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Review

The complexity of p53 stabilization and activation

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Abstract

A number of proteins are activated by stress stimuli but none so spectacularly or with the degree of complexity as the tumour suppressor p53 (human p53 gene or protein). Once stabilized, p53 is responsible for the transcriptional activation of a series of proteins involved in cell cycle control, apoptosis and senescence. This protein is present at low levels in resting cells but after exposure to DNA-damaging agents and other stress stimuli it is stabilized and activated by a series of post-translational modifications that free it from MDM2 (mouse double minute 2 but used interchangeably to denote human also), a ubiquination ligase that ubiquitinates it prior to proteasome degradation. The stability of p53 is also influenced by a series of other interacting proteins. In this review, we discuss the post-translational modifications to p53 in response to different stresses and the consequences of these changes.

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Abbreviations: p53, human p53 gene or protein; UV, ultraviolet light; Chk1/2, checkpoint kinases; MDM2, mouse double minute 2 but used interchangeably to denote human also; HAUSP, herpes virus-associated ubiquitin-specific protease; NQO1, NAD(P)H quinone oxidoreductase

Introduction

The response of cells to DNA-damaging agents is complex involving recognition and repair of the lesions in DNA to minimize the risk of genetic instability. Thus, it is not surprising that mutations that interfere with the function of key steps involved predispose to genome instability, cancer and other pathologies.[1] A central player in protecting the integrity of the genome is p53 (human p53 gene or protein), which is present at low levels under unperturbed conditions but becomes rapidly stabilized and activated in response to a variety of stimuli including DNA damage.[2] The importance of the role of p53 in maintaining genome stability is exemplified by the findings that this molecule is mutated in approximately 50% of tumours and these tumours respond poorly to therapy.[3,4] Once activated, p53 can either cause cell cycle arrest by transactivation of p21 or induce cells to undergo apoptosis by both transcription-dependent and -independent mechanisms.[5] P53 exerts its control on the cell cycle primarily through the G1/S checkpoint but it has also been shown to regulate the G2/M checkpoint.[6] While the exact mechanism of stabilization of p53 remains unclear, it involves a series of post-translational modifications to both itself and MDM2 (mouse double minute 2 but used interchangeably to denote human also), which facilitate the dissociation of the MDM2–p53 complex that is primarily responsible for keeping the levels of p53 in check. However, it is evident that a multitude of other proteins also influence the stability of p53. Some of these appear in Figure 6 and their involvement in p53 stabilization will be discussed in greater depth later in this chapter. The capacity of p53 to induce specific proteins to regulate the passage of cells through the cell cycle is of key importance in fulfilling its role in genome stability, but its involvement in nucleotide and base excision repair pathways may also contribute.[7–9] The objective of this contribution is to describe the post-translational changes that occur in p53 that contribute to its stabilization and activation particularly in response to DNA damage. We will examine its role in control of the G1/S checkpoint and its implications for minimizing genome instability and cancer. Consideration will also be given to the influence of other proteins on p53 stabilization.

p53 is Stabilized by Different Stresses

Since the structural and functional domains of p53 are adequately covered elsewhere in this volume, only minimal coverage of this topic will be provided here in the context of the post-translational modifications that arise as a consequence of cellular stress. The major modifications to p53 in response to stress are located at the N-terminus of the molecule, a region responsible for the transactivation capacity of p53 and where interaction occurs with MDM2, and in the C-terminal regulatory domain.[10] These post-translational modifications include phosphorylation, acetylation, methylation, ubiquitination and sumoylation and are observed at approximately 24 different sites.[11] A variety of different stresses stabilize and activate p53. Maltzman and Czyzyk[12] showed that ultraviolet light (UV) radiation stimulated the levels of p53 cellular tumour antigen, as it was known then, in mouse cells. Kastan *et al.*[13] later demonstrated an increase in p53 protein levels in response to DNA double-strand breaks induced by ionizing radiation. They showed that this was primarily due to an effect at the post-translational level rather than due to transcriptional control. Subsequent studies revealed that a variety of other

DNA-damaging agents were capable of stabilizing p53 protein. Hypoxia, which does not induce any detectable level of DNA lesions, also leads to the accumulation of p53.[14] This appears to occur in response to DNA replication arrest since it is only observed in S phase. Activation of AMP-activated protein kinase (AMPK) as a consequence of nutrient deprivation or overexpression of the protein increased endogenous levels of p53 protein and phosphorylation of p53.[15,16] Interference with ribosome biogenesis causes growth arrest or apoptosis. Since p53 represses Pol1 transcription on the rRNA promoter, this may represent another level of cell cycle control by p53 in response to stress.[17] Stabilization and activation of p53 is also achieved in response to oncogenic stimuli.[18,19] Transformation of cells with c-myc or Ras induces the tumour suppressor $p19^{ARF}$, which associates with mdm2 and blocks degradation of p53 and when cells are depleted of ribonucleotide pools activation also occurs.[20] Stabilization of p53 also occurs in the presence of telomeric (TTAGGG) repeats.[21] This effect was found to be specific for this sequence and provides support for the possible activation of p53 by shortening telomeres which leads to senescence. A number of other stresses that include cytokine stimulation, anchorage, cell-to-cell contact, viruses and various metabolic changes also activate p53.[22]

Post-translational Modification

As outlined above, a number of different stimuli, acting at different levels of cellular physiology, stabilize and activate p53. In all cases this involves a series of post-translational modifications, some of which are known to influence the interaction between p53 and MDM2, the major mechanism for controlling p53 stability.[11] These modifications are depicted in Figure 1. Phosphorylations dominate these changes with 10 being observed within 100 amino acids of the N-terminus and approximately the same number towards the C-terminus of the molecule. These phosphorylations are brought about by a number of protein kinases that respond to different stress stimuli including ATM (mutated in ataxia-telangiectasia); ATR (A-T and Rad3-related), the checkpoint kinases, Chk1 and Chk2; Jun NH_2-terminal kinase (JNK), p38 and others. A comprehensive list of the phosphorylation and other sites modified, together with the protein kinases involved can be accessed through the website of Carl Anderson www.biology.bnl.gov/cellbiol/anderson.html (see Figure 1). The most frequently described phosphorylation is on Ser15 (Ser18 in the mouse) of p53 and occurs in response to different stress signals. This phosphorylation occurs rapidly in response to DNA double-strand breaks and is carried out by ATM.[23–25] Indeed ATM mediates phosphorylation at multiple sites on p53 in response to ionizing radiation.[26] These include ser 6, 9, 15, 20, 46 and Thr 18 (Figure 1). Not surprisingly, considerable overlap in the sites of phosphorylation on p53 exists when the different agents employed are compared. The patterns of phosphorylation on p53 after genotoxic and nongenotoxic stresses are also available on www.biology.bnl.gov/cellbiol/anderson.html (see Figures 2 and 3). The sites most commonly phosphorylated are Ser46, Ser15, Ser20 and Ser33. On the other hand, phosphorylation at Thr81 is only seen after UV or H_2O_2 treatment and it is observed at Thr18 in response to DNA breaks and replica tive senescence. JNK is responsible for phosphorylation of p53 at Thr81, an event that stabilizes and transcriptionally activates the molecule.[27] Some sites on p53, Ser p376 and Ser p378, are constitutively phosphorylated and undergo dephosphorylation in response to radiation

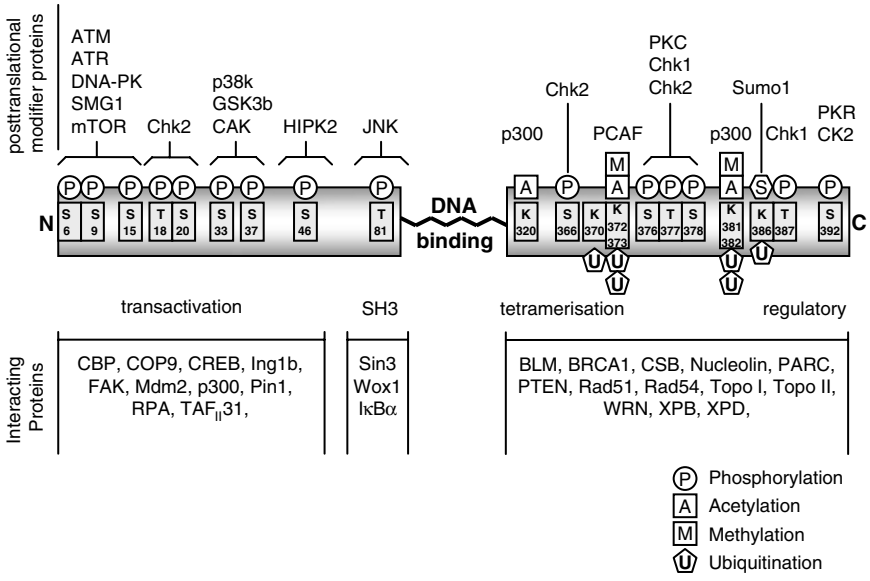

Figure 1 Post-translational modification of p53. In the upper part of the schematic representation of p53, the proteins that phosphorylate, acetylate or methylate the molecule are indicated. Where modification occurs at the same or adjacent sites this is shown, for example, at K381, K382 methylation and acetylation. Ubiquitination also occurs at these sites which is shown under the p53 schematic. The transactivation, SH3, DNA binding, tetramerization and regulatory domains of p53 are outlined, as well as the proteins that interact with these domains

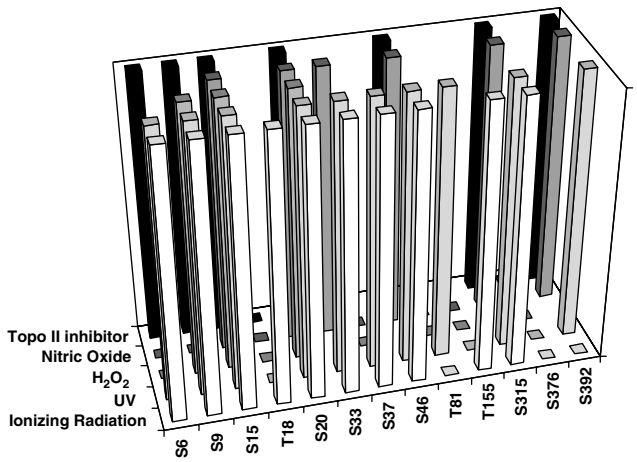

Figure 2 Post-translational modification of p53 in response to different genotoxic stresses. The modifications indicated are phosphorylations at serine(s) and threonine(T) amino acids. The pattern of modification for each stress is depicted by bars

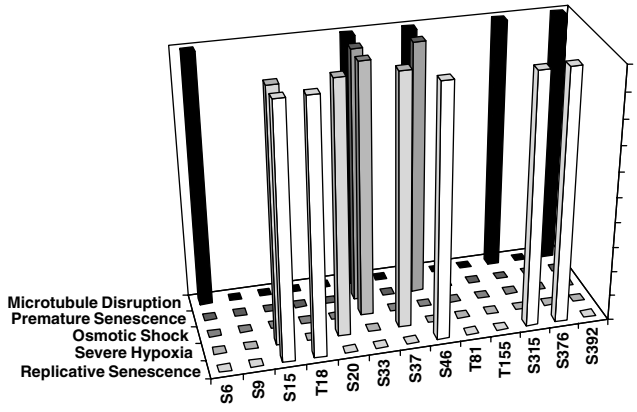

Figure 3 Post-translational modification of p53 in response to nongenotoxic stresses. As in Figure 2, phosphorylations at serine(s) and threonine (T) residues are shown and the patterns for each treatment are indicated by bars

damage.[28] The loss of phosphorylation from Ser p376 creates a consensus binding site for 14-3-3 proteins and in turn increases the affinity of p53 for sequence-specific binding sites on DNA.

Agents that alter the conformation of the C-terminal region of p53 enhance its DNA-binding activity.[29] This appears to be due to an unfolding of the C-terminus, exposing the DNA-binding domain. Acetylation of several lysine resides in this region also enhances DNA-binding activity *in vitro*. The histone acetylase family members p300/CBP bind p53 and enhance its transcriptional activity[30] and p300 has been shown to acetylate p53.[31] The histone acetylase P/CAF can also acetylate p53.[32] In response to a variety of cellular stresses, acetylation was demonstrated at five sites near the C-terminus.[33–35] More recently, it has been shown that acetylation is not required for DNA binding by p53 to the p21 promoter [36] and substitution of lysine with arginine at four sites (K320R, K373R, K381R and K382R) did not affect its binding to the promoter.[37] However, contradictory data have been presented on the influence of C-terminal acetylations on the transcriptional activation of p53.[36,37] At best, acetlylation may have a modest effect on transcriptional activity of p53.[38] Acetylation of p53 has also been linked to its localization to promyelocytic leukaemia (PML) bodies.[33] Interaction of p53 with Sir2, an NAD-dependent histone deaceylase, could provide a mechanism for downregulation of p53.[39] Recent evidence for a fine tuning role for p53 acetylation in homeostasis was provided by Krummel *et al*.[40] They generated mutant mice in which seven C-terminal lysine residues were substituted with arginine and showed that the mice were largely phenotypically normal. However, they showed that the mutant form of p53 was more readily activated by DNA damage in the thymus. It has also been suggested that lysine acetylation of p53 protects the molecule from degradation since some of these residues are also targets for ubquitination by mdm2 and its subsequent degradation by the proteasome.[41]

It seems likely that there exists an ordered pattern and interdependence of stress-induced modifications to p53. Ser15 phosphorylation of p53 occurs rapidly in response to DNA damage and appears to represent a 'priming event' for the subsequent series of modifications.[42] It acts as a precursor for the subsequent phosphorylation of Thr18 by another kinase such as Chk2 and may also have a bearing on Ser9 and Ser20 phosphorylations.[43] Phosphorylations at Ser15, Thr18 and Ser20 stimulate the recruitment of other factors including p300, CBP and P/CAF that promote C-terminal acetylation and as mentioned above the ensuing acetylations to p53 may prevent ubiquitination and degradation.[41] That order exists in the series of post-translational modifications to p53 is also exemplified by the involvement of the prolyl isomerase Pin1, an enzyme that controls the function of p53.[44,45] Phosphorylation of p53 on Ser33, Thr81 and Ser315 (all of which are present in Ser/Thr–Pro motifs) in response to stress leads to efficient interaction with Pin1 which in turn brings about conformational change in p53 that enhances its activity. Pin1-deficient cells are defective in accumulation and activation of p53 protein and show impaired cell cycle checkpoint activation in response to DNA damage. Thus, it is evident that only when a number of stress kinases alter p53 at specific sites does Pin1 bind to it efficiently to complete an additional step in the process of activation. It is also of interest that Pin1 associates with p73 after genotoxic stress to promote its acetylation by p300 and increase the stability of p73.[46]

While it seems likely that the complex web of stress-induced modifications to p53 is important in generating a functional molecule, there is also evidence that p53 can be activated without these changes. Blattner *et al*.[47] mutated a series of known stress-induced phosphorylation sites on p53 and demonstrated that these mutant forms could be stabilized. Mutation of the 30 C-terminal amino acids of p53, a region predicted to be important for interaction with p300 and other acetylases, also failed to prevent damage-induced stabilization. It was concluded that the single prerequisite for induced stabilization of p53 was its prior destabilization by MDM2 and subsequent disruption of the complex. Further evidence pointing to a lack of requirement for post-translational modification in p53 stabilization comes from the use of small molecule inhibitors of the p53–MDM2 interaction.[48]

They identified a series of *cis*-imidazoline analogs they named Nutlins in a screen of a library of synthetic chemicals that displaced recombinant p53 from its complex with MDM2. They subsequently showed that p53 accumulated in cells treated with Nutlin-1 followed by an increase in the levels of both p21 and MDM2 consistent with activation of the p53 pathway. Unlike DNA damage, nutlins did not induce phosphorylation of p53 but this stabilized, unphosphorylated form of p53 was equally efficient at sequence-specific DNA binding and the induction of apoptosis[49]

Post-translational Modification and the p53-MDM2–MDMX Axis

The amount of p53 protein present in unstressed cells is low and this is determined by its rate of degradation rather than by translation from mRNA. This degradation is ensured by autoregulatory negative feedback loops in the form of three ubiquitin ligases, MDM2, Pirh2 and Cop-1.[11] Pirh2 interacts with p53 and promotes its ubiquitination independent of MDM2.[50] Cop-1 also increases p53 turnover by targeting it for degradation by the proteasome in a ubiquitin-dependent fashion.[51] There is also evidence that the topoisomerase 1 and p53-binding protein, topors, functions as a ubiquitin ligase for multiple transcription factors including p53.[52] Because of the involvement of MDM2 in several of the regulatory loops that control the function of p53, it seems likely that it plays the most central role in controlling p53 activity.[11] In addition, knockout of MDM2 in mice is embryonic lethal and when combined in a double knockout with p53 this phenotype is rescued suggesting that no back-up p53 ubiquitin ligase is operational during development.[53]

MDM2 is a proto-oncogene amplified in a significant number of tumours. This molecule regulates the activity of p53 by ubiquitinating it, transporting it to the cytoplasm and promoting its degradation by the proteasome (Figure 4). MDM2 is an E3 ubiquitin ligase capable of targeted ubiquitination of p53 but it also self-ubiquitinates.[54] Exposure of cells to stress reduces sumoylation of MDM2, and causes an increase in self-ubiquitination and degradation, thus favouring p53 stabilization.[55] There is also evidence that post-translational modification of MDM2 destabilizes its interaction with p53 to contribute to p53 stabilization in response to stress. Khosravi *et al*.[56] demonstrated that MDM2 is rapidly phosphorylated in an ATM-dependent manner in response to specific DNA-damaging agents. Subsequent studies revealed that ATM phosphorylates MDM2 on Ser 395 *in vitro* and a decrease in binding of a monoclonal antibody to a motif containing this site after DNA damage is consistent with this being the site phosphorylated *in vivo*. The phosphorylated form of MDM2 appears to be less capable of nucleo-cytoplasmic shuttling of p53, suggesting that the MDM2 phosphorylation destabilizes the interaction with p53.[57] ATM also plays a key role in phosphorylating p53 on Ser 15 and other sites in response to DNA double-strand

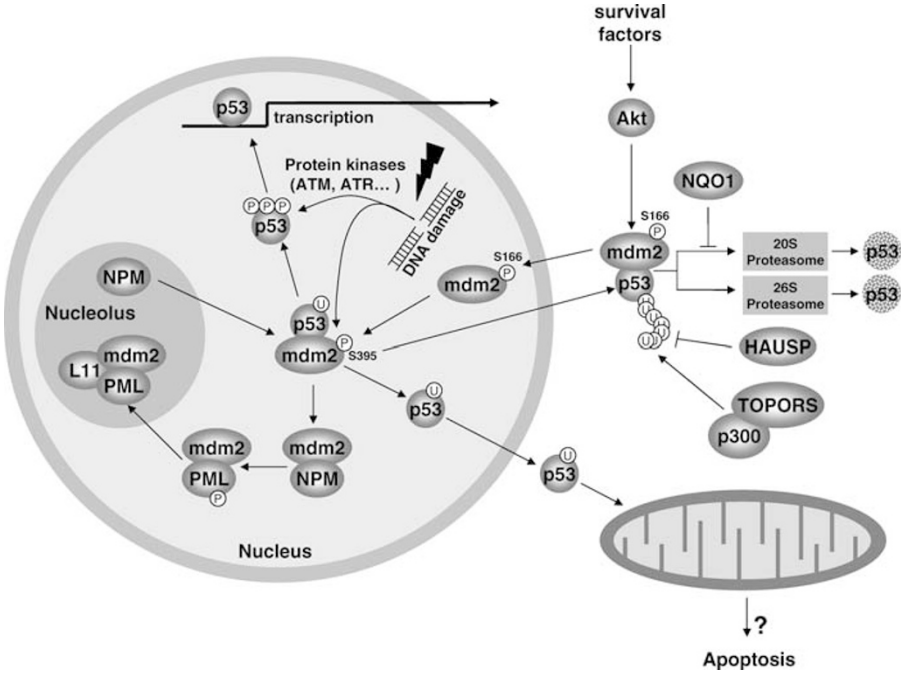

Figure 4 Control of p53 stabilization by MDM2. Under nonstress conditions, MDM2 binds to p53 and monoubiquitinates it prior to exporting it to the cytoplasm where it is polyubiquitinated. Akt-dependent phosphorylation of MDM2 on S166 facilitates this export. Topors or p300 are implicated in the polyubiquitination reaction. Once it is polyubiquitinated, p53 is then degraded by the 20S proteasome. NAD(P)H quinone oxidoreductase (NQO1), one of the p53 interacting proteins, prevents p53 degradation by the proteasome by acting as a gatekeeper of this process. Exposure of cells to DNA damage and other stresses activates PI3 kinases such as ATM and ATR which facilitate the stabilization of p53 by reducing the interaction between MDM2 and p53. The nucleolus is also implicated as a sensor of stress releasing nucleophosmin which acts as a negative regulator of the MDM2–p53 complex. Promyelocytic leukaemia (PML) protein also enhances the stability of p53 by sequestering MDM2 to the nucleolus. Once stabilized, p53 can activate cell cycle checkpoints or apoptosis. There is evidence that part of its apoptotic function may reside in its ability to localize to the mitochondrion

breaks.[23–26] In addition, ATM-dependent phosphorylation on Ser 20 of p53 is mediated by Chk2.[58] All of these post-translational modifications of p53 contribute to its stabilization. Thus, it seems likely that modifications to both p53 and MDM2 are responsible for stabilization and activation of the p53 pathway.

The story does not end there since another player MDMX also gets in on the act. Like MDM2, MDMX interacts with p53 but it does not ubiquitinate it, instead it inhibits its transactivation activity.[59] MDM2 and MDMX interact through their RING finger domains,[60] protecting p53 from MDM2-mediated degradation.[61] This appears to be achieved by MDMX, preventing the nuclear export of p53.[62] On the other hand, MDMX stabilizes MDM2 by preventing its self-ubiquitination. MDM2 and MDMX are functionally dependent in controlling p53. In the absence of MDMX, the capacity of MDM2 to control p53 is reduced because of a shorter half-life.[63] MDMX is in turn dependent on its binding to MDM2 to enter the nucleus where it inhibits p53 function. Li *et al*.[41] demonstrated that MDMX is translocated from the cytoplasm to the nucleus in response to DNA damage. This might appear to be counter-intuitive since DNA damage stabilizes and activates p53. This issue was resolved by Pereg *et al*.[64] who showed that DNA damage-induced phosphorylation of HDMX mediated its MDM2- and ATM-dependent degradation. In response to DNA damage, three sites were phosphorylated on HDMX, one of which, Ser 403, was a direct ATM target. The other two phosphorylation sites, Ser 342 and Ser 367, have been identified and shown to require Chk2, also implicating this kinase in MDMX degradation.[65] It seems likely that these phosphorylation events control the stability of MDMX since mutation of these sites inhibited to varying degrees MDM2-mediated ubiquitination, but this was not due to reduced MDM2–MDMX binding.[64] Furthermore, DNA damage-induced phosphorylation of MDM2 at Ser 367 creates a binding site for 14-3-3 protein which controls binding of MDM2 and in turn degradation of MDMX.[66] To add further complication to the mechanism for control of p53 function, Meulmeester *et al*.[67] have recently shown that ATM-dependent phosphorylation of both MDM2 and MDMX lowers their affinity for the deubiquitinating enzyme herpes virus-associated ubiquitin-specific protease (HAUSP) which would result in decreased activity and stability of these proteins, favouring p53 activation.

The picture that emerges for control of p53 stabilization and activation in response to stress stimuli is a complex one that involves direct phosphorylations by ATM on several molecules including p53 itself, Chk2, MDM2 and MDMX. ATM also mediates its effect through Chk2 and other kinases. It should also be pointed out that this is dependent on the form of stress since ATM responds primarily to DNA double-strand breaks. Other lesions in DNA and indeed additional stressful events to the cell lead to the activation of several protein kinases including ATR, JNK, c-Abl, PI3 kinase and Akt, all of which influence the state of stability and activity of p53. Clearly, the whole process of p53 activation is sophisticated and fine-tuned to ensure that this molecule is maintained in a dormant state ordinarily and only activated with the appropriate response.

p53 Plays a Key Role in Activation of the G1/S Cell Cycle Checkpoint

Exposure of cells to radiation leads to a delay in progression of cells at different stages of the cell cycle.[68] Cell cycle checkpoints are activated by radiation to delay progression from G1 to S phase; to inhibit DNA synthesis as part of the intra-S phase checkpoint and to arrest passage of cells from G2 into mitosis.[69] ATM is the key regulator of all these checkpoints in response to DNA double-strand breaks.[70] It phosphorylates a series of proteins including p53 to mediate cell cycle control.[71] A major feature of ATM-mediated cell cycle control is the complexity and fine tuning of regulation at all three checkpoints. Multiple substrates are phosphorylated and control is achieved by parallel pathways. Stabilization and activation of p53 in G1/S checkpoint control is a good example of this 'tight' control. As pointed out earlier in this review, p53 is post-translationally altered at multiple sites as part of its activation. One of these modifications is ser15 phosphorylation of p53 which is directly phosphorylated by ATM.[23–25] This site is not essential for p53 stabilization or activation but rather is one of a series of changes that contribute to efficiency of the overall process.[72] There is evidence that phosphorylation at this site stimulates interaction between p53 and its transcriptional coactivators p300/CBP.[73] However, what is clearly evident is that this is an excellent marker site for ATM activation since the modification is markedly slower in A–T cells in response to radiation.[74]

A second site on p53, ser20, is also phosphorylated in response to DNA damage, not directly by ATM but rather mediated through Chk2.[58] As with the ser15 phosphorylation on p53, there exists conflicting data as to the importance of ser20 phosphorylation on the activation of p53. Substitution of this site with Ala (S20A) increased significantly the susceptibility of p53 to negative regulation of MDM2, as determined by apoptosis induction and transcription activation.[75] In another study, when the same site was substituted with Asp (S20D) it led to a constitutively activated form of p53 but this change did not affect p53 stability.[76] In yet another report, S20A substitutions alone or in combination with S15A and T18A failed to alter the degree of p53 stabilization in response to ionizing radiation.[77] Hirao *et al*.[78] showed that Chk2-deficient mice are unable to stabilize p53 in response to radiation exposure. Consistent with this defect they are also resistant to apoptosis and after low doses of radiation exhibit a G1/S checkpoint defect. On the other hand, MEFs from these mice showed no defect in p21 induction or in the G1/S checkpoint.[79] The cellular phenotype for a second Chk2$^{-/-}$ mouse was somewhat different revealing apoptosis resistance as in the first example, no obvious defect in the G1/S checkpoint, some reduction in p53 stabilization but normal phosphorylation on ser20 in response to radiation.[80] It seems likely that neither the ser15 nor ser20 phosphorylations are essential for p53 stabilization but contribute to an overall set of post-translational modifications that enhance the efficiency of this process. In response to radiation, both ATM and Chk2 are activated and work together to initiate this process (Figure 5).

As discussed previously, the stability of p53 is primarily determined by its interaction with the RING-finger proteins

MDM2 and MDMX.[64] Thus, any changes that interfere with those interactions would favour p53 stabilization. While controversy surrounds the importance of p53 ser20 phosphorylation in its stabilization, there is at least some evidence that this modification reduces interaction with MDM2.[75] In addition, we have seen that ser395-phosphorylated *MDM2 is less capable of nucleo-cytoplasmic shuttling of p53 pointing to reduced interaction between these molecules, resulting in a nett stabilization of p53.[57] ATM is responsible for this phosphorylation in response to DNA damage which represents an additional level of control of the G1/S checkpoint (Figure 5). ATM also phosphorylates MDMX on ser 403 after DNA damage leading to its degradation and preventing it from interfering with the transactivation activity of p53.[64] This enhancement of degradation is also mediated by Chk2.[65] As depicted in Figure 5, the control of the G1/S checkpoint through p53 is complex, involving a series of mediators. This is of course also applies to apoptosis control where activated p53 induces other downstream effector genes such as Puma and Noxa to mediate this process.

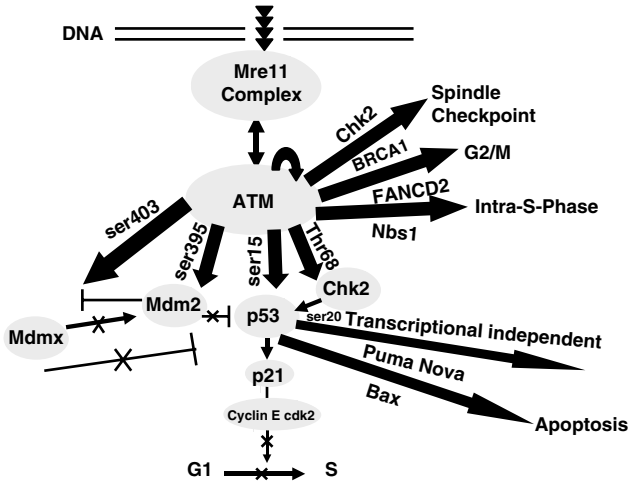

Figure 5 Role of p53 modification in cell cycle control through the G1/S checkpoint. Exposure of cells to ionizing radiation or radiomimetic drugs in this case causes double-strand breaks in DNA. These breaks are detected by the Mre11 sensor complex (Mre11. Rad 50. Nbs1) which in turn recruits and activates ATM by autophosphorylation. Once activated, ATM can phosphorylate a series of downstream substrates including p53, Chk2, MDM2, MDMX, BRAC1, FANCD2, Nbs1 and other proteins. In this case, ATM phosphorylates p53, MDM2, Chk2 and MDMX to facilitate activation of the G1/S checkpoint. The transcriptionally active form of p53 induces p21 which binds to and inhibits the cyclin E–Cdk2 complex to prevent downstream protein phosphorylation required for passage into S phase. ATM also activates other cell cycle checkpoints through Nbs1, Chk2, BRCA1 and FANCD2 as indicated. P53 is also implicated in the G2/M checkpoint

Multiple Proteins Influence the Stabilization and Activation of p53

The major mechanism for control of p53 stabilization and activation is dependent on its interaction with and ubiquitination by MDM2 prior to degradation by the proteasome.[11] However, as outlined in Figure 6, multiple proteins contribute to the stabilization of p53 in response to different stress stimuli. These include proteins that modify p53 for both stabilization and destabilization, proteins that reverse these modifications, proteins that enhance the translation of p53 mRNA and proteins that alter its subcellular localization. The intention here is not to cover an exhaustive catalogue of these

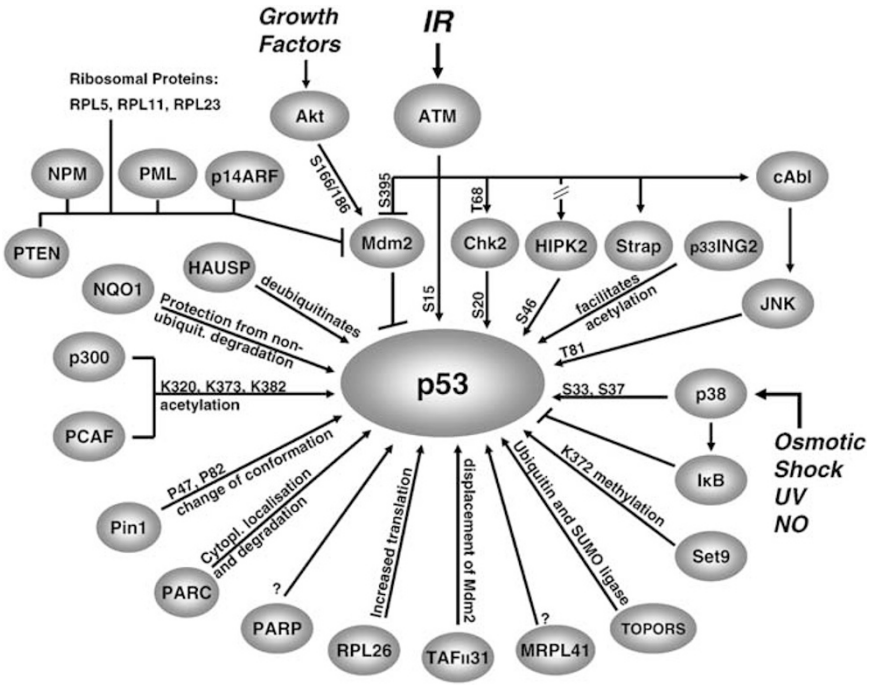

Figure 6 Proteins influencing the stabilization and activation of p53. In addition to MDM2, a variety of other proteins impact upon the stability of p53, constitutively and when cells are exposed to different stresses. These proteins include protein kinases, proteins that modify p53 in other ways, proteins that affect the subcellular localization of p53 and proteins that influence the p53–MDM2 interaction. Some of these are illustrated in this schematic and their specific effect on p53 is indicated

proteins but rather to provide examples of the different modes of control.

Preventing the ubiquitination of p53 by reducing its interaction with MDM2 is one way to stabilize p53. Another possibility is by removing the ubiquitin modification. Such an enzyme, HAUSP was identified by mass spectrometry as a p53-associated factor.[81] HAUSP stabilized p53 causing growth repression and apoptosis. On the other hand, disruption of HAUSP in human cells by targeted homologous recombination also caused stabilization and functional activation of p53. The explanation offered for the altered phenotype in the latter case was that HAUSP may be able to deubiquitinate other proteins such as MDM2 and that the steady-state level of p53 is determined by a balance between the deubiquitination of the different targets of HAUSP.[82] It has also been suggested that escaping MDM2-mediated degradation is not sufficient to protect p53 against 20S proteasomal degradation. Asher *et al*.[83] have reported that degradation of p53 also occurs in an MDM2- and ubiquitin-independent manner. They subsequently showed that this process is mediated by the 20S proteasome and is regulated by NAD(P)H quinone oxidoreductase (NQO1).[84] Both p53 and p73a interacted with NQO1 which was largely associated with the 20S proteasome and excess NQO1 and NADH, a cofactor of NQO1, prevented p53 degradation. The amount of p53 binding to NQO1 increased in response to DNA damage and this was prevented by dicoumarol, a drug that competes for NADH binding to NQO1. It seems likely that NQO1 acts as a gatekeeper to the 20S proteasome and associates with both p53 and p73α in a NADH-dependent manner to protect them from degradation.[84]

As outlined in Figure 6, several proteins other than MDM2 regulate stability of p53, some by influencing the interaction between MDM2 and p53 and others by mechanisms independent of MDM2. As mentioned above, DNA damage-induced phosphorylation of p53 increases its association with CBP/p300 transcriptional coactivators and as a consequence it increases p53 acetylation and leads to increased stability.[37,85] Under unperturbed conditions, MDM2 prevents interaction between p53 and p300.[86] A partner protein of p300, Strap, also increases the level and half-life of p53.[87] In response to DNA damage, Strap is phosphorylated by ATM leading to its relocalization to the nucleus, association with p300 and acetylation of p53. The nuclear protein p33ING2 also increases the acetylation of p53 at Lys382, inducing both G1/S checkpoint activation and apoptosis to negatively regulate cell growth and survival.[88] Another modification in the same region of the molecule, methylation, also regulates the activity of p53.[89] Overexpression of the Set9 methyltransferase caused hyperstabilization and activation of nuclear p53 and increased apoptosis. Set 9 methylated p53 on Lys 372 and restricted it to the nucleus.

The nucleolus and nucleolar proteins also impact on p53 stabilization. Rubbi and Milner[90] proposed a model in which nucleolar disruption was central to p53 stabilization. In this model, the nucleolus is a stress sensor for a variety of different agents that stabilize p53. They demonstrated that DNA damage *per se* does not stabilize p53 but required also nucleolar disruption. This model is further supported by the observations that the nucleolar protein, nucleophosmin, leaves the nucleolus in response to DNA damage, binds to p53 causing it to be stabilized and activated.[91] Its capacity to interact with MDM2 and act as a negative regulator of the p53–MDM2 complex is also key to this regulation.[92]

As is evident from Figure 6, a number of protein kinases influence the stabilization of p53. Overexpression of the homedomain-interacting protein kinase 2 (HIPK2) increases p53 stability which is further enhanced in the presence of doxorubicin.[93] While the exact mechanism of protection is not known, HIPK2-induced phosphorylation of p53 on ser46 may assist in dissociating p53 from MDM2 and reduced shuttling of p53 from the nucleus is also a likely influencing factor.[94] HIPK2 phosphorylation of p53 on ser46 is facilitated by axin which plays a role in activating the protein kinase.[95] JNK phosphorylates p53 at Thr81 in response to DNA damage and stress-inducing agents to stabilize and activate the molecule. On the other hand, Fuchs *et al*.[72] have shown that the extent of association of JNK with p53 is inversely correlated with p53 expression in unstressed cells. A mutant form of p53 incapable of binding to JNK had a prolonged half-life compared to wild-type p53, and a peptide corresponding to the JNK-binding site on p53 blocked ubiquitination of p53. These data indicate that JNK is an MDM2-independent regulator of p53 stability in unstressed cells.

The DNA damage response protein PARP is an essential component of base excision repair and has also been shown to be involved in apoptotic cell killing.[96] Its observed binding to p53 suggested that it might influence the stability and activity of p53. Indeed, this turned out to be the case in cells overexpressing PARP where it delayed the release of cells from the G1/S checkpoint due to increased stability of p53 and protected p53 from degradation.[97] Furthermore, constitutive levels of p53 were down in PARP$-/-$ mutant mice, DNA damage-induced stabilization of p53 was also defective[98] and inhibition of PARP activity delayed and attenuated the induction of the p53-responsive genes, p21 and MDM2.[99] Thus, PARP joins a long list of proteins that influence the stabilization and/or activation of p53.It remains unclear how this is achieved but its capacity to bind p53 may be pertinent.

The recent description of heterogeneous nuclear ribonucleoprotein K (hnRNP K) as an MDM2 target and transcriptional coactivator of p53 represents another level of control of p53 activation.[100] This protein is rapidly induced by UV damage in an ATR-dependent fashion. UV increased the half-life of hnRNP K by transient inhibition of its ubiquitin-dependent proteasomal degradation. A mutual dependence was demonstrated for p53 and hnRNP K for recruitment to p53 responsive genes and depletion of hnRNP K abrogated the transcriptional induction of p53 target genes. These data reveal a coordinating role for hnRNP K in controlling the transcriptional activity of p53 in response to DNA damage.

This review has considered primarily post-translational modification as the major means of bringing about p53 stabilization after DNA damage. A recent report by Takagi *et al*.[101] has demonstrated that increased translation of p53 mRNA can also contribute to the induction of p53 protein in irradiated cells. They demonstrated that the length of the 5'UTR of p53 had a significant impact on the amount of p53 protein produced in response to DNA damage. Furthermore, they also demonstrated that the ribosomal protein RPL26 and

nucleolin bound to the 5′UTR of p53. Overexpression of RPL26 increased directly p53 translation by changing the distribution of p53 mRNA on polysomes and knockdown of endogenous RPL26 with siRNA markedly attenuated p53 induction in response to different DNA-damaging agents. Downregulation of RPL26 also diminished radiation-induced apoptosis and overexpression of this protein caused a marked G1 arrest. On the other hand, the nucleolar protein, nucleolin, blunted the radiation-induced translation of p53 protein. These data support an additional form of regulation of p53 at the translational level in response to DNA damage.

Conclusions

The tumour suppressor protein p53 plays a key role in minimizing genetic damage and protecting against cancer. Since this protein functions by reducing cellular proliferative capacity by delaying passage of cells through the cycle, by inducing apoptosis or senescence, it is important that it be carefully regulated. This is achieved by its interaction with MDM2 and a host of other cellular proteins. These interactions are in turn controlled by a series of post-translational modifications that facilitate the stabilization and activation of p53. This form of control is very aptly illustrated in the p53-activation pathway leading to G1/S checkpoint control. While a large number of proteins have been shown to impact on the stabilization of p53, it is evident that others wait to be described and that the regulation of p53 has a few more surprises in store.

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