CHK2 pathway, which phosphorylates p53 in Ser20, mediated part of the cellular response to DNA damage. Activated p53 induces the expression of *DUSP6* [69]. The loss of DUSP6 causes an accumulation of phosphorylated CHEK2 and regulates drug sensitivity in DDR by increasing the cytotoxicity of EGFR inhibitors [70]. Nevertheless, the effects of DUSP6 loss are complex, since there is also an activation of p38 [70], and the balance among different signalling pathways have not been characterized.

4.4.3. Dephosphorylation of p53-activating kinases

Other mechanisms have a delayed effect and are those requiring de novo gene expression and protein synthesis, which leave open a time window for other effects in this intervening period. Among the p53-activating kinases are ATM-CHK2, ATR-CHK1, VRK1, HIPK2 and JNK, that target different residues in the p53 N-terminal domain [7,24], but the functional interplay among them is not well known [7]. The targeted residues in p53 have different phosphorylation kinetics depending on the type of damage to which cells were exposed [25]. The activation of p53-activating kinases requires a phosphorylation, which is triggered by different signalling pathways in response to different cellular stimuli. As part of their autoregulation, deactivating phosphorylation of phosphatases is mediated by several p53-activating kinases including ATM-CHK2, ATR-CHK1, VRK1 and HIPK2. The dephosphorylation of p53, or its activating kinases, requires the participation of phosphatases belonging to several groups. However, their interdependence is unknown. Some directly target p53, but p53 also regulates their gene expression resulting in autoregulatory loops with a temporal organization for their effects.

5. Inactivation of kinases by proteolysis

An alternative mechanism of p53 functional inactivation implicates the active proteolytic degradation of p53-activating kinases. Proteolysis is an additional mechanism by which kinases are degraded that has received relatively little attention as downregulatory mechanism. In cells, kinases might be activated in response to a variety of mechanisms, such as mitogenic signals, as is the case of VRK1 that is activated by serum [31] and DNA damage [14,28]. VRK1 forms a pre-assembled basal complex with p53 that is activated in response to UV-induced DNA damage and results in DNA-damage dependent phosphorylation of p53 in Thr18 [14]. Activated p53, in addition to induction of *MDM2* expression, also induces the expression of *DRAM* (death regulated autophagic modulator) [71]. DRAM contributes to the removal of VRK1 by facilitating its degradation in cytosolic lysosomes [36,72]. This mechanism is sensitive to knockdown of either DRAM or Beclin-1, and is inhibited by lysosomal inhibitors [36]. By this mechanism, p53 induces proteolytic degradation of VRK1 and in that way facilitates the restoration of p53 protein to its basal level, which is the result of the balance among the different regulatory signals, including dephosphorylation and ubiquitylation (Fig. 2).

6. Deacetylation of p53

Several p53 lysine residues are the targets of different modifications, mainly ubiquitylation or acetylation (Table 2). Each of these modifications has a different, usually opposed, functional role that affect a common lysine residue. Acetylation is associated to p53 transcriptional activation and requires a previous phosphorylation. Ubiquitylation is associated to p53 downregulation in the proteasome and requires deacetylated lysine residues. The acetylation of p53 prevents its ubiquitylation by mdm2 [73]. Therefore, dephosphorylation of p53 will facilitate the loss of acetylation that makes lysine residues available for ubiquitylation and in that way reverts p53 responses roles [74]. Acetylation, like ubiquitylation, takes place in the p53 C-terminal domain and affects common residues [7]. Acetylation plays a key role in activation of p53 transcriptional roles, such as that of p21 [75] and others such as ubiquitin, are marks for specific degradation pathways. In this context downregulation of p53 should involve, not only N-terminal dephosphorylation, required for binding to mdm2, but also the

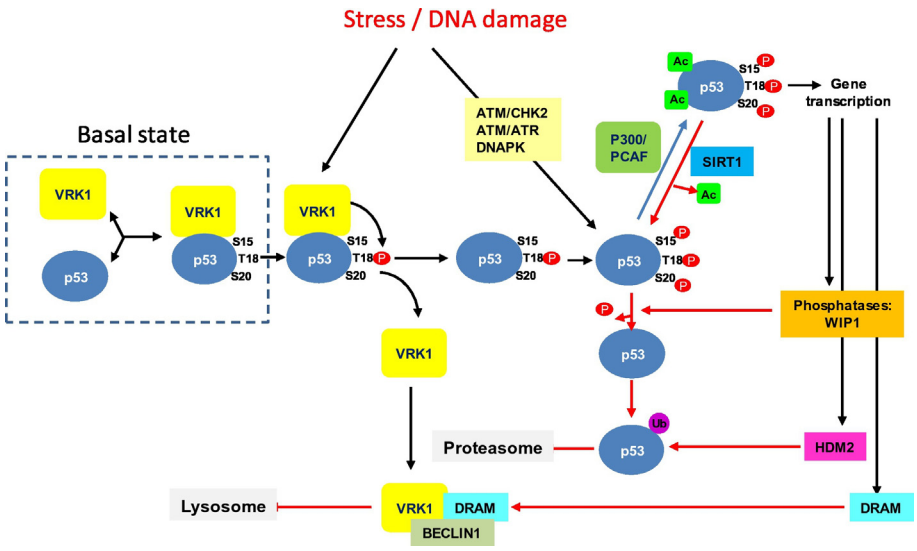

Fig. 2. Model of the sequential modifications and implication of p53 as a function of time after induction of DNA damage or cellular stress centred on the VRK1 activating-kinase that mediates the switch from binding to either ubiquitin ligases or transcriptional cofactors.

removal of C-terminal marks since both, acetylation and ubiquitylation, modify basic residues as lysines and arginines. In the p53 C-terminus several lysine residues are both acetylated or ubiquitylated (Table 2). These include K320 deacetylated by HDAC1, K370 by LSD1(KMD1), K373 by HDAC1 and K382 by HDAC1 and SIRT1. These deacetylases have to target p53 before their ubiquitylation can take place. Therefore, activation of histone deacetylases (HDAC) also facilitates downregulation of p53 activity, probably by indirectly switching p53-dependent promoters to an inactive state. In this context, activated p53 should regulate expression of deacetylase genes. Deacetylases targeting p53 have been mostly studied in different biological processes, such as chromatin remodelling and DNA damage responses [76] and are targets of novel inhibitors used for cancer treatment [77].

6.1. Sirtuins (transcription factor deacetylases) have an oncogenic role

Before p53 is targeted for degradation, it needs to be deacetylated and this deacetylation permits that shared lysine residues become available for ubiquitylation. Sirtuins are the most likely candidates to participate in this deacetylation, particularly SIRT1, which is the main sirtuin deacetylase in nuclei [78]. SIRT1 is also implicated in responses to oxidative stress, oncogenic stimulation and genotoxic responses [78], all functions in which p53 participates [6,20].

SIRT1 is overexpressed in many tumours and behaves like an oncogene [78], because deacetylation of p53 prevents its transcriptional activity and consequently there are no damage responses. This is conceptually analogous to overexpression of Hdm2 in tumours, behaving like an oncogene, where it facilitates p53 degradation and thus a loss of p53 functions [39]. P53 is acetylated in K120, a residue important for apoptotic signalling, but binding to HDAC5 prevents this acetylation [79]. SIRT1 activity and specificity is regulated by its phosphorylation mediated by cyclinB/CDK1 [80], DYRK1A and DYRK3 [81]; phosphorylation by JNK2 stabilizes SIRT1 [82], but phosphorylation by JNK1 facilitates its ubiquitylation-mediated degradation [83]. Signals mediated by cAMP induce p53 deacetylation by SIRT1 and HDAC [38,84]. Deacetylation of p53 by SIRT1 also contributes to maintain the stem cell pool and the pluripotency of human embryonic stem cells [85]. As expected, loss of *SIRT1* expression facilitates p53 acetylation causing developmental defects [86].

In some tumour types, as in acute promyelocytic leukemia the oncogenic PML-RAR protein recruits deacetylases that facilitate p53 deacetylation and thus can promote oncogenesis, not only by the translocation product, but also by eliminating functional p53 [87].

7. Ubiquitylation of p53

The best well-known downregulatory mechanism of p53 involves its ubiquitylation by hdm2, whose gene expression is induced by the accumulated p53 and this process has been very extensively reviewed recently [88,89]. However, this activation of *HDM2* gene expression requires an active, or phosphorylated, p53. This dependence on de novo gene expression permits a temporal separation of consecutive steps, opening up a time window for p53 actions before its activated state is reverted. However, ubiquitylation of p53 requires its previous dephosphorylation, since only dephosphorylated p53 will be able to interact with hdm2 and become its substrate [21]. In some tumours, such as those with infection by human papillomavirus, the functional inactivation of p53 is mediated by viral E6 ubiquitin ligase [90], as it occurs in cervical carcinomas without p53 mutations.

Table 2
Lysine residues and their modification in the C-terminal regulatory domain of p53.

P53 lysine residue	Acetylase	Deacetylase	Ubiquitin ligase	Methylase	Demethylase	Sumoylase
K305	p300 (KAT3B), CBP (KAT3A)					
K320	pCAF(KAT2B)	HDAC1	E4F1			
K370			Mdm2, Ub/N8	SMYD2 (KMT3C)	LSD1/KMD1	
K372	p300 (KAT3B)		Mdm2, Ub/N8	SMYD2 (KMT3C)		
K373	p300 (KAT3B)	HDAC1	Mdm2, Ub/N8			
K382	p300 (KAT3B)	HDAC1, SIRT1	Mdm2			
K383	p300 (KAT3B)	HDAC1	Mdm2			
K386			Mdm2			SUMO

Based on Toledo et al. [24] and Meek and Anderson [7].

7.1. Mdm2 phosphorylation affects its interaction with p53

The interaction between p53 and mdm2 is regulated by the covalent modification of mdm2 that alters the interaction between these two proteins. Kinases that phosphorylate mdm2/hdm2 promote its delocalization and degradation, which will result in the stabilization and accumulation of p53. Therefore, kinases targeting mdm2 should participate early in the response to cellular stress or DNA damage as one of the indirect p53-activating mechanisms. Later in the stress response, mdm2 phosphorylation has to be reverted in order to permit the interaction with p53 and facilitate its downregulatory role by p53 ubiquitylation. Hyperphosphorylation of mdm2 within the region 244–260 reduces its ability to degrade p53 and results in p53 accumulation [91]. Several kinases targeting mdm2/hdm2 have been identified, although their temporal and spatial order is not known. Mdm2 is phosphorylated in serine residues by ATM [37], by ATR in Ser407 [92], aurora A [93], casein kinase I in Ser240, Se-242, and Ser-246, as well as in Ser383 [94]. Mdm2 phosphorylation in tyrosine 394 by c-abl impairs p53 degradation [95]. Phosphorylation of mdm2 in Thr216 by cyclinA/cdk complexes in the S-phase weakens its interaction with p53 and facilitates binding to p19 [96]. Phosphorylation of mdm2 in Ser166 and Ser186 by Akt are necessary for mdm2 translocation from the cytosol to the nucleus, thus interference with this phosphorylation will result in the mislocalization of mdm2 and accumulation of p53 in nuclei [97].

7.2. Mdm2-RPL11 interaction protects p53 from degradation

A novel mechanism for p53 activation is the consequence of proteins that interact and compete with the protein complexes containing mdm2, which when disrupted result in their release and thus can contribute to tumorigenesis. Recently, it has been shown that the ribosomal protein RPL11 forms a complex with mdm2 and inhibits its ubiquitin ligase activity contributing to the accumulation of p53 [98], which the growth inhibitory properties of RPL11. In that way interphase cells, which are non-dividing but transcriptionally active, can maintain a basal level of p53 ready to respond to stress situations. The nucleolar GRWD1 (glutamate-rich WD40 repeat containing 1) protein is released into nuclei as part of the response to nucleolar stress. This nuclear GRWD1 interacts with RPL11 and releases mdm2 from the mdm2-RPL11 complex; in that way facilitates degradation of p53 by ubiquitylation. In tumours, high levels of GRWD1 have an oncogenic role and is associated with a poorer prognosis in patients with low-grade gliomas [98].

8. P53 and autophagy

The reversal of p53 activation that can be effectively resolved by the cell is usually minor damage, and this response may be partly a consequence of the activation of autophagy. By this mechanism, altered proteins are selectively degraded within cells. Autophagy is a basic mechanism induced by p53, but p53 by itself is not a direct target of autophagy. In the context of limited cellular damage that induces a p53 mediated cell cycle arrest, autophagy plays a role in the removal of damaged proteins and allows for resumption of normal cell viability. This response is quantitatively and qualitatively different from those situations in which the cellular damage is irreversible. The of p53 transcriptional targets have different activation thresholds depending if the gene targets are associated to cell cycle arrest or to induction of cell death, which are reversible and irreversible respectively [99].

P53 by itself is not a direct target of autophagy, but in manageable cell damage, p53 in addition to the activation of *HDM2* expression, also induces genes associated with autophagy, such as *DRAM* (death-related autophagic modulator). DRAM contributes to the restoration of p53 basal protein levels by eliminating its activating kinase VRK1 [36], and permitting its dephosphorylation and making it available to hdm2-mediated degradation of p53 and reliving the cell cycle arrest.

In stress responses that result in the activation of nuclear p53, a fraction of the phosphorylated p53 molecule is translocated to the cytosol, as a consequence of its phosphorylation in Ser46 by HIPK2 [100], where it can contribute to the activation of apoptosis (9) by acting on mitochondria and endoplasmic reticulum [101]. The proapototic effect of activated p53 in mitochondria is mediated by blocking the antiapoptotic effect of Bcl-1 and Bcl-xL and by the activation of proapoptotic proteins, such as Bax and Bak [102,103]. Furthermore, activated p53 in cytosol has a proapoptotic role by acting on the endoplasmic reticulum [104]. All these cytoplasmic effects of activated p53 in cytosol can be reverted because of phosphatase gene expression induced by the nuclear p53. However, the reversal of the cytoplasmic effects of p53 to resume with cell life need further studies before they are fully understood.

In the case of severe cellular damage, p53 has a more complex and dual role, both as protector or facilitator of tumorigenesis depending on cellular context. These roles of p53 in the regulation of autophagy have been recently reviewed [104–106].

9. Autoregulatory loops of p53 intracellular levels

The regulation of intracellular levels of p53 is a complex process in which multiple participants play a role at different times in the cellular response to stress. These participants include all enzymes that can modify either p53 or its covalent modifications. An outline of this complex regulatory web, in which the participants in a loop centred on p53 activation by VRK1 in response to cellular stresses, is shown in Fig. 2. However, on this network additional components have to be added mediating regulation by other participating kinases. All steps in these loops that involve enzymatic activities, such as kinases, phosphatases, ubiquitin ligases, acetylases and deacetylases are potential targets for manipulation of p53 mediated responses.

10. Pharmacological targeting of p53 downregulatory pathways

The rescue of p53 dependent functions can be therapeutically used in tumours with normal p53. One way by which p53 activity can be enhanced is by inducing its stability and facilitating an increase in its intracellular concentration. This can be achieved by inhibition of its different downregulatory mechanisms, which are multiple.

Manipulation of p53 activity, can be achieved by targeting its regulators instead of p53 itself. In situations in which p53 is wild type, interfering with its different downregulatory mechanisms, such as ubiquitylation, phosphorylation and deacetylation, can lead to a sustained increase in p53 levels that can be of use in cancer treatment, since this accumulation can induce either cell cycle arrest or cell death. These compounds might also potentiate or sensitize cells to other drugs used in chemotherapy, This approach is theoretically feasible for tumours harbouring wild-type p53 and if used in combination with other drugs, which are toxic due to their mechanism of action, might permit a reduction of their dose. These potential approaches have received very different attention. While interference with ubiquitylation has led to new drugs, such the proteasome inhibitor bortezomib [107], but less is known about the role of phosphatases and deacetylases inhibitors in this context. Nevertheless, it is likely they will have an important role in development of new drugs.

10.1. Inhibition of ubiquitylation

The possibility of inhibiting p53 ubiquitylation to prevent its proteasomal degradation has led to development of drugs that interfere with the interaction between p53 and its ubiquitin ligase mdm2/hdm2. Nutlins block the interaction of p53 with the hydrophobic pocket in mdm2, resulting in the accumulation of p53. Consequently, nutlins have been shown to potentiate control of tumours cells by induction of p53 dependent effects [108,109], such as senescence [110] or

increased sensitivity to chemotherapy [111]. Some of these nutlins are already in preclinical trials [112].

Some proteasome inhibitors not directed to p53 are currently in clinical use. But there are more than six hundred E3 ubiquitin ligases and their specificity is not well known [113,114]. P53 can be protected from Hdm2 ubiquitylation by treatment with MG132. Some inhibitors, like bortezomid is already in use for treatment of multiple myeloma [115]. The large number of ubiquitin ligases [113] and the ubiquitin code [114] are likely to lead in the future to novel drugs whose therapeutic potential remains to be realized.

10.2. Phosphatase inhibitors

Phosphatase inhibitors have not been used as drug targets. This is likely to be a consequence of the complex regulation of specificity of the phosphatase catalytic subunit, which is controlled by the regulatory subunit and by the difficulty in blocking the action on specific protein targets.

10.3. Protein deacetylation inhibitors

The p53 protein is the target of several deacetylases, although they have additional targets, such as histones, which account for most of their interest due to their role in chromatin remodelling. However, transcriptional factors are also acetylated. Therefore, it is likely that their function can also be manipulated with deacetylase inhibitors. Inhibition of p53-deacetylation will enhance its transcriptional activity and dependent functions, which can be of therapeutic use.

Inhibition of deacetylase with valproic acid will maintain p53 in a phosphorylated and acetylated state that is transcriptionally active [116]. Thus, inhibition of histone deacetylases [117] can be used as a therapeutic target. These inhibitors may partly function through their effect on p53 [118]. Thus, the DWP0016 inhibitor facilitates p53-dependent transcription and inhibit cell growth of glioblastoma cells [119]. Treatment with histone deacetylase inhibitors also suppresses the expression of MDM2 and consequently leads to accumulation of p53 [118]. These inhibitors of deacetylases can have an additive with those targeting ubiquitin ligases, thus the combination of entinostat or vorinostat, two HDAC inhibitors, combined with nutlin-3 resulted in an enhanced antitumor effect [120], and also combinations of vorinostat with bortezomid improve the response in aggressive B-cell lymphomas [121]. Similarly, both also improve cell sensitivity to cis-platinum, a DNA-damage drug, in resistant ovarian carcinomas [122] by facilitating induction of cell death. In addition, the inhibition of Sirt2 facilitates apoptosis in non-small lung cancer [123].

11. Summary

In the absence of p53 mutations, a complex web of signals that require addition or removal of different and sequential covalent modifications in either p53 or its regulatory molecules very tightly regulates the p53 pathway. The pharmacological targeting of this complex signalling web can facilitate development of novel treatments aiming to activate p53 in tumour cells.

Transparency document

The Transparency document associated with this article can be found, in online version.

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