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Phosphorylation and Degradation of MdmX is Inhibited by Wip1 Phosphatase in the DNA Damage Response

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Abstract

MdmX and Mdm2 regulate p53 tumor suppressor functions by controlling p53 transcriptional activity and/or stability in cells exposed to DNA damage. Accumulating evidence indicates that ATM-mediated phosphorylation and degradation of Mdm2 and MdmX may be the initial driving force that induces p53 activity during the early phase of the DNA damage response. We have recently determined that a novel protein phosphatase, Wip1 (or PPM1D), contributes to p53 regulation by dephosphorylating Mdm2 to close the p53 activation loop initiated by the ATM/ATR kinases. In the present study, we determine that Wip1 directly dephosphorylates MdmX at the ATM-targeted Ser403 and indirectly suppresses phosphorylation of MdmX at Ser342 and Ser367. Wip1 inhibits the DNA damage-induced ubiquitination and degradation of MdmX, leading to the stabilization of MdmX and reduction of p53 activities. Our data suggest that Wip1 is an important component in the ATM-p53-MdmX regulatory loop.

Keywords

Wip1; MdmX; Stabilization; Dephosphorylation

Introduction

The wildtype p53-induced phosphatase 1, Wip1 (or PPM1D), is a member of the type 2C serine/threonine phosphatases (PP2C delta). Wip1 has oncogenic activities, and can cooperate with other known oncogenes to induce transformation of rodent primary fibroblasts(1;2). Furthermore, the *Wip1* gene is present in amplified copy numbers and is overexpressed in many human cancer types, including breast carcinomas, ovarian clear cell adenocarcinomas, neuroblastomas, pancreatic adenocarcinomas, gastric carcinomas, and medulloblastomas(1; 3-8). In rodent primary fibroblast transformation assays, Wip1 cooperates with known oncogenes to induce transformation foci(2). Recent identification of Wip1 targets has provided mechanistic insights into its oncogenic functions. Wip1 acts as a homeostatic regulator of the DNA damage response by dephosphorylating ATM/ATR target proteins. Three of the Wip1

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targets now identified are kinases that phosphorylate and activate p53 (Chk1, Chk2, and p38 MAPK)(9-11). In addition, Wip1 targets p53 itself at Ser15, implicating Wip1 as a major inhibitor of p53 function(11). Studies on *Wip1* null mouse embryonic fibroblasts (MEFs) corroborate this Wip1 inhibitory function. MEFs lacking *Wip1* displayed reduced proliferation, enhanced p53 transcriptional activity, and an enhanced DNA damage- induced G1 checkpoint (12).

Negative regulation of p53 is manifested chiefly through Mdm2-mediated p53 ubiquitination and proteasomal degradation. Interestingly, p53 not only transcriptionally regulates genes involved in cell cycle arrest or apoptosis, but also induces expression of its negative regulator, Mdm2. Thus, p53 and Mdm2 participate in an auto-regulatory feedback loop(13). MdmX (or Mdm4) was identified as a p53-binding protein that was related to Mdm2, but lacked ubiquitin-ligase function. Similar to Mdm2, MdmX deficiency in mice causes early embryonic lethality rescued by p53 loss(14;15). Thus, MdmX and Mdm2 have non-redundant roles in the regulation of p53, and recent *in vitro* and *in vivo* studies have suggested that Mdm2 controls p53 transcriptional activity by regulating p53 protein stability, whereas MdmX functions as a p53 transcriptional inhibitor without altering p53 levels(13).

Recent results from our laboratory showed that Wip1 interacts with and dephosphorylates Mdm2 at serine 395, a site phosphorylated by the ATM kinase(16). Dephosphorylated Mdm2 has increased stability and affinity for p53, facilitating p53 ubiquitination and degradation. Thus, Wip1 may act as a gatekeeper in the Mdm2-p53 regulatory loop by stabilizing Mdm2 and promoting Mdm2-mediated proteolysis of p53(17). Several groups reported that MdmX is also phosphorylated and destabilized in response to DNA damage stress. Three phosphorylation sites identified are Ser342, Ser367 and Ser403(18-20). While Ser403 is directly phosphorylated by ATM, the other two sites are phosphorylated by Chk1 and Chk2, two kinases that are well-established cell cycle regulators known to be activated by ATM/ATR (18-21). Here, we present evidence that Wip1 specifically dephosphorylates MdmX at Ser403 and indirectly suppresses phosphorylation of MdmX at Ser342 and Ser376. Wip1 increases the stability of MdmX and extends its half-life. Our results suggested that Wip1 suppression of p53 signaling by augmenting the stability of MdmX may be an important component of its oncogenicity.

Materials and Methods

Cell lines and cell culture

U2OS (p53 wildtype) cell line is a human osteosarcoma line that was obtained from the American Type Culture Collection. Primary *p53*-/-, *p53*-/-*Mdm2*-/- and *p53*-/-*MdmX*-/- mouse embryonic fibroblasts were harvested and cultured as previously described(12;22;23). The A-T cell line GM9067 was obtained from the Coriell Cell Repositories. To obtain cells stably expressing MdmX, expression vector expressing HA-tagged wildtype or mutant human MdmX was transfected into U2OS cells and stable cells were selected with 1000 μg/mL G-418. Positive colonies were isolated, amplified, and checked for expression of HA-MdmX protein individually.

Plasmid Constructs

Expression vectors expressing mouse and human Wip1 used in these experiments have been previously described(24). The p21-Luciferase construct was obtained from Dr. G. Lozano. The vectors expressing wildtype and mutant MdmX were previously described(20). Wip1 shRNA expression vector (#RHS3979-9571552) was purchased from Openbiosystems.

Cell Transfection with plasmid DNA

Cells were transfected with plasmid DNA by Lipofectamine and Plus reagents (for transformed cell lines, Invitrogen, catalog # 18324-012 and #11514-015) or Lipofectamine 2000 reagent (for mouse embryonic fibroblasts, Invitrogen, catalog # 11668-019). Transfection experiments were performed following the instruction manuals provided with reagents.

Luciferase Assays

Cells were cotransfected with a p53 expression vector, p21-Luciferease-, Renilla luciferase-, and Wip1 expression vector. Cells were harvested and lysed. Luciferase activity was measured using a Turner 20/20n luminometer and normalized to Renilla luciferase and cell number according to the instructions provided with the dual-luciferase assay kit (Promega).

Western Blot Analysis, Antibodies, and Purified Proteins

Immunoprecipitations, Western blot analysis, and immunoprecipitation-Western blot analyses were performed by standard methods described previously(11). Anti-actin (#1616), anti-ubiquitin (#8017), HRP-anti-goat IgG (#2020), HRP-anti-rabbit IgG (#2302), and HRP-anti-mouse IgG (#2302) were purchased from Santa Cruz; Anti-HA (A190-108A) and anti-MdmX (#A300-287A from Bethyl Laboratories for human MdmX and #M0445 from Sigma-Aldrich for mouse MdmX) were purchased from Bethyl Laboratories; Anti-Wip1 (#AP-8437b) was purchased from Abgent; anti-MdmX (pS403), anti-MdmX (pS367) and anti-MdmX (pS342) were generated and described previously(18;20).

Protein stability measurement

Cell stably expressing wildtype or mutant HA-MdmX were transfected with Wip1 or control expression vector DNA. 24 h after transfection, cells were treated with 500 ng/mL NCS and 50 μg/mL cyclohexamide. Cells were harvested at indicated time points after treatment, and cell lysates were immunoblotted with anti-HA antibody. Levels of MdmX at each time point were quantitated by phosphorimager for MdmX bands on immunoblots.

In vitro phosphatase assays

The in vitro phosphatase assays and control peptide sequences have been described previously (24). MdmX phosphopeptides were custom synthesized by New England Peptide. Their sequences are: MdmX(pS342): Ac-LTHSLpSTSDIT-amide; MdmX(pS367): Ac-CRRTIpSAPVVR-amide; MdmX(pS403): Ac-AHSSEpSQETIS-amide.

Ubiquitination assays

Cells were treated with proteasomal inhibitors MG101 (25 μmol/L) and MG132 (25 μmol/mL). Six hours after treatment, cells were harvested and lysed in the lysis buffer (20mmol/L HEPES, pH7.4, 240 mmol/L NaCl, 0.1 mmol/L EDTA, 0.5% TritonX-100, 1mmol/L PMSF, 1mmol/L DTT and Complete Mini protease inhibitor tablet from Roche). Ubiquitinated MdmX were immunoprecipitated with anti-HA antibodies, and then were Western blot analyzed with anti-ubiquitin antibody.

Results

Wip1 affects the DNA damage-induced phosphorylation of MdmX

Recent studies have reported that MdmX is destabilized by the DNA damage-induced phosphorylation(18;20;21). To determine if phosphorylation and destabilization of MdmX is reversed by protein phosphatases, we generated a library of mammalian expression vectors encoding 23 protein serine/threonine phosphatases (or catalytic subunits if multimeric)

including 11 PPPs and 12 PPMs(25). Using this library, we screened for protein phosphatases that increase MdmX levels in human osteosarcoma U2OS cells treated with DNA damaging agent, neocarzinostatin (NCS). 5 out of 23 phosphatases were shown to increase MdmX protein levels in the DNA damage response, one of which was the Wip1 phosphatase (Fig. 1A). We then examined the phosphorylation of MdmX in U2OS cells with overexpression or knockdown of Wip1. Levels of global phosphorylated MdmX were increased in cells treated with NCS, consistent with previous reports(18). In similar amounts of total MdmX, overexpression of Wip1 significantly reduced phosphorylation of MdmX, while reducing endogenous Wip1 levels increased MdmX phosphorylation (Fig. 1B). The interaction between Wip1 and MdmX was detected in the reciprocal immunoprecipitation western blot analyses. The results showed that endogenous Wip1 binds to endogenous MdmX (left panels, Fig. 1C). To date, three DNA damage-induced phosphorylation sites on MdmX have been identified: Ser342, Ser367, and Ser403(18-20). We further examined whether phosphorylation of MdmX contributes to the MdmX-Wip1 interaction. A mutant form of MdmX with all of the three phosphorylation sites mutated to alanines retained the same level of Wip1-binding capacity as wildtype MdmX, suggesting that phosphorylation is not essential for MdmX's binding to Wip1 (right panel, Fig. 1C). We also tested whether Wip1 affects the phosphorylation of each serine site by using phospho-specific antibodies and cell lysates containing similar amounts of MdmX. Phosphorylation of all three sites was remarkably inhibited by Wip1 overexpression, suggesting that Wip1 may be responsible for the dephosphorylation of MdmX (Fig. 1D, left panel). To determine whether Wip1 directly dephosphorylates MdmX, we performed *in vitro* phosphatase assays by incubating purified Wip1 proteins with MdmX-derived phosphopeptides. The MdmX Ser403 phosphopeptide was dephosphorylated by Wip1 and the Wip1 activity on this phosphopeptide was magnesium dependent and okadaic acid insensitive, consistent with the known properties of the type 2C phosphatase (Fig. 1D, right panel). Although Wip1 inhibited the phosphorylation of MdmX at Ser342 and Ser367 *in vivo*, the MdmX peptides containing pSer342 or pSer367 were not dephosphorylated by Wip1 in the phosphatase assays, indicating that Wip1 regulates these two phosphorylation sites indirectly.

Wip1 augments MdmX stability after DNA damage

MdmX stability is regulated by ubiquitination and proteasomal degradation(26). We tested whether Wip1 regulates MdmX levels in cells in the presence of DNA damage stress. ATM-mediated phosphorylation of MdmX at Ser403 was increased immediately after NCS treatment, and levels of MdmX were reduced simultaneously in cells. Wip1 overexpression attenuated MdmX phosphorylation and increased MdmX levels, while knockdown of Wip1 by its shRNA enhanced MdmX phosphorylation and accelerated MdmX degradation (Fig. 2A). Wip1-mediated stabilization of MdmX was further confirmed in primary *Wip1*+/+ and *Wip1*-/- mouse embryonic fibroblasts (MEFs) harvested from littermate embryos. MdmX was remarkably more unstable in *Wip1*-/- MEFs than in *Wip1*+/+ MEFs in the presence of NCS treatment. Inhibiting ATM and ATR kinases by CGK733 dramatically enhanced the stability of MdmX in *Wip1*+/+ MEFs, but not in *Wip1*-/- MEFs, suggesting the regulation of MdmX by Wip1 is dependent on the kinase activity of ATM/ATR (Fig. 2B). We then measured the half-life of endogenous MdmX with or without overexpressed Wip1. The half-life of MdmX in unstressed cells was as long as 4.4 h, which was dramatically reduced to 24 min in NCS-treated cells. Altered Wip1 levels had no significant effects on the stability of MdmX in the absence of DNA damage stress. However, overexpressed Wip1 extended the half-life of MdmX to 51 min in the stressed cells, while knockdown of Wip1 further decreased MdmX stability with the half-life about 12 min (Fig. 2C & 2D). These results showed that Wip1 stabilizes MdmX in the DNA damage response.

We next tested whether Wip1 affects the ubiquitination of MdmX. To this end, we generated U2OS cell lines that stably express HA-tagged wildtype or mutant MdmX, of which one or

more of the DNA damage targeted serines are mutated into alanines, mimicking non-phosphorylated forms of MdmX. We examined the effects of altered Wip1 levels on MdmX ubiquitination. In cells expressing wildtype MdmX, overexpression of Wip1 lowered MdmX ubiquitination while silencing Wip1 by shRNA enhanced MdmX ubiquitination compared to control cells. Ubiquitination of MdmX(S403A) was also decreased by Wip1, but the effect of Wip1 was significantly diminished compared to that on the wildtype MdmX, suggesting that Ser403 is one of the major phosphorylation sites regulated by Wip1. As expected, much lower levels of ubiquitination were observed on the triple phosphorylation mutant MdmX (S342/367/403A). Altered levels of Wip1 had no effects on the ubiquitination of this mutant MdmX (Fig. 3A & 3B).

Wip1 stabilization of MdmX is dependent on the phosphorylation of MdmX

We further examined to what extent Wip1 promotes the stability of MdmX. We assessed the half-lives of wildtype and mutant forms of MdmX that were stably expressed as HA-tagged proteins in U2OS cells (Fig. 4A & 4B). The half-life of wildtype HA-MdmX was about 26 min and was extended to 53 min in the presence of overexpressed Wip1, which is consistent with the half-life of endogenous MdmX (Fig. 2D). MdmX(S367A) and MdmX(S403A) both had prolonged half-lives, of about 51 min and 41.5 min respectively, which is likely due to their partial defective phosphorylation state in the DNA damage response. Overexpressed Wip1 augmented the stability of mutant MdmX to a lesser extent compared to that of wildtype MdmX. In the presence of overexpressed Wip1, the half-lives of MdmX(S367A) and MdmX (S403A) were increased only by 26.5% and 44.6% compared to the control, in contrast to 104% of increase observed in the half-life of wildtype MdmX. Interestingly, if all of the three phosphorylation sites were mutated to alanine in MdmX(S342/376/403A), MdmX became extremely stable with a half-life about 80 min. Wip1 had no significant effects on the stability of the triple mutant MdmX, indicating that these sites are probably the major Wip1 regulated phosphorylation sites on MdmX. These results are also consistent with the ubiquitination assays (Fig. 3).

Wip1 was shown to inactivate several key kinases in DNA damage signaling pathway, including p38MAPK, Chk1 and Chk2(9-11). Since the phosphorylation of Ser342, Ser367 and Ser403 is mediated by ATM or its downstream targets – Chk1 and Chk2, we hypothesized that Wip1 regulates MdmX degradation in an ATM-dependent manner. An ATM-deficient human fibroblast cell line (GM9607) was used to determine whether increased levels of Wip1 promote MdmX accumulation in the absence of functional ATM signaling (Fig. 4B). MdmX remained relatively stable in NCS-treated GM9607 cells regardless of Wip1 levels. No significant reduction on the MdmX levels was observed during 4 h post-damage. When ATM was reintroduced into these cells, MdmX became more unstable following NCS treatment. Addition of Wip1 helped to stabilize MdmX in the ATM-containing cells, indicating that Wip1 stabilizes MdmX by reversing the destabilizing effects of ATM-mediated phosphorylation. It should be noted that overexpression of Wip1 slightly increased the levels of MdmX even in GM9607 cells (in particular, at times 0 and 1 h post treatment). Although ATM plays a primary role in the DNA damage response to double-stranded DNA breaks, ATR also contributes to the process by activating some of the common ATM/ATR targets(27;28). In ATM-deficient cells, Wip1 may affect the stability of MdmX by inhibiting these ATR targets, such as Chk1 and Chk2.

MdmX and Mdm2 are promptly destabilized immediately in DNA damage stressed cells, resulting in a rapid induction of p53(18). In Fig. 4C, the levels of Mdm2 and MdmX in U2OS cells bottomed at 1 h and 4 h after 5 Gy of ionizing radiation respectively, while the levels of Wip1 appears to be induced in a delayed pattern. Its level only has a mild increase 2h after ionizing radiation, and then increases gradually to the peak around 8-12 h post damage. This

pattern is consistent with the role of Wip1 as a homeostatic regulator in stressed cells. As expected, overexpression of Wip1 disturbed this homeostatic regulation, resulting in a high stability of MdmX at the early stage of DNA damage response (0 – 8 h).

Wip1 inhibition of p53 is dependent on MdmX and Mdm2

Previous study showed that Mdm2 is a critical E3 ubiquitin ligase that targets MdmX for degradation in cells(29). Here, we attempted to examine whether Wip1 regulates the stability of MdmX in an Mdm2- dependent manner. First, we examined the stability of MdmX in the presence or absence of Mdm2. In cyclohexamide-treated *p53*-/-*Mdm2*+/+ MEFs, MdmX showed a significantly higher stability in Wip1-overexpressing cells than in control cells. In contrast, MdmX was extremely stable in both of the control and Wip1-overexpressing *p53*-/-*Mdm2*-/- cells, suggesting the effect of Wip1 on MdmX may be mediated by Mdm2 in cells (Fig. 5A). We next tested whether Wip1 regulates the activity of p53 in the presence or absence of Mdm2 or MdmX. As both Mdm2 and MdmX knockout mice are embryonic lethal in the presence of wildtype p53(15;22;30), we tested the Wip1 effects in *p53*-/-, *p53*-/-*Mdm2*-/-, *or p53*-/-*MdmX*-/- MEFs that all lacked functional p53. These MEFs were transfected with p53 and with or without Wip1. Levels of p53 and p53-induced p21 were measured 4 h post-NCS treatment. Consistent with previous reports(11;17), Wip1 overexpression inhibits the DNA damage-mediated induction of exogenous p53 activity in *p53*-/- MEFs. However, effects of Wip1 on p21 induction were remarkably reduced in *p53*-/-*MdmX*-/- MEFs and *p53*-/-*Mdm2*-/- MEFs. The results suggested that both MdmX and Mdm2 are important mediators for Wip1 in regulating the transcriptional activity of p53 (Fig. 5B). To confirm and better quantitate the effects of Wip1 on p53 induction, we performed a luciferase reporter assay in the above MEFs. Cells were transfected with p53 expression vector, p21-luciferase expression vector, and either control or Wip1- expression vector. Cell growth was not much influenced by Wip1 overexpression during the experimental period and the luciferase activity peaks around 18 h post-NCS treatment (Supplemental Fig. S1). The results showed that Wip1 negatively regulates p53 specifically in the DNA damage response (Fig. 5C). Nutlin-3 is a potent Mdm2 inhibitor that interferes with p53-Mdm2 interaction, and Nutlin-3 treatment of *p53*-/- cells profoundly induced the activity of exogenous p53. This increase in activity was not inhibited by overexpressed Wip1. Moreover, loss of Mdm2 also abolished Wip1's effects on p53 activity, as shown in *p53*-/-*Mdm2*-/- cells. These results confirmed that the effects of Wip1 on MdmX are at least partly dependent on Mdm2, suggesting that Wip1-mediated stabilization of MdmX is likely Mdm2-dependent. We further examined the effects of Wip1 in the *p53*-/-*MdmX*-/- cells. Wip1 overexpression suppressed p53 activity in the stressed cells even in the absence of MdmX, but the reduction of p53 activity in the *p53*-/-*MdmX*-/- cells was relatively modest (33%) in contrast with a more profound reduction (61%) observed in the *p53*-/- cells (Fig. 5C). The data indicated that Wip1 synergistically modulates both Mdm2 and MdmX in the p53 signaling pathway.

Effects of Wip1 on MdmX-USP7 interaction

Our previous studies have shown that Mdm2 is dephosphorylated and stabilized by Wip1 (17). Since Mdm2 appears to be the primary E3 ligase for MdmX degradation(29), it is presently unclear how Wip1 stabilizes both Mdm2 and MdmX during the DNA damage response. One hypothesis is that phosphorylation of MdmX changes its binding capacity to Mdm2 and USP7 (ubiquitin specific peptidase 7) that stabilizes MdmX by deubiquitination (31). Dephosphorylated MdmX appears to be a preferable substrate for USP7, but not for Mdm2. The hypothesis is supported by the binding assays shown in Fig 6. Phosphorylation defective mutants of MdmX (single-, double-, or triple- phosphorylation mutants) have reduced binding ability with Mdm2 but have enhanced binding activity with USP7 in the DNA damage stressed cells (Fig. 6A), suggesting that DNA damage-induced phosphorylation of MdmX may influence binding of MdmX to Mdm2 and USP7. We next assessed the effects of Wip1 on

these protein interactions using cell lysates containing similar amounts of MdmX. After DNA damage, the relative amount of MdmX bound to USP7 was reduced, but this reduction was offset by overexpressed Wip1. In contrast, the Mdm2-MdmX interaction was increased after DNA damage, but was inhibited by Wip1 overexpression (Fig. 6B). Further structural information for these interactions shall provide a better understanding of Wip1 functions.

Discussion

Wip1 amplification and overexpression have been identified in many types of human cancers (1;3-8). Interestingly, in cancers where Wip1 was examined, p53 mutation rates were invariably low and poorer prognoses were associated with Wip1 amplification. The infrequent nature of p53 mutations in tumors with Wip1 amplification suggests that Wip1 may promote human tumors through its ability to inhibit p53, circumventing selective pressure to mutate p53 during tumor progression. Wip1 amplification in this context is reminiscent of tumors with MdmX or Mdm2 amplification, as only a small subset of these tumors also exhibit p53 mutation (32). It was recently reported that the Wip1's negative feedback between p53 and ATM is essential for maintaining a functional DNA damage response in cells(33). Our present study reveals that Wip1 also modulates the p53 regulatory loop by regulating MdmX. DNA damage-induced phosphorylation of MdmX is inhibited by Wip1 in two ways: 1) ATM-phosphorylated Ser403 is directly dephosphorylated by Wip1; and 2) Wip1 deactivates Chk1/Chk2 by dephosphorylating their ATM-targeted sites and thus Chk1/Chk2-mediated phosphorylation of Ser342 and Ser367 on MdmX is reversed. Overexpressed Wip1 extends the half-life of wildtype MdmX. However, Wip1's effects were not observed with an MdmX protein of which all three phosphorylation sites were mutated, suggesting that dephosphorylation of these three sites has a synergistic effect on the stabilization of MdmX.

Given its critical role in the ATM -p53 signaling pathway, Wip1 is anticipated to be regulated in response to DNA damage. Wip1 is transactivated in a p53-dependent manner(34;35). In U2OS cells treated with ionizing radiation, both Mdm2 and MdmX are promptly destabilized after treatment (0-4 h), resulting in a rapid induction of p53. In turn, p53 induces Mdm2 by transactivation. Wip1 appears to be induced in a delayed pattern. Wip1 protein level only has a mild increase during the first two hours, and then increases gradually to peak at around 8-12 hr post IR (Fig. 5C). This pattern is consistent with the role of Wip1 as a homeostatic regulator in stressed cells. In normal cells, the base level of Wip1 is about 4-fold lower than its peak level. Thus, Wip1 may need to be inhibited in the early stage of DNA damage response to facilitate rapid MdmX degradation. We observed that Wip1 is also phosphorylated in an ATM-dependent manner immediately after DNA damage (data not shown). Post-translational modifications such as phosphorylation may modulate the activity of Wip1 or access to its targets. We propose that dephosphorylation of MdmX and Mdm2 by Wip1 increases their stability, leading to a reduction of p53 activity back to normal homeostatic levels after DNA damage is repaired (Fig. 6C).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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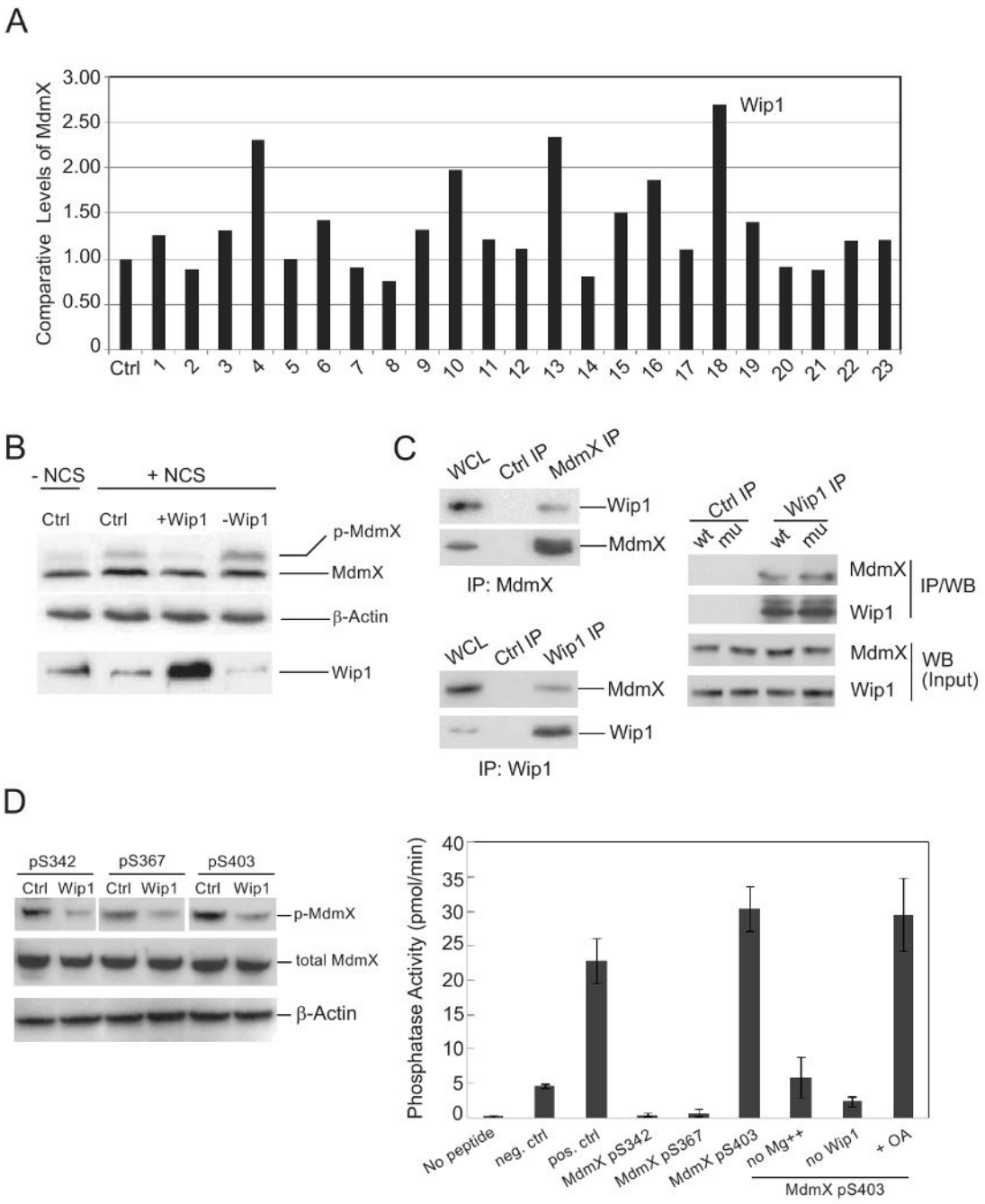

Figure 1.

Wip1 interacts with and dephosphorylates MdmX. (A) The effects of overexpressed Ser/Thr protein phosphatases on the levels of MdmX. U2OS cells were transfected with control or phosphatase expression vector, treated by NCS (500 ng/mL), and then harvested 2 h after treatment. Levels of MdmX were detected by immunoblotting and quantitated according to the intensity of MdmX bands. (B) Wip1 inhibits the phosphorylation of MdmX. U2OS cells were transfected with control or Wip1 expression vector. Cell lysates were harvested 2 h after NCS (500 ng/mL) and MG132 (25 mmol/L, proteasomal inhibitor) treatment. Protein levels were determined by immunoblotting. (C) Wip1 interacts with wildtype and mutant MdmX. Immunoprecipitates from U2OS cell lysates using control or anti-MdmX antibody were

analyzed by immunoblotting using anti-Wip1 antibody (upper left panel). A reciprocal experiment utilizing Wip1-containing immunoprecipitates confirmed the Wip1-MdmX interaction (lower left panel). U2OS cells were also transfected with vector DNA expressing wildtype or mutant MdmX with three phosphorylation sites (S342, S367, and S403) mutated to alanines (right panel). (D) Wip1 inhibits the phosphorylation of MdmX and directly dephosphorylates MdmX pSer403 *in vitro*. Phospho-specific MdmX antibodies were used to measure the effects of Wip1 on the phosphorylation of MdmX (left panel). Phosphopeptides from p38 MAP kinase (pT180, positive control), UNG2 (pT31, negative control), and MdmX (pS342, pS367, or pS403) were incubated with purified Wip1 proteins in *in vitro* phosphatase assays. Reactions on MdmX (pSer403) were also performed in the absence of magnesium or peptide, or in the presence of okadaic acid (right panel).

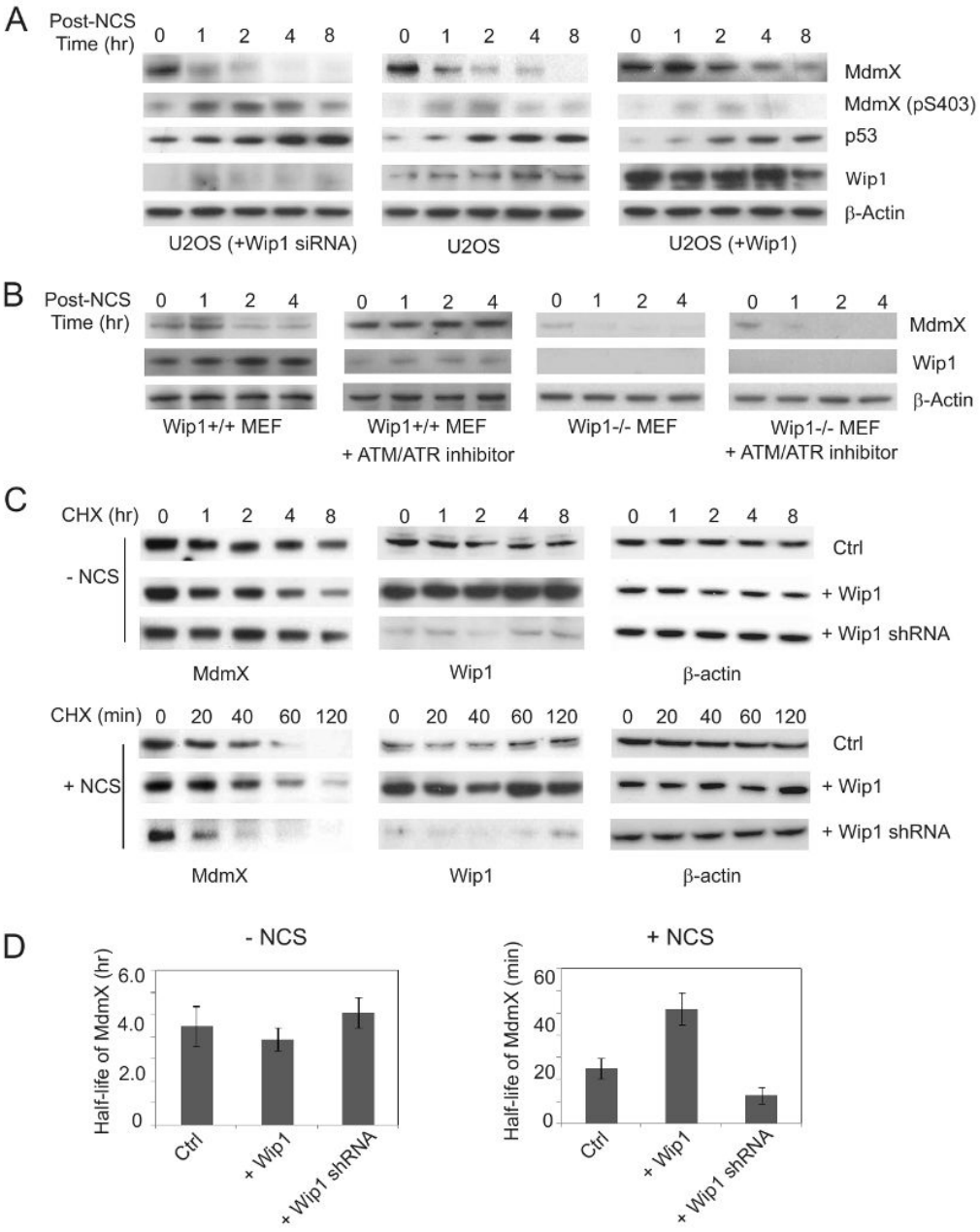

Figure 2.

Wip1 stabilizes MdmX in the DNA damage response. (A) MdmX is dephosphorylated and stabilized by Wip1. U2OS cells were transfected with Wip1, Wip1 shRNA, or control expression vector, and then treated with NCS (200 ng/mL). Protein levels were detected by immunoblotting at the indicated time point. (B) *Wip1*+/+ and *Wip1*-/- MEFs were treated with NCS (200 ng/mL) and with or without ATM/ATR inhibitor, CGK733 (4.5 μmol/L). Protein levels were detected by immunoblotting. (C) Overexpressed Wip1 stabilizes MdmX in the DNA damage response. U2OS cells were transfected with Wip1, Wip1 shRNA, or control expression vector. Cells were treated with cyclohexamide (CHX, 50 μg/mL)) to inhibit protein synthesis. Protein stability of MdmX was measured in cells treated with or without NCS (200ng/mL). (D) Levels of MdmX at each time point in the Fig. 2*C* were quantitated and half-life of MdmX was calculated (from two separate experiments).

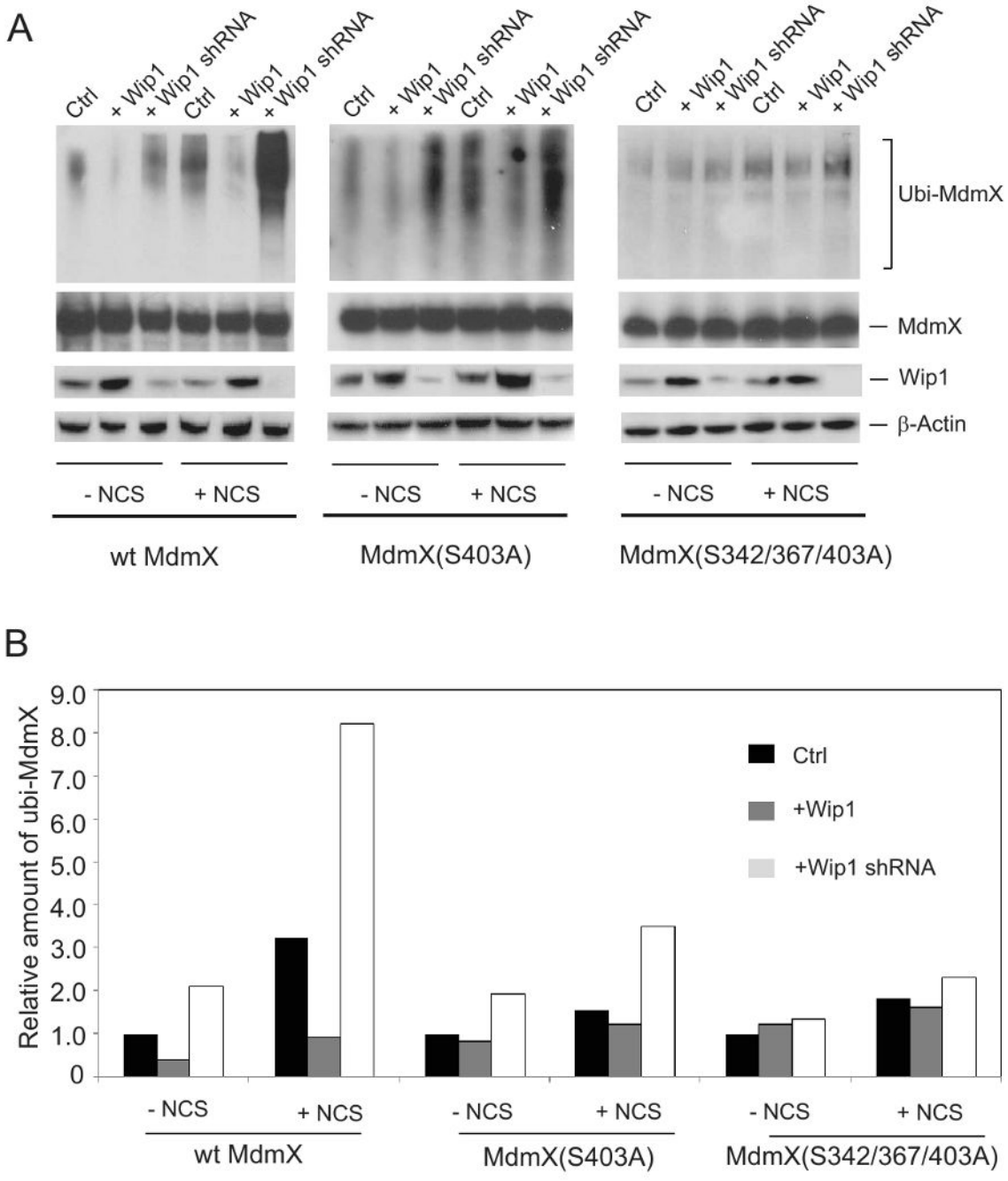

Figure 3.

Wip1 inhibits the ubiquitination of MdmX. (A) U2OS cells stably expressing HA-MdmX, HA-MdmX(S403A), or HA-MdmX(S342/367/403A) were transfected with Wip1, Wip1 shRNA, or control expression vector. Transfected cells were treated with NCS (200 ng/mL) and protease inhibitors MG132 and MG101, and harvested 4 h after NCS treatment. Cell lysates were immunoprecipitated with anti-HA antibody and immunoblotted by anti-ubiquitin antibody. (B) Levels of ubiquitinated MdmX under each condition in Fig. 3*A* were quantitated.

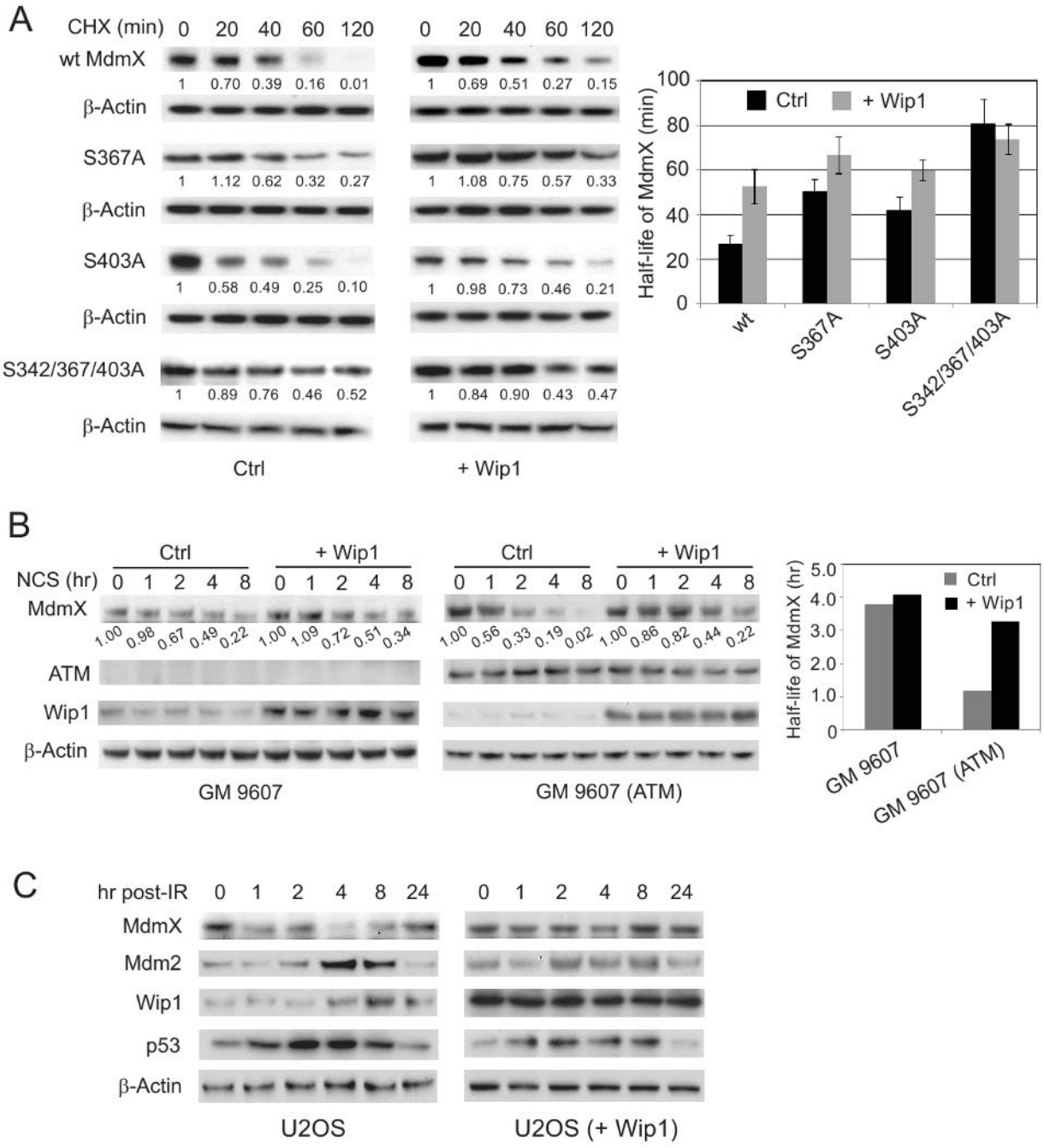

Figure 4.

Stabilization of MdmX by Wip1 is dependent on ATM-mediated phosphorylation. (A) Wip1 extends the half-lives of wildtype and mutant forms of MdmX. Protein levels of wildtype or mutant forms of MdmX were measured as described in Fig. 2C. Values below the MdmX blots indicate protein levels relative to the time zero point in each set (left panel). Half-lives of wildtype and mutant forms of MdmX were calculated (right panel). Error bars correspond to standard deviation of the mean. (B) Wip1 stabilizes MdmX in an ATM-dependent manner. A-T cells (GM9607) were transfected with control or Wip1 expression vector, together with ATM expression or control vector. Cells were then treated with NCS (200 ng/mL), harvested and immunoblotted as indicated. Levels of MdmX at each time point were quantitated. Values

below the MdmX blots indicate protein levels relative to the time zero point under each condition (left panels). Half-life of MdmX was determined for the graph (right panel). (C) Induction of Wip1 has a delayed onset compared to the destabilization of MdmX and Mdm2. U2OS cells were treated with 5 Gy of ionizing radiation and cell lysates were harvested and analyzed by immunoblotting.

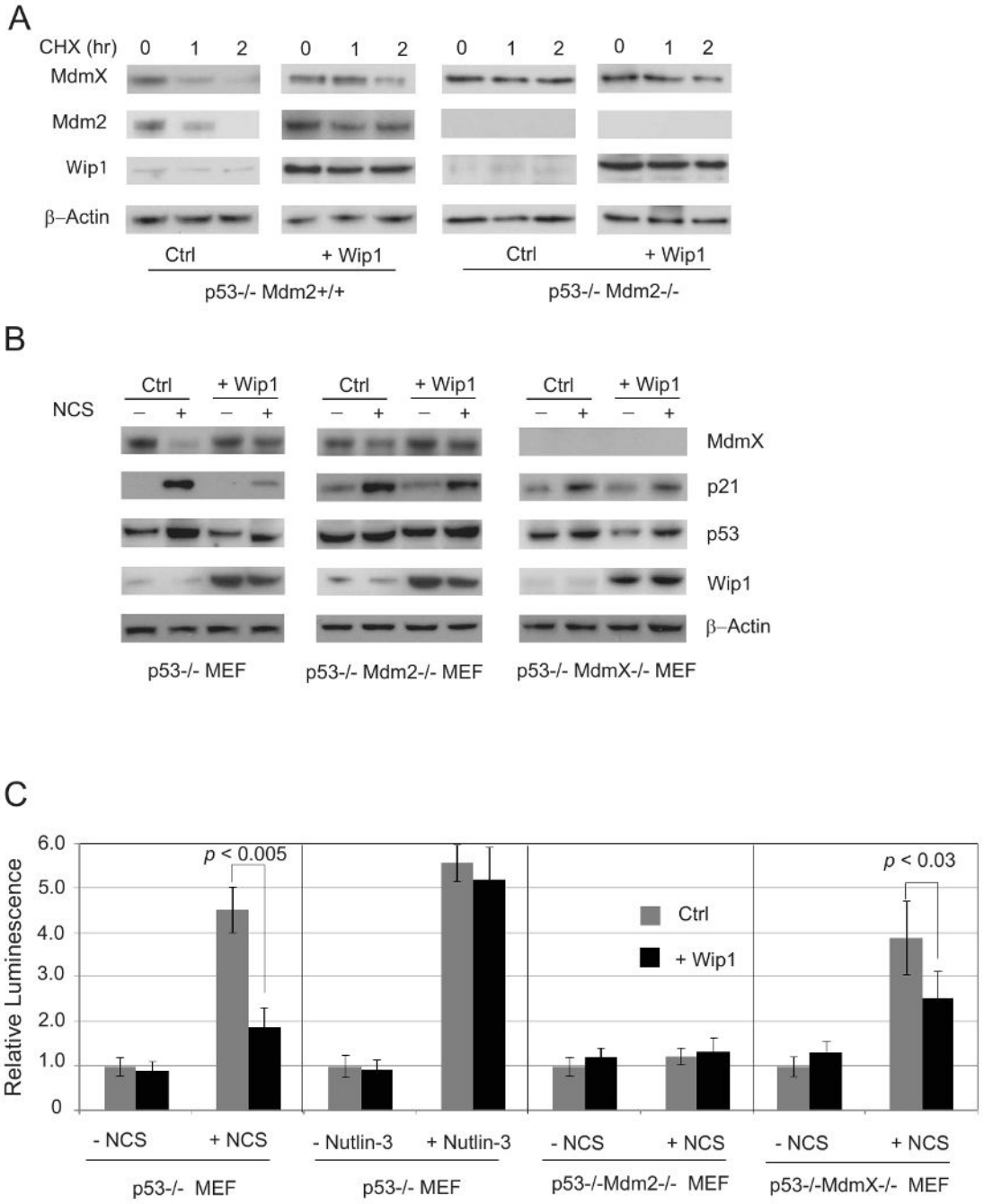

Figure 5.

Wip1 regulates the activity of p53 in an Mdm2- and MdmX- dependent manner. (A) Effect of Wip1 on MdmX is dependent on Mdm2. MEFs were transfected with control or Wip1 expression vector, treated with NCS (200 ng/mL) and cyclohexamide (100 μg/mL), harvested and immunoblotted as indicated. (B) p53 activity shown by p21 induction is inhibited by Wip1 in an Mdm2- and MdmX- dependent manner at the early stage of DNA damage response. MEFs were transfected with p53 expression vector, and control or Wip1 expression vector. 24 h after transfections, cells were treated with NCS (200 ng/mL), harvested 4 h after treatment and immunoblotted as indicated. (C) MEFs were transfected with p53 expression vector, p21-Luciferase expression vector, Renilla luciferase control vector, along with Wip1 or control

expression vector. Cells were treated with NCS (200 ng/mL) or Nutlin-3 (10 μmol/L) as indicated. 18 h after treatment, cells were assayed for relative luciferase activity (versus non-treated cells transfected with control vector DNA in each set) to assess the transcriptional activity of p53. Error bars correspond to standard deviation of the mean of three independent experiments. Two group comparisons were analyzed by Student's *t*-test.

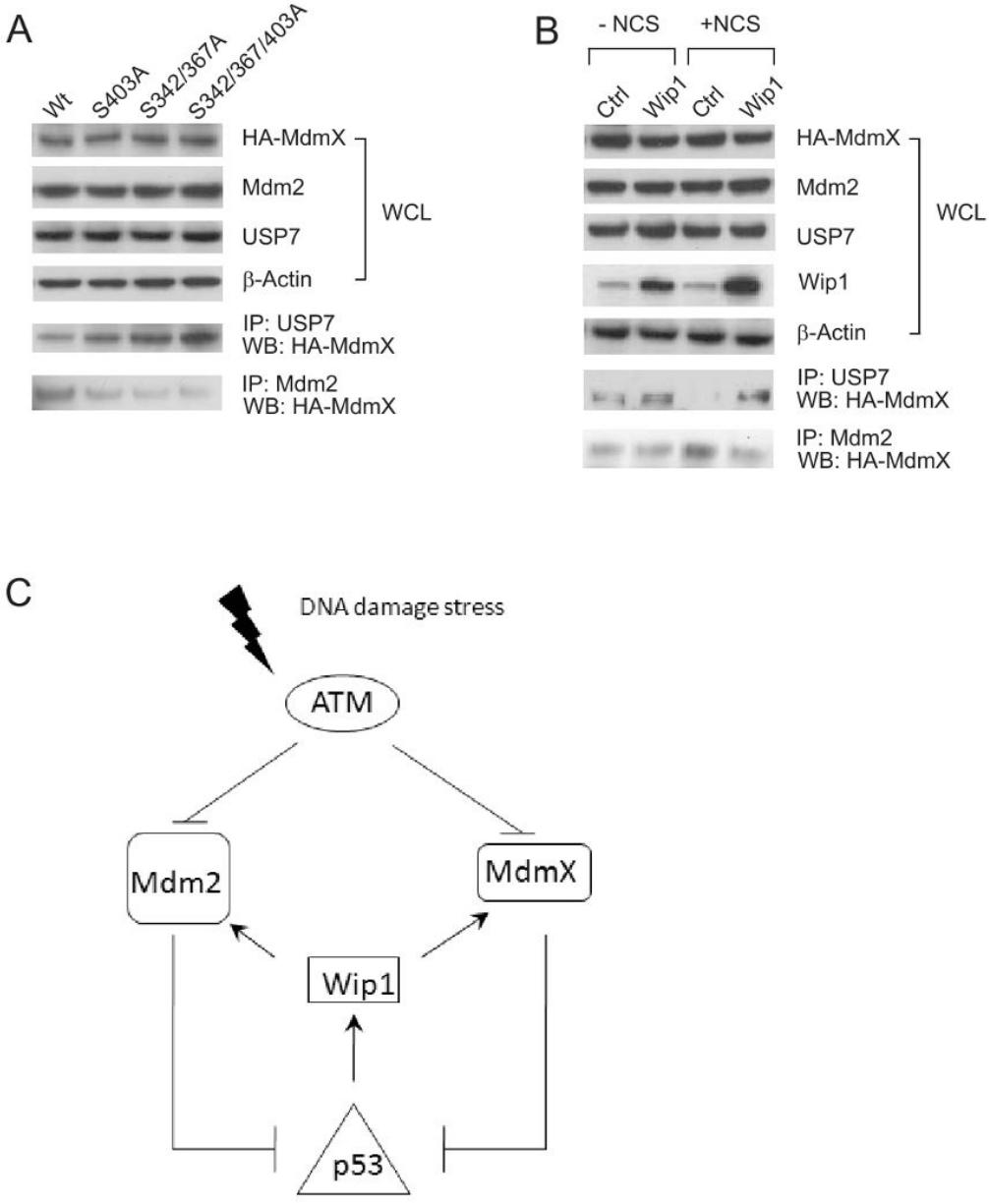

Figure 6.

Wip1 interferes with Mdm2-MdmX interaction but enhances USP7-MdmX interaction. (A) Phospho-mutant forms of MdmX have reduced binding ability with Mdm2 but have enhanced binding activity with USP7. 293 HEK cells were transfected with HA-tagged wildtype or mutant MdmX expression vector, treated with NCS (200 ng/mL), and harvested 2 h after treatment. Cell lysates were immunoprecipitated with anti-Mdm2 or anti-USP7 antibody. MdmX in the immunoprecipitates and various proteins in whole cell lysates (WCL) were assessed by immunoblotting as indicated. (B) Overexpression of Wip1 inhibits Mdm2-MdmX interaction but promotes USP7-MdmX interaction. 293 HEK cells that express inducible Wip1 with doxycycline were treated with or without NCS (200ng/mL) and MG132 (25 mmol/L), harvested and lysed 2 h after treatment. Immunoprecipitation and immunoblotting were performed as described in Fig. 6*A*. (C) Wip1 suppresses the induction of p53 through dephosphorylating and stabilizing Mdm2 and MdmX. Upon DNA damage stress, ATM is

activated to phosphorylate MdmX and Mdm2. Phosphorylation of MdmX and Mdm2 expedites their destabilization and degradation. p53 is rapidly stabilized and activated to initiate a variety of cell activities. Induced by p53, Wip1 dephosphorylates MdmX and Mdm2, resulting in their stabilization and p53 reduction.