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Heavy Metal-induced Metallothionein Expression Is Regulated by Specific Protein Phosphatase 2A Complexes*

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Background: The molecular mechanism and key signaling pathways underlying MT expression in response to metal stress remains elusive.
Results: Upon metal stress, PP2A PR110 complexes bind to and dephosphorylate MTF-1 at Thr-254, leading to the transactivation of MTs.
Conclusion: Specific PP2A complexes regulate metal-induced MTs expression.
Significance: Delineate a novel pathway regulating metal-induced cytotoxicity and clarify the role of PP2A in cellular stress response.

Induction of metallothionein (MT) expression is involved in metal homeostasis and detoxification. To identify the key pathways that regulate metal-induced cytotoxicity, we investigate how phosphorylated metal-responsive transcription factor-1 (MTF-1) contributed to induction of MT expression. Immortal human embryonic kidney cells (HEK cells) were treated with seven kinds of metals including cadmium chloride ($CdCl_2$), zinc sulfate ($ZnSO_4$), copper sulfate($CuSO_4$), lead acetate (PbAc), nickel sulfate ($NiSO_4$), sodium arsenite ($NaAsO_2$), and potassium bichromate ($K_2Cr_2O_7$). The MT expression was induced in a dose-response and time-dependent manner upon various metal treatments. A cycle of phosphorylation and dephosphorylation was required for translocation of MTF-1 from cytoplasm to nucleus, leading to the up-regulation of MTs expression. Protein phosphatase 2A (PP2A) participated in regulating MT expression through dephosphorylation of MTF-1. A loss-of-function screen revealed that the specific PP2A complexes containing PR110 were involved in metal-induced MT expression. Suppression of PP2A PR110 in HEK cells resulted in the persistent MTF-1 phosphorylation and the disturbance of MTF-1 nuclear translocation, which was concomitant with a significant decrease of MT expression and enhanced cytotoxicity in HEK cells. Notably, MTF-1 was found in complex with specific PP2A complexes containing the PR110 subunit upon metal exposure. Furthermore, we identify that the dephosphorylation of MTF-1 at residue Thr-254 is directly regulated by PP2A PR110 complexes and responsible for MTF-1 activation. Taken together, these findings delineate a novel pathway that determines cytotoxicity in response to metal treatments and provide new insight into the role of PP2A in cellular stress response.

Toxic heavy metals such as arsenic, cadmium, lead, and mercury are ubiquitous, have no essential role in maintaining cellular homeostasis, and are known to exert multiple organ toxicities and contribute to a variety of chronic diseases (1–3). To date, environmental heavy metal contamination becomes an increasingly serious threat to human health. Currently, high level exposure to heavy metals in some regions of China remains a serious issue. For example, the Dabaoshan mine in the southeast of Guangdong Province is at high risk of multimetal pollutant discharge into a local river, Hengshihe, and the surrounding area. Previously, our group reported the high level exposure to cadmium, zinc, and lead in local environmental samples (water and crops) and blood of local residents. In addition, heavy metal exposure was associated with increased risk of behavioral disorders in school-aged children. The epidemiological data revealed that high level exposure to multiple heavy metals within the environment significantly increased the risk of mortality from cancer, such as stomach, esophageal, and lung cancers (4–6). Previous studies have demonstrated that exposure to heavy metals can cause many adverse health effects through the formation of free radicals, DNA damage, lipid peroxidation, and consumption of protein sulfhydryls, etc. (7). However, the molecular mechanism and critical signaling path-

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ways underlying the toxicity of heavy metals still remains elusive.

Previous studies have demonstrated that heavy metal-induced acute toxicity mostly depended upon enzymatic inhibition, antioxidants metabolism, or oxidative stress. However, exposure to heavy metals triggers a number of adaptive responses such as induction of metallothionein (MT),[4] which confers cells with resistance to heavy metal-induced toxicities (8). Upon heavy metals stimuli, *metallothionein* genes are rapidly transcriptionally activated and function in protecting cells from damage (9, 10). MTs are a group of intracellular low molecular (6–7 kDa), cysteine-rich, metal-binding proteins, acting as scavengers of toxic metal ions or reactive oxygen species. MTs have been implicated in the regulation of cell proliferation and apoptosis (11, 12), suggesting a role for MTs in cell survival. MT function in heavy metal detoxification primarily depends on the high affinity binding between the heavy metals and MTs, leading to the sequestration of metals away from critical macromolecules (13, 14). Moreover, the studies conducted in MT transgenic mice or MT-null mice models provide strong evidence that MTs play an essential role in protecting cells from acute heavy metal poisoning (15–18). It is evident that MTs can be a useful biomarker for the prediction of heavy metal toxicity and adverse biological outcome (19, 20).

MT expression can be transcriptionally induced by a variety of environmental stressors such as metals, oxidative stress, or hypoxia (21, 22). Metal-responsive transcription factor 1 (MTF-1) is considered to be a major activator for *MT* gene expression (22, 23). Previous reports have indicated that MTF-1 activity is mainly regulated by phosphorylation (24, 25). Although protein kinases such as protein kinase C (PKC), c-Jun N-terminal kinase (JNK), or phosphoinositide 3-kinase (PI3K) have been reported to be involved in modifying the MTF-1 signaling pathway (24, 25), the dynamic changes of phosphorylated MTF-1 in transactivation of MT remains to be defined. Because specific dephosphorylation of this transcription factor contributes to its activation (24), it is crucial to identify the specific protein phosphatases involved in transcriptional activation of MTF-1 under heavy metal stress.

Protein phosphatase 2A (PP2A) holoenzymes are ubiquitously expressed serine/threonine phosphatases, each containing a catalytic C subunit, a structural scaffolding A subunit, and a variable B regulatory subunit. The dynamic interaction of the B subunits with the core AC dimer contributes to the target specificity and subcellular localization of individual PP2A holoenzymes (26), and it is evident that specific PP2A complexes mediate particular physiological processes (27, 28). Previous studies have revealed the crucial roles for PP2A in cellular signaling pathways including transcriptional activation, cell cycle progression, apoptosis, DNA damage response, and cell transformation (27, 29–31). Our preliminary results provided evidence that inhibition of PP2A resulted in a down-regulation of MT, suggesting a role for PP2A during this process. Hereby, we speculate that PP2A may regulate cellular responses to metals through modification of the phosphorylation status of key targets such as MTF-1, in turn altering the expression of MT and metal-induced acute cytotoxicity.

In this study, we investigated the role of PP2A in the cellular stress response against the heavy metals and identify specific PP2A complexes containing the PR110 subunit that functions in regulating MT expression through dephosphorylation of MTF-1. Our results indicate the involvement of PP2A in the modulation of cellular response.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The following primary antibodies were used: mouse anti-MT (GeneTex), mouse anti-phosphoserine/threonine (BD Biosciences), mouse anti-myc tag, rabbit anti-HA tag, rabbit anti-GAPDH, rabbit anti-Lamin B1 (Cell Signaling Technology), mouse anti-PP2A Cα (1D6; Upstate Biotechnology), rabbit anti-PR110 (Proteintech Group), and rabbit anti-B56β were purchased from Novus Biologicals.

Cadmium chloride ($CdCl_2$), zinc sulfate ($ZnSO_4$), copper sulfate ($CuSO_4$), and nickel sulfate ($NiSO_4$) were purchased from Sigma. Sodium arsenite ($NaAsO_2$) was obtained from Sigma. Lead acetate (PbAc) and potassium bichromate ($K_2Cr_2O_7$) were purchased from Guangzhou Experiment Reagent (Shanghai, China). All of the chemicals were of >99% purity.

Plasmid Construction and Establishments of Stable Cell Lines—To create an HA epitope-tagged version of PP2A PR110, we performed PCR using the pEGFP-N3 wild type striatin vector (generously provided by Dr. David C. Pallas, Emory University, Atlanta) as a template and subcloned the fragment into a retroviral vector pBabe to generate a retroviral vector pBabe-puro-HA-PR110. The pBabe-puro-HA-MTF-1 and pBabe-puro- HA-B56β were generated by RT-PCR with specific primers (Table 1). The human embryonic kidney cells expressing simian virus 40 LT antigen (LT) and the telomerase catalytic subunit (*hTERT*) (HEK cells), and shRNAs against each PP2A subunit were generously provided by Dr. William C. Hahn (Dana Farber Cancer Institute, Harvard Medical School, Boston). To generate stable HEK cells, pBabe-HA-MTF-1 or pLKO-shRNAs were introduced into HEK cells by lentiviral infection and selected with puromycin (1 μg/ml).

Detection of Phosphatase Activity—The protein phosphatase activity in PP2A C immune complexes was determined as previously described (32).

Measurement of Cytotoxicity—HEK cells were seeded in 96-well plates with a density of 8×10^3 per well. After 24 h, the HEK cells were treated with seven kinds of heavy metal for 24 h. The concentrations for each heavy metal compound were $CdCl_2$ (0, 10, 20, 40, 80, and 160 μM); $ZnSO_4$ (0, 25, 50, 100, 200, and 400 μM), $CuSO_4$ (0, 50, 100, 200, 400, and 800 μM), PbAc (0, 50, 100, 200, 400, and 800 μM), $NiSO_4$ (0, 100, 200, 400, 800, and 1600 μM), and $NaAsO_2$ (0, 3.13, 6.25, 12.50, 25.00, and 50.00 μM), and $K_2Cr_2O_7$ (0, 3.13, 6.25, 12.50, 25.00, and 50.00 μM). Cytotoxicity was measured by modified MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2-H-tetrazolium bromide) assay using a cell proliferation kit (WST-1). The IC_{50} (the concentration that caused 50% growth inhibition) was calculated by the modified Karbers method (33) according to the formula: IC_{50} =

[4] The abbreviations used are: MT, metallothionein; MTF-1, metal-responsive transcription factor-1; p-MTF-1, phosphorylated MTF-1; PP2A, protein phosphatase 2A; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; co-IP, co-immunoprecipitation.

TABLE 1
Primer sequences used in cloning
Underlined sequences represent a site for restriction enzyme.

Plasmid	Vector	Restriction enzyme	Primer (5′-3′)
4HA	*pBabe-puro*	BglII	GGAAGATCTATGGCTTACCCATACGATG
		SnaBI	GGCCTACGTAAGCGTAATCTGGAACGTC
myc-MTF-1	*pBabe-puro*	SnaBI	GGCTACGTAATGGAGCAGAAACTCATCTCTGAAGAGGATCTGGGGGAACACAGT
		SalI	GGCGGCGTCGACTCACTTGGAGAAGCT
HA-MTF-1	*pBabe-4HA*	SnaBI	GGCGGCTACGTAATGGGGGAACACAGT
		SalI	GGCGGCGTCGACTCACTTGGAGAAGCT
HA-PR110	*pBabe-4HA*	SnaBI	GGCGGCTACGTAATGGACGAGCAGGCG
		SalI	GGCGGCGTCGACTCATACAAAGACTTT
HA-B56β	*pBabe-4HA*	SnaBI	GGCGGCTACGTAATGGAGACGAAGCTG
		SalI	GGCGGCGTCGACCTAGCTCTGACC
MT1A	*pGL3*	XhoI	GGCGGCCTCGAGGGTGGCTGTGAATGA
		HindIII	GGCGGCAAGCTTCCAAGCAAGAAGTTG
HA-MTF-1-S5A	*pBabe-4HA*	SnaBI	ATGGGGGAACACGCTCCAGACAAC
		SalI	GTTGTCTGGAGCGTGTTCCCCCAT
HA-MTF-1-T252A	*pBabe-4HA*	SnaBI	AAGCACATTCGAGCTCATACAGGGGAA
		SalI	TTCCCCTGTATGAGCTCGAATGTGCTT
HA-MTF-1-T254A	*pBabe-4HA*	SnaBI	ATTCGAACTCATGCAGGGGAAAAGCCA
		SalI	TGGCTTTTCCCCTGCATGAGTTCGAAT
HA-MTF-1-S305A	*pBabe-4HA*	SnaBI	AGCACTCAATACAGTCTCAAAAGTCAC
		SalI	GTGACTTTTGAGACTGTATTGAGTGCT
HA-MTF-1-S620A	*pBabe-4HA*	SnaBI	CAGATTGGCCTCGCTGTTCCTGTGATC
		SalI	GATCACAGGAACAGCGAGGCCAATCTG

$\log^{-1}[X_k - i\,(\Sigma P - 0.5)]$, in which X_k represents the logarithm of the highest chemical concentration, i is that of the ratio of adjacent concentration, and ΣP is the sum of the percentage of growth inhibition at various concentrations.

Extraction of Cytoplasmic and Nuclear Fractions—Nuclear extract was prepared by the NE-PER TM Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL). Briefly, harvested cells were suspended in Cytoplasmic Extraction Reagent I and incubated on ice for 5 min before Cytoplasmic Extraction Reagent II was added. After shaking vigorously for 10 s, the homogenate was centrifuged at 16,000 × *g* for 5 min at 4 °C, and the supernatant was defined as the cytoplasmic fraction. The pellet was resuspended in ice-cold nuclear extraction reagent and rocked vigorously at 4 °C for 40 min. The mixture was centrifuged at 16,000 × *g* for 10 min, and the supernatant was collected as the nuclear fraction.

Immunoblotting Analysis—HEK cells were suspended in ice-cold lysis buffer (150 mmol/liter NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, and 50 mmol/liter Tris (pH 7.4)) containing protease inhibitors (Roche Applied Science). The lysates were centrifuged at 12,000 × *g* for 20 min at 4 °C. The supernatant was removed and transferred to a new tube and stored at −80 °C. For analysis of the content of metallothionein, HEK cells were lysed directly on the plate using 2× SDS sample buffer (125 mM Tris-base, 138 mM SDS, 10% β-mercaptoethanol, 20% glycerol, bromphenol blue (pH 6.8)). Soluble proteins (50 μg) were subjected to 8–16% gradient acrylamide gel for SDS-PAGE before immunoblotting.

Co-immunoprecipitation (Co-IP)—For immunoprecipitation, 293FT cells were lysed in a 0.3% CHAPS lysis buffer. Cell lysates (3 mg) were incubated with the HA tag or PP2Ac (clone 1D6) antibody overnight at 4 °C followed by the addition of protein G-Sepharose beads (GE Healthcare) for 2 h at 4 °C. The protein G beads were eluted in 2× SDS sample buffer followed by SDS-PAGE and immunoblotting.

Laser Scanning Confocal Microscopy Analysis—HEK cells were grown overnight on the coverslips. After treatment with 40 μM $CdCl_2$ for 12 h, the cells were fixed in 4% formaldehyde for 15 min, washed in PBS, permeabilized in 0.2% Triton X-100, washed and blocked with PBS containing 0.3% FBS for 1 h, and followed by an incubation with specific antibody against the HA tag overnight. Alexa Fluor 488-conjugated goat anti-rabbit IgG second antibody (1:1000) was incubated for 1 h then counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/ml) and observed with a LSM510 META laser scanning confocal microscope (Carl Zeiss) under oil with ×100 magnification.

Vector Construction and Luciferase Reporter Assay—To create luciferase reporter construct pGL3-MT1A-promoter, cDNA (100 ng) from HEK cells served as a template to amplify MTIA promoter (NC_000016.9) and was cloned into the XhoI and HindIII sites downstream of the luciferase reporter gene in pGL3 plasmid.

For the luciferase reporter assay, HEKSHGFP, HEKSHB56β, and HEKSHPR110 cells expressing HA-tagged MTF-1 were grown in a 96-well plate for 24 h and then transiently co-transfected with 50 ng pGL3-MT1A-promoter and 25 ng of pRL-TK by Lipofectamine 2000 (Invitrogen). pRL-TK was used as an internal control (Promega, Madison, WI). 24 h after transfection, the cells were treated with or without 40 μM $CdCl_2$ for 12 h and analyzed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instructions.

Statistical Analysis—The results are presented as the mean ± S.D. for at least three replicate experiments. Differences between treatment groups were analyzed by a one-way analysis of variance followed by LSD multiple comparison tests. Differences were considered statistically significant when $p < 0.05$.

RESULTS

MT Expression Is Induced by Heavy Metals—It has been demonstrated that the induction of MT expression increased by several-fold in response to many kinds of heavy metals or oxidative stress (34, 35). We first examined the effect of seven kinds of heavy metal compounds including cadmium chloride

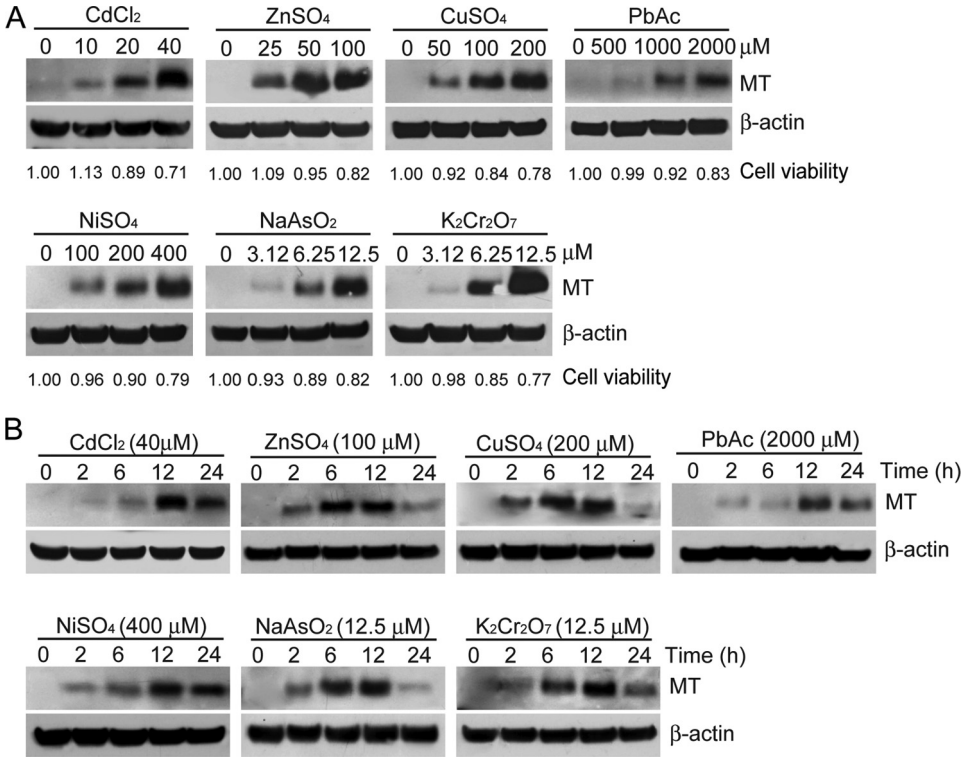

FIGURE 1. **MT expression is induced by metals.** *A*, dose-dependent induction of MT expression by metals. HEK cells were treated with $CdCl_2$, $ZnSO_4$, $CuSO_4$, PbAc, $NiSO_4$, $NaAsO_2$, and $K_2Cr_2O_7$ at the concentrations indicated for 12 h and followed by immunoblotting analysis with an antibody against MT. Cytotoxicity was measured by using MTT assay at the indicated concentrations. The relative cell viability corresponding to the amount of MT is calculated as the ratio of each dose of a particular metal to that observed in control cells treated with a vehicle and is indicated *under each lane*. *B*, time-dependent induction of MT proteins. HEK cells were treated with metals at the indicated concentrations for 2, 6, 12, or 24 h, and the cell lysates isolated at each time point were subjected to immunoblotting analysis using the antibodies indicated.

($CdCl_2$), zinc sulfate ($ZnSO_4$), copper sulfate($CuSO_4$), lead acetate (PbAc), nickel sulfate ($NiSO_4$), sodium arsenite ($NaAsO_2$), and potassium bichromate ($K_2Cr_2O_7$) on the induction of MT expression. In parallel, we measured the cytotoxicity of these heavy metals in HEK cells using MTT assay. The IC_{50} values for $CdCl_2$, $ZnSO_4$, $CuSO_4$, PbAc, $NiSO_4$, $NaAsO_2$, and $K_2Cr_2O_7$ were determined as 72.5, 225.4, 413.8, 463.1, 854.0, 40.2, and 22.6 μM, respectively. The highest concentration of heavy metals used in induction of MT expression was ~50% of the IC_{50}. As shown in Fig. 1, the levels of MT proteins in HEK cells increased upon exposure to seven kinds of heavy metals in a dose-response and time-dependent manner. Correspondingly, the cell viability displayed dose-dependent effects ($p < 0.05$) (Fig. 1, *A* and *B*). Similar results in MT expression were also observed in HEK cells treated with six other heavy metals (Fig. 1*B*). These results indicate that the level of MT expression is a good marker for the exposure and the cellular adaptive response to heavy metals.

MTF-1 Activity Determines MT Expression through Dephosphorylation and Nuclear Translocation—MTF-1 is the key trans-activating factor for MT expression (22, 24, 36). To examine whether the amount of phosphorylated MTF-1 (p-MTF-1) determines the transactivation of MT, we transfected 293FT cells with a vector encoding HA-MTF-1. 48 h after transfection, 293FT cells were treated with diverse heavy metals for 2 h before harvesting. The cell lysates were co-immunoprecipitated with an antibody against the HA tag and followed by detection of the amount of p-MTF-1 with an antibody against Ser/Thr-phosphorylated protein. As a result, we found that these metals with the exception of $CuSO_4$ could induce phosphorylation of MTF-1, indicating a role in regulation of MT expression (Fig. 2*A*). Given the result that $CuSO_4$ had no impact on level of p-MTF-1, we speculated that there was an alternative way for copper-responsive MTs induction, which was consistent with previous findings showing that copper did not activate MT-1 transcription in Nrf-null cells (37). Because the treatment of $CdCl_2$ and $ZnSO_4$ exhibits profound effects on induction of MT expression in a relatively low concentration (Fig. 1*A*), we chose them as the representative heavy metals for the following experiments. Next, we revealed that p-MTF-1 exhibited a dynamic change in response to 40 μM $CdCl_2$ and 100 μM $ZnSO_4$ treatments. Phosphorylated MTF-1 peaked at the 2 h time and then gradually declined to a basal level at 12 h (Fig. 2, *B* and *C*). In contrast, MT expression was up-regulated gradually with the time and reached at the highest level at 12 h upon $CdCl_2$ treatment, which was inversely correlated with the reduction of p-MTF-1 (Fig. 2, *B* and *C*). These observations are in agreement with the previous findings (24) that the dephosphorylation of MTF-1 plays a central role in regulation of heavy metal-induced MT expression.

It has been revealed that MTF-1 primarily localizes in the cytoplasm and translocates to the nucleus to activate *MT* gene expression upon zinc or cadmium stimuli (38, 39). Next, we determine whether the nuclear translocation of MTF-1 can be induced by heavy metals. To this end, we established the HEK cell lines stably expressing HA-tagged MTF-1 (named as

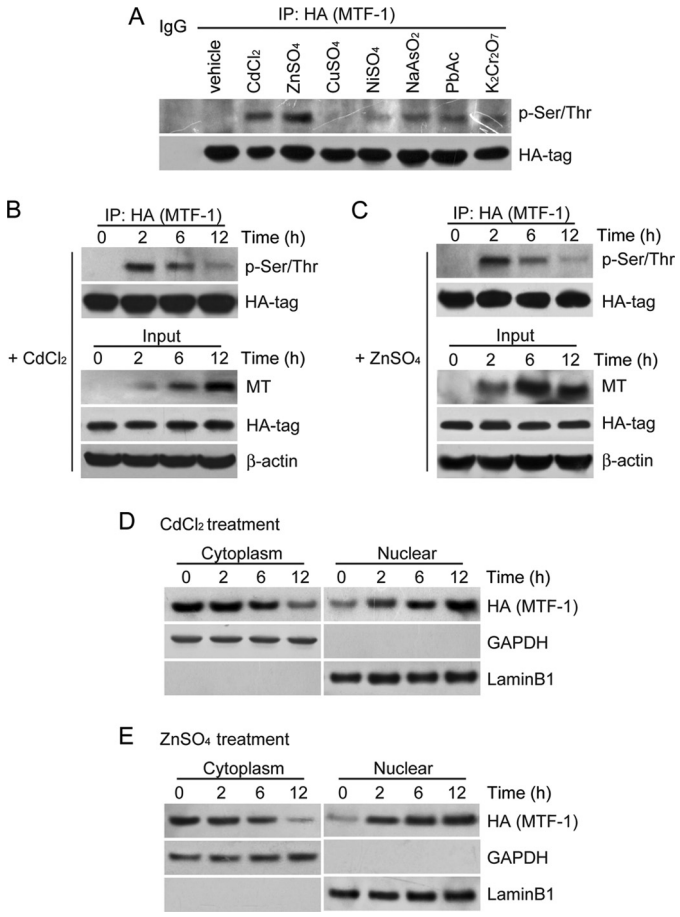

FIGURE 2. **MTF-1 activity regulates MT expression.** 293FT cells were transfected with a vector encoding HA-tagged MTF-1 for 48 h and followed by the treatment of 40 μM $CdCl_2$, 100 μM $ZnSO_4$, 200 μM $CuSO_4$, 400 μM $NiSO_4$, 12.5 μM $NaAsO_2$, 2000 μM PbAc, and 12.5 μM $K_2Cr_2O_7$ for 2 h (*A*) or 40 μM $CdCl_2$ (*B*) and 100 μM $ZnSO_4$ (*C*) for different time intervals (2, 6, and 12 h) before harvested. The co-IP assay was performed with an antibody against the HA tag and followed by immunoblotting using antibodies indicated. The *lower panels* show the input of each protein indicated at the different time points. A retroviral vector encoding HA-tagged MTF-1 was introduced into HEK cells to generate stable HEK-MTF-1 cells. The HEK cells were treated with 40 μM $CdCl_2$ (*D*) or 100 μM $ZnSO_4$ (*E*) for different time intervals (2, 6, and 12 h). Cytoplasmic and nuclear fractions were isolated, and the protein expression was examined by immunoblotting. GAPDH and Lamin B1 were used as the loading controls for cytoplasmic and nuclear fractions, respectively.

HEKHA-MTF-1 cells) and isolated cytoplasmic and nuclear fractions from cells exposed to 40 μM $CdCl_2$ at different time points (2, 6, and 12 h). Immunoblotting analysis revealed that the amount of MTF-1 in the nuclear fraction increased by 1.5-fold at 6 h and was retained at a high level at 12 h (Fig. 2*D*). Accordingly, the level of MTF-1 in the cytoplasmic fraction declined at 6 h after $CdCl_2$ treatment. A similar pattern was observed by exposing cells to 100 μM $ZnSO_4$ (Fig. 2*E*). Consistent with these findings, MT expression gradually increased and reached the highest level at 12 h in response to $CdCl_2$ or $ZnSO_4$ treatments (Fig. 2, *B* and *C*). Taken together, these observations suggest that the reduction of phosphorylated MTF-1 and subsequent nuclear import of MTF-1 are required for the induction of MT expression.

PP2A Is Involved in Dephosphorylation of MTF-1—Because the dephosphorylation of MTF-1 is responsible for up-regulation of MTs expression, we assess whether the MTF-1 dephosphorylation is mediated by PP2A, a large family of holoenzymes that accounts for the majority of Ser/Thr phosphatase activity in eukaryotic cells. We first examined whether MT expression was affected by PP2A activity in HEK cells stably expressing two independent PP2A Aα-specific shRNAs (named as HEKSHAα-1 and HEKSHAα-2 cells). Two shRNAs decreased Aα expression by ~50% compared with the control cells (HEKSHGFP) as determined by immunoblotting analysis (Fig. 3*A*), resulting in a significant reduction of the PP2A-attributable phosphatase activity by 46.5% and 54.3%, respectively (Fig. 3*B*). Notably, suppression of Aα led to a decrease in MT induction in HEK cells treated with $CdCl_2$ or $ZnSO_4$ in a dose-dependent manner (Fig. 3, *C* and *D*). In line with the MT expression, cell viability in HEK cells expressing two independent shAα showed a 17.0 $\pm$ 3.3 or 16.2 $\pm$ 6.1% decrease compared with the control cells with the treatment of different concentrations of $CdCl_2$ or $ZnSO_4$ ($p < 0.05$) (Fig. 3, *C* and *D*). These findings indicate that suppression of PP2A activity sensitized cells to heavy metal-induced cytotoxicity.

To explore whether PP2A participates in MTF-1 dephosphorylation, we analyzed the amount of p-MTF-1 in 293FT cells expressing two shRNAs against Aα (named as shAα-1 and shAα-2 cells). As shown in Fig. 3*E*, we found that the levels of p-MTF-1 in shAα-1 or shAα-2 cells were elevated by 2.3~2.5-fold, respectively. Consistent with these findings, we observed that the induction of MT reduced by 50% and 75%, respectively (Fig. 3*E*). These results suggest that PP2A activity may be involved in regulation of MT expression through dephosphorylation of MTF-1.

Identification of Specific PP2A Complexes in Regulation of MT Expression—To identify which PP2A complexes participated in the regulation of MT expression, we performed a loss-of-function screen in HEK cells expressing shRNAs against each of the PP2A regulatory subunits. Two independent shRNAs that targeted different sequences of each PP2A regulatory subunit were chosen to eliminate the off-target effects. The effects of gene suppression by introduction of shRNAs were examined by immunoblotting analysis (Fig. 4). In addition, we analyzed cytotoxicity in HEK cells expressing shRNAs treated with heavy metals by MTT assay and found that suppressing the expression of PP2A B56β, B56δ, PR130, or PR110 subunit in two independent cell lines resulted in a reduced cell viability compared with HEKSHGFP cells. Immunoblotting analysis of MT in HEK cells expressing shRNAs after heavy metal treatments showed that suppressing the expression of PP2A B56δ or PR130 led to an increase in MT expression in response to $ZnSO_4$, suggesting that B56δ or PR130 regulated heavy metal-induced cytotoxicity in a MT-independent manner. However, we observed that PP2A B56β or PR110 suppression resulted in a 62.5 $\pm$ 6.4 or 78.6 $\pm$ 4.2% declines in MT expression in HEK cells treated with 40 μM $CdCl_2$ or 100 μM $ZnSO_4$, respectively (Fig. 5*A*). Consistent with these observations, we found that PP2A B56β and PR110 suppression led to a 13.5 $\pm$ 3.7 or 23.3 $\pm$ 2.1% reduction in cell viability ($p < 0.05$) compared with HEKSHGFP cells treated with either 40 μM $CdCl_2$ or 100 μM $ZnSO_4$. Similar results were obtained when we measured cytotoxicity in a human hepatic L02 cells expressing shB56β or shPR110 (Fig. 6). Taken together, PP2A holoenzyme containing B56β

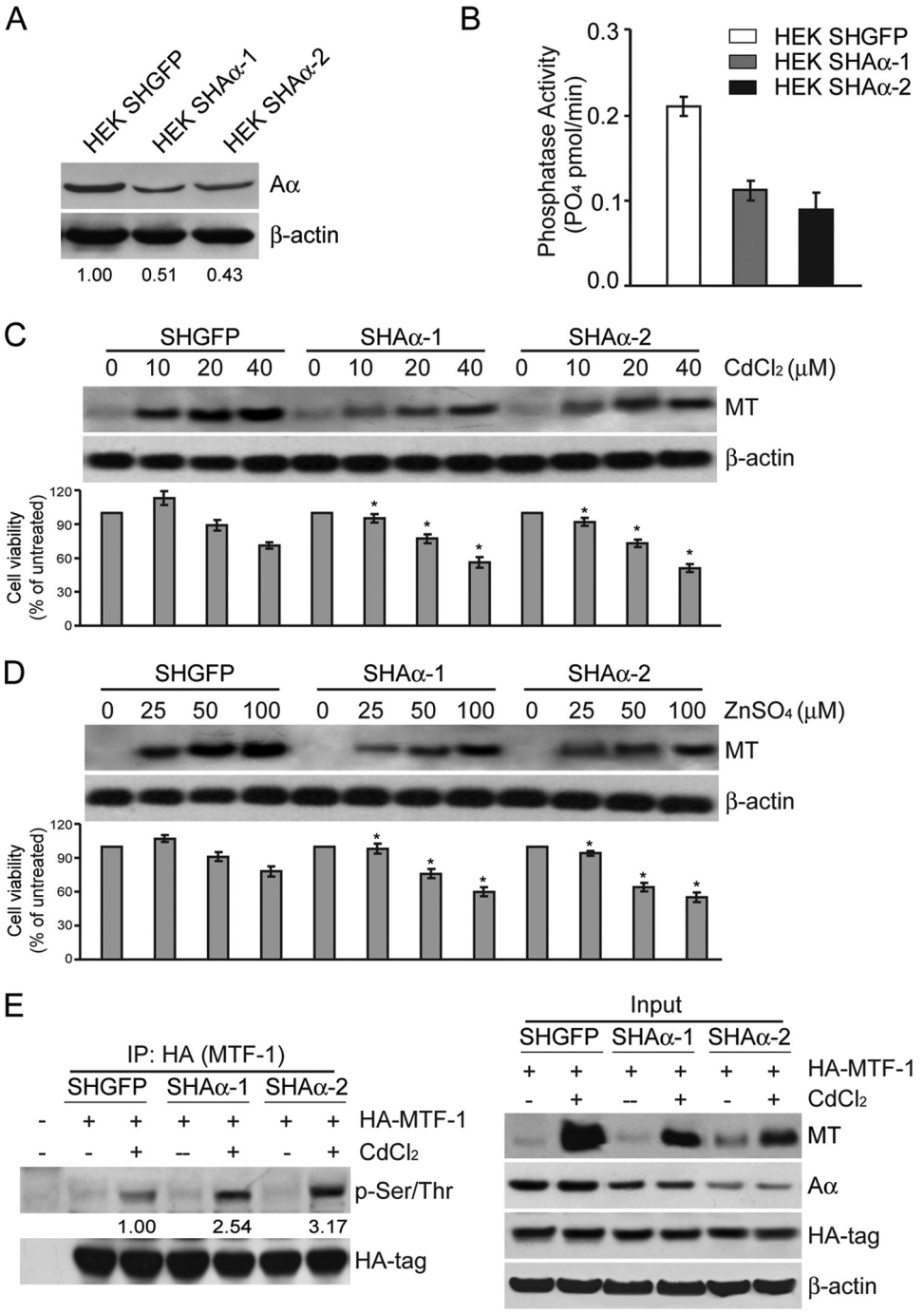

FIGURE 3. **PP2A regulates MT expression through dephosphorylation of MTF-1.** *A*, lentiviral vectors encoding two independent shRNAs targeting Aα were introduced into HEK cells, generating stable cell lines, HEKSHAα-1 and HEKSHAα-2. The *value under each band* indicates -fold change of the Aα level normalized to β-actin expression relative to control cells. *B*, PP2A-attributable activity was detected in HEKSHGFP, HEKSHAα-1, and HEKSHAα-2 cells. Shown is the immunoblotting analysis of MT expression in HEKSHGFP, HEKSHAα-1, and HEKSHAα-2 cells exposed to $CdCl_2$ at concentrations of 0, 10, 20, and 40 μM, respectively, for 12 h (*C*) or $ZnSO_4$ at concentrations of 0, 25, 50, and 100 μM, respectively, for 12 h (*D*). The corresponding cell viability at indicated concentrations was measured by MTT assay and is indicated under each lane. *, $p < 0.05$ compared with the SHGFP control group. *E*, HA-tagged MTF-1 was co-expressed with two independent shRNAs that targets Aα subunit (shAα-1 and shAα-2) in 293FT cells. The 293FT cells were treated with 40 μM $CdCl_2$ for 12 h. 3 mg of the cell lysates were subjected to co-IP with an antibody against HA tag and followed by immunoblotting analysis with specific antibodies indicated. The *value under each band* indicates -fold change of p-MTF-1 level normalized to HA-tag expression relative to SHGFP cells.

or PR110 may be putatively involved in regulating heavy metal-induced cytotoxicity through modification of MT expression.

MTF-1 Is a Direct Target of PP2A PR110 Complexes—To determine whether suppression of expression of PP2A B56β or PR110 subunit has an impact on dephosphorylation of MTF-1, we co-transfected 293FT cells with shRNA vector targeting GFP, B56β, or PR110 and a vector encoding HA epitope-tagged MTF-1. 48 h after transfection, we treated 293FT cells with 40 μM $CdCl_2$ for 6 h. The co-IP was performed with an antibody against HA tag. As a result, we found that the suppression of PR110 resulted in a 158.4 $\pm$ 63.2% elevation in p-MTF-1 after

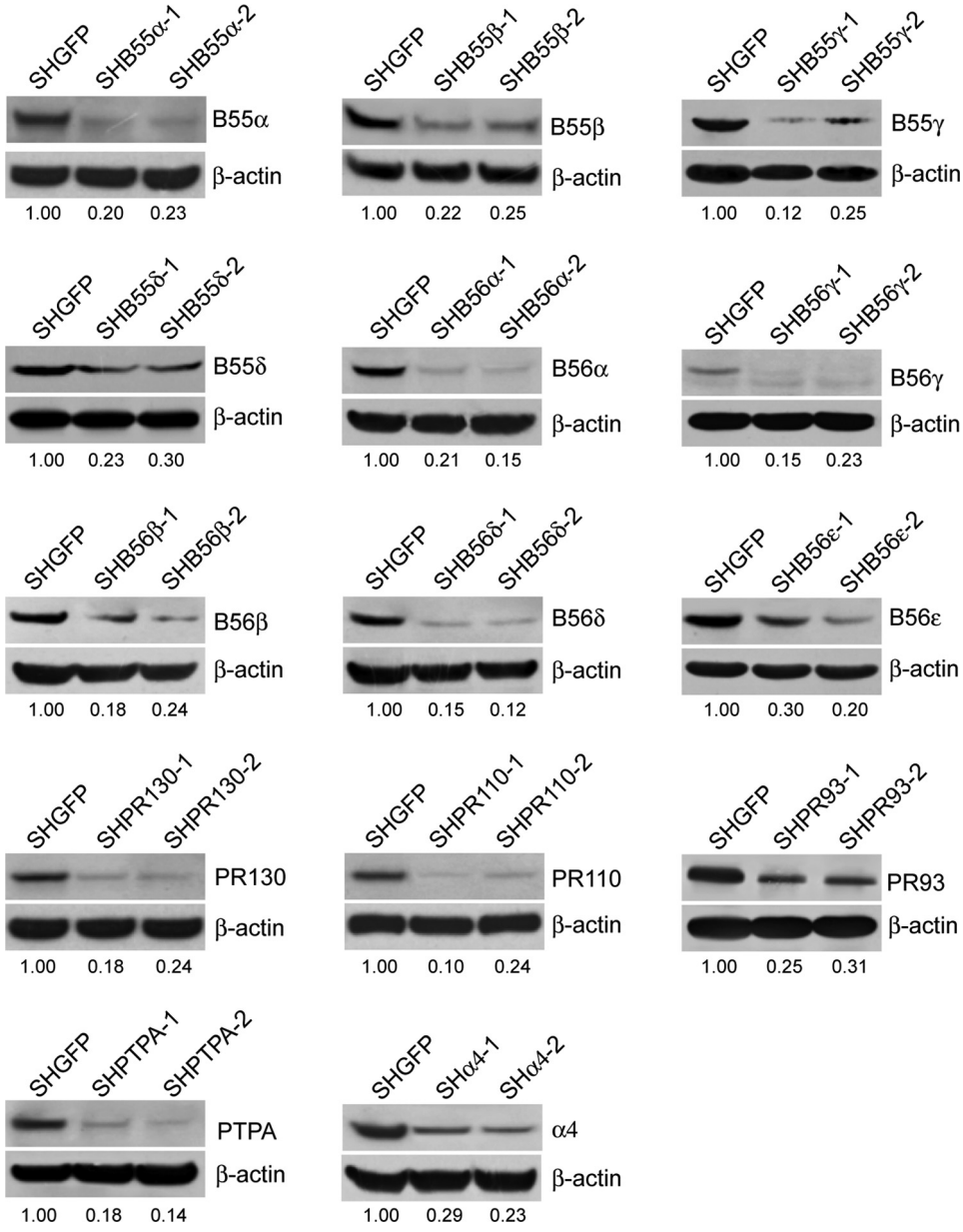

FIGURE 4. **Establishment of stable HEK cells expressing shRNAs targeting respective PP2A subunits.** HEK cells were infected with two independent shRNAs that target a specific subunit of PP2A to generate stable cell lines indicated. The suppression of PP2A subunits was confirmed by immunoblotting with the specific antibodies indicated.

$CdCl_2$ treatment (Fig. 5*B*). In concert with this observation, we detected a remarkable decrease in MT expression (Fig. 5*B*), reinforcing the notion that dephosphorylation of MTF-1 is required for transactivation of MT. Although the MT suppression was also presented in 293FT cells expressing shB56β, we failed to detect a direct interaction between MTF-1 and B56β and an increase in p-MTF-1, indicating there were alternative pathways involved in control of MT expression (Fig. 5*B*).

To address whether the direct interaction existed between PP2A complexes and MTF-1, we transfected 293FT cells with a retroviral vector encoding HA-MTF-1 for 48 h and followed by 40 μM $CdCl_2$ or 100 μM $ZnSO_4$ treatment for 6 h. The co-IP results revealed that MTF-1 was in complex with PP2Ac catalytic subunit in 293FT cells upon exposure to $CdCl_2$ or $ZnSO_4$ (Fig. 5*C*). Notably, we detected PR110 subunit presenting in the MTF-1 complex (Fig. 5, *C* and *D*), indicating a role of PP2A PR110 on dephosphorylation of MTF-1.

PP2A PR110 Complexes Are Involved in the Regulation of MTF-1 Activity—Prior studies reveal that the translocation of MTF-1 from cytoplasm to the nucleus is a prerequisite for activation of MT (36, 40). To assess whether dephosphorylation of MTF-1 by PP2A PR110 complexes mediated the nuclear import of MTF-1, we generated HEK cell lines stably expressing HA-MTF-1 in HEKSHGFP, HEKSHAα-1, HEKSHAα-2, HEKSHB56β, or HEKSHPR110 cells and visualized the localization of MTF-1 upon heavy metal treatment. Cytoplasmic and nuclear fractions of HEK cells were isolated after exposing cells to 40 μM $CdCl_2$ for 12 h. As shown in Fig. 7*A*, in addition to Aα, the suppression of PP2A PR110 resulted in a 63.0 $\pm$ 7.4% decrease in the amount of MTF-1 translocation upon $CdCl_2$

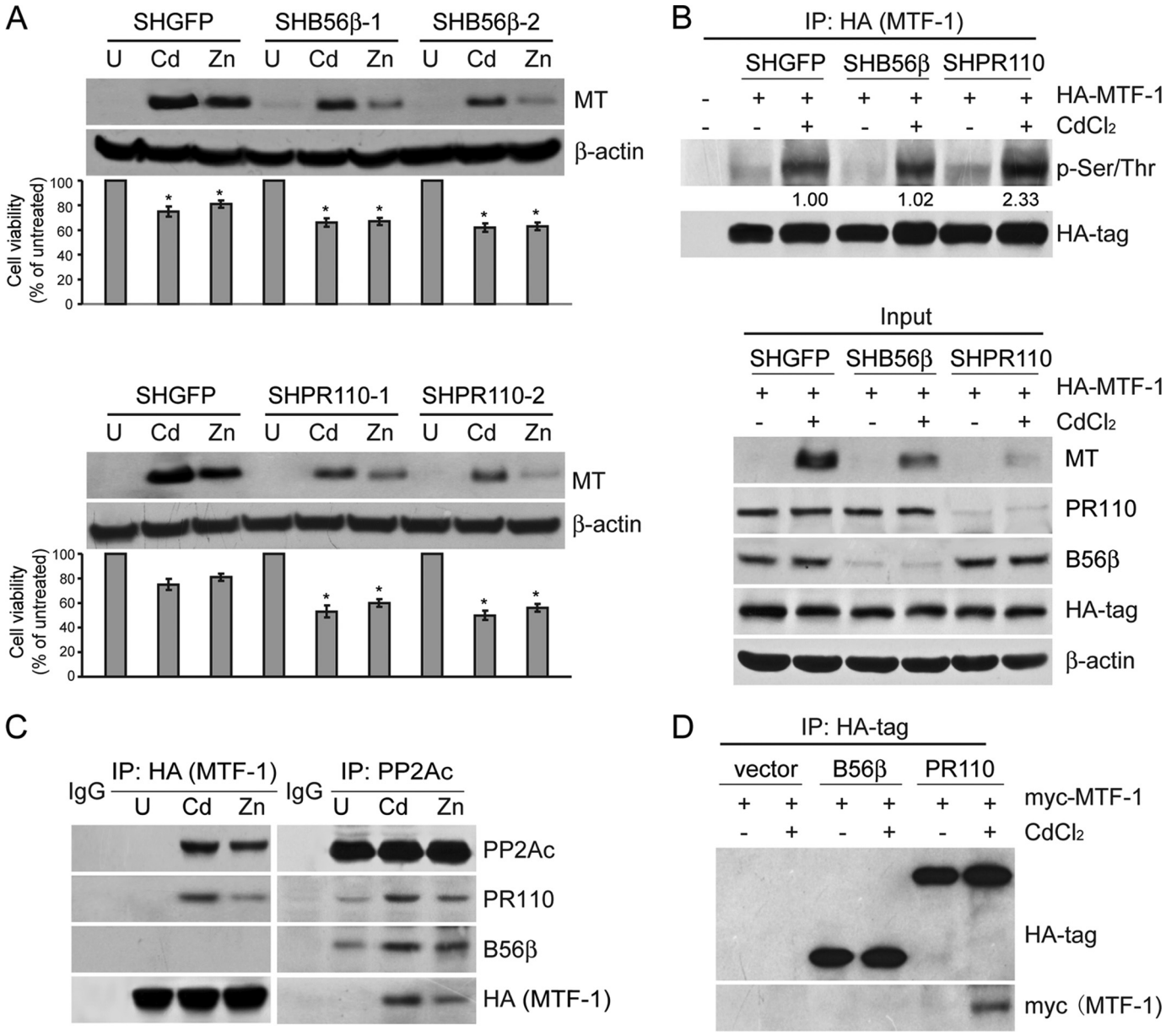

FIGURE 5. **Identification of specific PP2A complexes in regulation of MT expression.** *A*, immunoblotting analysis of MT expression in HEK cells expressing shRNA that targets GFP or expressing two independent shRNAs that target B56β or PR110 with the treatment of 40 μM $CdCl_2$ or 100 μM $ZnSO_4$. A MTT assay was performed to measure the cytotoxicity at the indicated concentrations. The corresponding cell viability is indicated *under each lane*. *, $p < 0.05$ compared with the SHGFP control cells. *B*, HA-tagged MTF-1 was co-expressed with shRNAs targeting GFP, B56β, and PR110 in 293FT cells and followed by a treatment of 40 μM $CdCl_2$ for 12 h. 3 mg of the cell lysates were co-immunoprecipitated with an antibody against the HA tag and followed by immunoblotting analysis with specific antibodies indicated. The *lower panel* shows the input of each protein indicated corresponding to the IP assay. The *value under each band* indicates -fold change of p-MTF-1 level normalized to HA-tag expression relative to SHGFP cells. *C*, 293FT cells were transfected with vectors encoding HA-tagged MTF-1 for 48 h and treated with 40 μM $CdCl_2$ or 100 μM $ZnSO_4$ for 6 h before harvesting. 3 mg of the cell lysates was subjected to co-IP using antibodies against the HA tag and PP2Ac and followed by immunoblotting using antibodies against B56β, PR110, PP2Ac, and the HA tag. *D*, Myc-tagged MTF-1 was co-expressed with HA-tagged vectors encoding B56β or PR110 in 293FT cells for 48 h and followed by a treatment of 40 μM $CdCl_2$ for 6 h. The co-IP assay was performed with an antibody against the HA tag and followed by immunoblotting using specific antibodies against the myc tag and the HA tag.

treatment. Moreover, we visualized the MTF-1 translocation under the laser scan confocal microscopy and found that the MTF-1 primarily localized in the cytoplasm in HEKSHGFP cells treated with a vehicle. However, the treatment of $CdCl_2$ resulted in a translocation of HA-MTF-1 from the cytoplasm to the nucleus. Notably, we found that the suppression of PP2A Aα or PR110 subunit disturbed this translocation (Fig. 7*B*), indicating that PP2A activity and PP2A PR110 complexes were indispensible for activation of MTF-1. In contrast, B56β suppression had no impact on the translocation of MTF-1 (Fig. 7, *A* and *B*). Consistent with these observations, the luciferase reporter assay results revealed that PR110 deficiency suppressed the transcriptional activity of MTF-1 by 34.1 $\pm$ 7.6% compared with the SHGFP cells ($p < 0.05$) upon $CdCl_2$ treatment. No difference was observed in the cells expressing shB56β (Fig. 7*C*). These observations indicate that PP2A PR110 complexes specifically regulate the activity and cellular translocation of MTF-1.

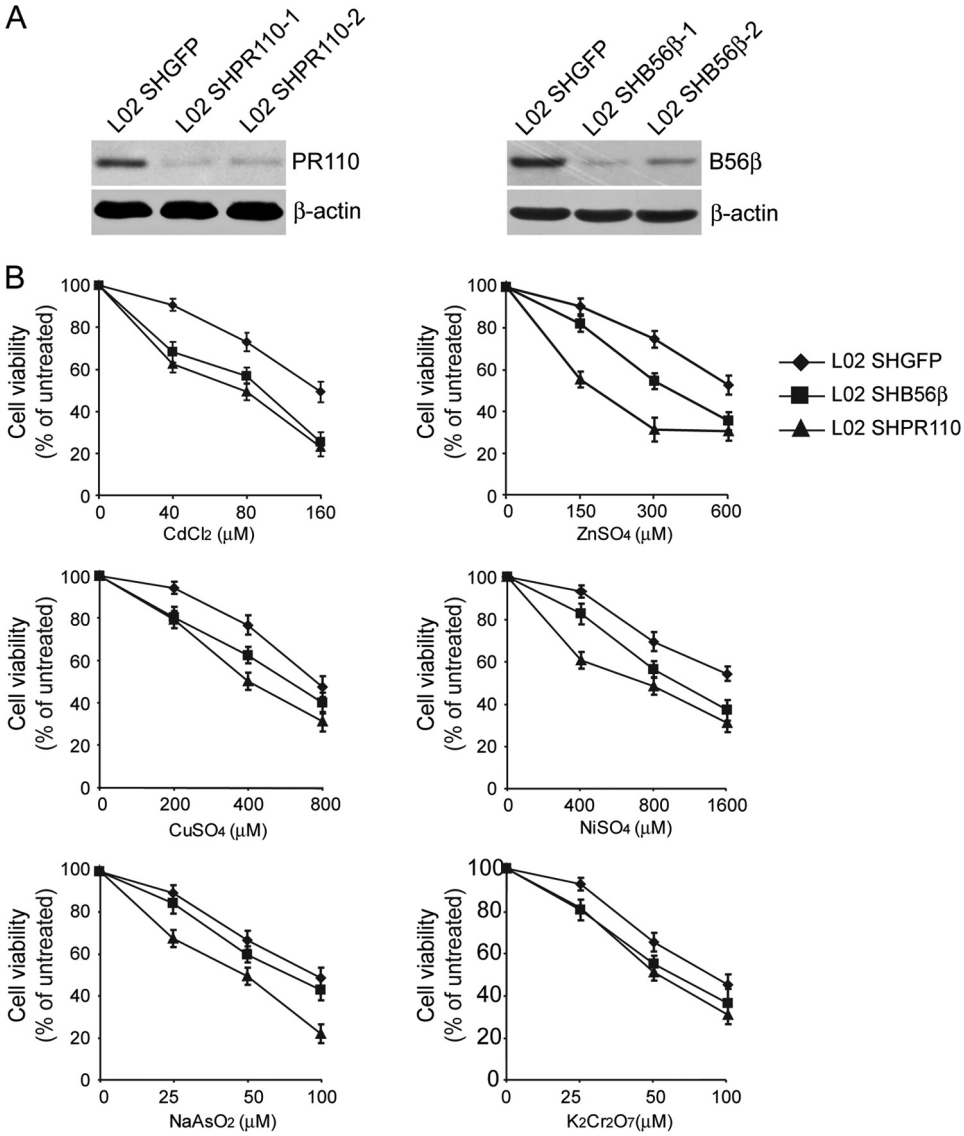

FIGURE 6. **The effects of suppression of PP2A B56β or PR110 on cytotoxicity induced by various metals.** *A*, L02 cells were infected with vectors encoding shRNAs targeting B56β and PR110 to generate stable cell lines indicated. Immunoblotting assay was performed to detect the gene suppression. *B*, the cell viability was measured upon various metal treatments at different concentrations in L02 cells with PP2A B56β or PR110 suppression. Data are presented as the mean ± S.E. from three experiments.

MTF-1 Thr-254 Is Responsible for MTF-1 Activation—It has been reported that MTF-1 can be phosphorylated at multiple sites (41). To determine which phosphorylated serine/threonine residue of MTF-1 interacts with PP2Ac, we generated vectors encoding wild type HA-MTF-1 (WT) or mutants HA-MTF-1 at S5A, T252A, T254A, S305A, and S620A (Fig. 8*A*). We expressed these vectors in 293FT cells and performed co-IP assays with an antibody against the HA tag. As a result, we found that the levels of p-MTF-1 reduced by 55∼62% in Mut-MTF-1-bound complexes at residue S5A, T254A, or S305A (Fig. 8*B*). However, we only observed a 64.3 ± 5.9% decline in the amount of PP2A PR110 complexes interacting with Mut-MTF-1 T254A upon $CdCl_2$ treatment (Fig. 8*B*). These results imply that other phosphatases may be involved in dephosphorylation of MTF-1.

To further address whether p-MTF-1 Thr-254 played a critical role in regulation of MT transactivation, we generated HEK cells stably expressing HA-MTF-1 (WT) or mutants HA-MTF-1 (Mut-MTF-1) at residue S5A, T252A, T254A, S305A, and S620A, respectively. We analyzed MT expression on these cells treated with 40 μM $CdCl_2$ for 12 h. As shown in Fig. 8*C*, the overexpression of HA-MTF-1 (WT) in HEK cells resulted in a 25% increase in MT expression. In accord with HA-MTF-1 (WT), expression of Mut-MTF-1 at residue S5A, T252A, S305A, or S620A in HEK cells led to an up-regulation of MT by 20∼30%, suggesting that the defective phosphorylation of MTF-1 at these residues did not affect MT induction. However, we failed to observe an additional transactivation of MT in HEK cells expressing Mut-MTF-1 T254A, indicating that this mutant was functionally defective in MT induction. Consistent with these results, the laser scan confocal microscopy analysis revealed that the disturbance of MTF-1 nuclear translocation only occurred in HEK cells expressing Mut-MTF-1 T254A (Fig. 8*D*). Taken together, these findings indicate that the phosphor-

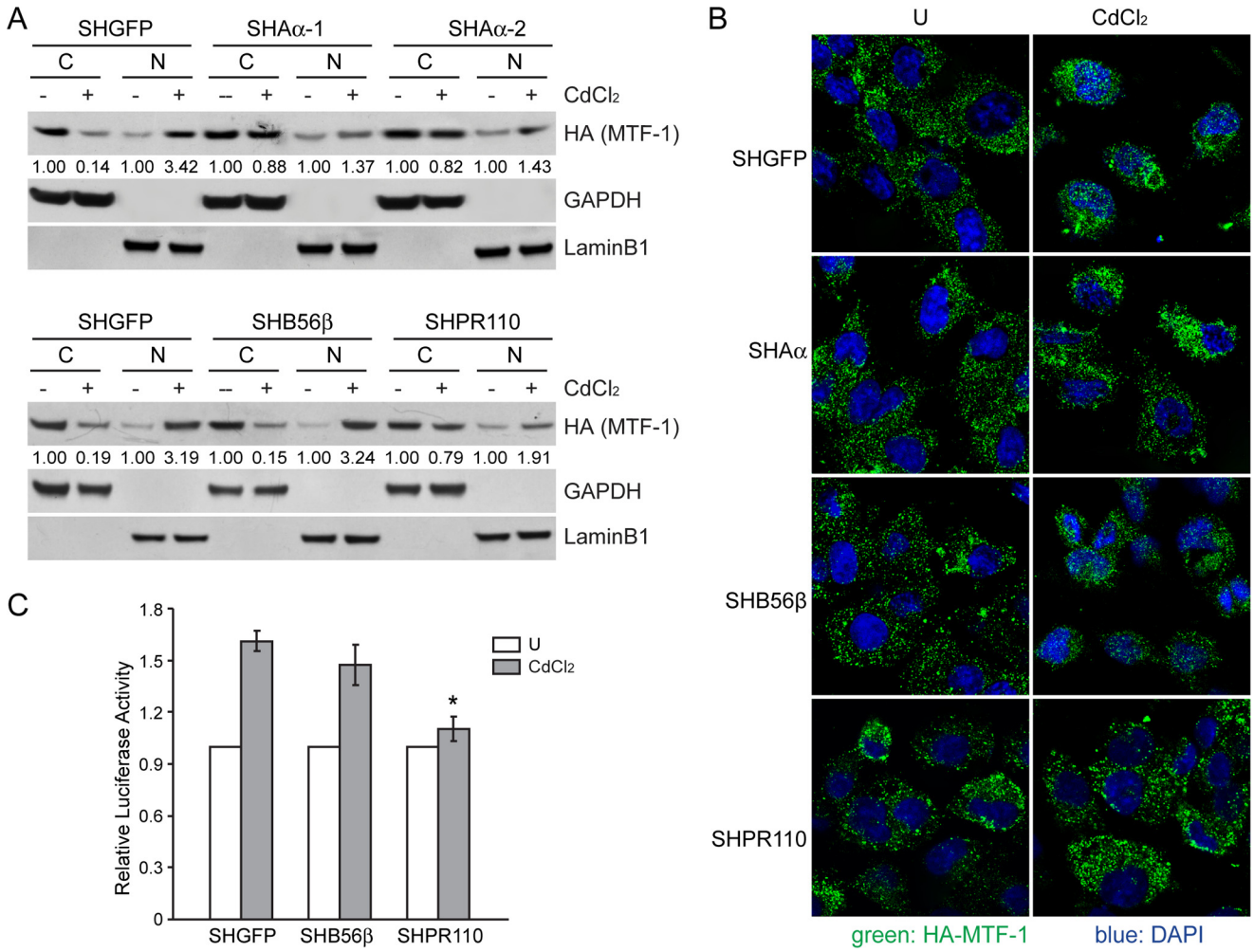

FIGURE 7. **Dephosphorylation of MTF-1 is directly regulated by PP2A PR110 complexes.** A retroviral vector encoding HA-tagged MTF-1 was introduced into HEKSHGFP, HEKSHB56*β*, and HEKSHPR110 cells. *A*, HEK cells generated were treated with 40 μM $CdCl_2$ for 12 h. Immunoblotting analysis was performed on cytoplasmic (indicated as *C*) and nuclear fractions (indicated as *N*) using specific antibodies indicated. The *value under each band* indicates the -fold change of MTF-1 level normalized to GAPDH or LaminB1 expression relative to vehicle control. *B*, HEK cells generated were treated with 40 μM $CdCl_2$ for 12 h. *U*, untreated. Immunofluorescence analysis was conducted using an antibody against HA-tag (*green*), and the representative images were taken under a laser-scanning confocal microscopy. The nuclei (*blue*) were stained with DAPI. *C*, these cells were co-transfected with pGL3-MT1A-promoter and pRL-TK for 24 h and treated with 40 μM $CdCl_2$ for an additional 12 h. Relative luciferase activity was measured. *, $p < 0.05$ compared with the SHGFP control cells.

ylation of MTF-1 at residue Thr-254 is responsible for MTF-1 activation and subsequent MT induction.

DISCUSSION

The understanding of how environmental chemicals affect cellular responses and toxicity pathways will lead to a better prediction of toxicity and adverse health outcome. In this study, we identify particular PP2A complexes containing PR110 that participate in regulation of MT expression through direct dephosphorylation of MTF-1 at Thr-254. The perturbation of this regulatory pathway sensitizes cells to heavy metal-induced cytotoxicity. Our findings uncover a key event mediated by protein phosphatases 2A in determination of cellular response to heavy metals.

Cellular response to various stresses may change the gene expression profile, which allows cells to repair the damage. MTs, a group of stress response proteins induced at a high level by reactive oxygen species or heavy metals, is generally considered to be a critical defense mechanism in several organisms (35). Previous studies have demonstrated the important roles of MTs in numerous biological effects including zinc and copper homeostasis (42, 43), metal detoxification (14, 44), and oxygen radical scavenging (45, 46) and promote carcinogenesis (47, 48). Moreover, the amount of MTs is considered as a potential biomarker for monitoring heavy metal exposure and predicting the toxic effects based on the strong correlation between MT expression and environmental heavy metal burden (20, 49). In an effort to clarify the molecular mechanism that triggers the induction of MT expression, we identify a novel pathway that is specifically involved in regulation of MT expression and plays an important role in control of cytotoxicity. A critical event in this pathway is the dephosphorylation of MTF-1 by the specific PP2A complexes. In response to heavy metal stress, alternative phosphorylation/dephosphorylation of MTF-1 triggers the translocation of MTF-1 from cytoplasm to nucleus and the following MT transactivation. This proposed regulatory model is supported by the evidence that suppressing a particular PP2A subunit PR110 or mutation at a specific residue, Thr-254, of MTF-1 abolishes the MTF-1 nuclear import and up-regulation

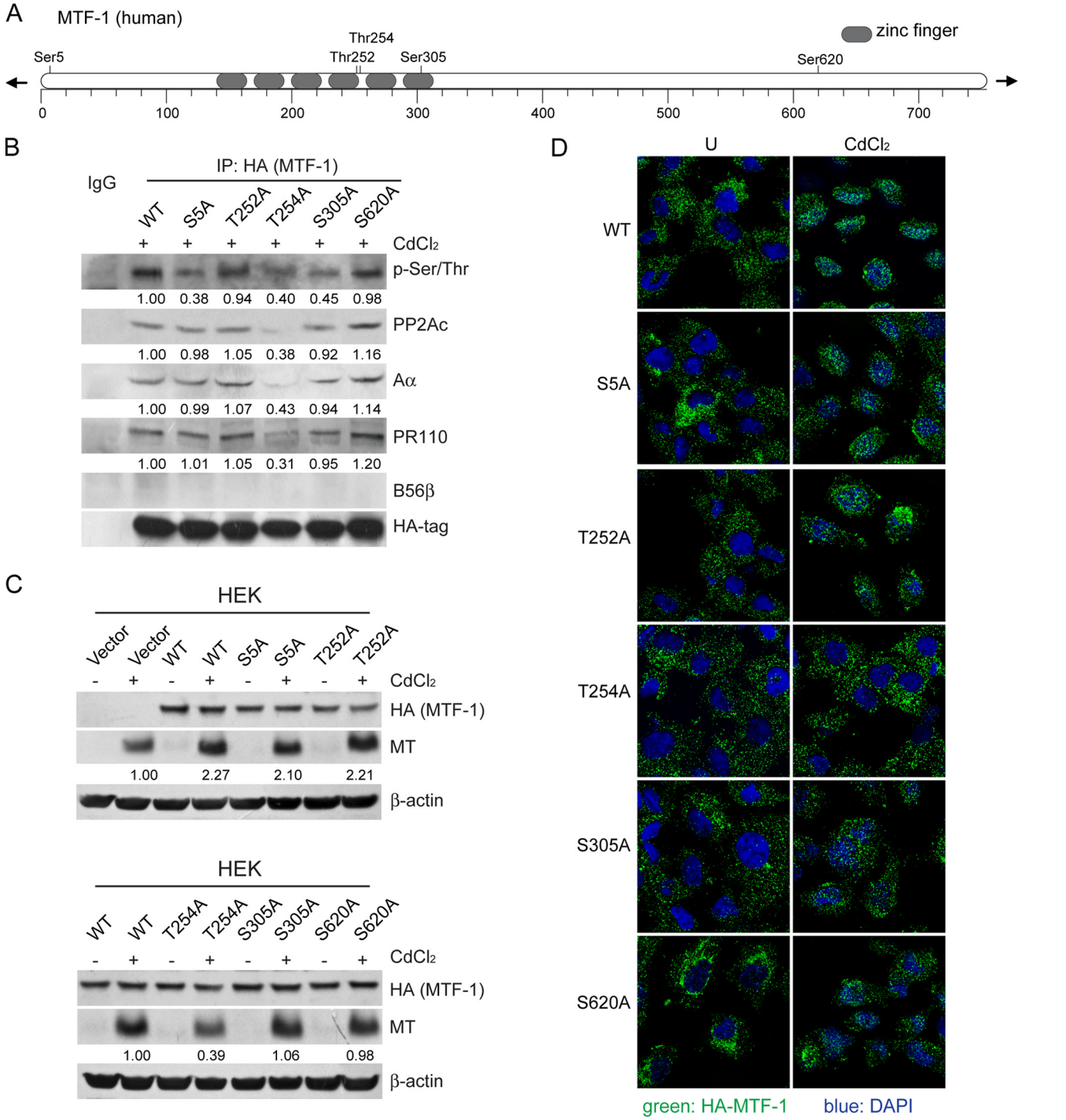

FIGURE 8. **The dephosphorylation of MTF-1 Thr-254 is responsible for MTF-1 activation.** *A*, schematic display of serine/threonine sites of the MTF-1 predicted using PhosphoSitePlus software. *B*, 293FT cells were transfected with vectors encoding wild type HA-tagged MTF-1 (HA-MTF-1 WT) or each HA-MTF-1 mutant at residue S5A, T252A, T254A, S305A, and S620A for 48 h followed by 40 μM $CdCl_2$ treatment. Co-IP was performed with an antibody against HA tag followed by immunoblotting using specific antibodies indicated. Vectors encoding HA-MTF-1 (WT) or mutants HA-MTF-1 (Mut-HA-MTF-1) S5A, T252A, T254A, S305A, and S620A were introduced into HEK cells, respectively. These cells were treated with 40 μM $CdCl_2$ for 12 h. The *value under each band* indicates the -fold change of the indicated protein levels normalized to HA-tag expression relative to WT cells. *C*, immunoblotting analysis was performed using antibodies indicated. The *value under each band* indicates the -fold change of MT level normalized to *β*-actin expression relative to Vector or WT cells. *D*, immunofluorescence analysis was performed using an antibody against the HA tag (*green*), and the images were visualized under the laser-scanning confocal microscopy. The nuclei (*blue*) were stained with DAPI.

of MT expression. Importantly, we identify that PP2A PR110 complexes directly bind to and dephosphorylate MTF-1 at Thr-254.

MTF-1 has been considered as the major transcription factor in the induction of MT by heavy metals, hypoxia, or reactive oxygen species (24, 36). The dysfunction of MTF-1 confers cells

highly sensitive to the heavy metal-induced toxic effects (36, 50). Upon heavy metal exposure or stress stimuli, MTF-1 translocates from the cytoplasm into the nucleus. Nuclear MTF-1 binds to specific DNA sequences termed as metal response elements (MREs) and in turn activates MT transcription (22, 24). It has been reported that MTF-1 activation requires stress-induced posttranslational modifications including phosphorylation (25). Although previous study demonstrates that the dephosphorylation of MTF-1 leads to its activation (24), the dynamic regulatory pattern remains unknown. In this study, we reveal that the phosphorylation and coupled dephosphorylation of MTF-1 at Thr-254 occur in response to heavy metal stress and are critical for MTF-1 activation. At an early stage, the action of phosphorylation is predominant and may reach a plateau at a certain time point. Afterward, the overwhelming dephosphorylation of MTF-1 allows the nuclear translocation of MTF-1. Moreover, we showed that T254A mutant attenuated the phosphorylation and activation of MTF-1. Similarly, in an effort to assess whether the phosphomimetic mutant T254E led to the induction of MT, we failed to observe an interaction between PP2Ac and MTF-1 and transactivation of MT (data not shown), indicating that the dephosphorylation of p-MTF-1 at Thr-254 was prerequisite for MTF-1 activation. Based on our observations, we speculate that the dynamic phosphorylation/dephosphorylation provide a signal for MTF-1 nuclear translocation. Without a signal, the unphosphorylated MTF-1 will remain in the cytoplasm and has no impact in MT induction.

In this study, we identify a PP2A holoenzyme containing PR110 as a key regulator in the cellular stress in response to heavy metals. PR110 subunit is classified as one of the PP2A regulatory B‴ subunits, also named striatin. To date, the function of the PP2A PR110 complexes and their regulatory targets remain largely unknown. A previous study showed that PP2A bound directly to and regulated the activity of estrogen receptor α (51). In the course of regulation, PR110 subunit functioned as critical molecular anchors targeting estrogen receptor to the cell membrane and served as a scaffold for the assembly of proteins, facilitating estrogen-induced activation of endothelial NO synthase (eNOS) (52). Several lines of evidence also suggest that PP2A PR110 complexes are involved in regulating vesicular trafficking (53, 54) and the remodeling of cellular cytoskeleton (55). Here, we revealed a novel role of PP2A PR110 complexes in regulation of MT expression by directly dephosphorylating MTF-1. Given the results that suppression of PR110 expression leads to abolishment of MTF-1 nuclear translocation, we conclude that MTF-1 dephosphorylation and relocation are a prerequisite for the transcriptional activation of MT.

In this study, we also showed that PP2A B56β suppression led to a down-regulation of MTs and enhanced heavy metal-induced cytotoxicity. However, we fail to find the impact of B56β on the phosphorylation of MTF-1, suggesting that PP2A B56β regulated MT expression in a MTF-1-independent manner. Consistent with these observations, a previous study reported that the activation of PI3K/AKT pathway was involved in suppression of MT expression in primary hepatocellular carcinoma (HCC) cells through inhibition of glycogen synthase kinase-3/C/EBPα signaling (56). PP2A B56β has been implicated in the dephosphorylation of AKT (57, 58). Because glycogen synthase kinase-3 and C/EBPα are the downstream targets of PP2A, which plays a role in the regulation of cell growth and survival (59, 60), we thus speculate that PP2A B56β may affect MT expression via PI3K/AKT pathway.

In summary, we discover a novel signaling pathway in which PP2A is involved in response to the regulation of heavy metals stress. The suppression of PP2A PR110-sensitized cells to heavy metal-induced cytotoxicity is attributable to the down-regulation of MT expression. The dephosphorylation of MTF-1 at residue Thr-254 is responsible for its translocation and activation. Our study also revealed that suppression of other PP2A regulatory subunits including PR130 and B56δ subunits led to an enhanced cytotoxicity of heavy metals, indicating that alternative pathways are involved in regulation of cellular toxicity independent of MTs expression. Further study is required to elucidate the comprehensive mechanism by which PP2A contribute to cellular stress response.

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