Review

Control of p53 Ubiquitination and Nuclear Export by MDM2 and ARF1

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Abstract

p53 and ARF-INK4a are the two most frequently altered loci in human tumors. The activity of p53 protein is inhibited during normal cell growth by the proto-oncoprotein MDM2 through either repression of p53-mediated transcription in the nucleus or proteasomal degradation of p53 protein in the cytoplasm. Responding to oncogenic signal-activated cell hyperproliferation, ARF-mediated antagonism of MDM2 inhibition results in p53 becoming active and its protein levels rising. The biochemical mechanisms of ubiquitination and nuclear export that underlie the functions of ARF and MDM2 in p53 control continue to emerge.

Introduction

The discovery and investigation of ARF3 has roots that intertwine with, and indeed arise from, those of p16INK4a, a 16,000 human protein that binds to and inhibits CDK4 (1, 2). Shortly after its initial cloning, p16INK4a received intense attention after reports that the p16 locus is mutated in a remarkably high frequency in tumor-derived immortalized cell lines (3, 4) as well as in primary tumors of different types (see recent compilations in Ref. 5–7). It is now clear that the frequent alteration of this locus stems from its unique genomic structure, which also encodes the hidden second gene, ARF, expressed from a separate promoter with a distinct first exon (exon 1β) localized upstream of the exon1α of p16 (Refs. 8–11; Fig. 1A). Despite sharing coding sequence with p16 over exons 2 and 3, the exon 1β-specified transcript contains its own translation initiation AUG codon, resulting in production of a protein completely unrelated to p16 because of translation in an alternative reading frame. Indeed, not only is this peculiarly encoded and seemingly unrealistic protein actually synthesized in vivo, its ectopic expression, like that of p16, also inhibited cell cycle progression (11). More importantly, selective deletion of ARF exon 1β in mice, although retaining apparently normal expression of p16INK4a, resulted in development of spontaneous tumors at an early age (12). Compelling evidence from the analysis of a large number of human tumor samples supports both ARF and p16 playing critical roles in the prevention of tumorigenesis (5–7). However, the relative contributions of p16INK4a and ARF in suppressing tumor growth in mice remain unsettled. Mice lacking ARF alone (retaining functional p16INK4a) recapitulated almost all of the tumor phenotypes, including both the spectrum and the rate of tumor growth, that were seen in mice lacking both ARF and p16INK4a (12, 13). Genetic analysis of pure p16INK4a-deficient mice is still in progress, but preliminary studies have indicated that the p16INK4a-deficient mice that retain the expression of ARF are viable and do not develop tumors at an early age (14). Whether p16INK4a functionally collaborates with, or its loss is compensated by, other INK4 genes, especially p19INK4c (15, 16), will need to be determined to more clearly define the role of p16INK4c in suppressing tumor growth in mice.

Biochemical studies of p16INK4a and ARF have offered mechanistic foundations supporting both as tumor suppressors. Although its upstream regulation remains evasive, the biochemical mechanism of p16INK4a was elucidated quickly after its isolation and is relatively simple. p16INK4a binds to the catalytic kinase subunit CDK4 (and later CDK6; Ref. 2) and induces conformational changes that inhibit the binding of ATP and reduce substantially the CDK4/6-cyclin D interface (17–19), thereby maintaining Rb in its hypophosphorylated state and inducing G1 cell cycle arrest (20, 21). In contrast, the molecular mechanisms underlying ARF function appear to be more complex and remain a subject of active debate. In this review, we examine the biochemical properties and mechanisms of the ARF protein and its principal target MDM2, focusing on the control of p53 protein ubiquitination and nuclear export by these two proteins. The regulation of ARF gene expression in response to oncogenic signals and the genetic function of ARF in preventing cell transformation and organismal tumorigenesis have been reviewed extensively in several excellent articles (6, 7, 22).

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3 The abbreviations used are: ARF, alternative reading frame; CDK, cyclin-dependent kinase; INK4, inhibitor of CDK4; MDM2, murine double minute 2; HDM2, human homologue of MDM2; NLS, nuclear localization signal; NES, nuclear export signal; NoLS, nucleolus localization signal; GFP, green fluorescent protein; HECT, homologous to E6AP COOH terminus; SUMO, small ubiquitin-like modifier; LMB, leptomycin B; PML, promyelocytic leukemia.
The Origin and Biochemical Properties of ARF

ARF: A Late Invader of the p16 Locus. Thus far, the ARF gene has been identified in only four mammalian species (Fig. 1B), human (132 amino acid residues, p14), mouse (169 residues, p19), rat (160 residues), and gray opossum (155 residues). The gene has undergone relatively rapid evolutionary drift. Human ARF, for example, shares only 49 and 44% amino acid sequence identity with its mouse and opossum homologues, respectively. As a comparison, the human p16 protein is 63% identical to both mouse and opossum p16. Such a high degree of sequence divergence indicates that the ARF gene was not highly constrained during evolution, suggesting that ARF function was not initially evolved to perform functions important for cell survival or organismal development. This notion is consistent with the fact that many established cell lines have sustained homozygous deletion of the ARF-INK4a locus (3, 4), and that mice deficient for ARF spontaneously developed tumors early in their life but exhibited no obvious developmental defects (12, 13).

A single INK4 gene, p13CDKN2X, has been reported from the Southern platfish (Xiphophorus maculatus) genome and was linked to hereditary susceptibility to UV-induced melanomas (23, 24). The platfish INK4 gene contains an intron in the same position as mammalian INK4 genes and is equally related to the four mammalian INK4 genes, indicating that the fish INK4 gene might have evolved from a common ancestor that gave rise to the four mammalian INK4 genes after two gene duplication events. Rapid amplification of cDNA ends using primers within the second exon of p13CDKN2X failed to identify products representative of an ARF gene (24). Similarly, a single INK4 gene was identified in Japanese pufferfish (Takifugu rubripes; database accession CAC12811), but again, there was no evidence for the existence of an ARF gene product. Conceptual translation of exon 2 yields only a short ARF in the platfish (32 residues) and pufferfish (32 residues) INK4s, as well as in the human INK4b (32 residues), INK4c (14 residues), and INK4d (31 residues) genes, that bears no significant similarity to ARF and lacks
the conserved ARF COOH-terminal domain. These observations, together with the early origin of the anykrin motif during evolution (as early as the origin of bacteria), suggest that the ARF gene might have been a late invader of the p16 locus. The MDM2 gene, the major target of ARF, has been identified in frog (25), zebrafish (database accession AAB64176), and ascidian (database accession AV382318) and seems to have evolved earlier than ARF. Back further still, the Drosophila melanogaster genome contains a single p53 gene, but neither MDM2 nor ARF (Ref. 26). It therefore appears that the ARF-MDM2-p53 pathway evolved by the sequential addition of upstream regulators during evolution, first adding the control of p53 by MDM2 and then of MDM2 by ARF. One possibility is that the puzzling genomic arrangement of the ARF-INK4a locus was evolved by the insertion of a MDM2-binding domain into the existing p16 locus that conferred on cells the ability to couple the MDM2-p53 and INK4-Rb pathways through coordinated regulation of the chromatin structure at the locus and thus transcription of both genes.

**ARF Binds to MDM2.** Three lines of genetic evidence suggest that ARF may act upstream of p53 in a fairly direct fashion: (a) tumors that arose from ARF/INK4a-deficient mice lack p53 mutation/deletion (27); (b) ectopic expression of ARF inhibited S-phase entry in wild-type MEFs but not in several established fibroblast cell lines lacking p53 function (12); and (c) ARF inhibited cellular transformation only in the presence of functional p53 (28). ARF does not share significant sequence similarity to any known proteins and contains no obvious structural motifs, providing little initial clue to its biochemical properties in regulating p53. The only discernible feature common to ARF proteins is their unusual number of positively charged residues. As a result, the isoelectric points (pI) of the human (12.4), mouse (12.1), rat (12.3), and opossum (11.9) ARF proteins are remarkably high. The basic nature of ARF protein may be responsible for its binding to an acidic domain of MDM2 and for its nucleolar localization, two properties essential for ARF function.

The finding that ARF bound MDM2 and stabilized p53 was pivotal in advancing understanding of the molecular mechanism of ARF function (28–31). ARF has also been reported to bind p53 directly after baculovirus-mediated coexpression of both proteins in insect cells (31). This MDM2-independent ARF-p53 interaction, however, has not been observed in mammalian cells, and its significance, if genuine, remains to be elucidated. A truncated HDM2 containing residues 208–491 was capable of interacting with human ARF (29), and deletion of HDM2 residues 222–437 abolished its binding to ARF (30), mapping the ARF binding domain to the central or COOH-terminal portion of MDM2. Further deletion analysis showed that the region containing amino acids 210–244 of HDM2 possesses most, if not all, of the ARF binding activity (32). The p53 binding region was mapped previously to the NH2-terminal region of MDM2 between residues 17 and 125 (33–36), suggesting that ARF and p53 might bind to two separate regions of MDM2 in a noncompetitive manner. The assembly of ternary ARF-MDM2-p53 complexes confirmed this notion (28–31). The central and COOH-terminal region of MDM2 contains several functional domains, including a p300/CBP binding site (37), a NLS (38), a NES (39), a L5 ribosomal binding site (40), a zinc finger, and a RING finger (Fig. 1C), all of which could potentially be affected by ARF binding. Two MDM2 activities, nuclear export (41, 42) and RING finger-mediated p53 ubiquitination (28, 32, 43), are inhibited by ARF binding. The biochemical mechanisms underlying ARF inhibition of these MDM2 activities, however, are unknown. Deletion of either the NES or the RING finger did not affect the binding of MDM2 with ARF, disfavoring a simple mechanism of masking the NES from interaction with export machinery, or the RING finger from interaction with E2 ubiquitin-conjugating enzymes by competitive ARF binding.

Deletion of the exon 2-encoded COOH-terminal domain of ARF (residues 65–132) had no detectable effect on its binding to MDM2, and the deletion of the exon 1β-encoded NH2-terminal domain in both human and mouse ARF abolished MDM2 binding activity (29, 31), mapping MDM2-binding activity within the NH2-terminal domain of human ARF. One report found that the COOH-terminal domain of human ARF contained weak MDM2 binding activity (44). This weak ARF–HDM2 binding probably represents an electrostatic interaction resulting from the fusion of ARF with three tandem copies of the SV40 NLS. The same human ARF (65–132) fragment without a fused NLS (29), as well as a series of overlapping ARF peptides spanning this region (32), did not exhibit any detectable HDM2 binding activity. A synthetic peptide corresponding to the first 20 amino acids of human or mouse ARF, one of two highly conserved regions in ARF containing seven identical residues among the four mammalian ARF proteins (Fig. 1B), bound tightly to MDM2 or HDM2 (32). Conversely, deletion of this sequence from human ARF severely impaired its binding to HDM2.4 These results map the HDM2 binding activity to the NH2-terminal 20 amino acids in human ARF. Deletion of this sequence from mouse ARF, however, did not significantly reduce its binding to MDM2 (45), and a second MDM2 binding site was identified between residues 20 and 40, albeit with lower affinity than the NH2-terminal one (32, 45). This second MDM2 binding site of murine ARF is, however, not conserved in ARF from other species and contains only three identical and three similar residues (Fig. 1B). Peptides corresponding to human ARF residues 11–30 and 21–40 exhibited no significant MDM2 binding activity (32). Hence, mouse and human ARF proteins were structurally distinct in their interactions with MDM2/HDM2. It has yet to be determined whether the two MDM2 binding sites in mouse ARF allosterically cooperate with each other to increase the murine ARF–MDM2 binding affinity.

**ARF Localizes to the Nucleolus.** ARF protein normally localizes to the nucleolus (28, 41, 46), a subnuclear compartment that has long been recognized primarily for its function in ribosome assembly, but has recently been implicated in various other cellular activities (47, 48). Mapping of the NoLSs revealed another topographical difference between human and mouse ARF proteins, causing some confusion and conflicting interpretations regarding the biochemical mechanism of p53 stabilization by ARF. Deletion of mouse

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4 W. Yarbrough and Y. Xiong, unpublished data.
ARF residues 26–37 (KFVRSRRPRTAS, underlined in Fig. 1B) within the NH₂-terminal domain resulted in altered nucleolar localization and the loss of ability to stabilize p53 and to induce cell cycle arrest (46). On the other hand, human ARF residues 85–101 (QLRRPRHSHPTRARRCP, underlined in Fig. 1B) within the COOH-terminal domain was found to be necessary for the nucleolar localization of ARF (41, 49). Neither mouse ARF 26–37 nor human ARF 85–101 is conserved in other species. Unlike full-length human ARF protein, the COOH-terminal domain accumulated primarily, but not entirely, within the nucleolus and exhibited visible nucleoplasmic and even some cytoplasmic distribution. Fusion of human ARF 85–101 with a reporter GFP resulted in visible nucleolar accumulation of GFP; however, most GFP remained in the nucleoplasm (41). These observations led to the suspicion that some sequence(s) within exon 1 remained in the nucleoplasm (41). These observations led to the suspicion that some sequence(s) within exon 1β might also contribute to the nucleolar localization of human ARF. This surmise was later explained by the reports that human ARF residues 1–29, besides containing the HDM2 binding site, also contained a nuclear and nucleolar localization activity capable of mobilizing heterologously fused reporter proteins into the nucleoli (44, 49). Intriguingly, the second MDM2 binding site in mouse ARF overlaps with the nucleolar localization sequence (45). Hence, in both human and mouse cells, HDM2/MDM2 binding may potentially mask one or both nuclear/nucleolar localization sequences in human or mouse ARF, respectively. If this model holds, subnuclear distribution of the ARF-MDM2 complex could conceivably be affected by proteins that can bind to MDM2 and be assembled into an ARF-MDM2 complex. These possibilities have not yet been investigated but may provide insight to clarify some of the confusion about subnuclear localization of ARF-MDM2 complexes. Given their high degree of sequence divergence and these topographical differences, extension of interpretations between human and mouse ARF proteins should be made with caution.

There is one overlooked issue with regard to the experimental techniques that have been used to map sequence elements important for the nucleolar localization of ARF that also warrants cautious interpretation of the resulting data. Many experiments examined the localization of small ARF peptides that are tagged with various epitopes, including fusion with one or more copies of a NLS. Unlike the nucleus and other membrane-bound organelles, there is no physical barrier separating the nucleolus from the surrounding nucleoplasm that would require a signal peptide-mediated protein transport. No consensus nucleolar targeting sequence has been found. Rather, it is currently believed that stretches of basic amino acids in proteins that accumulate in the nucleolus bind to acidic proteins or nucleic acids residing in the nucleoli through electrostatic interactions (47). L7a (50) and L5 (51, 52) are two components of the major ribosomal subunit. Both contain three distinct NLSs, and each is capable of targeting an otherwise cytoplasmically localized β-galactosidase reporter to the nucleus but not to the nucleolus. Fusion with an additional NLS from other domains or from SV40 restored nucleolar localization of β-galactosidase. We have also noted that although fusion with one copy of a SV40-derived NLS localized reporter GFP into the nucleoplasm, fusion with two or three tandem copies of the same NLS resulted in an evident and almost exclusive nucleolar accumulation of GFP, respectively. Hence, multiple NLSs, ipso facto, could drive nucleolar localization of fused proteins.

**MDM2-mediated p53 Ubiquitination**

Growing evidence has identified MDM2 as a key regulator of p53 function in response to various forms of cellular stress (53, 54). MDM2 controls p53 through one of two different mechanisms: inhibiting the transcriptional activity of p53 (55) or promoting p53 degradation (56, 57). MDM2-mediated repression of the transactivating activity of p53 remains poorly characterized. A dual mechanism has been proposed: MDM2 binds to and masks the NH₂-terminal activation domain of p53 as well as directly interfering with the basal transcription machinery (34, 58). Support for the masking mechanism includes findings that mutations in the activation domain of p53 that impaired its binding with various components of the transcription machinery (reviewed in Ref. 53) also disrupted its binding with MDM2 (59), suggesting that MDM2 and the basal factors might competitively interact with overlapping sequences in p53. When recruited to a promoter by fusion with a heterologous DNA-binding domain, MDM2 was capable of repressing basal transcription in the absence of p53 via a sequence comprising residues 50–222 that appeared to be separate from its p53 binding site (58). Notably, this inhibitory domain overlapped with the domain encompassing MDM2 residues 102–222 shown to bind histone acetylase p300/CBP (37). Entering a complex including both proteins, MDM2 inhibited p300-mediated p53 acetylation and activation (60–62). Coexpression of ARF reversed the inhibition of p53 acetylation by MDM2 and induced p53 stabilization, despite the high levels of MDM2-p53 complex (62), suggesting the possibility that ARF may be able to inhibit MDM2 and activate p53 without dissociating MDM2-p53 binding. How acetylation stabilizes p53, by antagonizing ubiquitination or nuclear export, and how MDM2 inhibits p53 acetylation, by inhibiting p300 or bringing in a deacetylase, are not known. It also remains to be determined whether acetylation regulates the transcriptional activity of p53, beyond altering its stability. ARF is likely to participate antagonistically in these MDM2-mediated regulations of p53 acetylation.

**MDM2 Is a RING Finger Ubiquitin Ligase of p53.** p53 protein is kept at low levels during normal cell growth by its rapid turnover and is accumulated primarily through post-transcriptional regulation after various physiological stress conditions including oncogenic stimulation and DNA damage (63–65). Treatment of normal and papilloma virus infected cells with 26S proteasome inhibitors increased the half-life and steady-state levels of p53 protein with a correlative accumulation of ubiquitinated p53 (66), indicating a ubiquitin-mediated proteasomal degradation of p53. Through a cascade of enzymes involving ubiquitin activating

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5 Y. Zhang and Y. Xiong, unpublished data.
(E1), conjugating (E2), and ligation (E3) activities, the ubiquitin-mediated protein degradation pathways catalyze the formation of polyubiquitin chains onto substrate proteins via isopeptide bonds. Polyubiquitinated substrates are then rapidly delivered to and degraded by the 26S proteasome (67–69). E1 and E2 both represent structurally related proteins that are relatively well characterized biochemically. On the other hand, the molecular nature and regulation of E3 ubiquitin ligases, generally defined as containing both a ubiquitin ligase activity for catalyzing isopeptide bond formation and a substrate targeting function, are still under intensive investigation. Although p53 ubiquitination was first characterized with the E6-E6AP E3 ubiquitin ligase (70–72), this appears to represent a special pathway targeting p53 ubiquitination that is not involved in p53 stability control in the absence of E6 (73). It is generally accepted now that MDM2 is the principal p53 ubiquitin ligase (74). Unlike E6AP, the prototype member of the HECT domain family of E3 ubiquitin ligases (75), MDM2 belongs to the growing family of RING finger ubiquitin ligases (76). The ligase activity of MDM2 was completely abolished by mutations at each of eight cysteine and histidine residues involved in zinc coordination, and it could also be inhibited by a metal chelator (74, 77), indicating a requirement for the correct zinc-dependent folding of the intact RING finger. The ligase activity of MDM2, like that of another well-characterized RING finger protein, ROC1/Rbx/Hrt (78), was not inhibited by alkylating agents at a concentration sufficient to abrogate the activity of an HECT E3 ligase (77), suggesting that catalysis of isopeptide bond formation by RING finger ligases, unlike HECT ligases (72), does not involve an intermediate thioester linkage of ubiquitin to a cysteine residue in the RING. In vitro, the purified recombinant RING finger protein APC11 alone was capable of activating E2 to form polyubiquitin chains (79, 80). Whether MDM2 and other RING finger ubiquitin ligases, such as APC11, can also activate E2 without additional cofactors is still under investigation.

MDM2 also bound to and repressed the transcriptional activity of the p53 homologue, p73, but did not cause its destabilization (81–83). The basis for this discrimination is not entirely clear. The COOH-terminal region of p53 is not conserved in p73 and contains an NES, the mutation of which impaired the nuclear export and cytoplasmic degradation of p53 (84). The very COOH terminus of p53 also contains multiple lysine residues that were required for MDM2-mediated as well as E6AP-mediated ubiquitination and degradation (85, 86). Granting this domain onto p73, however, did not confer p73 sensitivity to MDM2-mediated degradation. Instead, domain swapping experiments identified a sequence element, consisting of residues 92–112 of p53, that remarkably converted p53 into being resistant, and p73 into being sensitive, to MDM2-mediated degradation (87). This sequence is conserved among p53 proteins from different species but not between p53 and p73 and does not share any obvious similarity to other characterized degradation signals such as destruction (88), KEN (89), or F boxes (90). Further investigation of this sequence, including identification of cellular proteins interacting with this sequence, is clearly needed and may provide insights not only as to how p53 is targeted for MDM2-mediated degradation but also how this degradation is inhibited by ARF.

Regulation of the Ubiquitin Ligase Activity of MDM2.

Three factors have been identified that directly interact with MDM2 and in turn affect its ubiquitin ligase activity toward p53; binding with ARF (32, 43), with MDMX, a homologue of MDM2 (91–94), and covalent modification by SUMO (95).

Binding of ARF to a central region of MDM2, separate from the p53 binding domain and the RING finger, substantially reduced the p53 ligase activity in vitro of MDM2 (32, 43), implying that a sequence in this region of MDM2 might contribute to ligase activity, either in-cis or by serving as the site for the binding of another factor. How ARF inhibits MDM2-mediated p53 ubiquitination is unknown at present. Neither preventing p53 and MDM2 interaction nor blocking their nuclear export seems to be the answer, because ARF can assemble into a ternary complexes with p53 and MDM2 (28–31), and MDM2-mediated p53 ubiquitination can occur in the nucleus (96, 97). A human ARF to p53 peptide that contains the primary HDM2 binding activity was sufficient to block the ubiquitin ligase activity of HDM2 (32). This peptide did not block formation of Ub-E1 or Ub-E2 thioesters, indicating that ARF inhibits the final step of isopeptide bond formation between ubiquitin and p53 or MDM2 (32).

MDMX shares a high degree of sequence similarity with MDM2, including the p53 binding domain, the central zinc finger, and the COOH-terminal RING finger, and similar to MDM2, can also inhibit p53-mediated transcriptional activation (98). Despite these conservations, however, MDMX is unable to target p53 degradation and does not interact with ARF. On the contrary, MDMX protected p53 from MDM2-mediated degradation as well as preventing the degradation of MDM2 itself, most likely through oligomerization and dimerization between the RING fingers in both proteins (91–94, 98). Whether MDMX lacks an intrinsic ubiquitin ligase, as counterintuitive as one might be, has not been determined formally. All eight cysteine and histidine residues are conserved within its COOH-terminal RING domain. One noticeable difference between the two RING fingers is in Lys-444 in MDM2 (Lys-446 in HDM2) as compared with Arg-444 in MDMX (Arg-445 in HDMX). Lys-446 of MDM2 has been shown to be the site of covalent modification by SUMO (95). MDM2K446R mutation abrogated autoubiquitination of HDM2 but increased its ligase activity toward p53, leading to the suggestion that Lys-446 is the major site of HDM2 autoubiquitination, which could be blocked by SUMO conjugation. Inhibition of MDM2 autoubiquitination would then allow it to become a more efficient ubiquitin ligase of its substrate p53 (95). Conceivably, differences at this Lys/Arg residue could contribute to, or even account for, the lack of self-ubiquitination activity in MDMX. Binding with MDMX, like SUMO conjugation, could block MDM2 self-ubiquitination. This model, however, does not explain how blocking the auto-ubiquitination of MDM2 brings about increased ligase activity toward p53 and how MDMX protects p53 from MDM2-mediated ubiquitination.

Export out of the nucleus is also necessary for p53 degradation (see below). In the region corresponding to the MDM2 NES, MDMX contains an altered sequence with four
extra amino acids, separating conserved hydrophobic residues involved in interaction with the export machinery as well as a nonconserved disruptive proline residue. Unlike a MDM2-EGFP fusion that shuttled between the nucleus and the cytoplasm, an MDMX-EGFP fusion remained constantly and exclusively in the nucleus and exhibited little nucleo-cytoplasmic shuttling activity, as demonstrated by heterokaryon assay (93). Lack of nucleo-cytoplasmic shuttling activity could in theory trap p53 in the nucleus, thereby inhibiting its cytoplasmic degradation by MDM2.

Nuclear Export of p53 and MDM2
Blocking p53 nuclear export inhibited MDM2-mediated p53 degradation and led to p53 stabilization (39, 100), indicating that p53 degradation occurs in the cytoplasm and implying p53 nuclear export as a major regulatory event in MDM2-mediated p53 degradation. ARF stabilizes p53 by blocking the nuclear export of both p53 and MDM2 (Refs. 41 and 42 and see below). Human tumor-derived mutations in the shared exon 2 of the ARF-INK4a locus selectively impaired ARF function in blocking p53 nuclear export, underscoring the importance of nuclear export in regulating p53 stability (41). Why p53 must be exported to be degraded is unclear. Neither access to proteasomes nor cytoplasmic ubiquitination is likely to be the answer, because proteasomes are present in both the nucleus and in the cytoplasm (101), and p53 can be ubiquitinated by MDM2 in both compartments (97). The mechanism governing p53 nuclear export has been under intense investigation but remains unclear and controversial. Three competing models have been proposed to explain p53 nuclear export.

MDM2-dependent p53 Export. The first model suggests that MDM2 binds p53 in the nucleus and shuttles p53 to the cytoplasm (Fig. 2A; Refs. 38, 39). This model is based on the observations that MDM2 shuttled between the nucleus and the cytoplasm via an intrinsic NES, the mutation of which abolished both the nuclear export and ability of MDM2 to degrade p53 (39). Corroborating this model, mutation in the NLS of MDM2 excluded it from nuclear entry and impaired its ability to degrade p53 (38). Treatment of various cell lines with LMB led to an increase in the half-life and steady-state levels of p53 protein (100). LMB forms covalent linkage with a conserved cysteine residue in CRM1 (exportin 1; Ref. 102), an evolutionarily conserved receptor for nuclear export signal of proteins, and abolishes CRM1-NES binding (103–105). LMB treatment also inhibited E6-E6AP-mediated p53 degradation in human papillomavirus-infected cell lines (100), implying that nuclear export of p53 is necessary for degradation by two different ubiquitin ligases but is not absolutely dependent on MDM2.

MDM2-independent Autonomous p53 Export. The second model proposes that p53 itself contains a functional NES capable of mediating its own nuclear export (Fig. 2B; Refs. 84 and 106). After injection into nuclei, fluorescein-labeled p53, but not NLS-tagged human serum albumin, was exported within minutes through an energy-dependent path-
way (106). A leucine-rich NES, spanning residues 339–352 of human p53, was identified, the mutations of which prevented p53 export. Fusion of this sequence with an otherwise nuclear localized BSA (p53<sup>339–352</sup>–BSA) resulted in cytoplasmic accumulation of BSA in an LMB-sensitive manner (84). Supporting this MDM2-independent p53 nuclear export model are the observations that a p53-GFP fusion protein localized both in the cytoplasm and nucleus when ectopically expressed in p53<sup>−/−</sup>–MDM2<sup>−/−</sup> doubly deficient MEF cells, whereas mutations in the p53 NES resulted in an exclusively nuclear localization of p53-GFP. Because this NES is located in the tetramerization domain of p53, it has been proposed that regulated p53 tetramerization might obstruct the NES, thereby ensuring nuclear retention of p53 in its DNA-binding form (84).

**Ubiquitination-dependent p53 Export.** The third model contends that MDM2 ubiquitinates p53 in the nucleus, and the ubiquitination of p53 promotes its nuclear export and subsequent cytoplasmic degradation (Fig. 2C; Refs. 96 and 107). This model was proposed based on the observations that coexpression of MDM2 resulted in cytoplasmic accumulation or, sometimes, nuclear exclusion of a p53-GFP fusion protein. MDM2-promoted p53-GFP nuclear exclusion was abrogated either by a mutation in the RING finger domain of MDM2 that inactivated its ubiquitin ligase activity or by a mutation in the E1 ubiquitin-activating enzyme. Supporting this model, inhibition of nuclear export by LMB treatment resulted in an accumulation of ubiquitinated p53 in the nuclear fraction, and mutations in the COOH-terminal p53 NES blocked p53 nuclear export but not ubiquitination (96, 97), suggesting that p53 ubiquitination could occur in the nucleus prior to export to the cytoplasm.

Critical to the clarification of these different models and the mechanism of p53 nuclear export is the role of MDM2 in p53 export. Two particularly confusing issues arose from the characterization of p53 nuclear export using p53 mutants that disrupted p53–MDM2 binding and from the use of MDM2 mutants with impaired nuclear export activity. Within the NH<sub>2</sub>-terminal region of p53 resides the MDM2 binding domain. Both p53<sup>L14Q/F19S</sup> and p53<sup>L22Q/W23S</sup> mutations disrupt MDM2–p53 binding (59) and confer p53 resistance to MDM2-mediated degradation (56, 57). Although a recent study reported that these MDM2 binding-deficient p53 mutants were fully capable of exporting a fused reporter GFP (84), others found that the same mutations (107) or deletion of the NH<sub>2</sub>-terminal sequence from p53 (96) blocked nuclear export of the p53-GFP fusion. The reason for this discrepancy is not entirely clear. In the study that showed active export of the MDM2 binding-deficient p53 (84), 75% of the cells expressing p53-GFP or p53<sup>L22Q/W23S</sup>–GFP fusion proteins had varying degrees of cytoplasmic GFP fluorescence, as opposed to the predominantly nuclear staining seen in nearly all cells transfected with unfused p53. It is possible that high levels of ectopic expression and/or the GFP moiety might have increased cytoplasmic retention of p53<sup>L22Q/W23S</sup>–GFP, which would then have entered the mouse nuclei in the heterokaryons and been scored incorrectly as positive nuclear export. Endogenous p53<sup>L22Q/W23S</sup> protein produced by a knock-in strategy was blocked from nuclear export and accumulated to very high levels in the nucleus (108), providing further support for the disruption of p53 nuclear export by L22Q/W23S mutations.

Apparently contradictory conclusions were made about whether p53 was exported when coexpressed with an export-deficient MDM2 mutant. It was initially found that mutations at two hydrophobic residues within the NES of MDM2 (HDM2<sup>L250A/I208A</sup>) impaired its abilities to export and to degrade p53 (39). Coupled with the finding that a NLS mutant of MDM2 also failed to degrade p53, these observations led to the notion that MDM2 shuttled p53 from the nucleus to the cytoplasm for its degradation (38). This conclusion was inferred from the effects of MDM2 mutations on the steady-state levels of p53 protein, not an actual measurement of p53 nuclear export. In separate studies, however, when the MDM2 NES mutant was coexpressed with p53-GFP, the cytoplasmic fluorescence (measuring p53 export) increased, and the steady-state levels of p53 protein decreased (96, 107). These results not only suggested that an intact NES in MDM2 is not required for nuclear export of p53 but also implied that nuclear-secluded MDM2 does not trap p53 in the nucleus. Instead, coexpression with a RING finger MDM2 mutant (HDM2<sup>C464A</sup>) that lacks ubiquitin ligase activity resulted in a nuclear exclusion of p53-GFP and had no effect on the steady-state levels of p53 protein. One complication in resolving these discrepancies was the use of different forms of p53 (p53<sup>versus</sup> p53-GFP fusion) and different assays for determining or assessing protein nuclear export (heterokaryon assay, steady-state levels, or nuclear exclusion of p53-GFP). Although all three models agree that nuclear export is necessary for p53 degradation, none provides any clue as to why p53 must go to the cytoplasm to be degraded. Whether p53 and MDM2 export can be differentially regulated and whether cells use nuclear export to regulate the nuclear concentration of p53 and MDM2 or even their interaction are yet to be explored. Investigations into these questions could potentially reveal a novel regulation of p53.

**Three Models of ARF Function in p53 Stabilization**

Regardless of whether nuclear p53 is shuttled from the nucleus to the cytoplasm by MDM2, exits independently, or exits dependent on MDM2-mediated ubiquitination, blocking p53 nuclear export or disrupting the MDM2–p53 complex would cause p53 stabilization. ARF inhibited MDM2-mediated p53 degradation, at least in part, by blocking the nuclear export of p53 and MDM2 (41, 42). Presently, there are three competing models concerning the molecular mechanism by which ARF inhibits MDM2-mediated p53 nuclear export: (a) by sequestering MDM2 into the nucleolus (Refs. 42 and 46; Fig. 2D); (b) by formation of ternary complexes in the nucleoplasm (Ref. 41; Fig. 2E); or (c) by inhibiting MDM2 ubiquitin ligase activity (Ref. 107; Fig. 2F). These models each have experimental support, but none can satisfactorily integrate all observations. They are compared and contrasted below.

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<sup>6</sup> Unpublished results.
**Nucleolar Sequestration.** The nucleolar sequestration model proposes that ARF sequesters MDM2 in the nucleolus, releasing p53 from MDM2 inhibition (Refs. 42 and 46; Fig. 2D). This model is based mainly on two observations: (a) a portion of MDM2 protein (but not all) was localized into nucleoli in HeLa cells cotransfected with plasmids expressing MDM2 and mouse ARF (42), in MEFs microinjected with plasmids encoding mouse ARF, and in aging MEFs, where both MDM2 and ARF protein levels are elevated (46); and (b) a mouse ARF mutant (ARF<sup>126–37</sup>) that was defective in nucleolar localization yet retained MDM2 binding activity was unable to mobilize MDM2 into the nucleolus and had decreased ability to stabilize p53 (46). The nucleolar sequestration model has been presented in two different versions. The first simply proposes that ARF relocalizes MDM2 from the nucleoplasm to the nucleolus, enabling p53 to accumulate in the nucleoplasm free from MDM2 inhibition (46). A more intricate version, rooted in the idea of MDM2-dependent p53 export, postulates that the normal nuclear export of MDM2 and MDM2-p53 complexes travels through the nucleolus and ARF tethers crossing MDM2 in the nucleolus, thereby blocking its export as well as that of p53 (42). This hypothesis was inspired in part by the observations that MDM2 bound to ribosomal protein L5 (40), a nucleolar protein, and that L5 itself is involved in the export of SS rRNA (109) and possibly other cellular and viral proteins (110). One piece of missing evidence, predicted by this hypothesis, is the nucleolar staining of not only MDM2 but also of p53 in the presence as well as absence of ARF. In theory, it is possible that nucleolar transit of MDM2 and p53 is too rapid to be detected.

A key premise of both versions of the nucleolar sequestration model is that ARF dissociates MDM2-p53 complexes, freeing p53 from MDM2-mediated degradation and transcriptional inhibition. Thus far, dissociation of the MDM2-p53 complex by ARF has not been observed in any setting. Rather, ARF and p53 bind to two separate domains in MDM2 and in vivo, ARF, MDM2, and p53 readily formed a ternary complex (28–31). In vitro, binding of human ARF protein (43) or ARF<sup>1–26</sup> peptide (32) to MDM2 inhibited its ubiquitin ligase activity and induced p53 stabilization. Expression of ARF<sup>1–65</sup> lacking the major NoLS (41) or GFP-ARF<sup>1–20</sup> (32) induced p53 stabilization and activity in vivo, suggesting that minimally ARF could activate p53 through a mechanism independent of nucleolar sequestration of MDM2. Additionally, because ARF blocks p53 nuclear export (41), the nucleolar sequestration model has to assume that free p53 is unable to exit from the nucleolus, a supposition that is not supported by the finding that p53 contained a functional NES capable of exporting p53 in the absence of MDM2 (84). Furthermore, in those cells where MDM2 and ARF were seen to colocalize in nucleoli, abundant MDM2 remained throughout the nucleoplasm (42, 45, 46). According to the nucleolar sequestration model, it is necessary to argue that in these cells, the amount of endogenously expressed p53 was comparable with that of ectopically expressed MDM2 and that after a portion of MDM2 was sequestered by ARF into the nucleolus, the amount of p53 must have exceeded that of MDM2 in the nucleoplasm. Otherwise, a novel mechanism must be invoked to explain why the MDM2 remaining in the nucleoplasm was not inhibiting p53. Lastly, both NoLSs in mouse ARF overlap with MDM2 binding (45), raising the question of how the NoLSs retain their ability to move ARF-MDM2 complexes into the nucleolus, rather than being masked by MDM2. This was explained by the proposal that MDM2 contains a cryptic NoLS within its RING finger domain (KKLKKRNNK, residues 466–473 in HDM2), which was unmasked upon ARF binding and contributed to the nucleolar localization of the complex (45, 111). How ARF binding to a central region of MDM2 revealed this cryptic NoLS and whether other proteins could also liberate this cryptic NoLS is not clear. Coexpression of ARF with HDM2<sup>466–473</sup> resulted in colocalization of both proteins in the nucleoplasm, suggesting the possibility that localization of ARF-MDM2 complex in the nucleus could be regulated by whether this cryptic NoLS was exposed or masked (e.g., by the binding with E2 or MDMX). This cryptic NoLS was uncovered through the observation that HDM2<sup>222–437</sup> mutant was localized mainly in the nucleolus, as opposed to the nucleoplasm such as the wild type, and that a fusion protein containing HDM2 residues 466–473, three copies of SV40 NLS and a thioredoxin reporter localized to the nucleolus (111). One concern is that some of the observed nucleolar localization might have resulted from electrostatic interactions, as opposed to specific targeting. Deletion of the central acidic domain from MDM2 brings the NLS (residues 181–185) close to the cluster of basic residues in the RING finger and creates a sequence, such as the fusion of residues 466–473 with SV40 NLS, with a high content of basic amino acids (from pI 4.6 in wild type to 8.6 in HDM2<sup>222–437</sup>).

**ARF-MDM2-p53 Ternary Complex.** The second model proposes that nucleolar ARF is relocalized by MDM2 to the nucleoplasm, where it forms a ternary complex with MDM2 and p53, thereby blocking nuclear export of both MDM2 and p53 (Ref. 41; Fig. 2E). When ARF is coexpressed with p53 in the absence of MDM2, neither the nucleolar localization of ARF nor the nucleoplasmic distribution of p53 was changed (41), consistent with the observations that ARF interacts with p53 through inhibiting MDM2. Ectopic expression of MDM2 in HeLa cells, which express high levels of ARF as the result of p53 inaction, resulted in relocalization of ARF throughout the nucleoplasm. When all three proteins were coexpressed, ARF, MDM2, and p53 colocalized to discrete nuclear bodies in the nucleoplasm (41, 49). Deletion of the exon 1β-encoded MDM2 binding domain or mutations in the nuclear/nucleolar localization signals in exon 2 of human ARF abrogated the ability of ARF to form nuclear bodies and reduced the activity of ARF to stabilize p53 (41), providing a correlative link between ternary complex formation/nuclear body formation and ARF-mediated p53 stabilization. Ectopic expression of E2F1, an activator of ARF gene expression (112), resulted in an accumulation of ARF in the nucleoplasm in normal cells or in cells with MDM2 gene amplification but exclusively in the nucleoli in p53-deficient Saos-2 cells that expressed undetectable levels of MDM2, providing physiological evidence for the ability of MDM2 to alter ARF localization and induce nuclear body formation in the presence of ARF and p53 (41).
There are two predictions of this model that remain to be tested:

(a) this model proposes that entry of ARF into the MDM2-p53 complex activates p53 and restores the transcriptional activity of p53. It implicitly suggests that MDM2-bound (and thus repressed) p53 can be derepressed upon ARF binding without dissociation from MDM2. Repression of the transcriptional activity of p53 by MDM2 can occur on p53-responsive promoter sequences (58). The biochemical mechanism underlying this MDM2-imposed repression of p53-mediated transcription is not clear but could involve inhibition by MDM2 of otherwise activating covalent modifications of p53 such as acetylation (60, 61), with a restoration of the transcription-promoting effects in the presence of ARF. Reversal of the MDM2-mediated inhibition of p53 acetylation by ARF without a detectable dissociation of the MDM2-p53 complex would be consistent with such a possibility (62). More direct evidence showing in situ reversible regulation of the transcriptional activity of p53 by MDM2 and ARF is needed.

(b) The second unsatisfactory aspect of this model is the lack of information regarding the protein composition and function of the ARF-MDM2-p53 nuclear bodies. Formation of such large nuclear aggregates also raises concerns as to whether they result from protein overexpression and/or inappropriate protein conformation. These nuclear bodies are clearly visible under a light microscope, suggesting that they are likely to contain a large number of additional proteins with complex function and regulation. The appearance of an ARF-MDM2-p53 body is reminiscent of the PML bodies (or PODs) stabilized and activated (119, 120). It is unclear whether the ARF-MDM2-p53 nuclear body is structurally related to the PODs, or whether ARF and PML mediate two distinct pathways of p53 activation.

Inhibiting p53 Nuclear Ubiquitination. The third model proposes that MDM2 ubiquitinates p53 in the nucleus, and that ubiquitination of p53 facilitates, and may even be required for, nuclear export and subsequent cytoplasmic degradation of p53 (107). Because binding with ARF inhibited the ubiquitin ligase activity of MDM2 (32, 43), ARF might prevent p53 nuclear export by blocking MDM2-mediated nuclear ubiquitination of p53 (Fig. 2F). This model is based on experiments showing that coexpression of MDM2 and p53-GFP, but not p53-GFP alone, resulted in p53-GFP cytoplasmic accumulation or even nuclear exclusion in some cells, an observation that was interpreted as evidence for MDM2-mediated p53 nuclear export. Relocalization of p53-GFP was abrogated by a mutation in the RING finger domain of MDM2 (HDM2C464A) that inactivated its ubiquitin ligase activity (96, 107), or by a temperature-sensitive mutation in the E1 ubiquitin-activating enzyme (107). Consistent with this model, inhibition of nuclear export by LMB treatment resulted in a notable accumulation of ubiquitinated p53 in the nuclear fraction relative to the cytoplasm fraction (96), suggesting that p53 ubiquitination might occur in the nucleus prior to translocation to the cytoplasm.

Direct evidence that ubiquitination of p53 is required for its nuclear export and that even ubiquitinated p53 actually undergoes nuclear export has yet to be provided. It was suggested that MDM2-mediated ubiquitination of p53 might cause dissolution of p53 tetramers and subsequently render the NES of p53 accessible to export machinery (96, 107). A mechanism explaining how ubiquitination of p53 promote, rather than impede, the interaction of p53 with export machinery is yet to be devised. In addition to the lack of evidence in support of the masking of the NES of p53 by tetramerization, this hypothesis is also inconsistent with the finding that p53 could exit from the nucleus in the absence of MDM2 (84). Both studies only examined the static distribution of the p53 and MDM2 proteins, rather than their dynamic movement as could have been determined by a heterokaryon assay, and did not exclude the possibility that the RING finger mutations in MDM2 attenuated its own nuclear export, with a resultant trapping of p53 in the nucleus. Use of p53-GFP fusions and transient plasmid transfection-mediated protein overexpression throughout both studies also raises concerns as to whether native proteins would behave the same. This model is compatible with either the nucleolar sequestration or the nuclear body model; separating MDM2 from p53 or the binding of ARF to p53-bound MDM2 would inhibit p53 ubiquitination by MDM2 and thereby block p53 nuclear export. If proven true, this model presents a novel function of ubiquitination, apart from proteolytic degradation, in regulating protein trafficking. It also represents an attractive mechanism for ARF function, coupling inhibition of p53 ubiquitination with a block of p53 nuclear export, which could further potentiate p53 activity in the nucleus than either action alone.

Concluding Remarks
Since the initial discovery of MDM2-p53 interaction and spurred on by the revealing of ARF-MDM2 binding, intensive research on the control of p53 by these two proteins has brought many advances in our understanding. The ARF-MDM2-p53 pathway is now firmly established, both genetically and biochemically, yet still remains a relatively new field. Much remains uncharted, and many of the initial explorations have provided opportunities for further study. In addition to the issues related to the in vivo functions, such as the transcriptional regulation of ARF and p16 gene expression, there are many more questions concerning the biochemical mechanisms of this pathway. How does ARF inhibit the ubiquitin ligase activity of MDM2? What are the biochemical mechanisms underlying MDM2 and p53 nuclear export and how does ARF inhibit both? Why must p53 be exported out of the nucleus for degradation? Are p53 nuclear export and ubiquitination two separate events or intrinsically coupled? Do other stress signaling pathways including DNA damage ac-
tivate p53 by inhibiting p53 ubiquitination? blocking p53 nuclear export? or both? With the ongoing active investigation and energetic debate, we can anticipate that many of currently confusing issues concerning the control of p53 ubiquitination and nuclear export by MDM2 and ARF will soon be clarified, and some of these questions answered.

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References


