The p53 protein and tumor suppression

Proper coordination of p53-responsive gene expression provides a crucial barrier to tumor development, and more than 50% of human cancers harbor mutations in p53 (1,2). Moreover, in cancers bearing wild type p53 alleles, p53 function is frequently compromised through mutations of associated positive regulators or amplification of negative regulators of p53 (1). A large number of p53-mutant mouse models have been developed to study p53 function and regulation. Mice homozygous for p53-null alleles rapidly developed spontaneous tumors (3-5). All $p53^{-/-}$ mice develop tumors by 10 months of age, with a mean time to tumorigenesis of approximately 4.5 months. These tumors are primarily lymphomas (~70–80%, largely T-cell in origin), with some incidence of sarcomas and other tumor types. $p53^{+/+}$ mice develop tumors later than $p53^{-/-}$ mice, with the earliest tumor presentation around 12 months of age (4-6). In contrast, the majority of $p53^{+/+}$ mice (~95%) develop tumors by 24 months of age, with a mean time to tumorigenesis of approximately 17 months. These p53-heterozygous animals present with lymphomas (primarily of B cell origin), osteosarcomas, soft-tissue sarcomas and a range of carcinomas (7). Interestingly, one study observed that while the remaining wild-type $p53$ allele is deleted in approximately half of $p53^{+/+}$ tumors, the other half retain a functional wild-type allele (8). Thus, a reduction in p53
dosage is alone sufficient to increase the susceptibility of p53<sup>−/−</sup> cells to tumorigenesis (9).

In agreement with early observations that p53 impaired the ability of oncogenes to transform cells (10,11), numerous studies have shown that combining the p53-null allele with mouse models of cancer, such as those driven by PTEN deficiency, activated BRAF<sup>V600E</sup>, or Myc overexpression, leads to accelerated tumor development (12-14). Early in vitro studies performed in human cells aimed at identifying the mechanism by which p53 impaired tumor formation identified p53 as a regulator of cell growth, senescence, and apoptosis (15-19). This was supported by subsequent work in mice, wherein p53 was found to regulate spontaneous immortalization of MEFs (20-22), and to be essential for oncogene-induced senescence in cells overexpressing Ras, E2F1 and constitutively active β-catenin (23-25). A role for p53-dependent senescence in tumor suppression in mice was highlighted using mice expressing a mutant p53 protein (p53<sup>R172P</sup>) that was defective for apoptosis but retaining growth arrest capabilities. These mice displayed delayed Eµ-myc-driven B cell lymphomagenesis compared to mice heterozygous for p53 (26).

The influence of p53 on growth arrest and senescence has been largely attributed to its ability to upregulate p21 expression. p21 protein levels increase in normal fibroblasts as they approach senescence (27) and p21<sup>−/−</sup> cells display reduced growth arrest in response to DNA damage (28,29). Furthermore, tumor cell lines lacking functional p53 failed to arrest in response to forced expression of p53 when the p21 gene was disrupted (30), and induced expression of p21 promotes senescence in tumor cells lacking functional p53 (31,32).

Studies in primary murine cells have also shown p53 accumulation and stabilization can promote increased apoptosis (33,34), and p53-dependent apoptosis has been shown to suppress Eµ-myc-driven B cell lymphomagenesis (35), E2F1-driven skin carcinomas (36), and brain tumorigenesis induced by a mutant SV40 T antigen (non p53-binding Tg<sub>T<sup>121</sup></sub>) (37). Interestingly, development of Eµ-myc-driven tumors is also accelerated in mice lacking the p53-responsive, pro-apoptotic genes Bax (38), or Puma (39-41).

Although p53-dependent growth arrest, senescence and apoptosis appear to be important tumor-suppressive mechanisms, the relative contribution of these mechanisms is likely to be tissue- or cell-type-dependent. This is exemplified by a series of studies in which p53 reactivation in established tumors resulted in apoptosis in lymphomas and senescence in sarcomas, respectively (42-44). More recently, a number of studies have implicated additional p53-dependent mechanisms in impairing tumor growth. Gu and colleagues have described a knock-in mouse in which three p53 acetylation sites in the p53 DNA-binding domain were mutated to arginine (p53<sup>3KR</sup> mice) (45). p53<sup>3KR</sup> mice and cells fail to transactivate the majority of p53 target genes, and induce growth arrest or apoptosis. However, these mice do not develop cancer, suggesting that spontaneous tumor suppression by p53 may occur independently of growth arrest or apoptosis. It is proposed that as these animals retain the ability to transactivate the metabolic targets Glc2 and TIGAR, p53 may display tumor suppressive activity through the regulation of energy metabolism and reactive oxygen species (ROS) levels (45). Similar findings have been reported using, triple knock-out mice deficient for p21, Puma, and Noxa (p21<sup>−/−</sup>puma<sup>−/−</sup>noxa<sup>−/−</sup> mice) (46). These mice are profoundly resistant to DNA damage-associated apoptosis and growth arrest, and largely (but not entirely) resistant to p53-dependent senescence, yet do not develop spontaneous tumors. These authors noted that induction of p53 target genes involved in DNA repair was unperturbed in p21<sup>−/−</sup>puma<sup>−/−</sup>noxa<sup>−/−</sup> mice, and propose that coordination of DNA repair is an essential tumor suppressive activity of p53. More recently, Gu and colleagues have identified the ability of p53 to transcriptionally repress the cysteine/glutamate antiporter SLC7A11 and induce ferroptosis [an iron-dependent mechanism of non-apoptotic cell death (47)] in response to ROS as another mechanism by which p53<sup>3KR</sup> mice may suppress tumorigenesis (48).

The role of p53 responses in the DNA damage response

Following the identification of p53 as a tumor suppressor protein, and in concert with the aforementioned studies elucidating the tumor suppressive activities of p53, a series of studies showed that p53 levels and activity increased in response to DNA damage. Treatment of cells with DNA damaging agents such as ultraviolet light (UV), ionizing radiation (IR), and numerous cancer therapeutic and/or DNA damage-inducing compounds such as diaminodichloroplatinum (cisplatin), mitomycin C, etoposide, hydroxyurea (HU), methyl methanesulfonate (MMS) and actinomycin D results in increased p53 protein levels and associated cell cycle arrest (49-52). Furthermore, p53<sup>−/−</sup> MEFs are resistant to oncogene-sensitized apoptosis in response to serum withdrawal or a variety of genotoxic agents (53,54). Analyses of p53<sup>−/−</sup> mice determined that p53 governs IR-induced apoptosis in both thymocytes (55-57).
and epithelial stem cells of the small intestine (58). Further studies have identified additional radiosensitive cell populations in the spleen, bone marrow, and hair follicles (59).

An early indication of the signaling pathways governing the p53 response to DNA damage came from analysis of from patients with ataxia-telangiectasia; an autosomal recessive disorder resulting in neuronal degeneration, sensitivity to IR, premature ageing, increased incidence of cancer and other pathologies. Patients' cells do not display increased p53 levels and activity following IR exposure (60), and mice null for ataxia telangiectasia mutated (ATM) are extremely sensitive to IR-induced lethality, and show profound defects in DNA damage-induced growth arrest and apoptosis (61-64). Similar to AT patient cells, p53 is not stabilized in ATM−/− MEFs or thymocytes following IR (63,65). Furthermore, these animals succumb to T-cell lymphomas by 6 months of age (61,62). Research aimed at identifying the mechanism by which ATM leads to p53 stabilization is discussed below.

### Regulation of p53 function

The chief negative regulator of p53 stabilization and activity is the Mdm2 oncoprotein. The murine double minute 2 (Mdm2) gene was initially identified as an amplified DNA sequence present in a spontaneously immortalized mouse 3T3 cell line (66). Mdm2 overexpression is capable of conferring tumorigenicity (67), and Mdm2 is amplified in a significant fraction (~30%) of soft tissue sarcomas (68-70). Further studies have identified Mdm2 amplification in a variety of other tumor types, including breast carcinomas (71), glioblastomas and astrocytomas (72), myeloid neoplasms (73), B cell lymphomas (74) and oral carcinomas (75).

Shortly after the identification of Mdm2's interaction with p53, mapping of the p53 and Mdm2 interaction domains determined that the N-terminus of Mdm2 bound to and inhibited the transactivation domain of p53 (76,77). Accordingly, Mdm2 overexpression cooperates with Ras in transforming primary cells (78), and inhibits p53-dependent growth arrest and apoptosis in various cell lines (79-81).

It was subsequently shown that Mdm2 can also promote the proteosomal degradation of p53 (82,83) by functioning as an E3 ubiquitin ligase and directing p53 polyubiquitination (84). This E3 activity of Mdm2 is dependent on its C-terminal RING finger domain (85), which also promotes the nuclear export of p53 by directing its monoubiquitination (86-89). It is proposed that low levels of Mdm2 activity induce monoubiquitination and nuclear export of p53, whereas high levels promote polyubiquitination and nuclear degradation of p53 (90).

The principle sites on p53 of Mdm2-ubiquitin ligation are a series of C-terminal lysines also targeted by acetylation, creating one of many layers of regulation of p53 stability and activity (91-94). Notably, the Mdm2 gene is also a target of the p53 transcription factor (95,96). As p53 becomes stabilized and active, it increases the expression levels of its own negative regulator Mdm2, forming an autoregulatory feedback loop that returns p53 protein and activity to basal levels. Furthermore, Mdm2 has also been shown to be capable of directing its own degradation (85,97).

The crucial role of Mdm2 in regulating p53 activity is illustrated by the p53-dependent lethality of Mdm2-null mice during early embryogenesis. Mdm2−/− mice display peri-implantation lethality due to unregulated p53 activity, and crossing these mice with the p53-null allele completely rescues Mdm2-null mice (98,99). Mice null for both Mdm2 and p53 develop spontaneous tumors of similar incidence and spectrum as p53-null mice (100). Furthermore, primary p53-null and Mdm2/p53 double-null cells display similar growth characteristics in culture and in their response to genotoxic agents (100). Mdm2−/+ mice display delayed Myc-driven lymphomagenesis (101), and hypomorphic-Mdm2 mice expressing reduced levels of Mdm2 displayed p53-dependent sensitivity to radiation-induced lethality and apoptosis in lymphopoietic tissues (102). Thus, perturbations in the levels of Mdm2 can significantly impact p53 responses to oncogenes and DNA damage.

In contrast with Mdm2-null mice, mice overexpressing an Mdm2 transgene are viable and succumb to spontaneous tumors (103). The rate of tumorigenesis in Mdm2-transgenic mice is slower than that observed in p53−/− or p53−/− mice, likely due to the relatively modest levels of Mdm2 overexpression (approximately 4 fold) in the Mdm2-transgensics. However, like p53-mutant mice, Mdm2-transgensics present with a large percentage of lymphomas and sarcomas. Notably, while presence of the Mdm2-transgene does not accelerate spontaneous tumorigenesis in p53−/− mice, it does increase their incidence of sarcomas, revealing a p53-independent contribution of Mdm2 overexpression to tumorigenesis. Mdm2 overexpressing mice also display accelerated Myc-driven lymphomagenesis (104). This same study showed that elevated levels of Mdm2 resulted in reduced p53 protein levels and activity in B cells, and reduced B cell apoptosis following IR.

Similar to Mdm2, the related protein MdmX (or Mdm4) is also capable of binding p53 and inhibiting p53 transactivation of target genes (105,106). MdmX and Mdm2 share 34%
protein homology and contain highly homologous p53-binding, acidic, zinc finger, and RING finger domains (105). As with Mdm2, the MdmX gene is amplified or overexpressed in a variety of tumor types (107-111). However, unlike Mdm2, MdmX does not possess the ability to directly ubiquitinate p53 (112,113).

Mice null for MdmX display a similar p53-dependent embryonic lethality as observed in Mdm2−/− mice, albeit later in development (E9.5-10.5), and are rescued by deletion of p53 (114-116). Notably, the lethality in MdmX−/− embryos appears to be predominantly associated with a lack of proliferation, as opposed to aberrant apoptosis [as reported for Mdm2−/− embryos (117)]. Accordingly, a subsequent study revealed that co-deletion of p21 can significantly delay the embryonic lethality of MdmX-null mice (118). MdmX−/− cells and mice display decreased oncogene-driven transformation and Eµ-myc-driven lymphomagenesis, respectively (119), and MdmX−/− mice are sensitized to radiation induced lethality (119).

Mdm2 and MdmX have been shown to interact via their C-terminal RING domains (120,121), and stabilization of Mdm2 by heterodimerization with MdmX increases the ability of Mdm2 to degrade p53 (122-125). Notably, the turnover of MdmX is mediated by Mdm2 (126-128).

Recently, a series of Mdm2 and MdmX knock-in mouse models have been generated that display altered Mdm2-MdmX interactions and/or Mdm2 E3 ligase activity (129-132). Analyses of these models have revealed that Mdm2-MdmX interactions are crucial for inhibiting p53 activity during development and tissue homeostasis, whereas the E3 ligase function of Mdm2 is vital for regulating p53 protein levels and activity in cellular and organismal responses to DNA damage (132).

**p53 stabilization and activation in response to cell stress**

The inhibitory role of MDM proteins on p53 protein stabilization and activities must be interrupted in order for p53 to become elevated and activated in response to DNA damage or other forms of stress.

An important mediator of oncogene-dependent activation of p53 is the tumor suppressor protein p19ARF (p14ARF in humans). Oncogenes including c-Myc, Ras and E1A induce ARF and cause p53-dependent growth arrest and apoptosis (133-135). ARF binds to Mdm2 and can block its ubiquitin ligase activity towards p53 (136-141) as well as sequester Mdm2 in the nucleolus (142,143). This facilitates p53 protein stabilization and activation in order to limit the transformative effects of aberrant oncogene activity.

**DNA damage-induced modifications of p53**

The cellular response to DNA damage is primarily governed by the PI3K-related serine/threonine kinases (PIKKs) ATM and ataxia telangiectasia and Rad3-related protein (ATR). ATM is activated by DNA damaging agents that create DSBs, while ATR is activated following recruitment to ssDNA regions. The related protein DNA-PK (DNA-dependent protein kinase) primarily regulates a smaller group of proteins involved in DSB end joining. Following the recognition of DNA damage by different sensor proteins, these kinases trigger the direct or indirect phosphorylation of numerous effector proteins involved in a multitude of signaling networks that promote different DNA repair processes, cell-cycle arrest and programmed cell death (144,145).

Included among the various PIKK substrates is p53. p53 is phosphorylated on a number of residues, primarily clustered in the N- and C-terminal regions (Figure 1), in response to various DNA-damaging agents (146,147). Phosphorylation of C-terminal residues is primarily thought to influence site-specific DNA binding by p53, whereas N-terminal phosphorylation events have been implicated in regulating the Mdm2-p53 interaction as well as p300/CBP recruitment (146,147). Seven serines (Ser6, 9, 15, 20, 33, 37, 46) and two threonines (Thr18 and 81) in the N-terminal region of human p53 are phosphorylated in response to exposing cells to IR or UV light (146). The majority of these phosphorylation events occur directly by ATM, ATR, or DNA-PK (148-151) or indirectly by the ATR- and ATM-activated checkpoint kinases Chk1 or Chk2 (152-154). Additionally, casein kinase 1 (CK1) has also been shown to phosphorylate a number of these residues (155,156).

Of particular interest in the search for the mechanism of p53 stabilization following DNA damage were residues Ser15 and Ser20 (Ser18 and Ser23 in mice). In vitro experiments revealed phosphorylation of Ser15, a target of both ATM and ATR, inhibits the p53-Mdm2 interaction (157) and coincides with p53 activation (158). Similar experiments have shown phosphorylation of Ser20, a target of Chk2, leads to reduced Mdm2-mediated degradation of p53 and increased p53 activity (153,159,160). Furthermore, Thr18 phosphorylation, which occurs through CK1 and can disrupt Mdm2-p53 binding, was shown to be dependent on prior Ser15 phosphorylation (155,161).
As these p53 residues are located within, or immediately adjacent to, the Mdm2-p53 binding interface (162), it was hypothesized that phosphorylation of these residues was sufficient to account for p53 stabilization and activation following DNA damage. However, analysis of various genetically engineered mouse models, which allowed for the examination of these phosphorylation events under endogenous conditions, revealed that these phosphorylation events were insufficient to account for the full effects of DNA damage on p53 stabilization and activation or for p53 tumor suppression.

*p53<sup>S18A</sup>* mice in which serine 18 (Ser15 in humans) is replaced with alanine show no significant defects in p53 protein stabilization in thymocytes or MEFs in response to DNA damage (163,164). MEFs from *p53<sup>S18A</sup>* mice show no defects in proliferation or growth arrest after DNA damage, while thymocytes show an intermediate (compared to *p53<sup>−/−</sup>* thymocytes) defect in apoptosis. However, the ability of p53 to transactivate a number of target genes is compromised in *p53<sup>S18A</sup>* mice. A recent report suggests that this may be due to a role for p53 Ser15 in transcription and promoter relaxation as opposed to p53 stabilization (165). Additionally, *p53<sup>S23A</sup>* mice develop *Eµ-myc*-driven B cell lymphomas at an accelerated rate, possibly due to their apoptotic defects (166).

*p53<sup>S23A</sup>* mice in which serine 23 (Ser20 in humans) is replaced with alanine, show a similar absence of defects in p53 stabilization or growth arrest MEFs (167,168). However, *p53<sup>S23A</sup>* mice do show reduced stabilization of p53 and apoptosis in thymocytes in response to IR, though intermediate compared to *p53<sup>−/−</sup>* thymocytes (168). Furthermore, *p53<sup>S18A/S23A</sup>* mice develop spontaneous tumors (predominantly B cell lymphomas) beginning at approximately 12 months, with 70% of animals having developed tumors by 24 months. Interestingly, *p53<sup>S18A/S23A</sup>* mice, in which both Ser18 and Ser23 have been substituted, display more profound deficiencies in p53 stabilization and function, indicating an additive effect of phosphorylation of these two residues in regulating p53 function (169). While still intermediate to the phenotypes observed in *ATM<sup>−/−</sup>* and *p53<sup>−/−</sup>* cells, thymocytes from *p53<sup>S18A/S23A</sup>* mice show more significantly impaired p53 stabilization, transactivation of target genes, and apoptosis in response to irradiation than either single-
mutant alone. However, p53 stabilization and activities are still unperturbed in MEFs. These mice are similarly tumor prone as reported for Ser23 mutant mice, and again present primarily with lymphomas.

**DNA damage-induced modifications of MDM proteins**

As the in vivo results obtained from p53 knock-in mice failed to replicate the profound defects in DNA damage-induced p53 stabilization and activity predicted by in vitro studies, additional signaling events must contribute to this process. In addition to p53, its primary negative regulators Mdm2 and MdmX are also subject to a multitude of phosphorylation events in response to DNA damage (Figure 1).

**Phosphorylation of MDM proteins by ATM**

In response to DNA damage, human MdmX is phosphorylated at Ser342 and Ser367 by Chk2 (170-173) and Ser403 by ATM (174). These phosphorylation events lead to MdmX degradation, concurrent with p53 stabilization and activation (170,171). This phosphorylation-dependent degradation of MdmX is proposed to be directed by Mdm2, and possibly mediated by changes in MdmX binding by 14-3-3 and the deubiquitinase HAUSP (171-173,175). Wahl and colleagues have generated an MdmX<sup>3SA</sup> mouse model in which all three of these serine residues are replaced with alanine (176). MdmX<sup>3SA</sup> mice display impaired p53 stabilization and decreased p53 activity in response to IR. Furthermore, MdmX<sup>3SA</sup> mice are resistant to lethal doses of IR, and though not prone to spontaneous tumorigenesis, these mice display increased Erb-myc-driven lymphomagenesis. Thus, DNA damage-induced phosphorylation of MdmX, a negative regulator of p53, can impact p53 stabilization and activity.

ATM-dependent phosphorylation of Mdm2 also precedes p53 stabilization after DNA damage (177). It was initially shown that ATM directly phosphorylates Ser395 of human Mdm2 (Ser394 in mouse) in response to DNA damage (178). Furthermore, in transfection-based assays, Mdm2 with an aspartic acid in place of Ser395 (mimicking phosphorylation) shows a decreased capacity to induce p53 degradation and nuclear export (178). The phosphatase Wild-type p53-induced phosphatase 1 (Wip1) can dephosphorylate Mdm2 Ser395, and dephosphorylated Mdm2 has increased stability and affinity for p53, facilitating p53 ubiquitination and degradation (179). This result trends with another study that showed that DNA damage-induced p53 stabilization is preceded by the destabilization of Mdm2, which is a phenomenon that could be inhibited with the PIKK-inhibitor wortmannin (180). However, a subsequent study identified five additional residues in the C-terminal region of human Mdm2 that are phosphorylated by ATM (Ser386, Ser407, Thr419, Ser425 and Ser 429) (181) and in vitro work in which all six residues were replaced with alanine or aspartic acid suggests that ATM phosphorylation of this series of residues inhibits RING domain oligomerization and E3 ligase activity (181,182). Thus, ATM phosphorylation of Mdm2 is proposed to influence both Mdm2 stability and activity towards p53.

In order to examine the impact of Mdm2 Ser395 phosphorylation under endogenous conditions, our lab has previously reported the generation and initial characterization of a mouse model wherein ATM phosphorylation of Mdm2 at serine residue 394 (the equivalent of human Mdm2 Ser395) was abolished (Mdm2<sup>S394A</sup> mice) (183). Cells and tissues in Mdm2<sup>S394A</sup> mice display profound defects in DNA damage-induced p53 protein stabilization and p53 target gene activation. This failure to induce a robust p53 response translates to less p53-dependent apoptosis in hematopoietic tissues, radio-resistance, and increased spontaneous lymphomagenesis. Furthermore, replacing Mdm2 Ser394 with aspartic acid (Mdm2<sup>S394D</sup> mice) and mimicking constitutive phosphorylation at this residue result in prolonged p53 activity following damage and suggest dephosphorylation of this residue is involved in resolving the p53 response (183). Therefore, ATM phosphorylation of the negative regulator of p53, Mdm2, can profoundly impact p53 stabilization and activation in response to stress in vivo. In a subsequent study using Mdm2<sup>S394A</sup> mice we showed that phosphorylation of Mdm2-S394 regulates p53 activity and the DNA damage response in lymphatic tissues in vivo by modulating Mdm2 stability (184). Intriguingly, while Mdm2-S394 phosphorylation delays lymphomagenesis in Erb-myc transgenic mice, and preventing Mdm2-S394 phosphorylation obviates the need for p53 mutation in Myc-driven tumorigenesis, irradiated Mdm2<sup>S394A</sup> mice display increased hematopoietic stem and progenitor cell functions, and decreased radiation-induced lymphomagenesis. These findings document contrasting effects of ATM-Mdm2 signaling on p53 tumor suppression (184).

**Phosphorylation of MDM proteins by c-Abl**

Similar to ATM, the c-Abl tyrosine kinase is activated by a variety of DNA damaging agents (185-187). c-Abl interacts with ATM and is phosphorylated on Ser465, leading to its
activation (188,189). This initially led to c-Abl activities in the DNA damage response being viewed as downstream of ATM. However, more recent work has shown c-Abl to phosphorylate both ATM and ATR, and that these phosphorylation events are required for maximal activity of either PIKK (187). Overexpression studies indicate c-Abl promotes growth arrest in a p53-dependent manner, and apoptosis by p53-dependent and independent mechanisms (190-193). c-Abl mediated p53-independent apoptosis is attributed to the p53 homolog p73, which is directly phosphorylated by c-Abl on Tyr99 (194-196). However, no c-Abl target residues have been identified on p53.

MdmX is phosphorylated by c-Abl on Tyr55 and Tyr99 in response to DNA damage. These residues are located within the p53 binding domain of MdmX, and Tyr99 phosphorylation impairs p53 binding in a transfection-based assay (197). Similar co-expression studies have shown that c-Abl protects p53 from Mdm2 mediated degradation, and overcomes the inhibitory effect of Mdm2 on p53 transcriptional activity and p53-dependent apoptosis (198). Additionally, c-Abl is required for maximal p53 accumulation in response to IR, doxorubicin, and mitomycin C in MEFs, and co-expression of c-Abl overcomes Mdm2 mediated ubiquitination and nuclear export of p53 (199). In vitro studies have shown that c-Abl phosphorylates human Mdm2 on Tyr394 (Tyr393 in mouse) as well as Tyr276 and Tyr405 (200,201), and that c-Abl phosphorylation of Mdm2 Tyr394 impairs Mdm2’s ability to inhibit p53’s stabilization and transactivation, and p53-mediated apoptosis (200).

It has since been proposed that c-Abl phosphorylation of Mdm2 increases Mdm2-MdmX binding and promotes Mdm2-directed MdmX ubiquitination. This increase in MdmX ubiquitination ultimately destabilizes the Mdm2-MdmX complex, promoting p53 stabilization (202). Recently, our lab has very recently generated Mdm2-Tyr393 knock-in mice to explore the physiological role of c-Abl phosphorylation of Mdm2 on p53 stabilization and activation. However, analysis of the DNA damage response and tumorigenesis in these mice is presently ongoing.

Other phosphorylation events on Mdm2

Located in the acidic domain of Mdm2 is a cluster of residues that are phosphorylated under homoeostatic conditions (203). Phosphorylation of these residues is known to occur through the activities of the kinases—glycogen synthase kinase 3 beta (GSK-3β), CK1, and CK2 (204-207). Phosphorylation of these residues improves Mdm2-mediated turnover of p53 in the absence of stress stimuli (204,207,208), and hypo-phosphorylation of this region of Mdm2 is reported to coincide with DNA damage-induced p53 stabilization (208). Accordingly, inhibition of GSK-3β leads to p53 stabilization in cells (207). Notably, GSK-3β is inhibited through phosphorylation by Akt, which is activated by DNA-PK following DNA damage (209). Akt-mediated inhibition of Mdm2 activity in this context contrasts with other reports in which the direct phosphorylation of Mdm2 Ser166 and Ser186 by Akt is proposed to inhibit p53 activity by facilitating Mdm2 translocation into the nucleus (210,211) and by inhibiting Mdm2 self-ubiquitination and degradation (212).

**Effects of Mdm2 phosphorylation on p53 function**

Previous studies have suggested that ATM phosphorylation of the analogous residue on human MDM2 (Ser395), either alone or in combination with several other ATM-target serine residues in the same region, impacts the ability of Mdm2 to promote p53 degradation and nuclear export, and governs Mdm2 RING-domain oligomerization and polyubiquitination of p53 (178,181). It is likely that DNA damage-induced p53 activity is caused not only by reduced Mdm2-mediated p53 degradation (due to destabilization of Mdm2) but also by reduced Mdm2 steric inhibition of p53. As Mdm2 binds to the amino-terminal, transcriptional activation domain of p53 and inhibits p53 target gene expression, reduced Mdm2-p53 complex formation after Mdm2 phosphorylation by ATM may account for an increase in p53 activity even when p53 protein stability is only modestly altered (76,213).

As discussed above, we and others have demonstrated that Mdm2 phosphorylation can reduce Mdm2 stability, but it remains unclear how Mdm2 phosphorylation facilitates Mdm2 destabilization. One study has suggested that Mdm2 stability is primarily mediated through self-ubiquitination (180). This study reached this conclusion by overexpressing an Mdm2 mutant with abrogated RING E3 activity (Mdm2C462A) in U2OS cells. However, a subsequent study in which MEFs from the corresponding Mdm2C462A knock-in mice were examined showed that Mdm2 RING E3 activity was in fact dispensable for Mdm2 destabilization after IR (129).

Degradation of Mdm2 in the absence of Mdm2 ubiquitin ligase function is indicative of other ubiquitin ligases being capable of regulating Mdm2 stability. One study has shown that the p300/CBP-associated factor (PCAF) is capable of promoting Mdm2 ubiquitination, and that PCAF can impact Mdm2 levels under unstressed conditions as well as under DNA damage conditions (212).
Mdm2 destabilization in response to DNA damage (214). Similarly, the anaphase-promoting complex/cyclosome (APC/C) E3 ubiquitin ligase complex has been shown to ubiquitinate Mdm2, with siRNA-mediated depletion of APC2, the Mdm2-binding member of the APC/C complex, leading to Mdm2 accumulation and diminished p53 stabilization in response to DNA damage (215). Other studies have identified the F-box proteins β-TRCP and FBXO31 as mediators of Mdm2 degradation by the SCF complex (216,217). β-TRCP mediated degradation of Mdm2 is dependent on Mdm2 phosphorylation by CK1 and promotes Mdm2 turnover in response to DNA damage (216). This is seemingly in contradiction with previous studies showing hypo-phosphorylation of CK1 target residues on Mdm2 following DNA damage (208). Notably, knockdown of β-TRCP does not appear to impact the initial DNA damage-induced stabilization of p53, but rather Mdm2 stability and p53 levels at later time points in the damage response (216). It has subsequently been shown that ATM phosphorylates CK1, and that this promotes CK1 nuclear localization and Mdm2 degradation (218). It is conceivable that the phosphorylation state of Mdm2 at CK1 target residues has opposing effects on Mdm2 stability depending on the damage response phase and differing active signaling events. Contrastingly, FBXO31 knockdown in cell lines abolishes the initial Mdm2 destabilization and p53 stabilization following DNA damage (217). FBXO31 can direct the polyubiquitination and degradation of Mdm2, and the FBXO31-Mdm2 interaction appears dependent on ATM phosphorylation of Mdm2 (217). However, the requirement for ATM phosphorylation was determined through the expression of an Mdm2 protein lacking all 6 proposed ATM target residues, and conversely, the treatment of lysates with phosphatase or cells with an ATM kinase inhibitor. Consequently, the precise mechanism of how ATM phosphorylation of Mdm2 Ser394 specifically promotes Mdm2 degradation under endogenous conditions remains to be determined.

In opposition to Mdm2 destabilization, a number of different mechanisms are proposed to promote Mdm2 stability. These mechanisms include modifications of Mdm2 with the small, ubiquitin-like proteins SUMO (Small ubiquitin-related modifier) and NEDD8 (Neural precursor cell expressed developmentally down-regulated protein 8), which are separately proposed to have Mdm2 stabilizing effects and to decrease in response to DNA damage (219,220). Additionally, Mdm2, MdmX and p53 can be deubiquitinated by the HAUSP (herpesvirus-associated ubiquitin-specific protease) protein (175,221,222). DNA damage has been shown to reduce the affinity of Mdm2 and MdmX for HAUSP, leading to their enhanced ubiquitination (175). A proposed mechanism for the dissociation of Mdm2 from HAUSP involves ATM phosphorylation of Daxx (death domain-associated protein 6) triggering its dissociation from Mdm2, and relieving Daxx mediated promotion of Mdm2-HAUSP interaction (223,224). MDM2 is also been reported to be deubiquitinated by the ubiquitin-specific proteases USP2a and USP15 (225,226). Notably, USP2a also acts as a deubiquitinating enzyme for MdmX, and is downregulated in response to cisplatin (227).

**Therapeutic implications**

That the overwhelming majority of tumors display loss of p53 function has led to massive efforts to develop cancer therapies that either exploits p53 tumor suppressive function, or its propensity for mutation or inactivation. These efforts have included, but are not limited to, the development of gene therapies involving viral delivery of p53 expression vectors into tumors (228), therapies employing siRNA-mediated knockdown of negative regulators of p53 such as viral E6 protein (229) and Mdm2 (230), and immunotherapies targeting elevated levels of p53 protein observed following p53 mutation (231).

As extensive research has provided an ever-improving understanding of the structures and activities of the various p53-interacting proteins involved in the p53 signaling network, a host of small-molecule and peptide therapeutics have also been identified that facilitate the pharmaceutical control of p53 signaling. Approximately 50% of tumors retain expression of wild-type p53. Accordingly, these tumors are potential targets for therapies that stimulate the tumor suppressive activities of p53 by freeing it from negative regulation. An obvious target for this approach is the interaction of p53 with its primary negative regulator, Mdm2. Aided by the compact, “druggable” p53-Mdm2 interface, which involves a short helical fragment of p53 binding in a deep hydrophobic pocket of Mdm2 (162), a series of small-molecule inhibitors of the p53-Mdm2 interaction have been developed. Both high throughput screening and structure based design methods have yielded three classes of small-molecule inhibitors, the Nutlin (232), the benzodiazepinediones (233), and the spiro-oxindoles (234). By interfering with p53-Mdm2 binding, these compounds have been shown to activate p53 function, and optimized
derivatives of these compounds are currently in a number of clinical trials (235). However, these compounds do not strongly inhibit the MdmX-p53 interaction, due to differences in the p53 binding pocket of MdmX as compared to Mdm2, and do not always show effects on cancer cells expressing high levels of MdmX (236,237). More recently, several small-molecules that target the p53-MdmX interaction have been reported (WK 298 and SJ-172550) (238,239), as well as molecules that inhibit MdmX transcription (NSC 207895) (240) or stability (17-AAG) (241). Furthermore, compounds that inhibit both p53-Mdm2 and p53-MdmX binding have been generated and are being studied (RO-5963) (242). Finally, the small-molecule RITA (reactivation of p53 and induction of tumor cell apoptosis) was identified in a high-throughput screen as inducing p53 accumulation and activation by binding p53 itself. RITA binding is proposed to induce a conformational change in p53, resulting in its dissociation from Mdm2 (243). However, it has also been shown that RITA causes DNA-protein crosslinks and is metabolized to a reactive species, leading to p53-independent toxicity (244-246).

A separate class of inhibitors of p53-Mdm2 and p53-MdmX binding are the stabilized small peptides termed “stapled peptides” (247,248). These compounds employ a chemical strategy termed “hydrocarbon stapling” that fixes an all-hydrocarbon crosslink within synthetic peptides to preserve their α-helical structure, confer protease resistance, and promote their cellular uptake (249). One of these compounds, SAH-p53-8 is capable of binding to both Mdm2 and MdmX with high affinity (247,248). However, despite a higher affinity for Mdm2 than Nutlin 3a, SAH-p53-8 is less potent at disrupting the p53-Mdm2 interaction in cells, possibly due to prevailing issues with bioavailability (250).

Compounds have also been developed that inhibit the ubiquitin ligase function of Mdm2, such as HLI98 compounds (251) and the related MPD compounds (252), and MEL23 and MEL24 (253). These compounds stabilize p53 and Mdm2 promote p53 transcriptional activation and growth arrest and apoptosis. However, these inhibitors also elicit some p53— independent cytotoxicity, particularly at higher concentrations, possibly due to inhibition of other RING domain-containing E3 ligases.

Separate from therapies aimed at combating tumor growth through p53 induction, research has also focused on regulating the p53 response in non-malignant tissues, in an effort to reduce the side effects of cytotoxic cancer therapies such as cytopenia and hair loss. In one approach, using the concept of cyclotherapy, an initial nongenotoxic p53 inducing compound is used to promote a reversible cell cycle arrest in normal proliferative tissues, before a second drug is employed to kill proliferating cells, presumably only the p53 mutant tumor cells. This approach has shown promise in cell lines, using Nutlin compounds for p53 induction (254,255). However, this approach is specific for chemotherapeutics that target S or M phase, such as nucleoside analogs β-D-arabinofuranoside (Ara-C) and gemcitabine, and paclitaxel, and does not show effectiveness when Nutlins are used in combination with doxorubicin or cisplatin. Conversely, the compound Pifithrin-α (PFT-α, an abbreviation for “p-fifty-three inhibitor”), a p53-inhibiting compound identified in a high-throughput screen, has been shown to protect p53 wild-type cells from apoptosis induced by irradiation and the cytotoxic drugs doxorubicin, etoposide, Taxol, and Ara-C (256).

In another approach that exploits the propensity for p53 inactivation in tumor cells, Yuan and colleagues have identified a phenomenon in which pre-treatment of cells with low-dose arsenic induces p53 stabilization and a p53-dependent metabolic shift from oxidative phosphorylation to anaerobic glycolysis which confers protection to normal tissues from 5FU and radiation-induced toxicities (257-259). This approach has recently shown hematopoietic protection in humans receiving myelosuppressive chemotherapy in a recently reported clinical trial (260).

Our recent findings suggest an avenue for an additional layer of pharmaceutical control of the p53 pathway. We have shown Mdm2 phosphorylation to significantly impact the capacity to repopulate bone marrow following irradiation and (in the case of Ser394 phosphorylation) simultaneously protect against lymphomagenesis induced by repeated IR exposure (184). Similar effects have been observed in the absence of appropriate MdmX phosphorylation (176). While broad inhibition of DNA damage responsive kinases such as ATM, Chk2 and c-Abl would likely be undesirable due to their involvement in additional processes such as DNA repair, small-molecule therapeutics that inhibit DNA damage-induced Mdm2 or MdmX phosphorylation events may be useful in reducing unwanted chemotherapeutic side effects without compromising p53 tumor suppressive function.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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