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P53 Licensed to Kill? Operating the Assassin

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Abstract The p53 protein is a key player in the cellular response to stress. Proper regulation of p53 is imperative for the suppression of tumor development. This regulation is largely governed by its master inhibitor, Mdm2, which both blocks p53 activities and promotes its destabilization. This tight regulation of p53 by Mdm2 must be interrupted under stress conditions in order for p53 to be stabilized in an active form. A combined action of partner proteins and modifying enzymes is essential for the relief of p53 from Mdm2. The recent revelation of p53 association with the PML-nuclear bodies provides one explanation of how this regulatory network is coordinated within the nucleus in response to certain stress conditions. Thus, it is not only the nature of the p53 regulatory complex but also the spatial and temporal context of this association that governs the output inhibitory signals mediated by p53. J. Cell. Biochem. 88: 76–82, 2003.

Key words: p53; Mdm2; PML; PML-NBs; phosphorylation; degradation; DNA damage

Over two decades of extensive research has been dedicated to exploring the intimate details of how the p53 tumor suppressor functions and is regulated. P53 performs a pivotal role in determining cellular responses to a variety of stresses including hypoxia, nucleotide deprivation, viral infection, heat shock, and oncogenic activation [reviewed by Giaccia and Kastan, 1998]. In apparent correlation with the severity of stress-induced damage sustained by a cell, p53 induces either growth arrest or programmed cell death (apoptosis), thereby preventing the replication of damaged DNA. Additional functions of p53 include contribution to the mitotic spindle checkpoint, DNA repair, senescence, and angiogenesis.

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The multi-faceted activities of p53 function to suppress tumor development. Critically, a high frequency of cancers, develop in mice lacking p53 and in Li-Fraumeni patients inheriting p53 mutations (reviewed by Vogt Sionov et al., 2001]. In fact, approximately 50% of human cancers have mutations in p53 and it appears that in the other 50%, p53 signaling is compromised by other mechanisms, such as inactivation of p53 cooperators or enhancement of p53 inhibitors. Thus, an increased predisposition for cancer development is associated with a lack of p53 function and the consequent failure to contain genetic aberrations.

The consequences of unscheduled activation of p53 targets are so severe, that p53 is kept under very tight regulation in healthy cells. In response to stress, however, p53 is accumulated in the nucleus, and through specific post-translational modifications, is rendered transcriptionally active. The p53 protein is regulated at three major levels, that of: protein stability, specific activity, and sub-cellular localization. This complex regulation is achieved by multiple positive and negative modulators of p53, often involving feedback loops. It is beyond the scope of this review to cover all aspects of p53 regulation, however, and we will focus on the regulation of p53 by its major inhibitor, Mdm2.

P53-Mdm2 INTERPLAY UNDER NORMAL AND STRESS CONDITIONS

In non-stressed cells, and in cells that have recovered from stress, p53 is expressed at low levels and is apparently biochemically inactive. P53 levels, transcriptional activity and consequent biological responses are negatively controlled, primarily through interaction with the Mdm2 oncoprotein. Importantly, Mdm2 is responsible for the degradation of p53 through the ubiquitin-proteasome pathway. The E3-ligase activity of Mdm2 is essential for p53 degradation. However, recent evidence points towards the involvement of additional proteins in the complete ubiquitination and targeted degradation of p53 [reviewed by Louria-Hayon and Haupt, 2002].

Mdm2 expression is induced by p53 upon activation, generating an auto-inhibitory circuit between these two proteins. The physiological relevance of this interplay has been clearly demonstrated in vivo using the mouse model. Embryos lacking *mdm2* die early during development. This early death is caused by massive p53-dependent apoptosis, and these embryos can be rescued by eliminating p53 [Parant et al., 2001; and references therein]. Interestingly, the Mdm2 analogue, MDMX (or MDM4), inhibits p53 without promoting its destabilization [Stad et al., 2000]. As in the case of *mdm2* null embryos, the early lethality of the *mdmx* embryos is p53-dependent. In contrast with the Mdm2 case, however, the death of *mdmx* embryos is caused by p53-mediated cell growth arrest [Parant et al., 2001; and references within]. These findings under physiological conditions demonstrate that the inhibitory activities of Mdm2 and Mdmx on p53 are complementary, rather than overlapping. The interesting possibility exists therefore, that these two inhibitors target distinct pools of p53 molecules, mediating different growth inhibitory signals. Due to space limitations, we will focus in this review only on the regulatory role of Mdm2.

MODULATION OF THE P53/Mdm2 INTERPLAY

The induction of *mdm2* expression along with the other p53 target genes that mediate p53 functions is enigmatic. Increased Mdm2 levels do not permit a sufficient time window for p53 to exert its inhibitory effects. It is imperative therefore, that the p53/Mdm2 autoregulatory loop be interrupted for p53 in order to be activated in a temporal and spatial manner. Over the past several years, extensive efforts have been devoted by many laboratories, including our own, to explore the molecular mechanisms underlying this interruption. Two major mechanisms have been identified, one involving post-translational modifications of p53 and Mdm2, and the other mediated through interaction with cooperating partner proteins. Elucidating the intricacies of the p53 and Mdm2 interaction that control this loop is an important key to our understanding of p53 regulation.

GENOTOXIC STRESS RESPONSES—P53 ACTIVATION THROUGH PHOSPHORYLATION

P53 Phosphorylation

The most extensively documented means of relieving p53 from Mdm2-mediated suppression, involves stress-induced phosphorylation. The principal phosphorylation sites identified in the p53–Mdm2 interplay are localized at the N-terminus of p53, within and near the Mdm2-binding site (amino acids 17–22) and these will be the focus of this review (depicted in Fig. 1). P53 is phosphorylated on serine 20 (Ser20) by distinct kinases depending on the incoming stress signals. Exposure to UV light triggers the checkpoint kinase 1 (Chk1) or JNK, while exposure to IR induces the ATM-activated Chk 2. Ser20 phosphorylation leads to p53 stabilization and activation by weakening the binding between p53 and Mdm2. The physiological importance of Chk2-induced p53 activation has been illuminated by the impaired accumulation and activation of p53 in the Chk2 null mice [reviewed by Bartek et al., 2001]. Recently, this impairment was determined to influence the apoptotic activity, but not the induction of G1 growth arrest by p53 [Jack et al., 2002]. Further, a low frequency of p53 mutations was found in cancers bearing germ-line or somatic mutations in the *Chk2* gene [Bartek et al., 2001].

The regulatory role of Ser20 has been challenged however, by one study in which the mouse equivalent Ser23, was substituted by alanine 23 (Ala23). This substitution in ES cells had no major effect on DNA-damage induced stimulation or stabilization of p53 [Wu et al., 2002], perhaps reflecting a difference in the role of Ser20 in human and Ser23 in the mouse. Besides Ser20, UV-irradiation induced

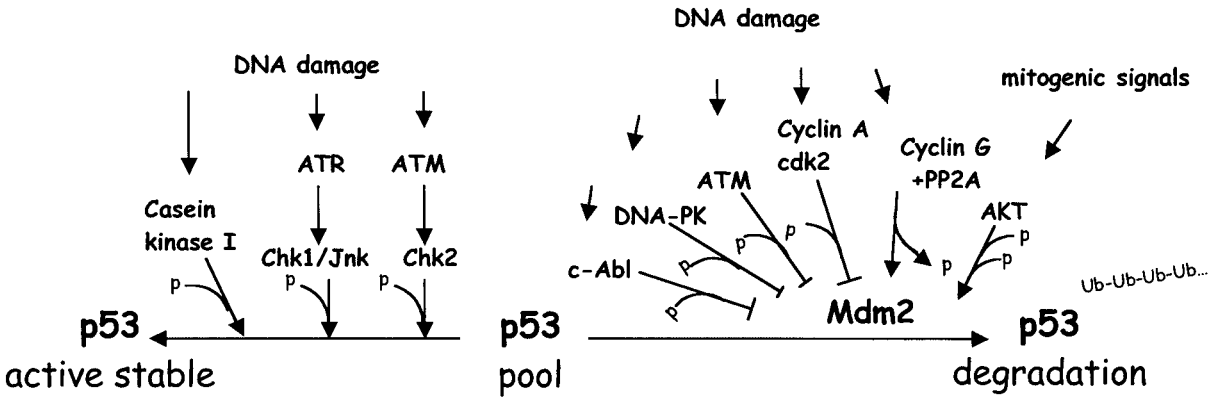

Fig. 1. Stress-induced p53 regulation by phosphorylation.

phosphorylation of human p53 at threonine 18 (Thr18) by casein kinase I reduces the p53/Mdm2 interaction [Bean and Stark, 2002]. The phosphorylation of Thr18 is preceded by phosphorylation at serine 15 (Ser15), demonstrating one of several post-translational modification cascades governing p53 regulation (reviewed by Appella and Anderson, 2001].

Mdm2 Phosphorylation

In addition to the stress-mediated phosphorylation of p53, ATM also phosphorylates Mdm2 on serine 395 (Ser395) in response to DNA damage. This phosphorylation impedes Mdm2-mediated degradation and nuclear export of p53 [Michael et al., 2002]. The critical importance of this region of Mdm2 for p53 regulation is emphasized by the activation of p53 following c-Abl tyrosine kinase phosphorylation of Mdm2 at the adjacent site, tyrosine 394 (Tyr394). Phosphorylation of this site impairs the ability of Mdm2 to promote the inhibition and destabilization of p53 [Goldberg et al., 2002]. Mdm2 inhibition of p53 is also weakened by the phosphorylation of Mdm2 at multiple sites by DNA-dependent protein kinase [DNA-PK; Mayo et al., 1997], and at threonine 216 (Thr216) by Cyclin A-cdk2 which enhances Mdm2-Arf binding [reviewed by Michael et al., 2002].

While phosphorylation at these described Mdm2 sites suppress Mdm2 inhibition of p53, the phosphorylation of Mdm2 by the survival promoting kinase, Akt, in contrast, enhances Mdm2 inhibition of p53. In response to mitogen-induced activation (i.e., serum stimulation), Akt phosphorylates Mdm2 on Ser166 and Ser186, thereby enhancing its nuclear localization and consequently the inhibition and degradation of p53 [Mayo and Donner, 2001]. Remarkably, p53 enhances the cleavage and destabilization of Akt, thereby creating another negative feedback loop. This loop is directed towards cell survival by Akt-dependent p53 degradation, or to apoptosis by p53-dependent Akt destruction [Gottlieb et al., 2002].

Mdm2 is also activated by an additional loop involving the downstream p53 target gene, *Cyclin G*. Cyclin G binds Mdm2 and recruits the PP2A phosphatase, thereby promoting the dephosphorylation of Mdm2 on Thr216, leading to activation of Mdm2 and destabilization of p53 [Okamoto et al., 2002]. Thus, the cooperation of two p53 target genes ensures an efficient termination of its growth inhibitory signals. Surprisingly, Cyclin G-PP2A also dephosphorylates Ser166 (the Akt site). Thus, PP2A and Akt have antagonistic effects on S166 phosphorylation, but with the same biochemical outcome. Further studies are needed to clarify this apparent contradiction.

P53 ACTIVATION THROUGH INTERACTION WITH PARTNER PROTEINS

Within the nucleus p53 may be rendered transcriptionally competent through interaction with partner proteins. P53 activation via protein–protein interaction maybe achieved either by direct enhancement of p53 transcriptional activity, or indirectly by relieving p53 from Mdm2-mediated suppression. Direct activation appears to involve binding of partner proteins to the C-terminus of p53. This has been demonstrated for several proteins, including BRCA-1, 14-3-3σ, c-Abl, and Ref-1. Interestingly, either removal of the p53 C-terminus, or the interaction of this region with a specific peptide or antibody induced p53 DNA-binding

and activation [Hupp et al., 1995]. This binding was believed to release the core DNA binding domain of p53 from negative effect imposed by the C-terminus. However, the mechanism for p53 activation via the C-terminus, in particular with respect to the regulatory role of p53 acetylation at this region is highly controversial [reviewed by Prives and Manley, 2001].

Avoiding p53 suppression by Mdm2 may be achieved by preventing their association, through interaction with additional proteins. Antibodies and peptides have been used to demonstrate the efficiency of this approach, and indeed, the p53 co-activator TAFII31 competes with Mdm2 for binding p53 in vivo [reviewed by Louria-Hayon and Haupt, 2002]. However, in most other cases, the partner proteins appear to induce p53 activation without interrupting the p53–Mdm2 interaction. The possibility that p53 is able to perform transcriptional activation with Mdm2 bound at its transcriptional activation domain is counter-intuitive, and one of two possible explanations maybe conjectured to resolve this enigma. One scenario is that binding of additional protein(s) to the p53–Mdm2 complex alters p53 configuration to expose the transcriptional activation domain. Alternatively, it has been suggested that a small fraction of p53 is transcriptionally active, but has not been detected in the background of the Mdm2–p53 pool. Improved techniques for the detection of low amounts of active p53, and better structural analyses of the various p53 complexes will help to distinguish between these possibilities.

ONCOGENIC ACTIVATION OF P53 IS FUNNELED THROUGH ARF

Onocogenic stimuli in a variety of normal cell types activate the tumor suppressor ARF (alternative reading frame of the INK4a/Arf tumor suppressor locus), leading to the induction of p53-dependent premature senescence, or apoptosis. Following activation, Arf, normally a resident of the nucleoli [Sherr and Weber, 2000], has been understood to trigger the importation of Mdm2 into the nucleoli. ARF appears to activate p53 by inhibiting the E3 ligase activity of Mdm2 [Honda et al., 1997]. Interestingly, however, the nucleoplasmic form of ARF can also neutralize Mdm2, although without relocating Mdm2 to the nucleoli, by an as yet unknown mechanism [reviewed by Llanos et al., 2001].

Deregulation of oncogenes such as, Ras, c-Myc, adenovirus E1A, and β-catenin induce *Arf* expression primarily through the activation of the E2F-1 transcription factor. Arf is also upregulated by the transcription factor DMP1 and repressed by Bmi-1 and Twist [Balint and Vousden, 2001]. Consistent with a number of other proteins that govern p53 activation, ARF is also engaged in a negative feedback loop involving p53. P53 represses Arf expression by abrogating E2F-1 activation [Sherr and Weber, 2000]. Prevention of inappropriate ARF activation induced by legitimate mitogenic signals is ensured, for example in the Ras–Raf growth promoting pathway, by the synchronized induction of Mdm2 expression in the context of p53 activation by Arf [Ries et al., 2000].

Further adding to the intrigue of p53 activation following exposure to oncogenic stress is the ability of a transgenic mouse tumor brain model to transmit an oncogenic response to p53 in an ARF-independent manner. This observation has been interpreted to indicate the existence of distinct cell-specific pathways that respond to similar stimuli [Tolbert et al., 2002]. In addition, ARF appears to be able to perform growth suppression independent of p53 stabilization [Korgaonkar et al., 2002] and in the absence of p53 and Mdm2 by targeting additional cell regulatory proteins [Sherr and Weber, 2000], however, as this is beyond the scope of this review, these Arf activities will not be expounded.

"LOCATION LOCATION LOCATION" PRINCIPLE OF P53 REGULATION

The nuclear localization of p53 is essential for it to act as a transcription factor. Manipulation of the p53 sub-cellular distribution therefore poses an important means of regulating p53 function. In normal non-stressed cells, p53 oscillates between the cytoplasm and the nucleus in a cell-cycle-dependent fashion [reviewed by Jimenez et al., 1999; Liang and Clarke, 2001]. Upon stress imposition, however, p53 accumulation in the nucleus is promoted. Interference with this nuclear uptake may effectively inactivate p53. Evidence of the efficacy of this approach to p53 inactivation is demonstrated by the common observation of cytoplasmic accumulation of p53 in tumors of the breast, colon, cervix, and in neuroblastomas [reviewed by Vogt Sionov et al., 2001].

The nuclear accumulation of p53 in response to stress may involve an enhanced nuclear import from the cytoplasm and/or inhibition of nuclear export. P53 is actively transported into the nucleus via the engagement of the p53 nuclear localization sequences (NLSs) with the nuclear transporter, importin-α. Truncation of this nuclear transporter results in the cytoplasmic accumulation of p53, which represents an effective means of inhibiting p53 [Kim et al., 2000]. However, the mechanisms by which p53 nuclear import is regulated, remains to be explored.

Nuclear export of p53 appears to be mediated by p53 nuclear export sequences (NESs) located at the C-terminal [Liang and Clarke, 2001] and N-terminal [Zhang and Xiong, 2001]. Of particular interest is the N-terminal NES located between residues 11 and 27, overlapping with the Mdm2 binding site and the phosphorylation sites, Ser15 and Ser20. It is attractive to speculate that nuclear export may be prohibited by the masking of this NES through phosphorylation of these two sites. The C-terminal NES overlaps the tetramerization zone of p53. The apparent masking of this region in the tetrameric form, but exposure in the monermeric/dimeric forms suggests an elegant means of controlling p53 nuclear export [Jimenez et al., 1999]. The need for two NESs is not yet clear, although one may postulate that the N-terminal NES may sense stresses such as DNA damage, through N-terminal phosphorylation, while the C-terminal NES may sense the p53/Mdm2 ratios within each cellular compartment at any given time.

The nuclear export of p53 is enhanced by Mdm2 or by HPV-E6 proteins [Thomas et al., 1999; Liang and Clarke, 2001]. Since both proteins promote p53 degradation, it was concluded that nuclear export is an integral step in p53 degradation. However, the simple exportation of p53 to the cytoplasm by CRM1 overexpression is not sufficient for promoting p53 degradation [Lohrum et al., 2001]. In fact, the major requirement for Mdm2-mediated p53 degradation is the co-localization of both proteins in the same compartment [Xirodimas et al., 2001]. Fascinatingly, it is now clear that p53 can be ubiquitinated and degraded both in the nucleus and the cytoplasm [Yu et al., 2000; Lohrum et al., 2001]. Compilation of these studies indicate that the ubiquitination and degradation of p53 by Mdm2 may be subject to independent regulation. Further, the observation that CRM1 can export p53 to the cytoplasm without affecting Mdm2 localization indicates that the export of the two proteins can be independent events [Lohrum et al., 2001]. While a big leap has been taken in understanding how the sub-cellular distribution of p53 is regulated, many questions remain unanswered. For instance, what is the link between p53 ubiquitination and its nuclear export?

ROLE OF PML IN P53 ACTIVATION

P53 activities appear to be regulated at a finer level than first emerged with the nuclear-cytoplasm division. In response to stress, within the nucleus itself, p53 is recruited into small structures defined as promyelocytic leukemia protein (PML) nuclear bodies (PML-NBs). Functional PML is essential for the formation of these sub-nuclear structures, and when it is fused to the retinoic acid receptor α, as in the case of acute promyelocytic leukaemia patients, the formation of these structures is impaired. Interestingly, p53 is recruited into the PML-NBs in response to Ras activation, UV-light and ionizing radiation (IR) [Carbone et al., 2002; Salomoni and Pandolfi, 2002]. A regulatory role for the PML-NBs was proposed when it was realized that important p53 modulators are also localized into these structures. The acetyl transferase CBP is recruited into the PML-NBs where it acetylates p53 and consequently enhances p53 transcriptional activity [Pearson et al., 2000], a process that can be reversed by the histone deacetylase hSir2 [Langley et al., 2002]. This acetylation is facilitated by the prior phosphorylation of p53 on Ser46 by the human serine/threonine kinase homeodomain-interacting protein kinase-2 (HIPK2) that is also localized to the PML-NBs. [D'orazi et al., 2001; Hofmann et al., 2001]. More recently, these modifications were also proposed to contribute to p53 stabilization [Bischof et al., 2002]. Further, the ubiquitin specific protease, HAUSP, has been recently shown to de-ubiquitinate p53 and consequently lead to p53 stabilization [Li et al., 2002]. However, the link between HAUSP and stress signals that activate p53 is yet to be demonstrated.

These modifications help to explain the critical regulatory role of PML in the activation of p53 as shown in the mouse model. The transcriptional activity of p53 in the PML null mice is severely impaired, and these mice are highly

resistant to IR-induced apoptosis [Salomoni and Pandolfi, 2002]. However, since Ser46 is not conserved between mice and humans and the regulatory role of the C-terminal acetylation is still controversial, it is likely that additional mechanism(s) exist(s) to explain the regulation of p53 by PML in association with the PML-NBs.

CONCLUSIONS

P53 is a loaded gun kept in check by the safety catch Mdm2. Deconstruction of the p53/Mdm2 loop has revealed multiple internal loops, all designed to assure accurate and timely gun firing to avoid unwanted consequences that can be either crippling or lethal depending on the target site. While the intricacies of how the loop is modulated are being unraveled, the exact role of Mdm2 in the ubiquitination and degradation of p53 is yet to be unequivocally demonstrated. What other factors are required for the polyubiquitination of p53 and for its recognition by the proteasome, remain to be explored. The revelation of the dynamic transportation of p53 and its modulators between the nucleus and cytoplasm, and within organelles opened a new window for the understanding of p53 regulation. In this regard, the link between p53 and PML and their association with the PML-NBs network has provided an example of how p53 that is dispersed within the nucleus can be become activated in response to specific stress signal in a rapid and coordinated manner. Much is yet to be understood about the role of the PML-NBs in p53 regulation; for instance, what other modifications of p53 occur in these structures, and where and how Mdm2 fits in the PML axis? Nevertheless, these new insights have raised the possibility that other networks may operate to coordinate p53 activation in response to specific signals. The recent trend to study p53 regulation at multiple levels, in space and time, has had an enormous contribution to this topic.

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