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ORIGINAL ARTICLE

Functional analysis and consequences of Mdm2 E3 ligase inhibition in human tumor cells

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Mdm2 is the major negative regulator of p53 tumor-suppressor activity. This oncoprotein is overexpressed in many human tumors that retain the wild-type p53 allele. As such, targeted inhibition of Mdm2 is being considered as a therapeutic anticancer strategy. The N-terminal hydrophobic pocket of Mdm2 binds to p53 and thereby inhibits the transcription of p53 target genes. Additionally, the C-terminus of Mdm2 contains a RING domain with intrinsic ubiquitin E3 ligase activity. By recruiting E2 ubiquitin-conjugating enzyme(s), Mdm2 acts as a molecular scaffold to facilitate p53 ubiquitination and proteasome-dependent degradation. Mdmx (Mdm4), an Mdm2 homolog, also has a RING domain and hetero-oligomerizes with Mdm2 to stimulate its E3 ligase activity. Recent studies have shown that C-terminal residues adjacent to the RING domain of both Mdm2 and Mdmx contribute to Mdm2 E3 ligase activity. However, the molecular mechanisms mediating this process remain unclear, and the biological consequences of inhibiting Mdm2/Mdmx co-operation or blocking Mdm2 ligase function are relatively unexplored. This study presents biochemical and cell biological data that further elucidate the mechanisms by which Mdm2 and Mdmx co-operate to regulate p53 level and activity. We use chemical and genetic approaches to demonstrate that functional inhibition of Mdm2 ubiquitin ligase activity is insufficient for p53 activation. This unexpected result suggests that concomitant treatment with Mdm2/Mdmx antagonists may be needed to achieve therapeutic benefit.

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INTRODUCTION

The p53 protein is most often considered a stress-activated tumor suppressor but its functions likely evolved for other purposes.[1] Recent studies indicate that p53 preserves genome integrity in the male germline, limits teratogen-induced developmental abnormalities, contributes to implantation and modulates metabolism.[2] Its tumor-suppressive role emerges when it is activated by genotoxic conditions or expression of oncogenes, whereupon it eliminates potentially tumorigenic cells via induction of cell cycle arrest, apoptosis, senescence or autophagy. Given the importance of p53 function in normal growth processes, as well as in response to tumor-initiating conditions, sophisticated mechanisms have evolved to limit p53 activity until it is absolutely required, and to then titrate its output in a stress and cell-appropriate fashion.

P53 activity is controlled by two broad mechanisms. First, is a complex array of post-translational modifications that affect its ability to engage with relevant co-activators or negative regulators. This in turn determines p53 stability and abundance, and its ability to productively engage chromatin to regulate downstream target genes and micro-RNAs.[3,4] Second, are mechanisms that control the stability and/or activity of its negative regulators, most significant of which are the related RING domain proteins, Mdm2 and Mdmx (also known as Mdm4).[5] Embryonic deletion of either protein engenders early lethality that is completely p53-dependent, underscoring their critical role during development. Conditional deletion of either Mdm2 or Mdmx indicates that while both restrict p53 activation in almost all tissues tested, Mdm2 loss often has a more profound effect.[6] These data suggest a modulatory, role for Mdmx in many somatic tissues, with possibly more important roles in actively dividing tissues.[7,8]

Both Mdm2 and Mdmx contain a RING domain, variants of which are found in several hundred human proteins. In the case of Mdm2, the RING domain possesses E3 ubiquitin ligase activity: it recruits one or more E2 ubiquitin-conjugating enzymes (hereafter E2)[9] and facilitates transfer of ubiquitin from E2 to p53 and other substrates,[10] leading to their proteasome-dependent degradation.[11] As p53 is a transcription factor, Mdm2-dependent degradation is therefore an effective mechanism to modulate activation of p53 target genes. The binding of both Mdm2 and Mdmx to p53 also directly inhibits p53 transactivation function by preventing recruitment of transcriptional co-activators.[8] Although Mdmx also has a RING domain, it does not possess intrinsic ubiquitin ligase activity.[12] RING-mediated oligomerization is a conserved function of this domain, and Mdm2 is able to homo-oligomerize, as well as hetero-oligomerize with Mdmx. By analogy with other RING/RING heterodimeric complexes (such as Brca1/Bard1, where only Brca1 has ligase activity),[13] a model in which Mdmx enhances Mdm2-dependent p53 ubiquitylation has been proposed.[12] However, the precise molecular mechanism(s) by which Mdmx modulates Mdm2 function remain to be defined.

Although Mdmx co-operates with Mdm2 to inhibit p53 function, Mdmx can also be targeted for Mdm2-dependent degradation.[14] Following genotoxic stress, Mdmx downregulation contributes to p53 activation. Indeed, attenuating damage-induced Mdmx degradation *in vivo* reduces both basal and

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stress-induced p53 activities. This engenders both remarkable radioresistance, and dramatically increases sensitivity to Myc-induced lymphomagenesis.[15]

In addition to the Mdm2 and Mdmx RING domains, residues at the extreme C terminus of each protein are also important for regulation of Mdm2 ubiquitin ligase function.[16,17] Structural and functional analyses predict that C-terminal aromatic residues in both Mdm2 and Mdmx have a critical role in the context of Mdm2/Mdmx hetero-oligomers.[16-19] Mdm2 point mutants in this region prevent p53 degradation, yet allow Mdmx degradation. Furthermore, Mdmx can restore Mdm2-directed ligase activity to these mutants, seemingly by providing the C-terminal residues in trans. These data suggest that the extreme C-terminus provides subtle structural elements that are critical for controlling p53 ubiquitylation; however, the mechanistic basis for these effects remains to be determined.

As both Mdm2 and Mdmx are potential therapeutic targets for cancer treatment,[5] insight into their molecular interplay may inform new drug discovery and development strategies. Here, we investigate the effects of Mdm2 ligase inhibition on the control of p53 stability and activity. We show that the Mdmx extreme C-terminus comprises a key regulatory element affecting the degradation of endogenous p53 and Mdm2; it is also required for degradation of Mdmx in response to DNA damage. Using a genetic approach, we show that the inhibition of Mdm2 ligase function leads to stabilization of transcriptionally inactive p53. Furthermore, the stabilized p53 can be reactivated by attenuation of the interaction of p53 with either Mdm2 or Mdmx. These findings indicate that drugs designed to selectively inhibit Mdm2 ligase activity may, if used alone, not activate p53 sufficiently to elicit adequate antitumor effects. Rather, as they do engender significant increases in p53 abundance, they may achieve therapeutic benefits if used in combination with Mdm2 and/or Mdmx antagonists.

RESULTS

Functional inhibition of Mdm2 stabilizes endogenous p53

By analogy with other heterodimeric E3 ligases, residues in the Mdm2 and Mdmx C-terminal tails may contribute to the correct structure for recruitment or processivity of the E2-conjugating enzyme(s) required for p53 degradation. While a previous study found that Mdm2 and Mdmx C-terminal point mutants ($Mdm2^{Y489A}$ and $Mdmx^{F488A}$, respectively) prevented Mdm2-dependent degradation of p53, the consequences for p53 activation were not explored.[17] We therefore initiated a genetic approach to evaluate the functional consequences of Mdm2 ligase inhibition by generating U2OS cell lines expressing doxycycline (Dox)-inducible wild type (WT) and $Mdm2^{Y489A}$ and $Mdmx^{F488A}$. U2OS was chosen as the host cell because it retains a WT p53 allele, and expresses a molecular excess of Mdm2 over Mdmx.[20] This provides a situation in which the excess Mdm2 is a relevant physiological target for evaluating the effects of exogenously expressed Mdm2 or Mdmx mutants. A relatively high dose (100 ng/ml) of Dox was used for comparisons between Mdm2 and Mdmx, because at lower doses we either failed to see robust increases in the levels of Dox-inducible Mdm2 or observed cell-to-cell heterogeneity in Mdm2 levels (data not shown). This is consistent with previous reports of differential expression of Mdm2 and Mdmx from the same promoter.[21] Importantly, $Mdmx^{WT}$ was downregulated by DNA damage at both low- and high-dose Dox (see Supplementary Figures 1C and D), indicating that the levels of induction achieved at the maximum Dox dose used for these studies is not saturating the capacity of the damage response system to induce Mdmx degradation.

Figure 1 shows the effects of $Mdm2^{Y489A}$ and $Mdmx^{F488A}$ overexpression on levels of p53 and its downstream target, p21.

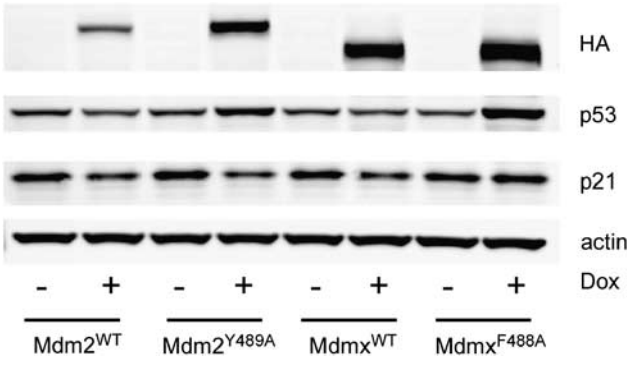

Figure 1. Expression of $Mdm2^{Y489A}$ or $Mdmx^{F488A}$ leads to stabilization of p53. Cell lines containing Dox-inducible Mdm proteins were treated with Dox (100 ng/ml) for 24 h and analyzed by western blot for the indicated proteins.

As expected, induction of $Mdm2^{WT}$ led to a decrease in p53, and corresponding decrease in p21. In contrast, $Mdm2^{Y489A}$ expression led to an increase in p53, likely due to inhibition of endogenous Mdm2 E3 ligase activity.[17] Note that despite the increase in p53 abundance, the levels of p21 were reduced by $Mdm2^{Y489A}$ expression (Figure 1 and Supplementary Figures 1E and F). Similarly, overexpression of $Mdmx^{F488A}$ increased p53 steady-state levels, yet p21 did not increase. The ability of $Mdmx^{F488A}$ to stabilize p53, yet suppress p53-dependent transactivation was also observed at low-dose (15 ng/ml) Dox (Supplementary Figures 1A, B, E and F). At this dose of Dox, exogenous Mdmx levels were approximately fourfold those observed in MCF7 cells, a breast cancer cell line with Mdmx gene amplification.[22] Together, these data suggest that while the extreme C-terminal aromatic residues of Mdm2 and Mdmx are required for p53 degradation, they are dispensable for suppression of p53 transcriptional activity (see below).

We next investigated the mechanism by which $Mdmx^{F488A}$ causes stabilization of p53. Figure 2 shows that $Mdmx^{F488A}$ expression increased the half-life of both endogenous Mdm2 and p53, and this was not accompanied by a change in Mdm2 mRNA levels (Figures 2a and b). This suggests that $Mdmx^{F488A}$ causes post-transcriptional stabilization of Mdm2 by inhibiting Mdm2 ubiquitin ligase activity, and this leads to concomitant p53 stabilization. We tested the hypothesis that $Mdmx^{F488A}$ inhibits ubiquitin ligase activity by determining the extent of p53 ubiquitylation in cell lines expressing equivalent levels of $Mdmx^{WT}$ or $Mdmx^{F488A}$. Consistent with this proposal, the total levels of p53 are higher in cells expressing $Mdmx^{F488A}$ compared with $Mdmx^{WT}$ and the amount of ubiquitylated p53 is significantly lower in cells expressing this mutant (Figure 2c). These data are consistent with a previous study in which p53 ubiquitylation was reduced by $Mdm2^{Y489A}$.[17]

$Mdmx^{F488A}$ binds Mdm2 and p53, but reduces p53 ubiquitylation

Like some other RING E3 ubiquitin ligase complexes, such as Brca1/Bard1, Mdm2 and Mdmx can hetero-oligomerize. As only Mdm2 possesses E3 ubiquitin ligase activity, a reasonable explanation for the above results is that $Mdmx^{F488A}$ hetero-oligomerizes with Mdm2 and reduces its ability to degrade p53. However, $Mdmx^{F488A}$ could also stabilize p53 by more indirect mechanisms. For example, $Mdmx^{F488A}$ might antagonize p53-dependent transactivation of *Mdm2*, leading to a reduction in Mdm2 levels. This is unlikely, because *Mdm2* mRNA levels are similar in the presence and absence of $Mdmx^{F488A}$ (Figure 2b).

The inability of $Mdmx^{F488A}$ to activate p53 while increasing p53 levels was surprising, and could be explained by its ability to

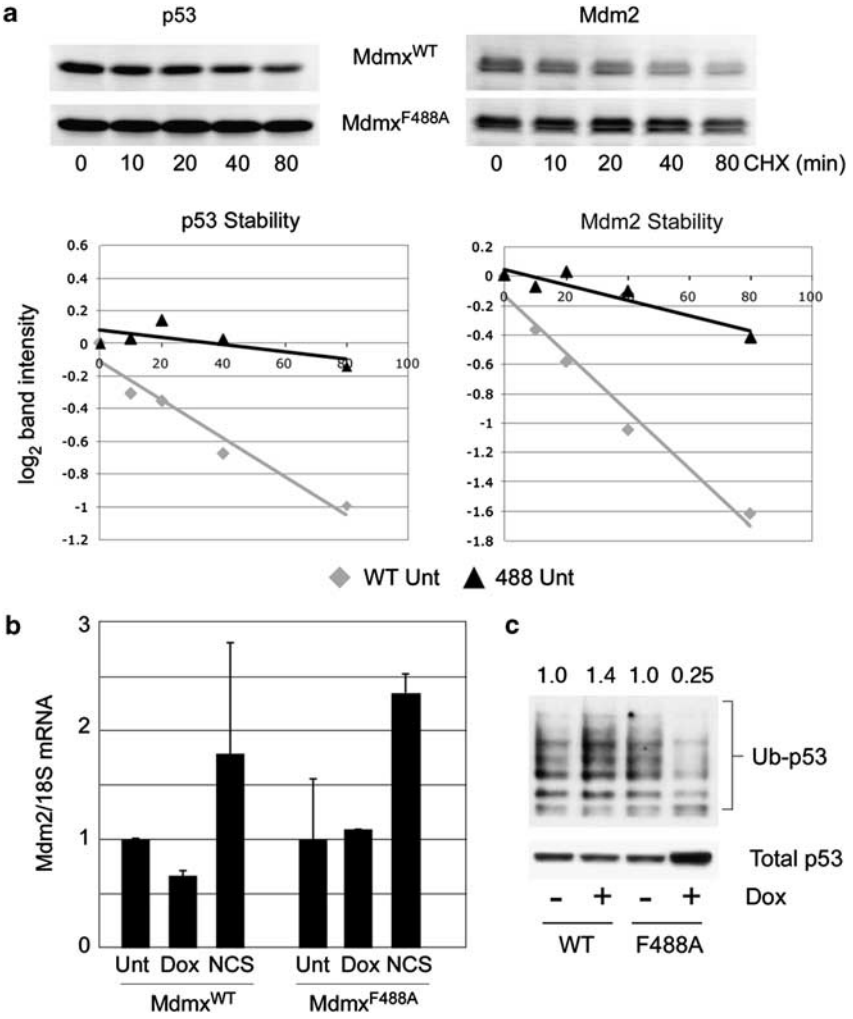

Figure 2. Stabilization of p53 on Mdmx^{F488A} expression is post-translational. (**a**) Following induction of Mdmx^{WT} or Mdmx^{F488A}, cycloheximide was added for the indicated times before blotting for p53 and Mdm2. Band intensities were calculated using the LiCor/Odyssey image analysis system, and plotted as $\log_2$ values. (**b**) *Mdm2* mRNA was analyzed by quantitative PCR following addition of Dox, or the damaging agent neocarzinostatin (NCS) (300 ng/ml) as a positive control. (**c**) Cells were transfected with Histidine-tagged ubiquitin and Dox was added to induce Mdmx^{WT} or Mdmx^{F488A}. After 24 h, cells were lyzed and ubiquitylated proteins were pulled down using Ni^{2+}-agarose beads. Following sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), p53 was detected using DO-1. Numbers above each lane represent the ratio of ubiquitylated p53 species to the total amount of p53 in the lysate.

suppress p53-dependent transactivation concomitant with binding to, and inactivation of, Mdm2 ligase function. To begin to explore this possibility, we first determined whether Mdmx^{F488A} could bind both Mdm2 and p53 using the proximity ligation *in situ* assay (PLISA, see Supplementary Figure 2 for a description of the method, and Soderberg *et al*.[23]).

PLISA confirmed that Mdmx^{F488A} binds endogenous Mdm2 and p53 in intact cells (Figures 3a and b). Induction of Mdmx^{WT} led to a small but detectable increase in the interaction between Mdmx and Mdm2, as shown by an increase in the number of PLISA foci (Figure 3a, panel ii). The PLISA signal for Mdmx–Mdm2 interaction was much more robust after Mdmx^{F488A} induction (Figure 3a, panel vi). This is in part due to the elevated Mdm2 levels in Mdmx^{F488A} expressing cells (see Supplementary Figure 3A and western blots in Figures 4 and 6). Interaction of Mdm2 with Mdmx^{WT} was detected in the nucleus, but appeared slightly enriched in the cytoplasm (Figure 3a, panel ii). This did not appear to be due to a preferential enrichment of either protein in the cytoplasm (Supplementary Figure 3C). In the case of Mdmx^{F488A}, the intensity of PLISA foci was greatest in the nuclei, although the Mdm2–Mdmx interaction was also detected in the cytoplasm (data not shown). These data confirm that the Mdmx^{F488A} mutation does not significantly interfere with Mdm2 binding, and that Mdm2 and Mdmx^{F488A} interact in both nuclear and cytoplasmic compartments *in situ*.

PLISA analyses revealed that Mdmx^{WT} and Mdmx^{F488A} also interact with p53 (Figure 3b). Again, the signal in the cells expressing Mdmx^{F488A} cell line was higher than in those expressing Mdmx^{WT} in part because induction of the F488A mutant leads to increased p53 levels. Standard immunofluorescence showed that both Mdmx^{WT} and Mdmx^{F488A} were distributed throughout the nucleus and cytoplasm, but p53 was predominantly nuclear following induction of Mdmx^{F488A} (Supplementary Figures 3B and C). This likely contributes to the preferential localization of p53/Mdmx PLISA signal in the nucleus. Cytoplasmic PLISA signals were also observed at a higher intensity in Mdmx^{F488A} cells compared with Mdmx^{WT} presumably due to the increased total p53 levels (data not shown). The PLISA signal faithfully reflects p53–Mdmx interaction because many fewer PLISA signals were observed in Dox-inducible cells expressing

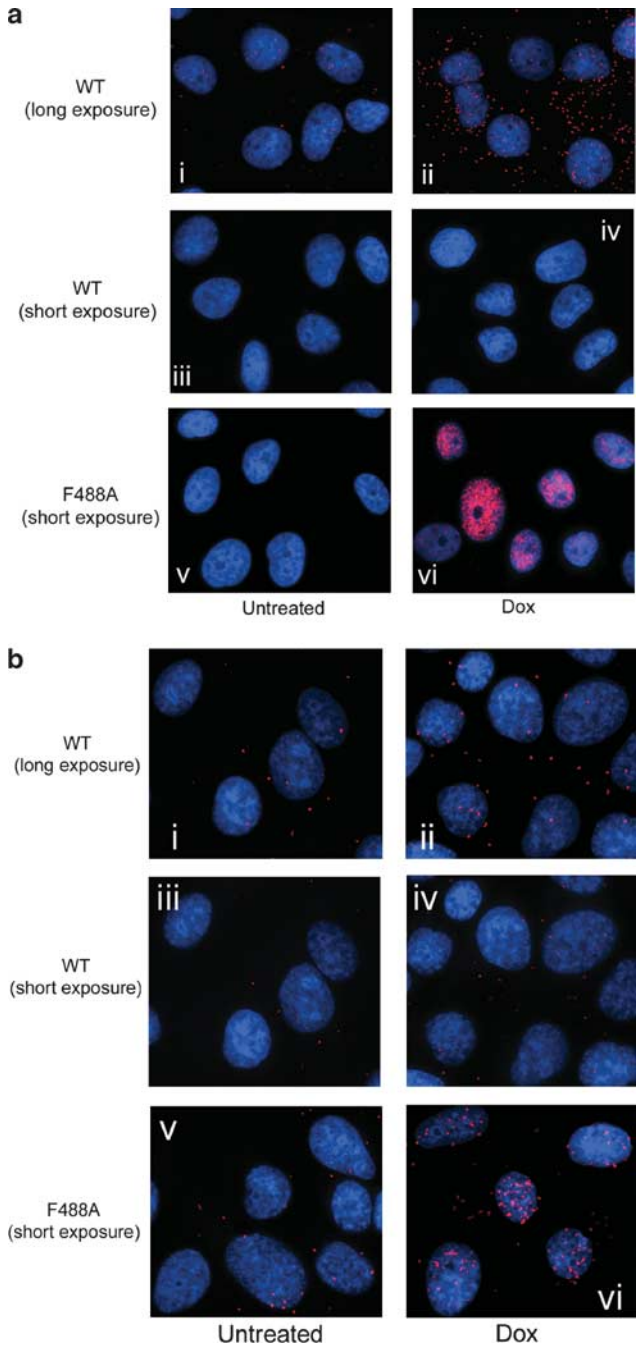

Figure 3. PLISA reveals Mdmx^{F488A} binds to p53 and Mdm2. (**a**) Both Mdmx^{WT} and Mdmx^{F488A} interact with endogenous Mdm2. Cells were treated for 24 h with Dox and then incubated with primary antibodies against Mdm2 and Mdmx. PLISA was then performed, and visualized under a fluorescence microscope. For Mdmx^{WT}, a longer exposure time was required to detect Mdmx/Mdm2 PLISA signals (panels i and ii). Panels iii and iv, (Mdmx^{WT}) and v and vi (Mdmx^{F488A}) were taken with the same exposure time. (**b**) Mdmx^{WT} and Mdmx^{F488A} Mdmx interact with p53. Cells were treated as in (**a**) and then processed for PLISA after incubation with primary antibodies to detect Mdmx and p53. Exposures were as in (**a**).

equivalent levels of the p53 binding-deficient Mdmx mutant, G57A (Supplementary Figure 3D). Conventional immunoprecipitation also confirmed that Mdmx^{F488A} is found in complex with Mdm2 and with p53 (Supplementary Figures 4A and B). Finally, we found that mutation of the Mdmx p53-binding site does not affect the ability of Mdmx^{F488A} to stabilize p53 (Supplementary Figure 4C and Figure 6d). Together, these data show that Mdmx^{F488A} binds both p53 and Mdm2, and suggest that the dominant-negative effect of Mdmx^{F488A} is due to direct perturbation of Mdm2 ligase function, rather than sequestration of p53.

Both Mdm2^{Y489A} and Mdmx^{F488A} suppresses p53 transactivation function

As an increase in p53 level is often associated with increased p53 activity, we next determined whether the p53 stabilized in cells expressing Mdm2^{Y489A} or Mdmx^{F488A} was transcriptionally active. As expected, induction of Mdm2^{WT} reduced p53 level and transcriptional activity (Figure 4a, lane 2 and Figure 4c). By contrast, induction of Mdm2^{Y489A} stabilized p53, yet was still able to suppress p53 activity (Figure 4a, lane 6 and Figure 4c). In fact, although Mdm2^{Y489A} expression and treatment with the DNA-damaging agent neocarzinostatin led to similar increases in p53 level, p53 transcriptional activity was only increased by damage (compare lanes 6 and 7). These data demonstrate that ligase-deficient Mdm2 retains the capacity to suppress p53-dependent transactivation, presumably by binding to and occluding the p53 transactivation domain. Similar results were obtained by comparing the effects of Mdmx^{WT} and Mdmx^{F488A} (Figure 4b). Specifically, while Mdmx^{F488A} expression led to an increase in p53 level, there was no corresponding increase in p53 transcriptional activity (Figure 4b, compare lanes 5 and 6 and Figure 4d). Again, although p53 levels following Mdmx^{F488A} expression or DNA damage were similar, p53 was only active in the latter case (Figure 4b, compare lanes 6 and 7 and Figure 4d). By normalizing the amount of *p21* mRNA to p53 protein, we found that both Mdm2^{Y489A} and Mdmx^{F488A} reduce p53-specific activity (Supplementary Figures 5A and B).

Following DNA damage, we observed that Mdmx^{WT} but not Mdmx^{F488A} was downregulated (Figure 4b, compare lanes 6 and 8). This is consistent with a dominant-negative function of Mdmx^{F488A}, because downregulation of Mdmx after DNA damage is an Mdm2-dependent process.[14,24] Similarly, Mdm2^{Y489A} appeared significantly more stable than Mdm2^{WT} following neocarzinostatin treatment (Figure 4a, compare lanes 2 and 4, 6 and 8). Intriguingly, neither Mdm2^{Y489A} nor Mdmx^{F488A} expression was sufficient to block damage-induced p53 activation (compare lanes 3 and 4, 7 and 8 in Figures 4c and d). Taken together, these data suggest that DNA damage signaling can counteract the suppressive effects of Mdm2 ligase inhibition on p53 activation. Additionally, the finding that DNA damage leads to downregulation of Mdmx^{WT} (Figure 4b) and destabilization of Mdm2^{WT} (Figure 4a and Supplementary Figure 6) indicates that, although these proteins are overexpressed, they are still subject to regulation by endogenous damage signaling pathways.

Antagonizing the interaction between p53 and Mdm2^{Y489A} or Mdmx^{F488A} derepresses p53 activity and elicits a cytotoxic response

Inhibition of Mdm2 ligase activity is suggested as a potential anticancer strategy in tumors with WT p53.[25,26] However, whether the ensuing p53 stabilization would be sufficient to elicit a cytotoxic response remains unclear. This is since inhibition of Mdm2 ligase activity would also stabilize Mdm2 itself, which could then interact with and antagonize p53. In this case, combinations of Mdm2 ligase inhibitors and Mdm2 antagonists, such as Nutlin-3a (hereafter Nutlin) may be required. We utilized the Mdm2^{Y489A} and Mdmx^{F488A}-inducible cell lines to investigate the potential for such an approach.

Although Mdm2^{Y489A} stabilizes p53, expression of the protein did not affect the growth of U2OS cells with WT p53 (Figures 5a and b). This is consistent with the ability of Mdm2^{Y489A} to block p53-dependent transactivation. By contrast, Nutlin suppressed

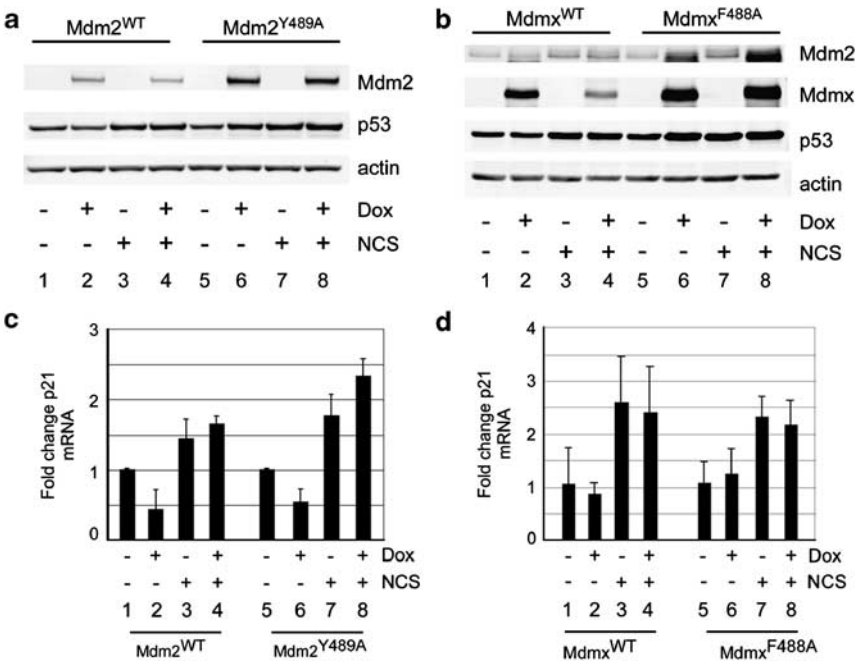

Figure 4. $Mdmx^{F488A}$ expression inhibits p53 ubiquitylation, but suppresses p53-dependent transactivation. (**a**, **b**) Cells expressing $Mdm2^{WT}$ and $Mdm2^{Y489A}$ (**a**) or $Mdmx^{WT}$ or $Mdmx^{F488A}$ (**b**) were treated with 100 ng/ml Dox for 24 h before addition of neocarzinostatin (NCS) (300 ng/ml, 5 h) and analyzed by western blot for the indicated proteins and quantitative PCR for *p21* mRNA expression (**c**, **d**).

colony outgrowth in the presence of both $Mdm2^{WT}$ and $Mdm2^{Y489A}$, although the increase in p53 abundance was similar (data not shown). Thus, while inhibition of Mdm2 ligase activity stabilizes p53, concurrent treatment with an Mdm2 antagonist is required for a maximal cytotoxic response.

A prediction from these results is that cells in which Mdm2 ligase activity is blocked by the $Mdmx^{F488A}$ mutant would not be sensitive to Nutlin, as numerous reports show that this drug interacts poorly with the Mdmx hydrophobic p53-binding pocket.[27,28] To address this, we repeated the colony outgrowth experiments with $Mdmx^{WT}$ and $Mdmx^{F488A}$. Consistent with previous studies, induction of $Mdmx^{WT}$ reduced basal levels of Mdm2 protein and mRNA by 50% (Figures 6a and b), whereas treatment with Nutlin alone lead to the expected increase in *Mdm2* mRNA and protein (Figure 6a, lane 3). Induction of $Mdmx^{F488A}$ or treatment with Nutlin both lead to similar increases in p53 and Mdm2 protein levels (Figure 6a, lanes 6 and 7), yet p53-dependent transactivation increased only with the Mdm2 antagonist (Figure 6b, bars 6 and 7 and Supplementary Figure 5D). This underscores the notion that $Mdmx^{F488A}$ retains the ability to antagonize p53-dependent transcription while concomitantly stabilizing both Mdm2 and p53 proteins.

As Nutlin is a less potent antagonist of Mdmx, Mdmx overexpression should attenuate Nutlin-induced p53 activation. Following Nutlin treatment, $Mdmx^{WT}$ marginally inhibited p53 activation, while $Mdmx^{F488A}$ expression had a more robust effect (compare Figure 6b lanes 3 and 4, and lanes 7 and 8). Consistent with this observation, only $Mdmx^{F488A}$ appeared to attenuate Nutlin-induced p53-specific activity (Supplementary Figure 5C).

The above results show that while $Mdmx^{F488A}$ blocks Mdm2 E3 ligase activity and stabilizes p53, its ability to inhibit p53-dependent transactivation remains intact. A prediction from the preceding results is that while p53 levels are elevated, cells expressing $Mdmx^{F488A}$ should continue to proliferate because of inhibition of p53 transactivation. Consistent with this, expression of $Mdmx^{F488A}$ alone did not inhibit growth, despite the high level of p53 under these conditions (Figure 6c). These findings suggest that inhibiting p53 degradation can be tolerated, providing the Mdmx–p53 interaction is preserved. Conversely, disrupting Mdmx/p53 binding under these conditions should lead to increased p53-dependent toxicity. In order to test this, we introduced a second mutation into $Mdmx^{F488A}$. This mutation (G57A) reduces the interaction of Mdmx with p53 (Wade *et al*.[29] and Supplementary Figures 3 and 7). Figure 6d shows that although $Mdmx^{F488A}$ and $Mdmx^{G57A/F488A}$ each lead to p53 accumulation, only $Mdmx^{G57A/F488A}$ causes an increase in p53 activity (measured as an increase in the level of p21 protein). Importantly, the elevated p53 activity in $Mdmx^{G57A/F488A}$ was not due to higher total p53 levels, or a low Mdmx:p53 ratio (Supplementary Figure 7). Interestingly, although $Mdmx^{G57A/F488A}$ and Nutlin stabilized p53 to similar levels, induction of p21 was much higher after Nutlin treatment. Additionally, although prolonged expression of $Mdmx^{G57A/F488A}$ Mdmx was associated with a marked decrease in cell viability compared with $Mdmx^{F488A}$, cell survival was significantly higher than following Nutlin treatment (Figure 6e, compare lanes 2 and 6). In theory, this could be due either to the increased Mdm2 levels resulting from $Mdmx^{G57A/F488A}$, inhibition of Mdm2 E3 ligase activity, or because $Mdmx^{G57A/F488A}$ has some residual binding to p53 (Supplementary Figure 7). We used Nutlin, an Mdm2-selective antagonist,[28] as a chemical probe to ascertain whether a putative Mdm2/$Mdmx^{G57A/F488A}$ heterodimer might bind to and inhibit p53 function. Indeed, we found that the viability of cells expressing $Mdmx^{G57A/F488A}$ was further decreased by Nutlin treatment (Figure 6e, compare lanes 6 and 8), indicating that the elevated level of stabilized Mdm2 is a significant determinant of p53 activity following induction of $Mdmx^{G57A/F488A}$. Together, these data suggest inhibition of Mdm2 ligase activity may be most effective when combined with either Mdm2 or Mdmx antagonists.

DISCUSSION

The functional co-operation between Mdm2 and Mdmx may be a critical component for regulating p53 levels in normal cells.

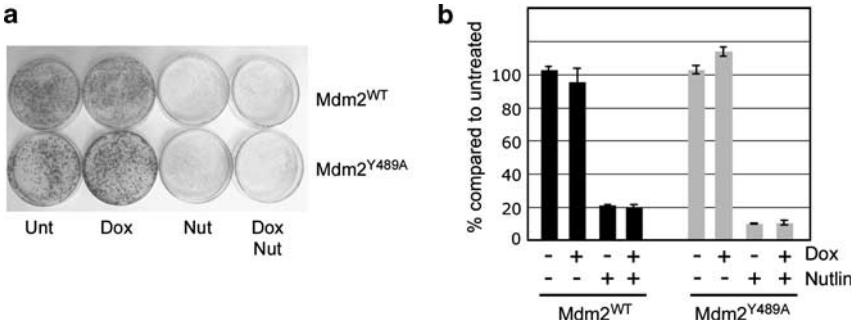

Figure 5. Mdm2^{Y489A} antagonizes p53-dependent transactivation and promotes cell survival. (**a**) Mdm2^{WT} or Mdm2^{Y489A} cells were seeded into 6 cm plates and colonies were allowed to form. Cells were then treated with 100 ng/ml Dox for 16 h before addition of Nutlin for a further 48 h. Drug was then replaced with regular medium. Following a further 6 days, cells were fixed and stained with crystal violet. (**b**) Following extraction with Sorenson's buffer (50 mM sodium citrate, 50 mM citric acid in 50% (v/v) ethanol solution), total crystal violet stain was quantified by measuring absorbance at 590 nm.

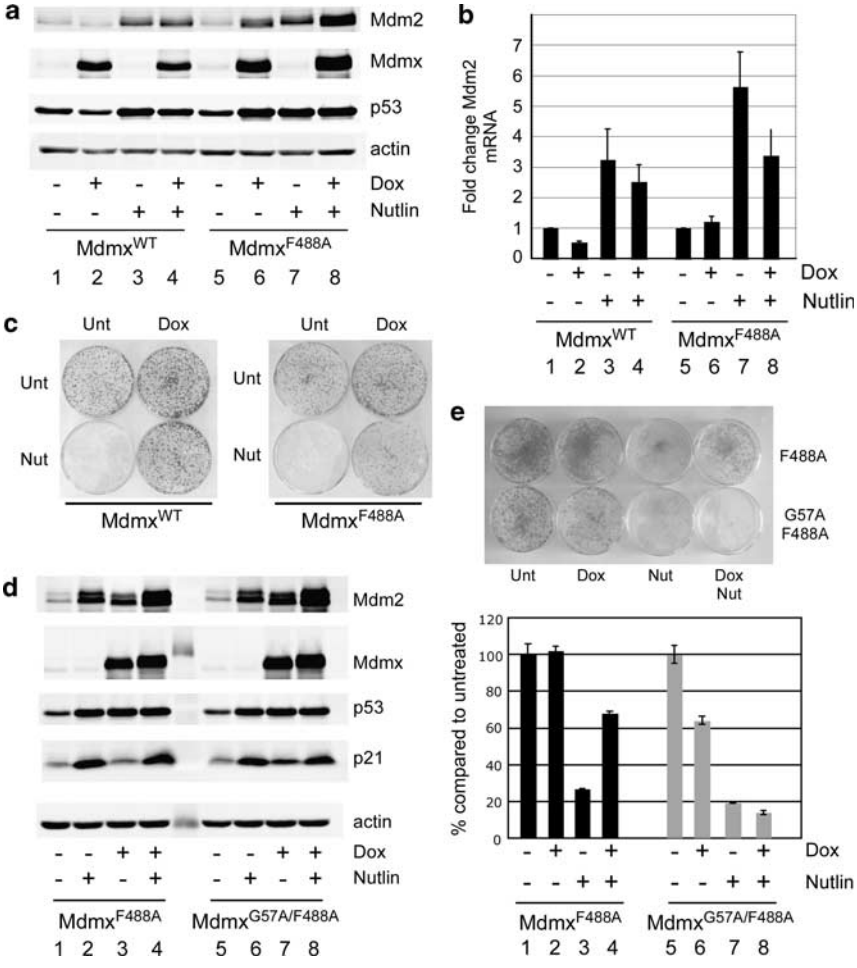

Figure 6. Mdmx^{F488A} antagonizes p53-dependent transactivation and promotes cell survival. (**a**) Following induction of Mdmx^{WT} or Mdmx^{F488A} for 19 h, cells were treated with 10 μM Nutlin for 24 h and analyzed for the indicated proteins. (**b**) Cells treated as in (**a**) were analyzed for *Mdm2* mRNA by quantitative PCR. Results are from three replicate experiments. (**c**) Mdmx^{WT} or Mdmx^{F488A} cells were seeded into 6 cm plates and colonies were allowed to form. Cells were then treated as in Figures 5a and b. (**d**) Mdmx^{F488A} and Mdmx$^{G57A/F488A}$ cells were treated and processed as in (**a**). (**e**) Mdmx^{F488A} and Mdmx$^{G57A/F488A}$ cells were treated and processed as in Figure 5b.

Therefore, understanding the mechanistic basis for this may aid rational design of Mdm2/Mdmx-targeted therapeutics. Mdm2/Mdmx binding proceeds via RING–RING domain interaction, which is utilized by a large number of other RING domain-containing human proteins. Thus, findings from studies of Mdm2 and Mdmx may provide greater insight into molecular mechanisms in other RING E3 ligase-associated pathways. In this study, we provide further insight into the regulation of p53 by Mdm2 and

Mdmx, and show that the co-operation between the two proteins is critical for p53 abundance control. Additionally, our data suggest that correct Mdm2/Mdmx hetero-oligomerization is critical for degradation of Mdmx following DNA damage. These findings indicate that regulating Mdm2/Mdmx hetero-oligomerization is likely to be a key determinant of p53-dependent responses to genotoxic stress.

Previous studies indicated that $Mdmx^{F488A}$ inhibits Mdm2-dependent degradation of p53.[17] We confirmed and extended these observations by showing that expression of $Mdmx^{F488A}$ leads to stabilization of non-ubiquitylated, endogenous p53. RING E3 ubiquitin ligases (including Mdm2) bind to E2s, and facilitate the transfer of ubiquitin to their target substrates (in this case, p53). As we show the interaction between Mdm2 and p53 is not perturbed in the presence of $Mdmx^{F488A}$, the most likely explanation is that transfer of ubiquitin to p53 is being inhibited. As structural studies suggest the $Mdmx^{F488A}$ mutation perturbs the function of the Mdm2/Mdmx heterodimer,[19] we infer this mutation may prevent the recruitment of E2 to the complex. Alternatively, E2 may be recruited, but unable to transfer its ubiquitin cargo to p53. This would be consistent with proposals that RING E3 ligases can allosterically activate E2 to facilitate ubiquitin transfer.[30,31] As $Mdmx^{F488A}$ can be detected in complexes containing both p53 and Mdm2, we suggest that $Mdmx^{F488A}$ induction is effectively a 'substrate trap', and may facilitate detection of intermediates in the degradation of p53. We also show that optimal Mdmx degradation after DNA damage requires an intact Mdmx extreme C-terminus. As damage-induced phosphorylation of $Mdmx^{F488A}$ and its binding to Mdm2 are unperturbed, the failure to degrade Mdmx is likely due to defects in Mdm2-dependent ubiquitylation. Recent studies have shown that following DNA damage, degradation of Mdmx is associated with an increase in p53 transactivation function.[15,32] In this study, p53 activation was equivalent in cells expressing both $Mdmx^{WT}$ and $Mdmx^{F488A}$, although the latter protein was not degraded after damage. It is likely that additional damage-induced modifications to p53 itself, and possibly to the RING domains of both Mdm2 and Mdmx are responsible for this effect.[33,34] Together with the finding that phosphorylation of the Mdm2 and Mdmx N-termini also contribute to p53 activation,[35,36] these data highlight the redundancy built into damage signaling pathways to ensure a 'failsafe' p53 response to genotoxic stress.

Earlier studies provided compelling evidence for the functional co-operation between Mdm2 and Mdmx.[16–19,37,38] Modeling this molecular interaction *in vivo* is beginning to provide further insight into its biological importance. For example, a point mutation of the Mdm2 RING domain that destroys RING structure (C462A) and blocks Mdm2 ligase activity triggers p53 stabilization and leads to p53-dependent embryonic lethality.[39] Additionally, mutation or deletion of the Mdmx RING also prevents Mdm2–Mdmx interaction and triggers p53-dependent lethality *in vivo*.[40,41] In these systems, Mdm2 remains unstable, likely explaining the inability to control p53 activity under these conditions. Therefore, it is still unclear whether complete inhibition of Mdm2 ligase activity, in the context of structurally intact Mdm2/Mdmx RING domains, would be sufficient to activate p53. Our *in vitro* data suggest that a high level of structurally intact but ligase-deficient Mdm2 can still block p53 activity. Whether this is recapitulated at physiological levels *in vivo* remains an open question. An *in vivo* study of $Mdmx^{F488A}$ and $Mdm2^{Y489A}$,[17] would also provide a direct test of whether ligase-independent RING domain activities of Mdm2 contribute to its biological function.

Our results also have implications for the design of drugs that block Mdm2 ligase activity by inhibiting the function of the Mdm2/Mdmx hetero-oligomer. We show here that perturbing the function of the hetero-oligomer increases p53 levels, suggesting ligase inhibition would be a viable anticancer strategy. However, in our system, p53 stabilization was not sufficient to trigger its activation, because it was antagonized by stabilized Mdm2 or high levels of exogenous Mdmx. Thus, we infer that increasing p53 level by blocking Mdm2 ligase activity may not be sufficient to trigger a robust p53 response. Previous studies lead to the discovery of the HLI series of compounds, which block Mdm2 ligase activity and lead to p53 stabilization.[25,42] In contrast to our results, treatment with HLI compounds also activated p53-dependent transcription. One explanation for this discrepancy is that the levels of exogenous Mdm2 or Mdmx used in our system exceed those present following treatment of tumor cells with HLI compounds. However, our results with $Mdmx^{G57A/F488A}$ show the increase in *endogenous* Mdm2 following Mdm2 ligase inhibition can also block p53-dependent transactivation. We suggest, therefore that there are cases in which Mdm2 ligase inhibition alone may not be sufficient to trigger p53 activation. In such instances, either treatment with antagonists such as Nutlin as single agents, or in combination with ligase inhibitors may be more effective.

MATERIALS AND METHODS

Cell culture and drug treatments

MCF7 cells were cultured in Dulbecco's modified Eagle's medium/10% fetal bovine serum with Ciprofloxacin (10 μg/ml). U2OS cells with Dox-inducible human Mdmx expression cassettes were generated as previously described[43] and cultured in Dulbecco's modified Eagle's medium/10% fetal bovine serum/Ciprofloxacin/G418 (400 μg/ml). Dox (SIGMA, St Louis, MO, USA) was dissolved in water as stocks of 500 μg/ml and frozen at −20 °C. Neocarzinostatin (stock 1 mg/ml in sodium acetate) was obtained from Robert Schultz at NCI, the proteasome inhibitor MG132 was from Calbiochem (La Jolla, CA, USA) and Nutlin-3a was a kind gift of Lyubomir Vassilev (Hoffman-La Roche, Nutley, NJ, USA). Unless otherwise stated, Nutlin-3a was used at a final concentration of 10 μM. Cycloheximide (US Biological, Salem, MA, USA) was dissolved in 0.1% ethanol and used at a final concentration of 100 μg/ml.

Western blotting, immunoprecipitation and antibodies

Cells were lyzed in Oren Buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 mM NaF and Complete Mini Protease Inhibitors (Roche, Nutley, NJ, USA)), at 4 °C for 30 min. Following sodium dodecyl sulfate–polyacrylamide gel electrophoresis, proteins were transferred to polyvinylidine fluoride membrane (Millipore, Billerica, MA, USA). The following antibodies were used for western blotting: p53, DO1 (Calbiochem, mouse, 1:1000) or FL-393 (Santa Cruz, Santa Cruz, CA, USA, rabbit, 1:1500); Mdmx, BL1258 (Bethyl Laboratories, Montgomery, TX, USA, 1:10 000, overnight); Mdm2, triple mouse monoclonal cocktail of IF-2 (Calbiochem), SMP-14 (Santa Cruz) and 4B2 (Calbiochem) (1:500 each, overnight); p21, C-19 (Santa Cruz, rabbit 1:1500 or CIP/Waf1 (Transduction Labs, Franklin Lakes, NJ, USA, mouse $IgG2_a$, 1:500); actin, (rabbit, 1:20 000, SIGMA). Peroxidase-conjugated secondary antibody was used at 1:10 000 (Jackson Immunochemicals, West Grove, PA, USA). For LiCor far-red western analysis (Li-Cor Biosciences, Lincoln, NE, USA), lysis was as above, but protein was transferred to Immobilon-Fluor (Millipore) before blocking with Bio-Rad (Hercules, CA, USA) phosphate-buffered saline/Casein Blocker per the manufacturer's instructions. Species-specific secondary antibodies for LiCor analysis were conjugate to Alexa Fluor 680 (Molecular Probes, Eugene, OR, USA) or IRD-800 (Rockland, PA, USA). Analysis was performed using Odyssey v3.0 software (Li-Cor Biosciences). For immunoprecipitation analyses, cells were harvested on the plate in Oren Buffer. Typically, 500 μg-1 mg of protein was used for immunoprecipitation. The antibodies used were: DO-1 or agarose bead-conjugated FL393 (p53), anti-HA (HA.11, Covance, Princeton, NJ, USA), BL-1258 (Human Mdmx, Bethyl Labs). In all, 2 μg of each antibody per milligram of protein was used. Immobilized recombinant protein A was used for antibody pulldown, and immunoprecipitates were washed and eluted as described.[44]

Proximity ligation *in situ* assay

U2OS cells expressing a Dox-inducible HA-HDMX construct[20] were seeded onto coverslips and treated with Dox for 24 h. Cells were fixed in

3.7% para-formaldehyde, washed in phosphate-buffered saline and permeabilized in 0.2% Triton X-100 for 5 min. Coverslips were then blocked in 10% normal goat serum in phosphate-buffered saline for 2 h. For Mdmx/p53 PLISA, primary antibodies HA.11 (Covance, 1:500) and FL-393 (Santa Cruz, 1:1000) were diluted in phosphate-buffered saline/EDTA/0.2% Triton X-100/2% normal goat serum and incubated at 4 °C overnight. For Mdmx/Mdm2 PLISA, primary antibodies BL-1258 (1:1000) and 5B10 (1:500) were used. Note that some background staining in the absence of Dox is observed using BL-1258, but the inducible nature of the system permitted clear distinction of Mdmx/Mdm2 complex formation. Following washes with TBS/0.05% Tween-20, a PLISA was performed according to the manufacturer's protocol (Detection Kit 613, OLink Bioscience, Uppsala, Sweden) with the following exception: goat anti-rabbit (minus) and anti-mouse (plus) PLISA probes were diluted in normal goat serum at 1:10 instead of 1:5. Coverslips were mounted on microscope slides and images acquired using OpenLab software (Perkin-Elmer/Improvision, Coventry, UK) and a Zeiss Axioplan 2 microscope with $\times 63$ magnification (Carl Zeiss Microscopy, Thornwood, NY, USA) Post-image capture, nuclear (Hoechst 33342 DNA stain) images were contrast-enhanced and all PLISA images (red foci) from untreated and Dox-treated cells were contrast-enhanced simultaneously to reduce background (non-focal) signals. RGB (red/green/blue) planes of nuclei and PLISA foci were then overlaid.

Ubiquitylation assay

U2OS were transfected with His-tagged ubiquitin using FuGene HD (Roche) at a 2:5 ratio (DNA to FuGeneHD). After 6 h, transfection reagent was replaced with regular medium in the presence or absence of Dox to induce $Mdmx^{WT}$ or $Mdmx^{F488A}$. After 24 h, cells were lysed, and pulldown of ubiquitylated proteins was performed using agarose-conjugated Ni^{2+} beads (Invitrogen, Carlsbad, CA, USA, licensed from Qiagen, Valencia, CA, USA). Lysis and wash conditions were as previously described.[45,46] Following sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transfer to polyvinylidine fluoride membrane, ubiquitylated p53 was visualized using DO-1 antibody.

Quantitative PCR

Total RNA was prepared using QiaShredder and RNeasy RNA isolation kits per the manufacturer's instructions (Qiagen). In all, 2 μg of total RNA per sample was used for complementary DNA synthesis with random hexamer primers, using SuperScriptIII Reverse Transcriptase system (Invitrogen). Real-time quantitative PCR was performed using an ABI PRISM 7700 Sequence Detection System, using Platinum SYBR SuperMix (Invitrogen) with ROX as an internal standard. Changes in gene expression were normalized to 18S mRNA. Primer sequences supplied on request.

Colony outgrowth

For colony formation assays, cells were plated at 3000/6 cm plate and colonies were allowed to form (~2 days) before addition of Dox for 24 h before treatment with 10 μM Nutlin (for 2 days). After a further 5–6 days, cells were fixed in 3.7% para-formaldehyde and stained with crystal violet. Images of colonies were taken on the plate, and then dye was extracted in Sorenson's buffer (50 mM sodium citrate, 50 mM citric acid in 50% (v/v) ethanol solution) and quantified by absorbance at 590 nm.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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