E3 ubiquitin ligase COP1 regulates the stability and functions of MTA1

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Metastasis-associated protein 1 (MTA1), a component of the nucleosome remodeling and histone deacetylation (NuRD) complex, is widely upregulated in human cancers. However, the mechanism for regulating its protein stability remains unknown. Here we report that MTA1 is an ubiquitinated protein and targeted by the RING-finger E3 ubiquitin–protein ligase constitutive photomorphogenesis protein 1 (COP1) for degradation via the ubiquitin–proteasome pathway. Induced expression of wild-type COP1 but not its RING motif mutants promotes the ubiquitination and degradation of MTA1, indicating that the ligase activity is required for the COP1-mediated proteolysis of MTA1. Conversely, depletion of endogenous COP1 resulted in a marked decrease in MTA1 ubiquitination, accompanied by a pronounced accumulation of MTA1 protein. MTA1, in turn, destabilizes COP1 by promoting its autoubiquitination, thus creating a tight feedback loop that regulates both MTA1 and COP1 protein stability. Accordingly, disruption of the COP1-mediated proteolysis by ionizing radiation leads to MTA1 stabilization, accompanied by an increased coregulatory function of MTA1 on its target. Furthermore, we discovered that MTA1 is required for optimum DNA double-strand break repair after ionizing radiation. These findings provide novel insights into the regulation of MTA1 protein and reveal a novel function of MTA1 in DNA damage response.

coregulator | DNA repair | ubiquitination

Regulation of fundamental cellular processes demands dynamic coordinated participation of transcription factors and their coregulators at the target gene chromatin (1, 2), and deregulation of such processes plays a critical role in the development of malignant phenotypes. One emerging family of ubiquitously expressed chromatin modifiers is the metastasis-associated protein (MTA) family, which has an integral role in nucleosome remodeling and histone deacetylation (NuRD) complexes that modify DNA accessibility for cofactors (2, 3). MTA1, the founding member of the MTA family, is widely upregulated in human cancers and plays an important role in tumorigenesis and tumor aggressiveness, especially tumor invasion and metastasis (4–6). MTA1 functions not only as a transcriptional repressor of estrogen receptor α (7), but also as a transcriptional activator on certain promoters, such as the breast cancer–amplified sequence 3 (BCAS3) promoter (8). In this context, MTA1 is acetylated at lysine 626 (K626) by histone acetyltransferase p300; such modification allows MTA1 to recruit RNA polymerase II (Pol II) on the *BCAS3* enhancer region and confers its coactivator function upon *BCAS3* (8). MTA1 is also a mechanistic mediator of c-Myc–regulated transformation as a downstream target of the oncogene c-Myc (9). Although a paramount role of MTA1 in cancer and coregulator biology, the mechanism for regulating its protein stability remains unknown.

Constitutive photomorphogenic 1 (COP1; also known as RFWD2, RING finger and WD repeat domain protein 2), an evolutionarily conserved RING-finger ubiquitin–protein ligase, has been defined as a central regulator of plant development by targeting critical positive regulators and/or the photoreceptors for ubiquitination and degradation (10–12). In mammals, COP1 is involved in regulation of cell survival, growth, and metabolism. COP1 functions as an E3 ligase for the tumor suppressor p53 to induce its degradation, consequently, regulates cell cycle progression and cell survival (13). COP1 also regulates lipid metabolism by targeting acetyl-CoA carboxylase (ACC), a rate-limiting enzyme in fatty acid synthesis, for degradation via its interaction with the pseudokinase tribbles 3 (TRB3), a pseudokinase and negative regulator of Akt in muscle and the liver (14–16). Recently, it was found that COP1 promotes the ubiquitination and degradation of the cAMP responsive coactivator transducer of regulated CREB activity 2 (TORC2), a key regulator of fasting glucose metabolism, and thereby regulates liver glucose metabolism (17, 18). COP1 also inhibits c-Jun transcriptional activity by recruiting c-Jun to an E3 complex containing de–etiolated-1, DNA damage binding protein–1, cullin 4A, and regulator of cullins–1 for c-Jun protein degradation (19, 20). Because c-Jun is a stress-responsive transcription factor, it has been speculated that COP1 may be involved in cellular stress responses (21). Indeed, recent studies revealed that ionizing radiation (IR) triggers an ataxia telangiectasia mutated (ATM)–dependent rapid autodegradation of COP1 by phosphorylating it on Ser 387, thereby stabilizing p53 after DNA damage (22). In addition to polyubiquitination of its substrates, COP1 also catalyzes its autoubiquitination for degradation as a part of an autoregulatory mechanism (19, 23, 24).

In this study, we provide evidence that the E3 protein-ligase COP1 targets MTA1 for degradation via the ubiquitin–proteasome pathway. MTA1, in turn, destabilizes COP1 by promoting its autoubiquitination, thus creating a feedback loop that regulates both MTA1 and COP1 protein stability. Furthermore, we observed that IR stabilizes MTA1 by disruption of the COP1-mediated proteolysis and increases MTA1 coactivator activity on its target *BCAS3*, and that MTA1 is required for optimal DNA double-strand break repair after IR treatment. These findings provide insights into regulation of MTA1 protein and its role in cellular response to DNA damage.

Results and Discussion

MTA1 Is an Ubiquitinated Protein. While exploring the role of the proteasome pathway in controlling the steady-state levels of MTA1, we found that MTA1 protein levels were dramatically increased by potent and selective proteasome inhibitors, such as MG-132 or lactacystin (25), in human osteosarcoma U2OS and lung cancer A549 cells (Fig. 1*A* and [supporting information (SI) Fig. S1]). To

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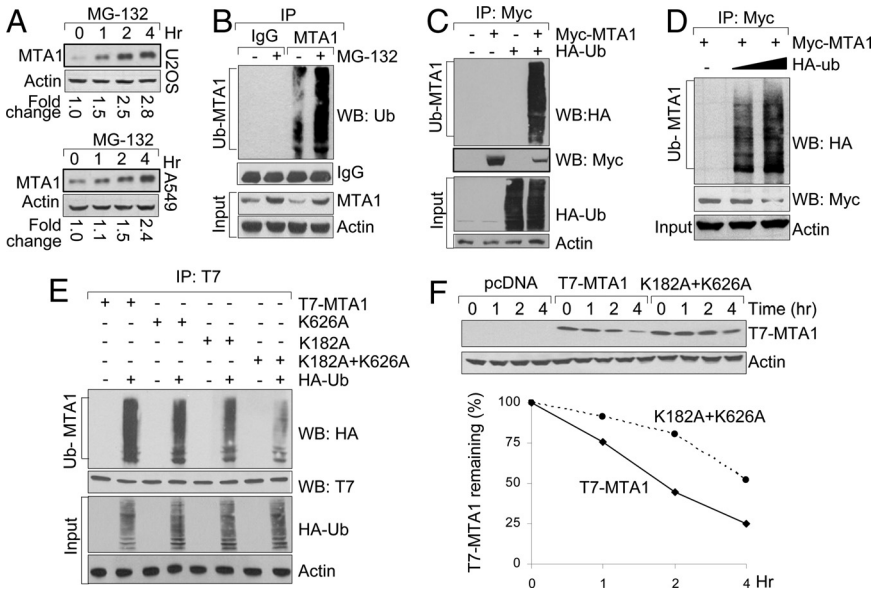

Fig. 1. MTA1 is an ubiquinated protein. (*A*) U2OS and A549 cells were treated with or without MG-132 and harvested at the indicated times for Western blot analysis with an anti-MTA1 antibody. The expression of the β-actin was used as a loading control. The density of bands was measured using the imageQuest program and normalized to that of β-actin. The fold change (MTA1/β-actin) is shown in the bottom of the figure. (*B*) U2OS cells were treated with or without MG-132, immunoprecipitated with an anti-MTA1 antibody or control IgG, and immunoblotted with an anti-ubiquitin antibody. (*C–E*) MTA1$^{-/-}$ MEFs (*C*) or HEK293 cells (*D* and *E*) were transfected with the indicated expression vectors, immunoprecipitated with an anti-Myc (*C* and *D*) or anti-T7 (*E*) antibody, and immunoblotted with the indicated antibodies. (*F*) U2OS cells were transfected with the indicated expression plasmids, treated with cycloheximide after 36 h of transfection, and collected at the indicated time points for Western blotting analysis using the indicated antibodies. Western blots were subjected to densitometric analysis and results were normalized based on actin expression levels, and reported in graphical form (*lower panel*).

test the existence of ubiquitination modification of MTA1 *in vivo,* U2OS cells were treated with or without MG-132 and subjected to sequential immunoprecipitation (IP)/Western blot analyses with the indicated antibodies. We noted the presence of a smear of polyubiquitinated MTA1 protein in the U2OS cells upon treatment with MG-132 (Fig. 1*B*), suggesting that endogenous MTA1 may be a target for proteasomal degradation in mammalian cells. To further test this notion, MTA1-knockout (MTA1$^{-/-}$) mouse embryonic fibroblasts (MEFs) (26) were transfected with expression vectors encoding Myc-tagged MTA1 (Myc-MTA1) and hemagglutinin (HA)–tagged ubiquitin (HA-Ub), either alone or in combination. Protein extracts were immunoprecipitated with an anti-Myc antibody and immunoblotted with an anti-HA antibody. We found that Myc-MTA1 was heavily ubiquitinated in the presence of HA-Ub (Fig. 1*C, last lane*). This was also true when these studies were repeated in HEK293 cells (Fig. 1*D*). These findings suggest that MTA1 is an ubiquitinated protein within cells.

Ubiquitin-dependent proteolysis occurs after covalent attachment of a polyubiquitin chains to a lysine residue in a given target protein, so the removal or modification of these residues generally leads to loss of ubiquitin ligation and resistance to proteasome-mediated degradation (27, 28). Based on our previous observations that lysine residue 626 (K626) of MTA1 is acetylated by p300 in breast cancer cells (8), and the fact that MTA1 contains another lysine residue (K182) which could potentially be modified, we next tested whether the two lysine residues are required for the ubiquitination of MTA1. Toward this aim, we generated various T7-tagged MTA1 expression vectors in which the lysine residues K182 and K626 were mutated to alanine (referred to K182A, K626A, or K182A+K626A mutant) and transfected these expression plasmids into HEK293 cells alone or together with HA-Ub expression vector. Sequential IP/Western blot analyses revealed that, although ubiquitination of K182A (*lane 4*) or K626A (*lane 6*) mutant was reduced as compared with its wild-type control (*lane 2*), a greater reduction was seen with the double mutant K182A+K626A (*lane 8*; Fig. 1*E*), indicating that K182 and K626 could be two of ubiquitination sites for MTA1. We further confirmed these results by measuring MTA1 half-life in the presence of cycloheximide, an inhibitor of protein biosynthesis. As shown in Fig. 1*F,* double mutant (K182A+K626A) increases the half-life of MTA1 as compared with its wild-type control. Briefly, these data strongly indicates that MTA1 is an ubiquitinated protein in cells and is subjected to degradation through the ubiquitin–proteasome pathway.

E3 Ubiquitin–Protein Ligase COP1 Targets MTA1 for Degradation. To determine which ligase is responsible for MTA1 ubiquitination, we screened a number of known E3 ligases by cotransfection experiments. We found that coexpression of Flag-COP1 dramatically increased MTA1 ubiquitination (Fig. 2*A*), whereas knockdown of endogenous COP1 by a specific siRNA against COP1 decreased the levels of MTA1 ubiquitination (Fig. 2*B*), suggesting that COP1 could be the primary E3 ubiquitin ligase for MTA1 ubiquitination. To further substantiate a role of COP1 on MTA1 ubiquitination, we generated two E3 ligase–defective mutants by substitutions of consensus Cys to Ser at the residues 136/139 (C136/139S) and 156/159 (C156/159S) within the RING domain (residues 136–174), which are required for COP1 E3 ligase activity (24), and tested their effects on MTA1 ubiquitination *in vivo*. We found that COP1 but not its E3 ligase-defective mutants promoted the appearance of inducible MTA1 ubiquitination (Fig. 2*C*), suggesting that COP1 ligase activity is required MTA1 ubiquitination.

Because ubiquitination of proteins is usually associated with their turnover, we next tested whether COP1 could regulate MTA1 protein abundance. As shown in Fig. 2*D,* coexpression of COP1 results in a dose-dependent decrease of MTA1 levels, and inclusion of proteasome inhibitor MG-132 abolished the effect of COP1 on the degradation of MTA1 (*last lane*), indicating a role of ubiquitin-dependent proteasome pathway in the COP1-mediated proteolysis of MTA1. In contrast, the E3 ligase–

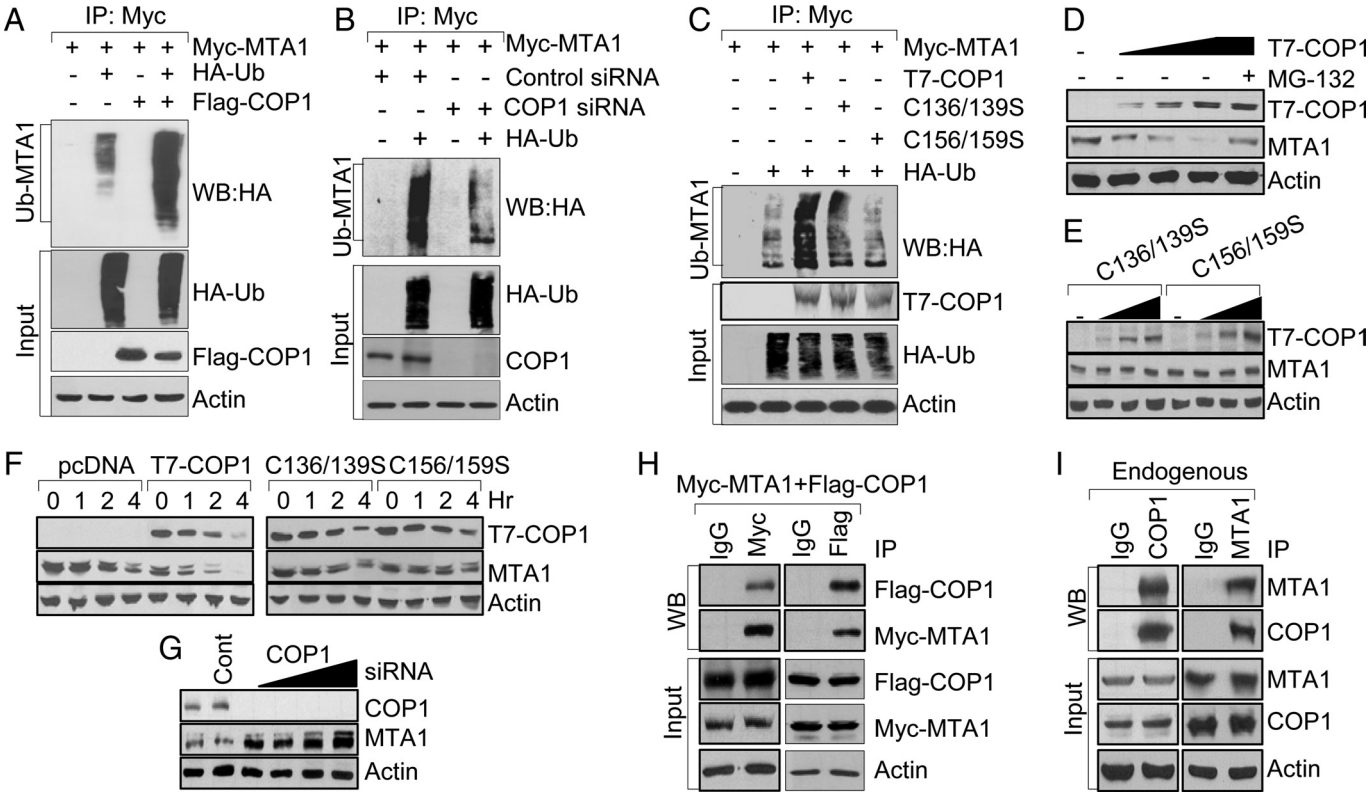

Fig. 2. COP1 targets MTA1 for degradation via the ubiquitin–proteasome pathway. (*A–C*) U2OS cells were transfected with the indicated expression vectors (*A* and *C*) or in combination with a specific siRNA against COP1 or control siRNA (*B*), immunoprecipiated with an anti-Myc antibody, and immunoblotted with the indicated antibodies. (*D*) U2OS cells were transfected with or without increasing amounts of a T7-COP1 expression plasmid, and treated or untreated with 20 μM of MG-132 for 6 h before harvesting for Western blot analysis with the indicated antibodies. (*E*) U2OS cells were transfected with expression vector encoding T7-COP1 C136/139S or T7-COP1 C156/159S, and immunoblotted with the indicated antibodies. (*F*) U2OS cells were transfected with the indicated expression plasmids, treated with cycloheximide, and collected at the indicated time points for Western blotting analysis as described above. (*G*) U2OS cells were transfected control siRNA or increasing amounts of COP1 siRNA, and harvested for Western blot analysis as Fig. 2D. (*H* and *I*) Protein extracts from the U2OS cells cotransfected with Myc-MTA1 and Flag-COP1 (*H*) or untransfected (*I*) were immunoprecipitated with the indicated antibodies or control IgG, and immunoblotted with the indicated antibodies.

defective mutants of COP1 were not able to alter the concentration of MTA1 (Fig. 2*E*). In support of these findings, cycloheximide half-life experiments revealed that COP1, but not its ligase-defective mutants, decreases the half-life of MTA1 (Fig. 2*F* and Fig. S2). In addition, we consistently demonstrated that depletion of COP1 using a specific siRNA against COP1 caused a pronounced accumulation of endogenous MTA1 protein (Fig. 2*G*). Taken together, these findings establish that COP1 ligase is a modifier of MTA1 ubiquitination and that MTA1 stability is regulated by COP1 through a proteasome-mediated process.

Given that COP1 could ubiquitinate MTA1, we next examined the possibility of a physical interaction between MTA1 and COP1. We found that transiently expressed Myc-MTA1 and Flag-COP1 in the U2OS cells could be coimmunoprecipiated with Flag or Myc antibodies, respectively (Fig. 2*H*). Furthermore, endogenous COP1 also coimmunoprecipitated with endogenous MTA1 in the U2OS cells (Fig. 2*I*), demonstrating that MTA1 and COP1 proteins interacts with each other *in vivo*. To further examine whether the interaction between MTA1 and COP1 is direct or indirect, we performed *in vitro* GST pull-down assays using the immobilized full-length GST-MTA1 and ^{35}S-labeled, *in vitro*–translated COP1 or NRIF3 as a positive control (29). Results showed that MTA1 was unable to directly bind to COP1 but did so with NRIF3 (29) (Fig. S3). These results suggest that COP1 and MTA1 could coexist within a native protein complex and that the observed MTA1-COP1 interaction *in vivo* may be mediated via other proteins. This notion has been already demonstrated by other studies. For example, a recent study (15) reported that COP1 regulates lipid metabolism by targeting acetyl-CoA carboxylase (ACC) for the ubiquitin-dependent degradation via its interaction with the pseudokinase tribbles 3 (TRB3), and that COP1 does not directly interact with ACC. TRB3 associates with both COP1 and ACC through distinct surfaces and mediates the interaction between COP1 and ACC and triggers ubiquitination of ACC by recruitment of COP1 to ACC (15).

MTA1 Destabilizes COP1 by Promoting Its Autoubiquitination. Like other RING finger ubiquitin ligases, COP1 catalyzes its autoubiquitination for degradation as a part of an autoregulatory mechanism (19, 23, 24). We next examined whether MTA1 affects the autoubiquitination activity of COP1. Interestingly, induced expression of MTA1 in the U2OS cells dramatically increased the levels of COP1 autoubiquitination (Fig. 3*A;* compare *lane 3* with *lane 2*), accompanied by decreased COP1 protein level. Furthermore, we found that induced expression of MTA1 led to a dose-dependent reduction in the protein levels of endogenous COP1 in the U2OS cells (Fig. 3*B*). The observed inhibitory effect of MTA1 on COP1 protein levels was independent of p53 and Mdm2, because MTA1 was also able to decrease the level of exogenous COP1 in the p53$^{-/-}$/Mdm2$^{-/-}$ double-knockout mouse embryonic fibroblasts (30) (Fig. 3*C*). In support of these findings, we further demonstrate that induced expression of MTA1 decreases the half-life of COP1 (Fig. 3*D*). These results suggest that COP1 targets MTA1 for the ubiquitin-dependent degradation; MTA1, in turn, destabilizes COP1 by

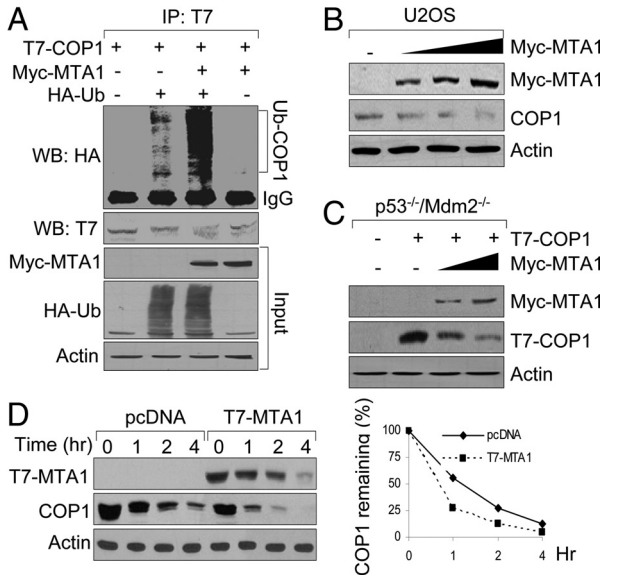

Fig. 3. MTA1 destabilizes COP1 by promoting its autoubiquitination. (*A*) U2OS cells were transfected with the indicated expression vectors, immunoprecipitated with an anti-T7 antibody, and immunoblotted with the indicated antibodies. (*B* and *C*) U2OS cells (*B*) or p53$^{-/-}$/Mdm2$^{-/-}$ double-knockout MEFs (*C*) were transfected with the indicated expression plasmids and immunoblotted with the indicated antibodies. (*D*) U2OS cells were transfected with the indicated expression plasmids, treated with cycloheximide, and collected at different time points for Western blot analysis as described above. Western blots were subjected to densitometric analysis, and results were normalized based on actin expression levels, and reported in graphic form (*right panel*).

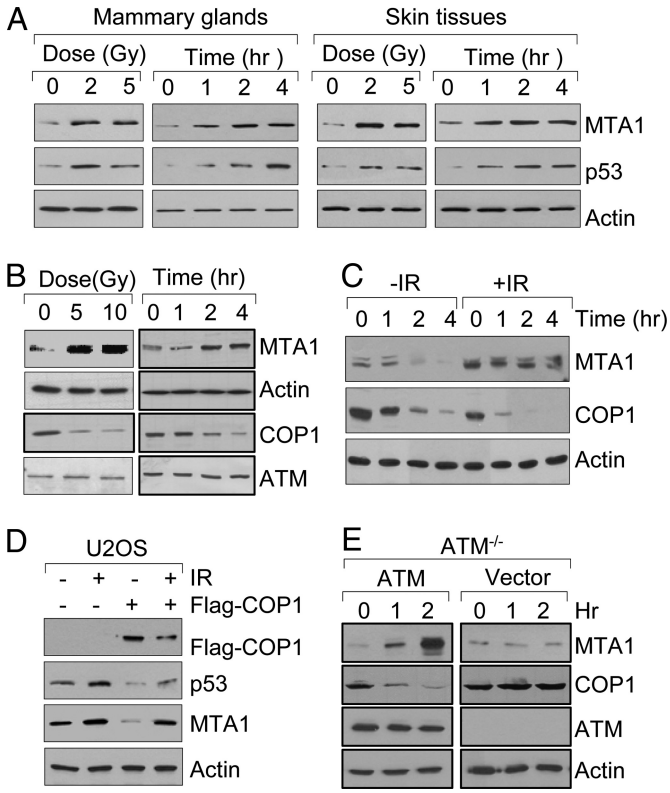

Fig. 4. Disruption of the COP1-mediated proteolysis led to MTA1 stabilization in an ATM-dependent manner. (*A* and *B*) Whole-body mice (*A*) or U2OS cells (*B*) were irradiated or mock-irradiated with IR at the indicated doses, and harvested at the indicated time points for Western blot analysis with the indicated antibodies. (*C*) U2OS cells were treated or untreated with 10 Gy of IR. After 1 h of IR treatment, cells were treated with cycloheximide and collected at different time points for Western blotting analysis as described above. (*D*) U2OS cells were transfected with a Flag-COP1 expression vector or empty vector. After 36 h of transfection, cells were treated or untreated with IR and harvested for Western blot analysis with the indicated antibodies. (*E*) ATM$^{-/-}$/vector or ATM$^{-/-}$/ATM fibroblasts were treated with 10 Gy of IR and harvested at the indicated time points for Western blot analysis using the indicated antibodies.

promoting its autoubiquitination, thereby creating an autoregulatory feedback loop that regulates the activity of both MTA1 and COP1 proteins.

MTA1 Is Stabilized in Response to Ionizing Radiation. Because IR triggers an ATM-dependent rapid autodegradation of COP1 (22) and COP1 targets MTA1 for the ubiquitin-dependent degradation (Fig. 2), we next determined whether MTA1 protein levels were affected by IR in a whole-animal setting. After exposure of whole-body mice to IR, the expression of MTA1, as well as p53 (positive control), protein was dramatically increased in the mammary glands, skin tissues (Fig. 4*A*), and thymus glands (Fig. S4). In support of these observations, we found that treatment of U2OS cells with IR resulted in a marked increase in the level of MTA1 protein in a dose-dependent (*left panel*) and time-dependent (*right panel*) manner (Fig. 4*B*). Consistent with previous reports (22), the levels of COP1 protein were reduced by IR in the same cellular lysates from the U2OS cells, which express high levels of endogenous ATM (31) (Fig. 4*B, lower panels*). The increased abundance of MTA1 protein in the U2OS cells after IR treatment was not attributed to an increase in MTA1 mRNA levels (Fig. S5), indicating a posttranscriptional regulation mechanism for MTA1 protein by IR. To further test this notion, we monitored the effect of cycloheximide on the rate of decline of the endogenous MTA1 in IR-treated cells versus controls. We found that IR led to a marked increase in the half-life of MTA1 in comparison to nonirradiated controls (Fig. 4*C* and Fig. S6). In contrast, IR decreases the half-life of COP1 (Fig. S6), as reported previously (22). These results suggest that IR upregulates MTA1 through its posttranslational modifications, possibly via inhibition of its degradation by COP1. To further test this hypothesis, U2OS cells were transfected with a Flag-COP1 expression vector and treated with or without IR. We found that IR rescued COP1-induced downregulation of MTA1 and p53, accompanied with a marked downregulation of COP1 (Fig. 4*D*), suggesting that IR stabilizes MTA1 by, at least in part, inhibiting the COP1-mediated proteolysis. Because IR induces rapid autodegradation of COP1 in an ATM-dependent manner (22), we next determined whether the noted stabilization of MTA1 in response to IR requires ATM protein using an A-T cell line (AT22IJE-T) that was stably transfected with plasmids encoding either wild-type ATM (ATM$^{-/-}$/ATM) or an empty expression vector (ATM$^{-/-}$/vector) (32). We found that MTA1 was upregulated by IR in the ATM$^{-/-}$/ATM but not in the ATM$^{-/-}$/vector cells (Fig. 4*E*), implying the need for ATM in the stabilization of MTA1 in response to IR. Collectively, these data indicated a mandatory requirement of the ATM-dependent degradation of COP1 for an increased stability of MTA1 by IR.

Modulation of the Coregulator Function of MTA1 by IR. As a transcriptional coregulator, MTA1 exists in both corepressor or coactivator complexes and can either repress or stimulate the expression of cellular genes (2). Because genotoxic stress increases the p300/HAT activity and MTA1-acetylation on K626 by p300 regulates its coactivator activity upon the *BCAS3* chromatin (8), we next determined the effect of IR upon the coregulatory activity of MTA1. For this purpose, U2OS cells were left untreated or treated with 10 Gy of IR and subjected to

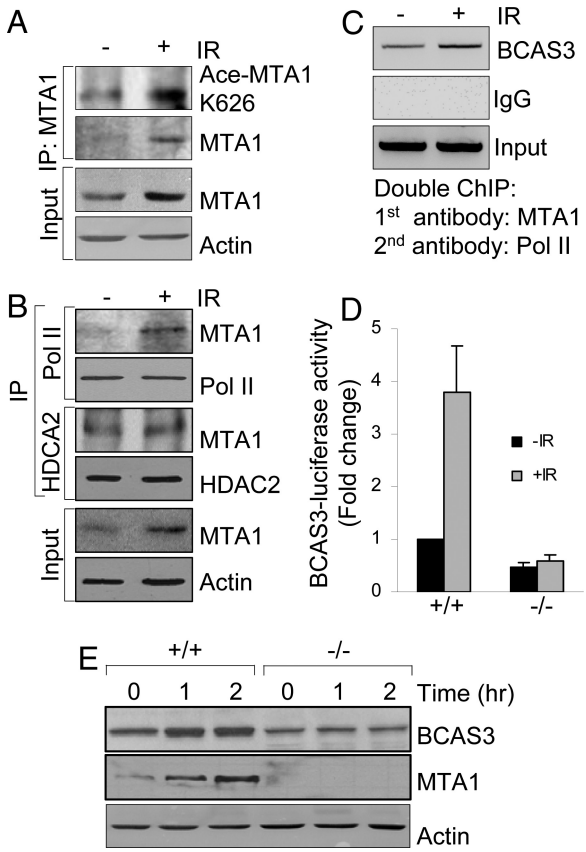

Fig. 5. Modulation of the coregulator function of MTA1 by genotoxic stress. (*A* and *B*) Protein extracts from U2OS cells treated or untreated with IR were subjected to IP analysis with the indicated antibodies, followed by Western blotting analysis with the indicated antibodies. (*C*) Sequential double-ChIP assay of recruitment of MTA1/Pol II complex to the BCAS3 promoter. U2OS cells were treated with or without IR and harvested after 2 h of IR treatment for double ChIP analysis as described in *SI Text*. The first ChIPs by an anti-MTA1 antibody were followed by the second ChIPs by an anti-Pol II antibody. (*D*) MTA1$^{+/+}$ and MTA1$^{-/-}$ MEFs were transfected with BCAS3-luciferase promoter expression vector and treated with or without IR. Cells were lysed after 2 h of IR treatment and luciferase activities were determined. (*E*) Western blot analysis of BCAS3 protein expression in MTA1$^{+/+}$ and MTA1$^{-/-}$ MEFs treated with or without IR.

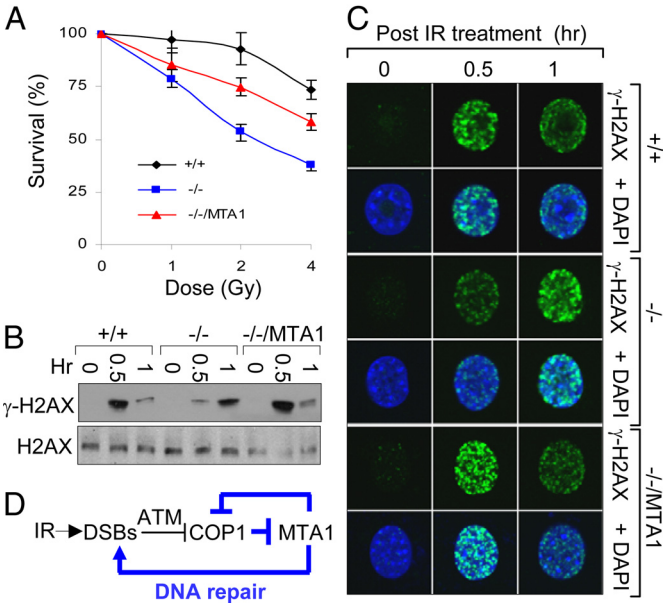

Fig. 6. MTA1 is required for optimal DNA repair. (*A*) Clonogenic survival assay of MTA1$^{+/+}$, MTA1$^{-/-}$, and MTA1$^{-/-}$/MTA1 MEFs treated with or without different doses of IR. Cells were counted, plated, and subjected to indicated doses of radiation and colonies formed over 14 days. Surviving colonies were plotted as a function of cells plated and normalized by the plating efficiency for each condition. (*B* and *C*) MTA1$^{+/+}$, MTA1$^{-/-}$, and MTA1$^{-/-}$/MTA1 MEFs were treated with or without IR and harvested at the indicated time points for Western blot analysis (*B*) or immunofluorescence staining (*C*) of γ-H2AX expression. (*D*) Proposed working model summarizing the findings is presented here.

the sequential immunoprecipitation followed by Western blot analyses. We found that IR-mediated increase in stability of MTA1 is also accompanied by a corresponding increase in the levels of K626 acetylated MTA1 (Fig. 5*A*) as well as an increased interaction between the MTA1 and Pol II (Fig. 5*B*). In contrast, IR exposure does not affect the interaction between MTA1 and HDAC2 in the U2OS cells (Fig. 5*B*), suggesting that IR affects the MTA1 coactivator but not corepressor function. In support of this notion, we showed that indeed, IR promotes the recruitment of MTA1/Pol II complex to the BCAS3 gene chromatin revealed by a sequential double-ChIP (chromatin immunoprecipitation) assay (Fig. 5*C*). Moreover, IR stimulates BCAS3-promoter activity in the wild-type MTA1 (MTA1$^{+/+}$) MEFs and that this effect was compromised in its knockout (MTA1$^{-/-}$) counterparts (Fig. 5*D*). Consistent with these findings, we found that IR increased the BCAS3 protein levels in the MTA1$^{+/+}$ but not MTA1$^{-/-}$ MEFs (Fig. 5*E*). Collectively, these findings reveal that IR may affect the functionality of MTA1 in mammalian cells.

Revelation of a Novel Function of MTA1 in DNA Repair. Because MTA1 is a DNA damage responsive protein (Fig. 4), we next investigated the possible role of MTA1 in the DNA double-strand break (DSB) repair following IR treatement. One of the hallmarks of defective DNA repair is increased radiation sensitivity. We first examined the effect of MTA1 deficiency (MTA1$^{-/-}$) on cell survival in response to IR by clonogenic survival assay (33). We found that MTA1$^{-/-}$ MEFs were hypersensitive to IR exposure and exhibited a decreased clonogenic survival compared to its wild-type controls (Fig. 6*A*), suggesting a defect in DSB repair in MTA1$^{-/-}$ MEFs. Interestingly, the noted hypersensitivity of MTA1$^{-/-}$ MEFs to IR treatment was efficiently rescued by stable reintroduction of MTA1 in the MTA1$^{-/-}$ MEFs (Fig. 6*A* and Fig. S7), indicating that MTA1 is critical for efficient DSB repair.

We next examined the effect of MTA1 deficiency on the levels of phosphorylated H2AX (γ-H2AX), an established surrogate marker for DSB signaling and the assembly of DNA repair complexes at the site or in the vicinity of DSBs (34–36). Western blot analysis using an anti–phospho-H2AX (Ser-139) antibody revealed that in response to IR the levels of γ-H2AX were greatly delayed in the MTA1$^{-/-}$ MEFs, whereas MTA1$^{+/+}$ controls exhibited the typical kinetics of γ-H2AX, with its level maximized at 30 min and declining afterward, reflecting DSB generation and repair (Fig. 6*B*). Interestingly, reintroduction of MTA1 in the MTA1$^{-/-}$ MEFs (MTA1$^{-/-}$/MTA1) effectively restored the delayed responsiveness of γ-H2AX to IR (Fig. 6*B*). Moreover, there was no change in total H2AX protein levels between MTA1$^{+/+}$ and MTA1$^{-/-}$ MEFs with or without IR treatment, indicating that MTA1 is critical for the efficient induction of H2AX phosphorylation in response to IR.

H2AX phosphorylation can be detected by immunofluorescence, resulting in individual foci within the cell nucleus that can be counted and are a measure of DSBs (37). We next examined whether MTA1 deficiency affects the formation of γ-H2AX foci by immunofluorescent staining using a phospho-H2AX (Ser-

139) antibody. After exposure of MTA1$^{-/-}$ MEFs to IR, we found a notable reduction in the number of γ-H2AX–containing repair foci and in the maximum achievable levels of γ-H2AX protein compared with values in MTA1$^{+/+}$ controls (Fig. 6*C* and Fig. S8). Of interest, the noted delay in responsiveness of the ability of MTA1$^{-/-}$ MEFs to form the γ-H2AX foci was efficiently rescued by stable reintroduction of MTA1 in the MTA1$^{-/-}$ MEFs (Fig. 6*C* and Fig. S8), suggesting that MTA1 is critical for the formation of γ-H2AX foci after DNA damage. As MTA1$^{-/-}$ MEFs still contain MTA2 and MTA3 (Fig. S9), these findings suggest that MTA1 is required for efficient DSB repair as well as cell survival after DNA damage.

Our collective findings provide evidence that MTA1, a transcriptional coregulator, is ubiquitinated and targeted for proteasomal degradation and that the process is mediated by a RING finger ubiquitin ligase COP1. Interestingly, we found that MTA1, in turn, destabilizes COP1 by promoting its autoubiquitination, thereby creating an autoregulatory feedback loop between MTA1 and COP1 for controlling their protein stability (Fig. 6*D*). Moreover, we show that MTA1 is a DNA damage responsive protein; it is stabilized and activated in response to IR through, at least in part, disruption of the COP1-mediated proteolysis. Importantly, such posttranslational modification of MTA1 affects the functionality of MTA1 as a coactivator on its target chromatin. The biologic significance of these findings was further revealed by the use of genetically engineered MTA1-knockout MEFs. We found that MTA1 is required for optimal DSB repair, and inactivation of MTA1 therefore increases the cellular sensitivity to IR-induced DNA damage. DNA damaging agents in the form of γ radiation and chemotherapeutic drugs are the mainstays of most current cancer treatment regimens. Given the fact that MTA1 is widely upregulated in human cancers and is closely associated with poor survival in patients with cancers (5, 6), this study shows that MTA1 is a potential therapeutic target that could be used to enhance the effectiveness of IR or DNA-damaging chemotherapy by inhibiting the action MTA1. These findings provide insights into the regulation of MTA1 protein and define a novel function of MTA1 in DNA damage response.

Materials and Methods

Human U2OS, A549, and HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA). AT22IJE-T (A-T), a fibroblast cell line derived from an ataxia-telangiectasia patient, and lines stably transfected with either an empty expression vector (ATM$^{-/-}$/vector) or full-length ATM cDNA (ATM$^{-/-}$/ATM) were gifts from Dr. Yosef Shiloh (Tel Aviv University, Tel Aviv, Israel) (32). MTA1$^{+/+}$ and MTA1$^{-/-}$ MEFs were generated in our laboratory from embryos at day 9 of development by using a standard protocol. To establish cell lines stably expressing MTA1, MTA1$^{-/-}$ MEFs were transfected with expression vector encoding pcDNA6/V5-MTA1 or empty vector using FuGENE HD Transfection Reagent (Roche Applied Science, Indianapolis, IN) and then subjected to selection after 24 h of transfection with 10 μg/ml of blasticidin (Invitrogen, Carlsbad, CA) for 2 weeks. The resulting colonies were isolated and analyzed for V5-MTA1 expression by immunoblotting. All of the cell lines were grown in the recommended media by the providers supplemented with 10% fetal bovine serum (FBS) and 1× antibiotic-antimycotic solution in a humidified 5% CO_2 at 37 °C. Cell culture medium and additives were obtained from Invitrogen (Carlsbad, CA) if not otherwise stated.

The detailed Materials and Methods are provided in the *SI Text*. Primers used for generating various mutants and quantitative real-time polymerase chain reactions are provided in Table S1 and Table S2, respectively.

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