Identification of XAF1–MT2A mutual antagonism as a molecular switch in cell-fate decisions under stressful conditions

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XIAP-associated factor 1 (XAF1) is a tumor suppressor that is commonly inactivated in multiple human neoplasms. However, the molecular mechanism underlying its proapoptotic function remains largely undefined. Here, we report that XAF1 induction by heavy metals triggers an apoptotic switch of stress response by destabilizing metallothionein 2A (MT2A). XAF1 directly interacts with MT2A and facilitates its lysosomal degradation, resulting in the elevation of the free intercellular zinc level and subsequent activation of p53 and inactivation of XIAP. Intriguingly, XAF1 is activated as a unique transcription target of metal-regulatory transcription factor-1 (MTF-1) in signaling apoptosis, and its protein is destabilized via the lysosomal pathway by MTF-1–induced MT2A under cytostatic stress conditions, indicating the presence of mutual antagonism between XAF1 and MT2A. The antagonistic interplay between XAF1 and MT2A acts as a key molecular switch in MTF-1–mediated cell-fate decisions and also plays an important role in cell response to various apoptotic and survival factors. Wild-type (WT) XAF1 but not MT2A binding-deficient mutant XAF1 increases the free intracellular zinc level and accelerates WT folding of p53 and degradation of XIAP. Consistently, XAF1 evokes a more drastic apoptotic effect in $p53^{+/+}$ versus isogenic $p53^{-/-}$ cells. Clinically, expression levels of XAF1 and MT2A are inversely correlated in primary colon tumors and multiple cancer cell lines. XAF1-depleted xenograft tumors display an increased growth rate and a decreased apoptotic response to cytotoxic heavy metals with strong MT2A expression. Collectively, this study uncovers an important role for XAF1–MT2A antagonism as a linchpin to govern cell fate under various stressful conditions including heavy metal exposure.

XAF1 | metallothionein | MTF-1 | heavy metal | apoptosis

Elimination of defective or potentially dangerous cells by apoptosis plays a fundamental role in the regulation of tissue homeostasis and prevention of malignant transformation and autoimmunity (1). X-linked inhibitor of apoptosis (XIAP)-associated factor 1 (XAF1) is a proapoptotic tumor suppressor that is identified as a negative regulator of XIAP based on its ability to bind and interfere with the anticaspase function of XIAP (2, 3). XAF1 expression is down-regulated in a broad range of human tumors mainly by aberrant promoter CpG site hypermethylation, and its reduction is associated with the advanced stage and high grade of many cancers (4–6). A recent study showed that PTEN-null mouse prostate tumors showing resistance to androgen-deprivation therapy have reduced levels of XAF1, and diminished expression of XAF1 by continuous androgen deprivation therapy is positively correlated with metastatic castrate-resistant tumor progression (7).

XAF1 sensitizes tumor cells to the proapoptotic effects of chemotherapeutic drugs, γ-irradiation, UV, H_2O_2, hypoxia, and growth factor deprivation, whereas its depletion increases cellular resistance to apoptotic stresses (5, 6). XAF1 was originally identified as a nuclear protein that could bind and sequester XIAP protein to the nucleus (3). It was thus proposed that loss of XAF1 may increase the functional pool of cytoplasmic XIAP,

which in turn deregulates the apoptotic process and contributes to tumorigenesis (8). However, it was shown that XAF1 evokes an apoptotic effect in $XIAP^{-/-}$ cells to the extent comparable in $XIAP^{+/+}$ cells, and XIAP is not sequestered to the nucleus in cells undergoing XAF1-induced apoptosis, indicating that XAF1's proapoptotic function is not solely dependent on the XIAP-interfering activity (6, 9). $XAF1$ was also identified as an IFN-stimulated gene that contributes to IFN-dependent sensitization of cells to tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL)-induced apoptosis (10, 11). Recently, we reported that XAF1 forms a positive feedback loop with the p53 tumor suppressor and acts as a molecular switch in p53-mediated cell-fate decisions favoring apoptosis over cell-cycle arrest (12). In this process, XAF1 appears to promote homeodomain-interacting protein kinase 2 (HIPK2)-mediated p53 phosphorylation by interrupting the HIPK2-targeting function of Siah2 and promotes zinc finger protein 313 (ZNF313)-induced $p21^{WAF1}$ ubiquitination (13). XAF1 was also shown to inhibit tumor angiogenesis by suppressing VEGF expression and regulate the cell cycle through modulating the G2/M checkpoint and interaction with checkpoint kinase 1 (14, 15).

Many heavy metals and metallic agents have biological activity but also cause many adverse health effects, such as carcinogenicity in humans (16). However, the mechanism underlying heavy-metal–induced tumorigenesis remains unclear. Metallothioneins (MTs) are low-molecular-weight (6–7 kDa), cysteine-rich, heavy-metal–binding proteins that play a crucial role in the regulation of intracellular metal metabolism and metal-induced stresses (17, 18). Upon heavy metals stimuli, MTs are rapidly

Significance

Epigenetic inactivation of XAF1 tumor suppressor is frequently observed in multiple human cancers. However, the mechanisms underlying its function remain largely undefined. Here, we present evidence that XAF1 plays a critical role in cell-fate decisions under heavy-metal–induced stress conditions through the mutual antagonism with MT2A. XAF1 is activated as a transcription target of MTF-1 and destabilizes MT2A through the interaction-directed lysosomal degradation, whereas it is destabilized by MT2A under cytostatic stress conditions. XAF1-mediated MT2A inactivation leads to elevation of free intracellular zinc level and up- and down-regulates p53 and XIAP, respectively, to promote apoptosis. XAF1–MT2A antagonism thus represents one critical regulator of cell-fate decisions, suggesting that it could be an attractive target for the therapeutic intervention of tumor progression.
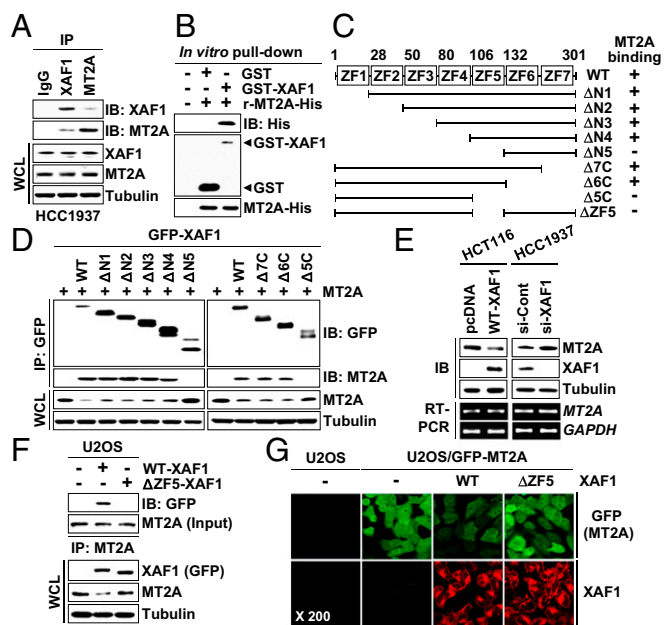
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Fig. 1. XAF1 binds directly to MT2A. (*A*) Interaction of endogenously expressed XAF1 and MT2A. IB, immunoblot; WCL, whole-cell lysate. (*B*) In vitro pull-down assay showing a direct interaction between XAF1 and MT2A. (*C*) XAF1 constructs and its MT2A-binding status. (*D*) Identification of the ZF5 domain as a MT2A-binding region of XAF1. (*E*) XAF1 down-regulation of MT2A protein expression. (*F*) A crucial role for XAF1 ZF5 domain in binding and downregulating MT2A. (*G*) Immunofluorescence microscopic analysis showing no MT2A-reducing activity of ΔZF5-XAF1.

transcriptionally activated by metal-regulatory transcription factor-1 (MTF-1) and function in protecting cells against heavy metals, particularly cadmium, zinc, copper, mercury, nickel, platinum, and silver primarily by acting as scavengers of toxic metal ions (19, 20). MTs also have crucial roles in cell proliferation and differentiation, and their activity to bind heavy metals and detoxify free radicals can help cancer cells to survive by inhibition of apoptosis (21, 22). Moreover, MTF-1 is elevated in human breast, lung, and cervical carcinoma-derived cell lines, supporting the importance of MTs in carcinogenesis (23).

Of the 11 human functional MT isoforms [MT-1 (A, B, E, F, G, H, M, and X), MT2A, MT-3, and MT-4], ubiquitously expressed MT1 and MT2A are extensively studied for their ability to attenuate metal-induced toxicity and as biomarkers in many human cancers (18, 24, 25). MT2A is the major MT isoform expressed in humans and has specific functions in regulating apoptosis, autophagy, and increasing risk of cancers (26–28). MT2A is up-regulated in many types of tumor tissues and cell lines, and its elevation is associated with adverse prognosis. MT2A induction decreases intracellular zinc availability whereas its silencing increases free intracellular zinc concentration, thus modulating the intracellular levels of bioavailable zinc (29). MT2A contributes to chemotherapy resistance by direct chelation of zinc and platinum-containing drugs and by indirect action on p53 zinc-dependent activity. It was reported that HIPK2 depletion induces MT2A up-regulation and p53 misfolding, but codepletion of MT2A restores p53 activity, showing that MT2A can modulate p53 through zinc exchange (30). Several studies demonstrated an association of MT2A elevation with grade and aggressiveness of tumors, including prostate cancer (27, 31, 32). Nevertheless, the molecular mechanism underlying the biological functions of MT2A and its regulation in the carcinogenic process has not been well understood.

In this study, we present evidence that XAF1–MT2A mutual antagonism via the interaction-directed lysosomal degradation

plays a crucial role in cell-fate decisions under various stressful conditions, including heavy metal exposure.

Results

XAF1 Directly Interacts with and Down-Regulates MT2A. In an attempt to understand the mechanistic basis of XAF1 proapoptotic function, we initially performed a yeast two-hybrid assay and identified MT2A as a XAF1-interacting protein (Fig. S1*A*). The XAF1–MT2A interaction was confirmed by immunoprecipitation (IP) and GST pull-down assays (Fig. 1*A* and Fig. S1 *B* and *C*). An in vitro pull-down assay using purified GST-XAF1 and recombinant MT2A proteins revealed that XAF1 binds directly to MT2A (Fig. 1*B*). Using a series of deletion mutants, we determined that zinc finger domain 5 (ZF5) of XAF1 is essential for the interaction (Fig. 1 *C* and *D*). Moreover, the MT2A protein level was markedly decreased and increased by XAF1 expression and depletion, respectively, suggesting that XAF1 may down-regulate MT2A via direct interaction (Fig. 1*E* and Fig. S1 *E* and *F*). As predicted, MT2A protein level was reduced by wild type (WT)-XAF1 but not affected by ΔZF5-XAF1, a mutant XAF1 lacking the ZF5 domain (Fig. 1*F*). Consistently, an immunofluorescence assay using U2OS/GFP-MT2A subline cells showed a profound reduction of GFP-MT2A fluorescence by WT-XAF1 but not by ΔZF5-XAF1 (Fig. 1*G*). Based on these observations, we asked whether XAF1 regulates MT1A, another ubiquitously expressed MT isoform, and found that XAF1 also interacts with and down-regulates MT1A (Fig. S1 *F–I*).

XAF1 Promotes Lysosomal Degradation of MTs. XAF1 controls proteasomal degradation of proteins through the interaction with ubiquitin E3 ligases (12, 13). Meanwhile, the protein stability of MTs has been known to be regulated mainly through the lysosomal pathway (33). We determined whether XAF1 down-regulation of MTs is affected by the proteasome inhibitor MG132 and/or the lysosome inhibitor Leupeptin. In HCT116 cells with a tetracycline-inducible system (Tet-XAF1), XAF1 down-regulation of MT2A and MT1A was blocked by Leupeptin but not affected by MG132 (Fig. 2*A* and Fig. S2*A*). A cycloheximide (CHX) chase experiment revealed that the half-life of MT2A
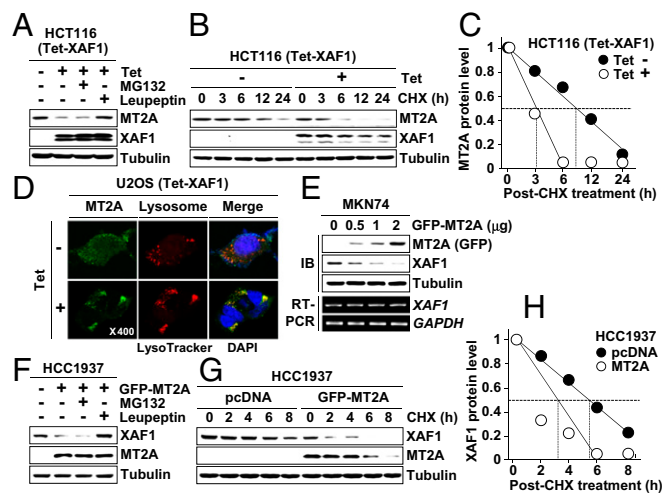

Fig. 2. XAF1 promotes lysosomal degradation of MT2A. (*A*) Blockade of XAF1-induced MT2A reduction by Leupeptin. Cells were treated with tetracycline (1 μg/mL) and then exposed to MG132 (10 μM) or Leupeptin (10 μM) for 6 h. (*B* and *C*) A CHX chase assay showing XAF1-mediated MT2A destabilization. Cells were treated with CHX (40 μM) for the indicated hours. (*D*) Immunofluorescence analysis showing XAF1 stimulation of MT2A lysosomal translocation. (*E*) MT2A down-regulation of XAF1 protein expression. (*F*) Blockade of MT2A-induced XAF1 reduction by Leupeptin. (*G* and *H*) A CHX chase assay showing MT2A-mediated XAF1 destabilization (*G*) and comparison of XAF1 protein levels in control and MT2A-overexpressed cells following CHX treatment (*H*).
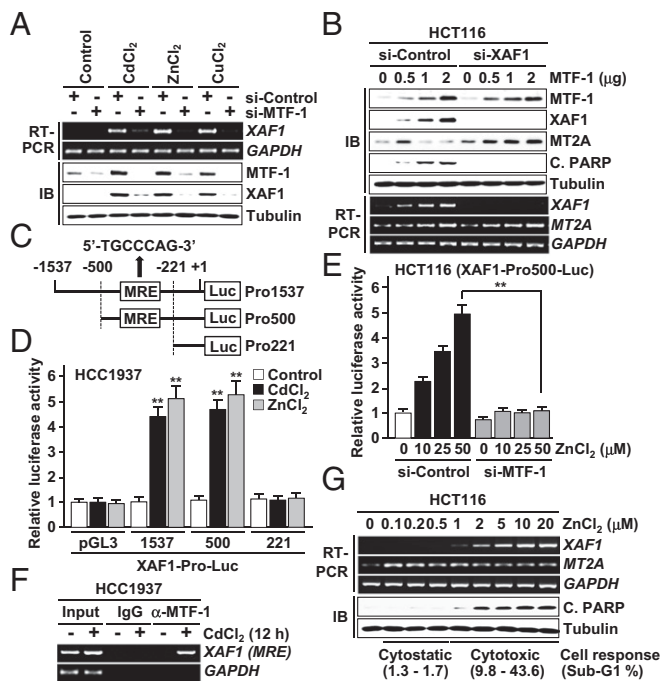
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Fig. 3. Identification of XAF1 as a direct transcriptional target of MTF-1. (*A*) Activation of *XAF1* mRNA expression by heavy metals. Cells were treated with heavy metal (100 μM) for 24 h. (*B*) MTF-1 induction of apoptosis and its attenuation by XAF1 depletion. (*C*) A MRE in the *XAF1* promoter and reporter construction for luciferase assay. (*D* and *E*) Metal activation of the *XAF1* promoter and its attenuation by MTF-1 depletion. (*F*) A ChIP assay showing MTF-1 interaction with the MRE in the *XAF1* promoter. (*G*) Activation of *XAF1* mRNA expression by cytotoxic but not cytostatic doses of ZnCl₂.

protein is shortened from 8.9 h to 3.1 h by XAF1 induction (Fig. 2 *B* and *C*). In U2OS/GFP-MT2A cells, GFP-MT2A fluorescence was decreased by XAF1, but this effect was abolished by Leupeptin (Fig. S2*B*). Moreover, XAF1 was shown to stimulate the lysosomal translocation of MT2A and MT1A, supporting that XAF1 facilitates lysosomal degradation of MTs (Fig. 2*D* and Fig. S2*C*). Intriguingly, XAF1 was also down-regulated at the protein level by MT2A and MT1A, and this effect was blocked by Leupeptin, indicating that both MTs promote lysosomal degradation of XAF1 (Fig. 2 *E* and *F* and Fig. S2 *D* and *E*). The half-life of XAF1 protein was shortened from 5.7 h to 3.3 h by overexpressed MT2A (Fig. 2 *F* and *G*). MT2A failed to decrease ΔZF5-XAF1, supporting that MT2A destabilizes XAF1 through the interaction (Fig. S2*F*). We next assessed whether interaction of XAF1 and MT2A results in their mutual degradation. Compared with individually transfected cells, cotransfected cells showed detectable reduction of both proteins, and this reduction of both proteins was blocked by Leupeptin (Fig. S2 *G–I*). Together, these results support the presence of XAF1–MT2A mutual antagonism.

XAF1 Is a Unique Transcriptional Target of MTF-1 in Signaling Apoptosis. Next we examined whether XAF1 is activated in response to heavy metals. In multiple human cells exposed to CdCl₂, ZnCl₂, and CuCl₂, XAF1 expression was markedly up-regulated at the mRNA level, and its induction showed an inverse correlation with MT2A at the protein level (Fig. S3 *A–D*). MTF-1 is a major transcriptional factor that protects cells from metal stress by activating metal-responsive genes, such as MTs, through the metal response element (MRE) in the promoters. Intriguingly, metal induction of *XAF1* mRNA was suppressed by MTF-1 depletion, and metal-induced apoptosis was blocked by either MTF-1 or XAF1 depletion (Fig. 3*A* and Fig. S3*E*). MTF-1 overexpression led to a dose-associated elevation of *XAF1* mRNA, and its apoptotic effect is abrogated if XAF1 induction

is blocked (Fig. 3*B*). In MTF-1–overexpressed cells, XAF1 induction suppressed MT2A protein but not mRNA induction. As expected, a putative MRE was identified in the 5′ upstream region (nucleotides −283/−277 relative to ATG) of the *XAF1* gene, and the reporters comprising this MRE displayed an approximately fivefold increase in luciferase activity in response to CdCl₂ and ZnCl₂ whereas this responsiveness was not detected in MTF-1–depleted cells (Fig. 3 *C–E*). A chromatin IP (ChIP) assay revealed that CdCl₂ exposure induces MTF-1 binding to the MRE in the *XAF1* promoter, indicating that the MRE is occupied by endogenous MTF-1 (Fig. 3*F*). Expression assays using increasing doses of ZnCl₂ treatment showed that *XAF1* expression is activated in response to cytotoxic (≥1 μM) but not cytostatic (0.1–0.5 μM) stress whereas *MT2A* is induced in response to both stresses (Fig. 3*G*).

XAF1–MT2A Antagonism Plays a Crucial Role in Cell-Fate Decisions Under Metal Stress Conditions. We investigated whether the XAF1–MT2A antagonism is linked to cellular response to metal stress. In cells exposed to cytostatic doses of CdCl₂ (<5 μM), ZnCl₂ (<1 μM), and CuCl₂ (<100 μM), MT2A was up-regulated and XAF1 was down-regulated (Fig. 4 *A* and *B*). By contrast, when the cells were exposed to cytotoxic doses, XAF1 was up-regulated and MT2A was down-regulated. The metal dose-associated reduction of XAF1 and MT2A was blocked by depletion of MT2A and XAF1, respectively, indicating that the opposite responses of XAF1 and MT2A to metal stress results from their mutual antagonism (Fig. 4*C*). Compared with control cells, XAF1-depleted cells manifested a substantially decreased apoptotic response to cytotoxic metal stress, and XAF1-overexpressed cells showed apoptotic response to cytostatic metal stress (Fig. 4*D* and Fig. S4 *A–D*). ΔZF5-XAF1 failed to evoke this effect. Either XAF1 overexpression or MT2A depletion directed apoptotic switch of cellular response to cytostatic stress, and either MT2A overexpression or XAF1 depletion protected cells from cytotoxic stress (Fig. 4 *C* and *E* and Fig. S4*D*). Moreover, MT2A depletion-induced apoptotic sensitization was debilitated when XAF1 was codepleted, and XAF1 depletion-induced cell protection was abolished when MT2A was codepleted (Fig. S4 *E–I*).
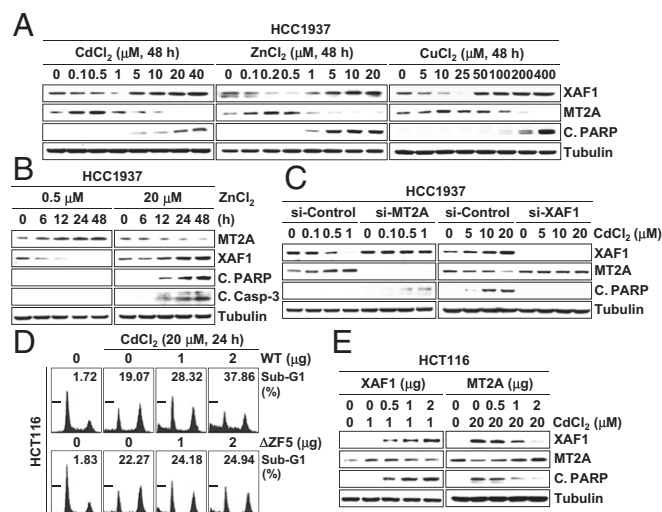

Fig. 4. XAF1–MT2A antagonism regulates cellular response to metal stress. (*A* and *B*) IB assays showing opposite responses of XAF1 and MT2A to cytostatic and cytotoxic metal stresses. (*C*) A crucial role for XAF1–MT2A mutual antagonism in cellular response to metal stress. Cells were treated with indicated doses of CdCl₂ for 24 h. (*D*) Enhancement of metal-induced apoptosis by WT-XAF1. (*E*) XAF1-mediated apoptotic switch of cellular response to cytostatic metal stress and MT2A attenuation of apoptotic response to cytotoxic metal stress.
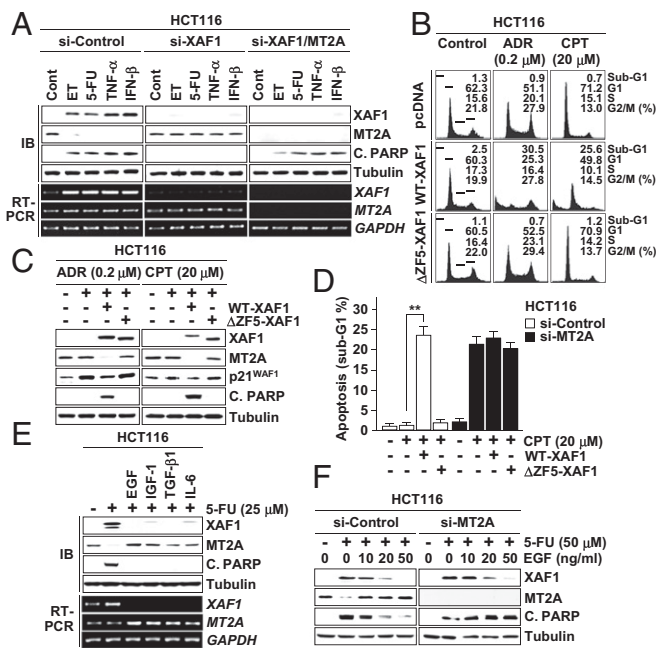
Fig. 5. XAF1–MT2A antagonism plays a key role in cellular response to nonmetal stresses. (*A*) A role for XAF1–MT2A interplay in cellular response to genotoxic and cytokine stresses. Cells were treated with ET (50 μM), 5-FU (25 μM), TNF-α (20 ng/mL), and IFN-β (200 U/mL) for 24 h. (*B* and *C*) XAF1-mediated apoptotic switch of cellular response to ADR and CPT. (*D*) Abrogation of XAF1-mediated switch of cell response to CPT by MT2A depletion. (*E*) Inhibitory effect of growth factors on 5-FU–induced XAF1 expression. Cells were pretreated with EGF (20 ng/mL), IGF1 (20 ng/mL), TGF-β1 (2 ng/mL), or IL-6 (2 ng/mL) and exposed 5-FU for 24 h. (*F*) Association of antiapoptotic function of EGF with its XAF1-repressing effect in 5-FU–treated cells.

MT2A-Targeting Function Is Closely Linked to XAF1's Apoptosis-Promoting Activity. Next we assessed whether the XAF1–MT2A antagonism plays a role in cellular response to nonmetal stresses. In HCT116 cells, apoptosis induction by etoposide (ET), 5-fluorouracil (5-FU), TNF-α, and IFN-β was accompanied by XAF1 induction and MT2A reduction (Fig. 5*A* and Fig. S5*A*). However, apoptosis induction and MT2A reduction were blocked when XAF1 expression was depleted, while apoptosis occurred when MT2A was codepleted. ET-induced apoptosis was strongly promoted by WT-XAF1 but not by ΔZF5-XAF1 (Fig. S5*B*). Upon exposure to adriamycin (ADR) and cisplatin (CPT), HCT116 cells exhibited p21^WAF1 but no XAF1 induction and manifested cytostatic response (G2 or G1 cell-cycle arrest) (Fig. 5 *B* and *C*). However, this cytostatic response was switched to apoptosis when WT-XAF1 was introduced and ΔZF5-XAF1 failed to induce this effect. Moreover, MT2A depletion caused apoptotic switch of cytostatic response, supporting that the MT2A-targeting role of XAF1 is crucial for its cell-fate–switching function (Fig. 5*D*). We next asked whether growth factors affect the XAF1–MT2A interplay to evoke their cell protection effect. In cells exposed to 5-FU, pretreatment with EGF, IGF1, TGF-β1, or IL-6 strongly inhibited XAF1 induction and MT2A reduction (Fig. 5*E*). Furthermore, XAF1 inhibition and antiapoptosis effects of EGF were impaired if MT2A restoration was blocked, indicating that growth factors exert their prosurvival effect through the regulation of the XAF1–MT2A axis (Fig. 5*F* and Fig. S5*C*).

XAF1 Increases the Free Intracellular Zinc Level to Promote Conformational Change of p53. MT2A is a prototype metal-binding protein that regulates intracellular zinc homeostasis, and its cell-protective function is linked to zinc-scavenging activity. To define apoptotic signaling pathways controlled by XAF1–MT2A antagonism, we examined the effect of XAF1–MT2A antagonism

on the intracellular zinc level and p53 protein, a representative apoptotic factor the conformation of which is regulated in a highly zinc-dependent manner (34). A spectrofluorimetric measurement of Zinquin revealed that the free zinc level is increased by WT-XAF1 but not by ΔZF5-XAF1 (Fig. 6*A*). Moreover, differential responses of XAF1 to cytostatic and cytotoxic doses were co-incident with the regulation pattern of p53 and expression of its target genes, such as *BAX*, *PUMA*, and *NOXA* (Fig. S6*A*). The XAF1 response to metals also correlated inversely with the expression level of XIAP, an antiapoptotic factor the stability of which is affected by heavy metals (35, 36). Expression assays of p53 using antibodies specific for WT (PAb1620) and mutant-type (PAb240) p53 revealed that XAF1 expression up- and down-regulates WT and mutant-type p53, respectively (Fig. 6*B*). Moreover, elevation of WT p53 conformation and its target expression by cytotoxic metal stress were impeded by MT2A (Fig. 6*C* and Fig. S6*B*). XAF1 regulation of p53 and XIAP was not detected in MT2A-depleted cells (Fig. S6*C*). IP assays also showed that the 5-FU–induced conformational shift of p53 is impeded when XAF1 is depleted (Fig. 6*D*). The opposite effects of XAF1 and MT2A on conformational shift of p53 were abolished when they were co-overexpressed (Fig. 6*E* and Fig. S6*D*). Comparative assays of HCT116 *p53^+/+* and *p53^−/−* sublines exposed to cytostatic dose of ADR revealed that the XAF1-induced apoptotic switch and its attenuation by MT2A are more drastic in *p53^+/+* versus *p53^−/−* cells (Fig. 6*F*). Likewise, XAF1 promotion of CdCl₂-induced apoptosis was more drastic in *p53^+/+* versus *p53^−/−* cells, further supporting that p53 is an important target of XAF1 in evoking its proapoptotic function (Fig. S6*E*).
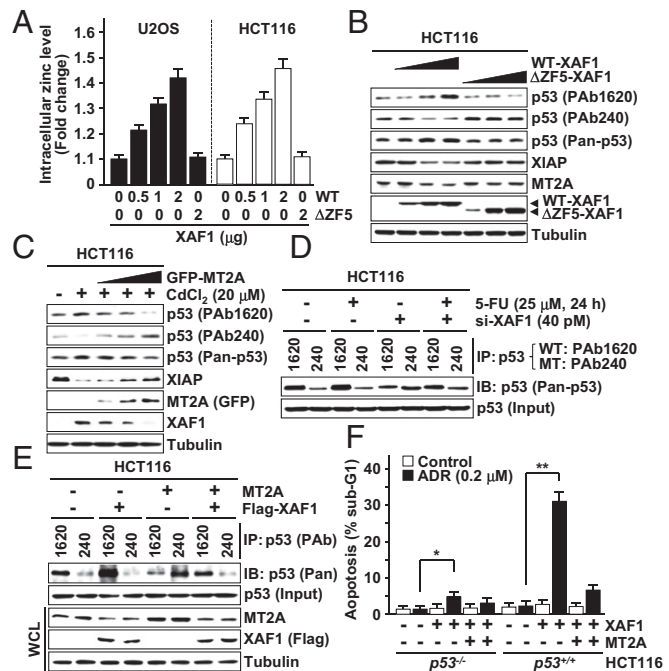

Fig. 6. A XAF1-mediated free zinc increase drives a WT shift of p53 conformation. (*A*) A spectrofluorimetric measurement of Zinquin showing a XAF1 dose-associated increment of the intracellular free zinc level. (*B*) Up-regulation of WT p53 conformation and down-regulation of XIAP level by WT-XAF1 but not by ΔZF5-XAF1. PAb1620 and PAb240 antibodies were used to detect WT and mutant type (under nondenaturing gel conditions) of p53 conformation, respectively. Pan-p53 antibody was used to determine total p53 protein level. (*C*) MT2A inhibition of cytotoxic metal stress-induced p53 conformational change and XIAP reduction. (*D*) IP assay showing a role for XAF1 in genotoxic stress-induced p53 conformational change. (*E*) Opposite effects of XAF1 and MT2A on p53 conformation. (*F*) Comparison of XAF1 ability to induce apoptotic switch of ADR-treated HCT116 *p53^+/+* and *p53^−/−* cells.
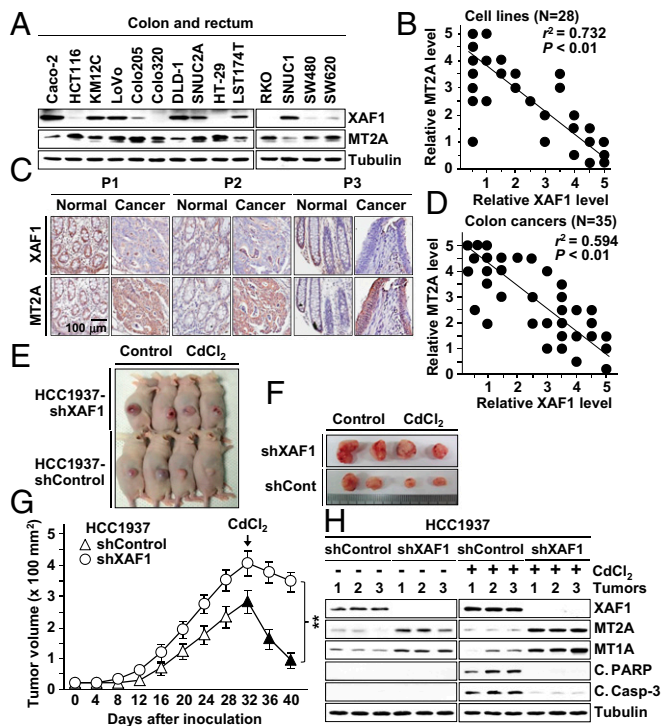
Fig. 7. Inverse correlation of XAF1 and MT2A expression in human cancers and XAF1 effect on tumor growth and response to metal stress. (*A* and *B*) Expression status of XAF1 and MT2A in human cancer cell lines. Relative expression levels were classified as levels 0–5. *r*, Pearson's correlation coefficient. (*C*) Immunohistochemical analysis of XAF1 and MT2A in human primary colon tumor and matched normal tissues. P, patient. (*D*) Inverse correlation of XAF1 and MT2A immunoreactivity in colon cancer tissues. Relative staining levels were classified as levels 0–5. (*E–G*) Mouse tumor xenograft assays showing the XAF1 depletion effect on tumor growth and response to metal stress. Data represent the mean ± SD (*n* = 6 per group; **P < 0.01). (*H*) IB assay of xenograft tumor tissues showing the XAF1 depletion effect on MT2A and cleaved PARP and caspase-3 levels.

XAF1 and MT2A Expression Levels Correlate Inversely in Human Cancers. To delineate the XAF1–MT2A interplay in human tumors, we characterized their expression status in 28 cancer cell lines of multiple tissue origins and 35 primary colon tumor tissues. A substantial fraction of cell lines and tumor tissues exhibited extremely low XAF1 and high MT2A expression, and a strong inverse correlation was detected between their levels (Fig. 7 *A–D* and Fig. S7 *A–C*). Compared with 35 normal tissues that we examined, 10 (28.6%) and 14 (40%) tumors were shown to express abnormally low XAF1 and high MT2A (Fig. 7*D* and Fig. S7*C*).

Moreover, high MT2A was detected in 8 of 10 (80%) low-XAF1 tumors but only in 6 of 26 (23.1%) normal XAF1 tumors, and low XAF1 was detected in 9 of 14 (64.3%) high-MT2A tumors but only in 2 of 21 (9.5%) normal MT2A tumors. Mouse tumor xenograft assays using XAF1-depleted HCC1937 subline cells revealed that XAF1-depleted tumors show substantially increased growth rate and delayed regression following injection of a cytotoxic dose of CdCl$_2$ (0.05 mg/g) (Fig. 7 *E–G*). Compared with control tumors, XAF1-depleted tumors showed higher MT2A and MT1A and lower cleaved PARP and caspase-3 levels (Fig. 7*H*). We next asked whether XAF1 induction could drive a switch of tumor response to cytostatic metal stress using HCT116 (Tet-XAF1) xenograft tumors. Upon exposure to low dose of CdCl$_2$ (0.02 mg/g), XAF1-induced tumors displayed drastic regression and undetectably low MT2A whereas control tumors showed only a delayed growth and elevated MT2A expression (Fig. S7 *D–F*).

Discussion

Upon heavy metal exposure, MT2A activated by MTF-1 sequesters metals to protect cells from metal cytotoxicity (19–22). However, the molecular mechanism by which MT2A is controlled under cytotoxic metal stress conditions remains undefined. MT2A degradation takes place mainly in the lysosomes, but the signaling pathways involved in the lysosomal trafficking of MT2A have been poorly understood (33). In the present study, we demonstrate first that XAF1 is a negative regulator of MT2A, the activation of which directs the apoptotic switch of cellular response to heavy metals. XAF1 has been shown to regulate protein stability through interaction with ubiquitin E3 ligases such as XIAP, cIAP1/2, ZNF313, and Siah2 (2, 12, 13, 37). Our data provide evidence that XAF1 binds to MT2A and accelerates its lysosomal relocalization, identifying its involvement in both the proteasomal and lysosomal degradation pathways. Intriguingly, it was also found that MT2A destabilizes XAF1 through the lysosomal pathway and that this activity is linked to its cell protection function. Therefore, our data support the notion that XAF1–MT2A antagonism functions as a linchpin to govern cell fate under metal stress conditions (Fig. 8).

XAF1 is a direct transcription target of p53 and IFN regulatory factor 1 (IRF-1), which is up-regulated by various apoptotic stresses (10–12, 38). In this study, we found that *XAF1* is a unique transcription target of MTF-1 in signaling apoptosis and counteracts MT2A, a prosurvival target of MTF-1. Given that MTF-1 can induce apoptosis in a highly XAF1-dependent fashion, it is plausible that MTF-1 provokes both prosurvival and proapoptotic effects depending on its target selectivity. In this context, MTF-1 might be modified differentially by cytostatic and cytotoxic stresses, allowing its selective or preferential binding to a subset of target promoters. Considering that both MTF-1 and MT2A are up-regulated and that XAF1 is down-regulated in many human cancers, alteration of the MTF-1–XAF1 and/or XAF1–MT2A axes might contribute to tumor resistance in heavy-metal–induced apoptosis.

In addition to its essential role for the sequestration of heavy metals away from critical macromolecules, MT2A contributes to chemotherapy resistance through the indirect action on p53 zinc-dependent activity (29, 30). MT2A is increased by HIPK2 knockdown, and this elevation correlates with p53 misfolding and cellular resistance to adriamycin-driven apoptosis (30). We have shown that XAF1 activates p53 by interfering with MDM2 interaction with p53 and also by activating HIPK2 (12). Our present study shows that XAF1 increases the free intercellular zinc level and activates p53 through MT2A destabilization. It is thus conceivable that XAF1 allows rapid amplification of the apoptotic program through Siah2-HIPK2 axis activation and
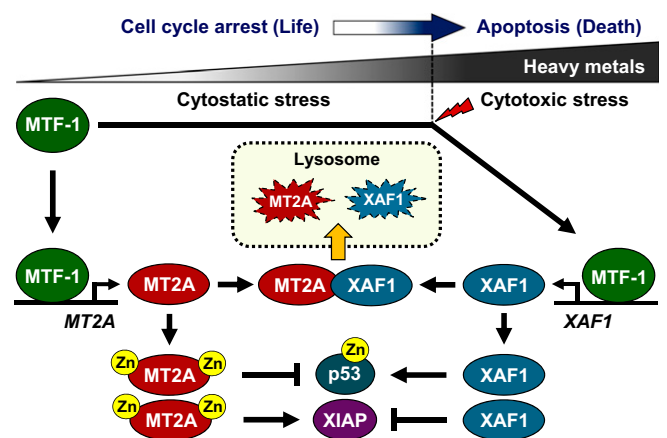

Fig. 8. Schematic representation of the antagonistic interplay of XAF1 and MT2A under metal stress conditions and its role in cell-fate decisions.

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MT2A inactivation. Consistently, we observed that the cell-fate decision function of the XAF1–MT2A axis is more drastic in $p53^{+/+}$ versus isogenic $p53^{-/-}$ cells.

XAF1 is originally identified to interact with and sequester XIAP to the nucleus (2, 3). However, XAF1's proapoptotic function is not solely dependent on the XIAP-interfering activity (6, 9). Our study shows that XAF1 induction by heavy metals leads to a drastic reduction of XIAP in a highly MT2A-dependent manner. Studies have shown that intracellular copper accumulation causes XIAP reduction through the copper-induced conformational change and that cadmium down-regulates XIAP via a proteasome-mediated mechanism, and this reduction coincides with an increased cellular sensitivity to TNF-α–induced apoptosis (35, 36). Given that MT2A presents a high affinity for copper, our study raises the possibility that XAF1-mediated MT2A destabilization increases the free intracellular copper level and thereby leads to conformational change and degradation of XIAP, lowering the apoptotic threshold and sensitizing the cell to apoptosis (39). Our study thus identifies an alternative mechanism of XAF1 inhibition of XIAP by attenuating MT2A stabilization of XIAP.

Collectively, this study demonstrates that XAF1–MT2A mutual antagonism represents one crucial molecular switch in cell-fate decisions under stressful conditions, adding a layer of complexity to the mechanisms by which stress response is regulated. Our data suggest that disruption of XAF1–MT2A interplay may contribute to the development and progression of various diseases, including malignant tumors.

Materials and Methods

Details regarding human tissues, cancer cell lines, cellular assays of cell cycle and apoptosis, expression constructs, ChIP, immunofluorescence, immunoblotting, GST pull-down, measurement of intracellular zinc concentration, and semiquantitative RT-PCR analysis are available in *SI Materials and Methods*. All animal studies were performed with the approval of Korea University Institutional Animal Care and Use Committee and Korea Animal Protection Law.

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