Metastasis-associated gene 1 (MTA1) enhances cisplatin resistance of malignant pleural mesothelioma by ATR-Chk1-mediated DNA repair

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Background: Malignant pleural mesothelioma (MPM) chemoresistance remains a challenge to oncologists. In our previous study, we demonstrated that the aberrant expression of metastasis-associated gene 1 (*MTA1*) is associated with carcinogenesis and metastasis in MPM. The aim of the present study was to investigate the mechanism of *MTA1* and chemo-resistance in MPM.

Methods: Western blotting and real-time polymerase chain reaction were used to analyze the protein and mRNA levels. A stable clone with a knockdown of *MTA1* was generated with shRNA via lentivirus technology in MPM cell lines. Cell Counting Kit-8 assay and crystal violet assay were used to measure cell viability. Immunochemical staining was employed to detect *MTA1* expression in MPM tissues. The cell cycle of MPM cells was determined by phosphohistone H3 staining and flow cytometric analysis.

Results: The *MTA1* protein was upregulated and enhanced cisplatin resistance in MPM. Cisplatin stabilized the expression of the *MTA1* protein by inhibiting its ubiquitination, and *MTA1* enhanced G2/M cell cycle delay and regulated and protected the tumor genome from chemotherapeutic drugs via participating in the phosphorylation of the ataxia telangiectasia mutated and rad3 related-checkpoint kinase 1 (*ATR-Chk1*) pathway.

Conclusions: These data suggest that *MTA1* enhances cisplatin resistance by *ATR-Chk1*-mediated DNA damage repairment and cisplatin stabilizes *MTA1* expression via affecting on the ubiquitination pathway of *MTA1* in MPM. Our findings indicate that *MTA1* could serve as a novel therapeutic target to overcome chemoresistance in MPM.

Keywords: Metastasis-associated gene 1 (*MTA1*); malignant pleural mesothelioma (MPM); chemoresistance; *ATR-Chk1*

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Introduction

Malignant pleural mesothelioma (MPM) is a rare malignant cancer with a poor prognosis and few treatment options (1). More than 95% patients are diagnosed at a later stage and the median overall survival time is less than 9 months (2). The primary cause of short overall survival is insensitivity to platinum-based chemotherapeutics, which are still the main treatment for MPM (3). Therefore, understanding the molecular mechanism of chemotherapy resistance in MPM remains a challenge for clinicians and scientists.

Multiple mechanisms are involved in the anti-cancer effects of cisplatin, yet the most important mechanism is to cause DNA replication disorder, thereby inhibiting the mitosis and proliferation of cancer cells (4). Although cisplatin treatment is initially effective, cells quickly develop chemotherapy resistance, leading to treatment failure (5). Nevertheless, DNA lesions induced by cisplatin can be repaired by multiple pathways, of which the 2 most important are the nucleotide excision repair and the mismatch repair systems (6,7). Once the systems are activated, ataxia telangiectasia mutated (*ATM*) and ATM and rad3 related (*ATR*) will be phosphorylated, thereby blocking cells in the S and G2 phases and re-establishing DNA integrity (8). *ATM* is mainly activated by the ionizing radiation-induced DNA damage response. As the downstream of *ATM*, *ATR* is not only activated by *ATM* but also activated by chemotherapeutic agents, such as cytotoxic agents and antimetabolites (9,10). When activated by DNA lesions, *ATR* phosphorylates its most prominent substrate and downstream effector, checkpoint kinase 1 (*Chk1*), which protects tumor genomes against damage caused by chemotherapeutic agents (11). The *ATR-Chk1* pathway then phosphorylates multiple substates, including DNA replication, DNA repair, and cell cycle proteins, which interrelates chemoresistance of tumor cells (12). For instance, *Chk1* inactivates the cyclin-dependent kinase/cyclin complex through M phase inducer phosphatase Cdc25 (*Cdc25*), rapidly halting DNA replication in the S phase and preventing cells with damaged DNA from entering mitosis (13). It has been well established that the *ATR-Chk1* pathway promotes homologous recombination by regulating the phosphorylation of RAD51 recombinase (*RAD51*), FA complementation group E (*FANCE*), and FA complementation group D2(*FANCD2*), which is a major pathway required for the repair of DNA double-strand breaks and collapsed replication forks (14-16). Therefore, the inhibition or knockdown of *ATR* and/or *Chk1* expression can increase the sensitivity of tumor cells to a mass of DNA-damaging agents, especially DNA cross-linking agents (cisplatin) (17).

MTA1, an important part of nucleosome remodeling and histone deacetylation complexes, is widely overexpressed in multiple cancers and participates in tumorigenesis, metastasis, and chemoresistance (18,19). In our previous study, we found that *MTA1* plays an oncogenic role in promoting the metastasis of MPM via E-cadherin, leading to the poor prognosis of patients (20). Interestingly, a previous study revealed *MTA1* takes part in the *ATR-Chk1*-mediated DNA damage checkpoint pathway, which reduces the sensitivity of tumor cells to Ultraviolet (UV)-induced DNA damage (21). According to the mechanism of the *ATR-Chk1* pathway which participates in chemoresistance and the association of *ATR* and *MTA1*, we hypothesized that *MTA1* may also play an important role in the chemoresistance and cell cycle regulation of MPM cells. In recent study, we observed that cisplatin promotes the expression of the *MTA1* protein by inhibiting its ubiquitination. However, *MTA1* enhances cisplatin resistance of MPM cells by enhancing G2/M cell cycle delay. Furthermore, *MTA1* regulates the phosphorylation of the *ATR-Chk1* pathway, protecting tumor genomes against damage caused by chemotherapeutic agents. Therefore, our findings indicate that *MTA1* enhances the chemoresistance of MPM cells by regulating the *ATR-Chk1* pathway. We present the following article in accordance with the MDAR checklist (available at http://dx.doi.org/10.21037/atm-21-941).

Methods

Patients and tissue samples

Tissue samples used in the present study were obtained from 39 MPM patients with new cases. The information of patients was collected in our previous study (20). The study was approved by the Ethical Committee of the First Affiliated Hospital of Soochow University (Suzhou, China), and written informed consent was obtained from each patient. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013).

Western blotting and immunoprecipitation

Cell protein was extacted and quantitated by protein assay (Beyotime, Shanghai, China). Proteins were fractionated by

sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene fluoride membrane, blocked in 4% dry milk at room temperature for 1 h, and immunostained with primary antibodies at 4 ℃ overnight using anti-*MTA1* (1:2,000; Abcam, Cambridge, MA, USA), anti-ATR (1:1,000; Abcam, USA), anti-p-ATR (Ser428, 1:1,000; Abcam, USA), anti-ATR-interacting protein (*ATRIP*) (1:1,000; Abcam, USA), anti-Chk1 (1:10,000; Abcam, USA), anti-p-Chk1(1 µg/mL; Abcam, USA), anti- RAD17 checkpoint clamp loader component (*RAD17*) (1:500; Abcam, USA), anti- chromatin-binding protein RAD9 (*RAD9*) (1:2,000; Abcam, USA), anti- DNA topoisomerase II binding protein 1 (*TopBP1*) (1:1,000; Abcam, USA), anti-Claspin (1:500; Abcam, USA), and anti-glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (1:1,000; KangChen, Beijing, China). The bands were detected via a chemiluminescent detection system (Thermo Fisher Scientific, Rockford, IL, USA) and exposed in the Molecular Imager ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA).

For the immunoprecipitation (IP) analysis, 30 µL of protein A/G PLUS-agarose (Santa Cruz Biotechnology, Dallas, TX, USA) and 1 µg of antibody were added to 1–2 mg of protein extracts. The immunoprecipitates were collected by centrifugation in a microcentrifuge at 14,000 g for 5 min, and the supernatant was discarded after overnight incubation at 4 ℃ on a rocker platform. Nonidet P-40 buffer (50 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40, 10% glycerol, 150 mM NaCl, 2 mM $MgCl_2$, and 1 mM ethylenediaminetetraacetic acid with protease inhibitors) washed the pellet 5 times and then dissolved in a sample buffer for polyacrylamide gel electrophoresis (SDS-PAGE).

Lentivirus production and transduction

MPM cells which were transfected by lentivirus containing pLL3.7-empty and pLL3.7-sh*MTA1* were obtained from D. Yun Chen from Nanjing Medical University. The uppercase letters represent *MTA1*-specific sequence, and lowercase letters represent hairpin sequences. Recombinant lentivirus was generated from 293 T cells using calcium phosphate precipitation. MSTO-211H and H2452 were transfected with lentivirus using polybrene (8 µg/mL).

Cell culture and establishment of cisplatin-resistant MPM cell lines

MSTO-211H, NCI-H2452, and 293 T cell lines [American

Type Culture Collection (ATCC), Manassas, VA, USA] were employed for this study. MSTO-211H and H2452 were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), while 293 T cells were cultured in Dulbecco's modified Eagle's medium and high glucose medium supplemented with 10% fetal bovine serum. To establish cisplatin-resistant subclones, MSTO-211H and NCI-H2452 cells were cultured with step-wise increasing concentrations of cisplatin [concentrations from the half-maximal inhibitory concentration (IC50) to 10 folds of IC50 of wildtype cells] for 15 months, and 2 cell cisplatin-resistant lines, MSTO-DDP and H2452-DDP, were established. All cells were cultured in a humidified 37 ℃ incubator with 5% CO_2.

Cell counting Kit-8 (CCK-8) assay

Cells were plated at a density of 30,000 cells per well in a 96-well plate at 37 ℃ with 5% CO_2 for 24 h. Then, 10% CCK-8 reagent was added to each well. The optical density at 450 nm was measured on a microplate reader after incubation for 4 h (Labsystems, Helsinki, Finland).

Immunohistochemical staining

Tissues which were fixed in 4% paraformaldehyde and cut from a paraffin block to 5-µm thickness were dewaxed with xylene and rehydration with a graded series of ethanol. The slides were heated in the autoclave for 3 min using citrate buffer (pH 6.0) and incubated with primary antibody *MTA1* (1:1,000; Abcam, USA) at 4 ℃ overnight. Blocking serum or antibody dilution buffer was prepared as the negative control. The primary antibodies utilized were all the same as for the Western blot analysis. Photographs were taken by microscope (ECLIPSE 50i; Nikon, Tokyo, Japan) and NIS-Elements version 4.0 (Nikon, Tokyo, Japan). Average values of integrated optical density were obtained from 5 random fields per slide by using Image-Pro Plus software (version 5.0) (Media Cybernetics, Maryland, USA). Every data was acquired from detections repeated 3 times at least.

Real-time polymerase chain reaction (PCR) analysis

The mRNA levels of *MTA1* were detected by real-time PCR using SYBR Premix Ex Taq (TAKARA, Tokyo, Japan). The expression of β-actin was prepared as the negative control. Amplification conditions, primers, and probes sequences for *MTA1* and β-actin were from a previous

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study (20). All procedures were done in accordance with the MIQE guidelines (22).

Crystal violet assay

Cells in a 6-well plate were incubated for 24 h at 37 ℃. The medium was aspirated and the cells were washed twice in a gentle stream of tap water. The water from the wells was immediately aspirated. A total of 1 mL of 0.5% crystal violet staining solution was added to each well, and the wells were incubated for 20 min at room temperature on a bench rocker with a frequency of 20 oscillations per minute. The plates were washed 4 times and the plate was inverted on filter paper. The plate was tapped gently for 2 h to remove any remaining liquid. A total of 4 mL of methanol was added to each well, and the plate with its lid on was incubated for 20 min at room temperature on a bench rocker with a frequency of 20 oscillations per minute.

Cell cycle analysis

Cells were trypsinized and then centrifuged. The pellet was washed 2–3 times with 1× phosphate buffer saline (PBS) to exclude the remaining culture medium. The cells were fixed with 90% methanol to –20 ℃ for 10 min. Afterwards, the cells were washed twice with 1× PBS and then incubated in a solution containing 50 µg/mL of propidium iodide and 100 µg/mL of RNase A for 30 min at 37 ℃ in the dark. After incubation, the cells were centrifuged and the pellet was resuspended in 1–1.5 mL of 1× Focusing Fluid (Thermo Fisher Scientific Inc., USA). Flow cytometry analysis was conducted using the Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific Inc., USA); in each run, at least 10,000 events were collected to 200 µL/min speed, using the channel BL2-A (574/26 nm filter). The histograms, dot plots, and density plots were made using Attune NxT SW version 1.1 software. The percentage of fibroblasts in the different phases of cells cycle (G0/G1, S, and G2/M) was estimated.

Phosphohistone H3 staining and assay

Cells were harvested at the indicated time points by trypsinization and fixed with 70% ethanol. The cell pellet was suspended in 100 µL of PBS containing 0.5% bovine serum albumin and 1:50 diluted phosphohistone H3 (Ser10) antibody, followed by staining with fluorescein isothiocyanate-labeled secondary antibody. The cells were

stained with 50 µg/mL propidium iodide and 10 µg/mL RNase A after 1 h of incubation at room temperature. Cellular fluorescence was determined by a BD Biosciences FACScan (BD Biosciences, San Jose, USA), and the data were analyzed using Cell Quest software (Becton Dickinson, San Jose, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 5.01; GraphPad Software, La Jolla, CA, USA) statistical software. The Student's t-test and paired t-test were used to analyze significance between independent groups and paired materials, respectively. The χ^2-test was used to test the significance of observed differences in proportions, except when the cell size was less than 5groups (Fisher's exact tests). P<0.05 indicated statistical significance.

Results

MTA1 protein was induced following exposure to cisplatin in MPM

To explore the clinical relevance of *MTA1* expression in the regulation of cisplatin resistance in MPM, we investigated 9 MPM patients with different pathological types who developed resistance to cisplatin. We found that *MTA1* protein level increased significantly after cisplatin resistance in the patients (*Figure 1A,B*). However, there was no change in the *MTA1* mRNA level (*Figure 1C*). Subsequently, to further confirm the effect of cisplatin on the *MTA1* protein, immunohistochemistry was used to detect tissues from 2 patients, and the results suggested that the expression of the *MTA1* protein was strongly positive after cisplatin resistance (*Figure 1D*). Thirty MPM patients were divided into 2 groups using a semiquantitative immunoreactivity scoring system, as reported in our previous study (*Table 1*) (20). Our findings indicated that after cisplatin-based chemotherapy, *MPM* patients with a high expression of *MTA1* protein had significantly reduced progression-free survival (PFS) time than patients with a low expression of *MTA1* protein (*Figure 1E*). These results suggest that the expression of *MTA1* protein can be induced by cisplatin chemotherapy and could be associated with the prognosis of MPM patients.

MTA1 enhanced cisplatin resistance of MPM cells

To further clarify the relationship between *MTA1* and cisplatin *in vitro*, 2 different cell lines (H2452 and MSTO)
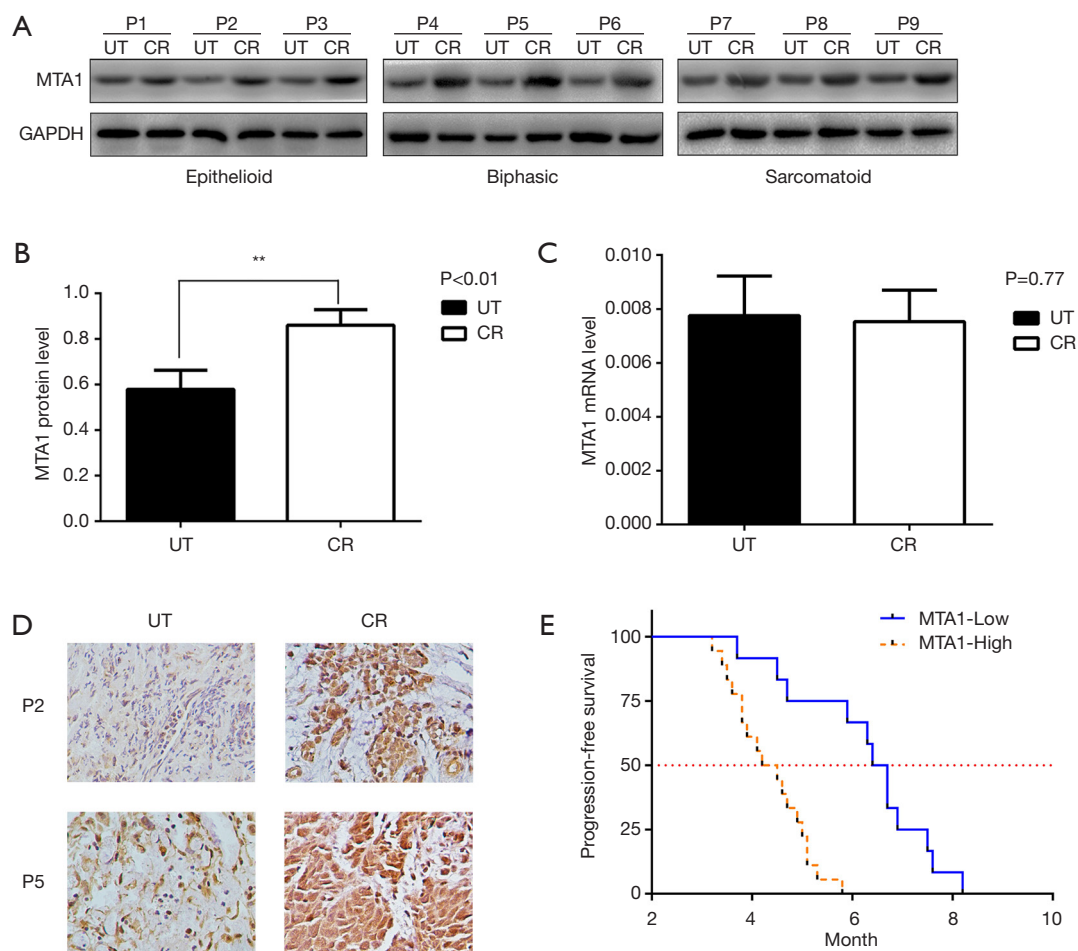
Figure 1 Metastasis-associated gene 1 (*MTA1*) protein is induced following exposure to cisplatin in malignant pleural mesothelioma (MPM). (A) Levels of *MTA1* protein, as determined by Western blotting in 9 primary human MPM cases. (B) Grayscale value from 3 independent Western blot was scanned using Image-J software. Related protein levels were verified by *t*-test. **P<0.01. (C) Levels of *MTA1* mRNA in 9 MPM cases relative to β-actin. (D) Immunochemical staining of *MTA1* in MPM tissues from 2 cases with avidin-biotin-complex (ABC) methods and magnified 40×. The arrows indicate the region of MTA1 protein expression. (E) MPM patients with a high expression of *MTA1* who received cisplatin chemotherapy had significantly shorter progression-free survival than patients with a low expression of *MTA1*. *P<0.05. Data are represented as mean ± standard deviation. CR, cisplatin resistance; UT, untreated.

were employed in our study. We successfully constructed 2 cell lines with a low expression of the *MTA1* protein (H2452-sh*MTA1* and MSTO-sh*MTA1*) by infecting with lentivirus plasmid (pLL3.7), as reported in our previous study (*Figure 2A*) (20). We then detected the IC50 values of cisplatin in each cell line via CCK-8 assay (*Figure 2B,C*). H2452 and MSTO showed 1.9- and 1.8-fold increased IC50 values for H2452-sh*MTA1* and MSTO-sh*MTA1* cells, respectively (*Figure 2D*). To further confirm the effect of *MTA1* on cisplatin toxicity, we established cisplatin-resistant cell lines (H2452-DDP and MSTO-DDP) and

detected *MTA1* protein expression. We found that the expression of the *MTA1* protein was significantly increased in H2452-DDP and MSTO-DDP compared with H2452 and MSTO (*Figure 2E*). Meanwhile, we detected the *MTA1* protein expression in MPM DDP-resistant cells transfected with si*MTA1*. As shown in *Figure 2F*, si*MTA1* reduced the level of *MTA1* protein expression in MPM DDP-resistant cells. Subsequently, we detected the IC50 values of cisplatin in H2452-DDP and MSTO-DDP transfected with si*MTA1* (*Figure 2G,H*). si*MTA1* showed reduced 1.8- and 1.7-fold IC50 values for H2452-

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Table 1 Correlation of metastasis-associated gene 1 (MTA1) expression and clinicopathological features of malignant pleural mesothelioma

Characteristics	All patients	MTA1 expression	
		Low	High
n	30	12	18
Sex			
Male	16	5	11
Female	14	7	7
Age (years)			
<60	14	5	9
≥60	16	7	9
Histology			
Epithelioid	5	3	2
Sarcomatoid	15	5	10
Biphasic	10	4	6
TNM stage			
III	8	4	4
IV	22	8	14
Lymph node metastasis			
Yes	8	3	5
No	22	9	13
Distance metastasis			
Yes	22	5	17
No	8	7	1

TNM, tumor node metastasis.

DDP and MSTO-DDP cells, respectively (*Figure 2I*). In clonogenic assays, we found that low *MTA1* expression cell lines (H2452-sh*MTA1* and MSTO-sh*MTA1*) treated with cisplatin caused a marked inhibition of proliferation compared with H2452 and MSTO (*Figure 2J,K*). Similarly, we also found that cisplatin combined with si*MTA1* could remarkably reduce the proliferation of H2452-DDP and MSTO-DDP cells (*Figure 2L*). We then examined the *MTA1* protein levels in H2452-DDP and MSTO-DDP cells, which were treated with cisplatin and si*MTA1* (*Figure 2M,N*). Collectively, these results suggest that *MTA1* protein expression is involved in the cisplatin resistance of MPM cells.

Cisplatin stabilized the expression of the MTA1 protein by inhibiting its ubiquitination

Cisplatin induces the *MTA1* protein in MPM patients, whereas the *MTA1* mRNA level has no change, indicating that a post-transcriptional mechanism might be involved in the regulation of the *MTA1* protein. To verify this, MPM cells were exposed to cisplatin at the indicated time points for Western blotting and quantitative real-time PCR assay. As shown in *Figure 3A*, *MTA1* protein expression gradually increased and reached its peak at 48 h, while the *MTA1* mRNA level did not change significantly. On the basis of this observation, we then examined the *MTA1* expression at the indicated doses of cisplatin for 48 h and found that *MTA1* protein expression increased with cisplatin dose, while the *MTA1* mRNA level did not change (*Figure 3B*). Interestingly, these results were consistent with the clinical data in *Figure 1*. Subsequently, a eukaryote protein synthesis inhibitor (cycloheximide) was employed to observe the effect on the degradation rate of the endogenous *MTA1* protein in MPM cells treated with or without cisplatin. We found that cisplatin significantly stabilized the expression of the *MTA1* protein compared with untreated controls (*Figure 3C*). These findings suggest that cisplatin induces the *MTA1* protein through its post-transcriptional regulation.

A previous study demonstrated that UV radiation stabilizes the *MTA1* protein by inhibiting its ubiquitination in HaCaT cells (aneuploid immortal keratinocyte cell line) (21). Therefore, we hypothesized that cisplatin might promote *MTA1* protein expression in the same way. To verify this, MSTO cells were pretreated with MG-132 (a proteasome inhibitor) to enhance the signal of ubiquitination of endogenous *MTA1*, as reported in a previous study (21), and then treated with or without cisplatin. The MSTO protein was subjected to IP analysis with an anti-*MTA1* antibody and immunoglobulin G control, followed by Western blot analysis with indicated antibodies. The findings indicated that cisplatin could significantly reduce the ubiquitination of endogenous *MTA1* (*Figure 3D*). Taken together, these results indicate that cisplatin promotes *MTA1* protein stability, at least in part, by inhibiting ubiquitin.

MTA1 enhanced G2/M cell cycle delay in MPM cells treated with cisplatin

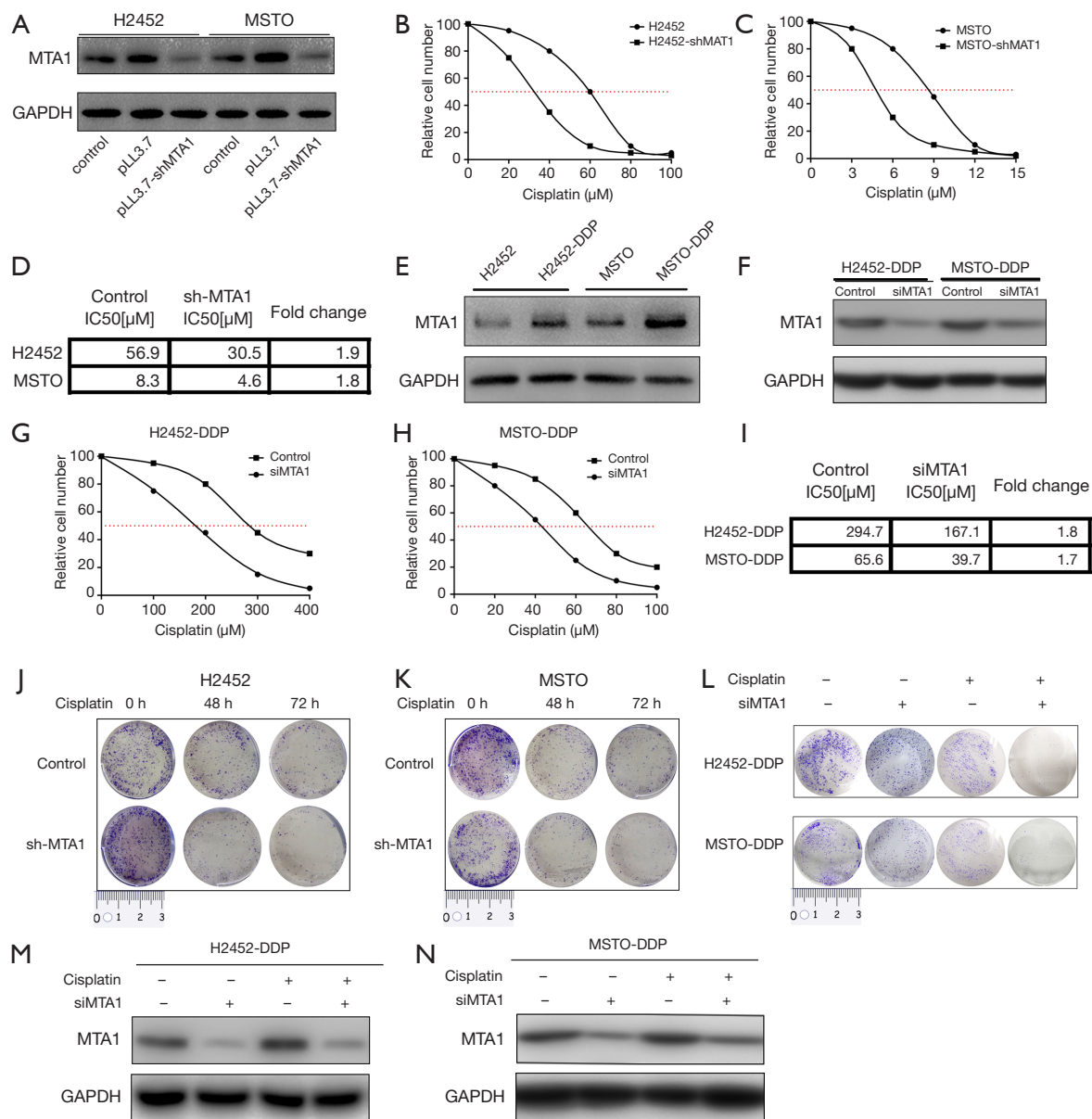DNA damage induced by chemotherapy can activate the

Figure 2 Metastasis-associated gene 1 (*MTA1*) enhances cisplatin resistance of malignant pleural mesothelioma (MPM) cells. (A) *MTA1* level of MPM wild-type cells (H2452 and MSTO-211H) and MPM cells transfected by lentivirus containing pLL3.7-empty and pLL3.7-sh*MTA1* by Western blotting. (B) The half-maximal inhibitory concentration (IC50) values for MPM cells wild-type cells (H2452 and MSTO-211H) and MPM cells transfected with pLL3.7-sh*MTA1*. (C,D) MPM wild-type cells (H2452 and MSTO-211H) and MPM cells transfected with pLL3.7-sh*MTA1* were treated with cisplatin at the indicated concentration for 48 h. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. (E) *MTA1* level of MPM wild-type cells and DDP-resistant cells were determined by Western blotting. (F) The indicates the IC50 values of MPM wild-type cells and DDP-resistant cells. (G,H) MPM wild-type cells and DDP-resistant cells were treated with cisplatin at the indicated concentration for 48 h. Cell viability was measured by MTT assay. (I) Table indicates the IC50 values of MPM wild-type cells and DDP-resistant cells. (J,K) MPM wild-type cells and MPM cells transfected with pLL3.7-shMTA1 were treated with cisplatin at the indicated concentration for 0, 48, 72 hours. Colonies were stained with crystal violet and imaged. (L) MPM DDP-resistant cells were treated with cisplatin and siMTA1 at the indicated concentration for 72 hours. Colonies were stained with crystal violet and imaged. (M,N) MTA1 level of MPM DDP-resistant cells treated with cisplatin and siMTA1 were determined by western-blot.

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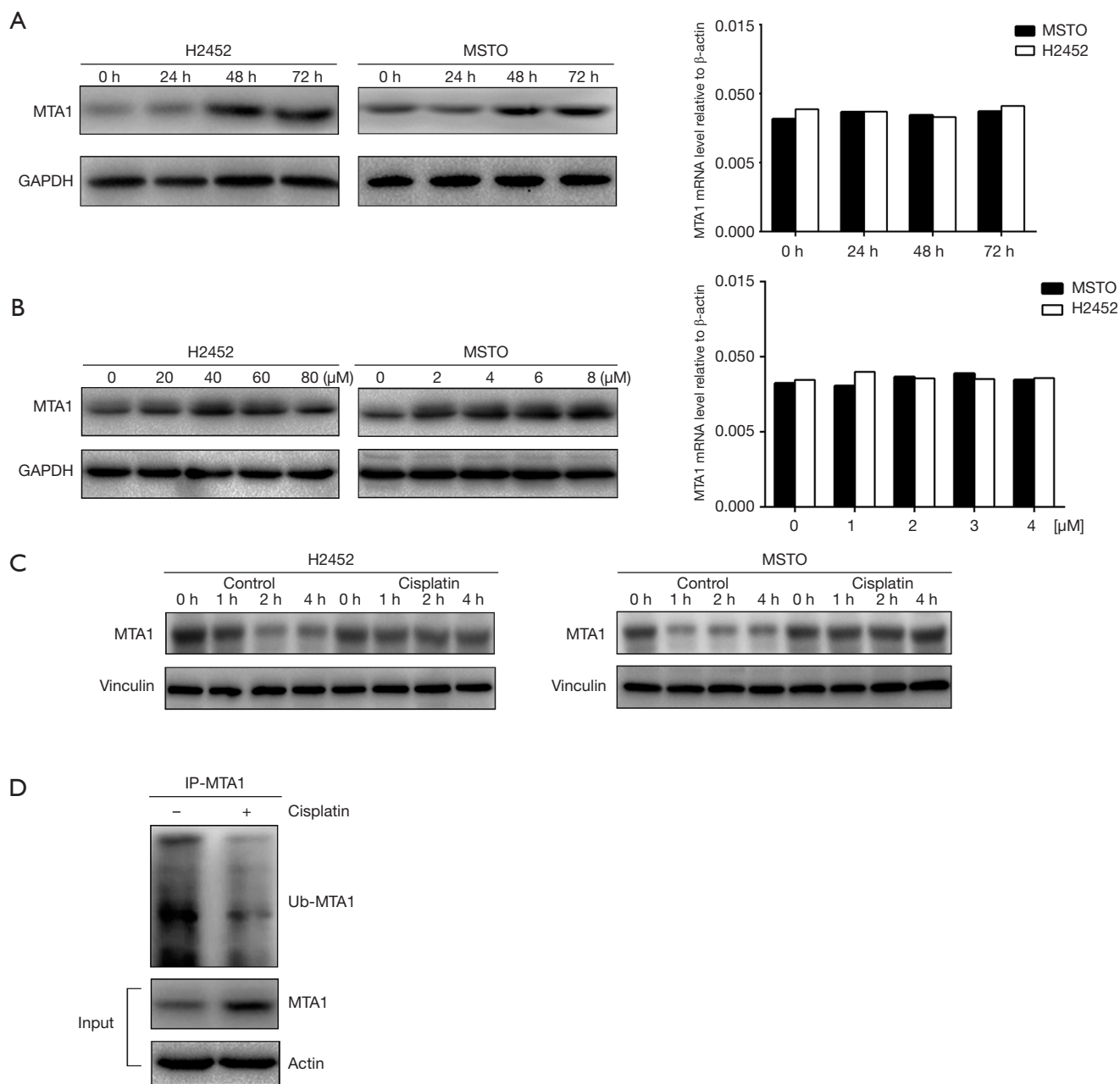Xu et al. MTA1 enhances cisplatin resistance of MPM

Figure 3 Cisplatin promotes the expression of the metastasis-associated gene 1 (*MTA1*) protein by inhibiting its ubiquitination. (A) Malignant pleural mesothelioma (MPM) cells were treated with cisplatin (2 µm) at the indicated time points. *MTA1* expression was detected by Western blotting (left and middle) and real-time polymerase chain reaction (PCR) (right). (B) MPM cells were treated with cisplatin at the indicated doses for 48 h. *MTA1* expression was detected by Western blotting (left and middle) and real-time PCR (right). (C) MPM cells were treated with or without cisplatin (2 µm) for 48 h and then incubated with 100 µg/mL cycloheximide. Cells were harvested at the indicated time points for Western blotting. (D) MSTO cells were treated with 20 µm MG-132 for 1 h and then treated with or without 2 µm cisplatin for 48 h. Protein extracts were subjected to immunoprecipitation with an anti-*MTA1* antibody, followed by Western blotting with the indicated antibodies.

cell cycle checkpoint, which arrests tumor cells in the S or G2/M phase for repair and prevent tumor cells with DNA damage from entering mitosis (23). We next sought to detect whether *MTA1* expression affects the cell cycle of MPM cells treated with cisplatin via flow cytometric analysis. As shown in *Figure 4A*, cisplatin successfully arrested the H2452 cells in the G2/M phase. Nevertheless, knocking down the expression of *MTA1* in H2452 cells attenuated this effect of cisplatin. The average value of the G2/M cell cycle from 3 independent experiments was analyzed (*Figure 4B*). These results were also confirmed in MSTO cells (*Figure 4C,D*). We then detected the cell cycle of MPM DDP-resistant cells treated with or without cisplatin and si*MTA1*. We found that the percentage of the G2/M cell cycle did not change significantly when MPM DDP-resistant cells were treated with cisplatin or si*MTA1* alone. However, once the cells were exposed to cisplatin combined with si*MTA1*, the percentage of G2/M cell cycle was significantly reduced (*Figure 4E,F,G,H*).

A previous study demonstrated that Phosphohistone-H3-positive cells could distinguish mitotic cells to G2 cells (24). Therefore, we detected the percentage of Phosphohistone-H3-positive cells in cisplatin-treated MPM cells by flow cytometric analysis. As shown in *Figure 4I*, H2452-sh*MTA1* and MSTO-sh*MTA1* cells presented a higher percentage of Phosphohistone-H3-positive cells compared with normal MPM cells at the corresponding time points after cisplatin treatment. Interestingly, the percentage of Phosphohistone-H3-positive cells increased significantly in MPM DDP-resistant cells when exposed to cisplatin combined with si*MTA1* (*Figure 4J*). This result indicated that reducing the level of *MTA1* expression can promote cisplatin-treated cells into the mitotic phase. In brief, these results suggest that *MTA1* is responsible, at least in part, for G2/M cell cycle delay in MPM cells with cisplatin treatment.

MTA1 regulated and protected tumor genomes from chemotherapeutic drugs via participating in the phosphorylation of the ATR-Chk1 pathway through protein-to-protein interaction

To further analyze the mechanism of *MTA1* participating in G2/M cell cycle delay, we detected the related protein expression of the *ATR-Chk1* pathway in MPM cells at different time points after cisplatin treatment by Western blotting. As shown in *Figure 5A*, compared with H2452 and MSTO cells, the expressions of *p-ATR*, *p-Chk1*, *Chk1*, and the Claspin protein were significantly reduced

in H2452-sh*MTA1* and MSTO-sh*MTA1* cells. The expressions of *ATR*, *ATRIP*, *RAD17*, *RAD9*, and *TopBP1* did not change significantly. As shown in *Figure 5B,C*, the interaction between *MTA1* and *ATR* was confirmed, and the upregulation of *MTA1* and *ATR* in MPM cells treated with cisplatin was able to be detected via IP assay in H2452 and MSTO cells, respectively. On this basis, to explore whether *MTA1* regulation of *ATR* depends on DNA-protein interactions, we then performed the IP analysis using anti-*ATR* antibodies in the presence of the DNA intercalator ethidium bromide (EtBR) and Micrococcal nuclease. EtBR disrupts protein-DNA interactions and Micrococcal nuclease digests single-stranded nucleic acids and cleaves double-stranded DNA or RNA (21). The results of the Western blot analysis indicated that Micrococcal nuclease and EtBR treatment had no effect on the binding of *ATR* to *MTA1* (*Figure 5D*), further indicating that the interaction between *MTA1* and *ATR* is independent on DNA. These results suggest that *MTA1* can affect the phosphorylation of the *ATR-Chk1* pathway through protein interactions, thereby reducing the negative impact of cisplatin on the genome of MPM cells.

Discussion

MPM, which originates from serosa tissue mesothelial cells (25), is an aggressive malignant tumor with a low survival rate (26,27). The first-line chemotherapy regimen of MPM based on cisplatin plus pemetrexed can extend the median overall survival of MPM patients to 16.1 months (28). One of the main reasons for tumor progression after chemotherapy is the resistance of tumor cells to cisplatin (29). Therefore, to elucidate the mechanisms of resistance of MPM cells to cisplatin can benefit the treatment outcome of patients.

In the present study, we demonstrated that the *MTA1* protein was markedly upregulated in MPM specimens of cisplatin-resistant patients, and can affect the PFS of patients in different ways. In addition, we demonstrated a vital role of *MTA1* in the process of enhancing cisplatin resistance of MPM cells. Of importance, we further confirmed that *MTA1* can protect tumor genomes from cisplatin by affecting the *ATR-Chk1* pathway and inducing G2/M cell cycle delay in MPM cells treated with cisplatin. Additionally, cisplatin can also stabilize the expression of the *MTA1* protein via inhibiting its ubiquitination. On the basis of these findings, we speculated that *MTA1* potentially plays an carcinogenic role in the chemotherapeutic insensitivity of MPM.
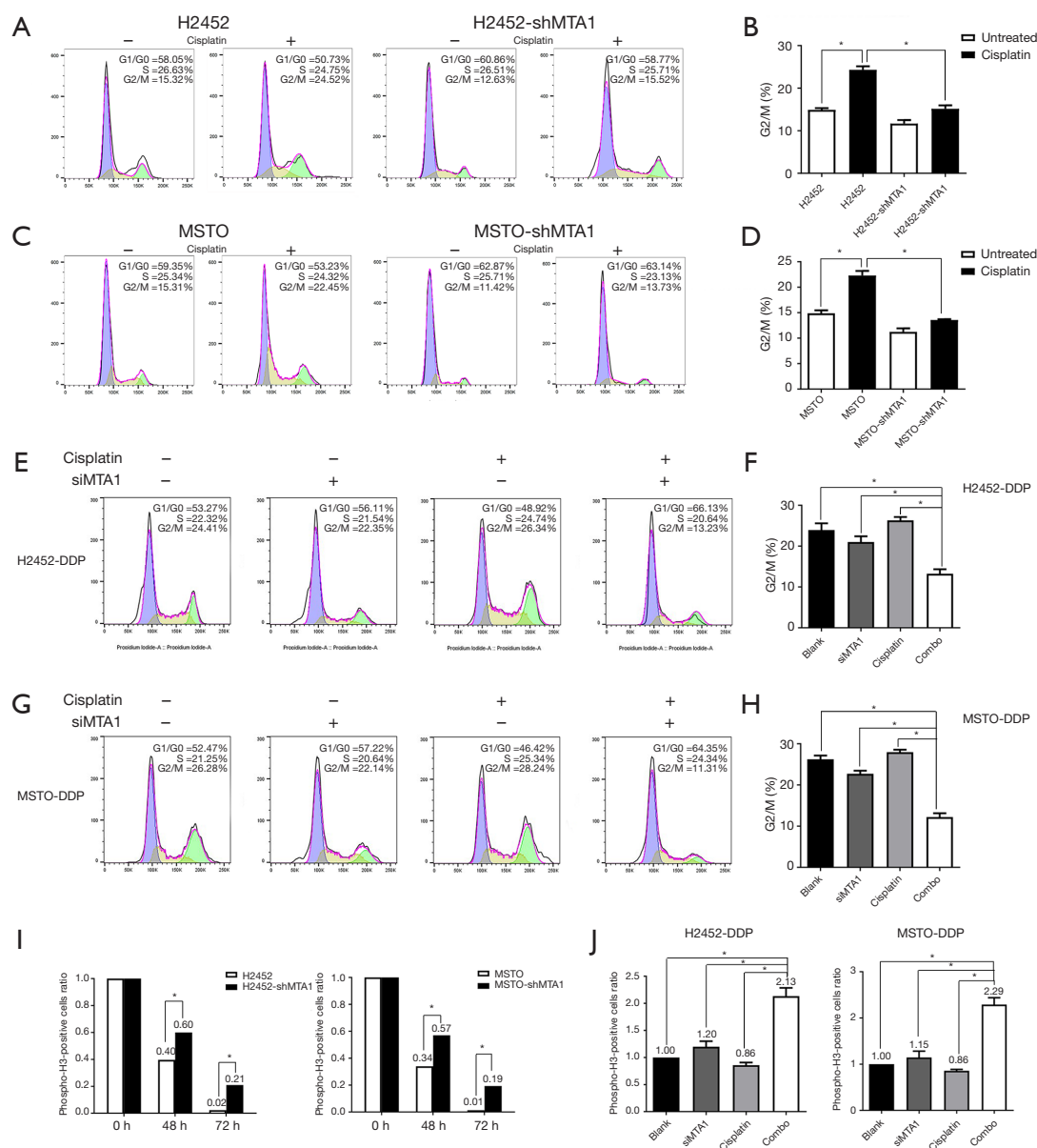
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Figure 4 Metastasis-associated gene 1 (*MTA1*) enhances G2/M cell cycle delay in malignant pleural mesothelioma (MPM) cells treated with cisplatin. (A) Cell cycle analysis of MPM wild-type cells transduced with *MTA1*-shRNA cells treated with 2 µM cisplatin for 48 h (left and middle). Graph data represent the average value of the G2/M cell cycle from 3 independent experiments (right). (B) MPM cells were treated with 2 µM cisplatin and harvested at the indicated time points. Cells were stained with anti-phosphohistone H3 (Ser10) antibody and subjected to fluorescence-activated cell sorting. Percentage of phosphohistone H3-positive cells in cisplatin-treated cells was normalized to that in untreated cells. All data were obtained from 3 independent experiments. *P<0.05. (C) Cell cycle analysis of MSTO cells and transduced with MTA1-shRNA cells treated with cisplatin for 48 h. (D) The graph data represent the average value of G2/M cell cycle from three independent experiments. (E) Cell cycle analysis of H2452 DDP-resistant cells treated with cisplatin and siMTA1. (F) The graph data represent the average value of G2/M cell cycle from three independent experiments. *P<0.05. (G) Cell cycle analysis of MSTO DDP-resistant cells treated with cisplatin and siMTA1. (H) The graph data represent the average value of G2/M cell cycle from three independent experiments. *P<0.05. (I) MPM cells were treated with cisplatin and harvested at the indicated time points. Cells were stained with anti-phosphohistoneH3 (Ser10) antibody and subjected to FACS. The percentage of phospho-H3-positive cells in cisplatin-treated cells was normalized to that in untreated cells. All data was obtained from three independent experiments. *P<0.05. (J) MPM DDP-resistant cells were treated with cisplatin and siMTA1 and harvested at the indicated time points. Cells were stained with anti-phosphohistoneH3 (Ser10) antibody and subjected to FACS. The percentage of phospho-H3-positive cells in treated cells (siMTA1, Cisplatin and Combo) was normalized to that in untreated cells (Blank). All data was obtained from three independent experiments. Combo: siMTA1 and Cisplatin. *P<0.05.
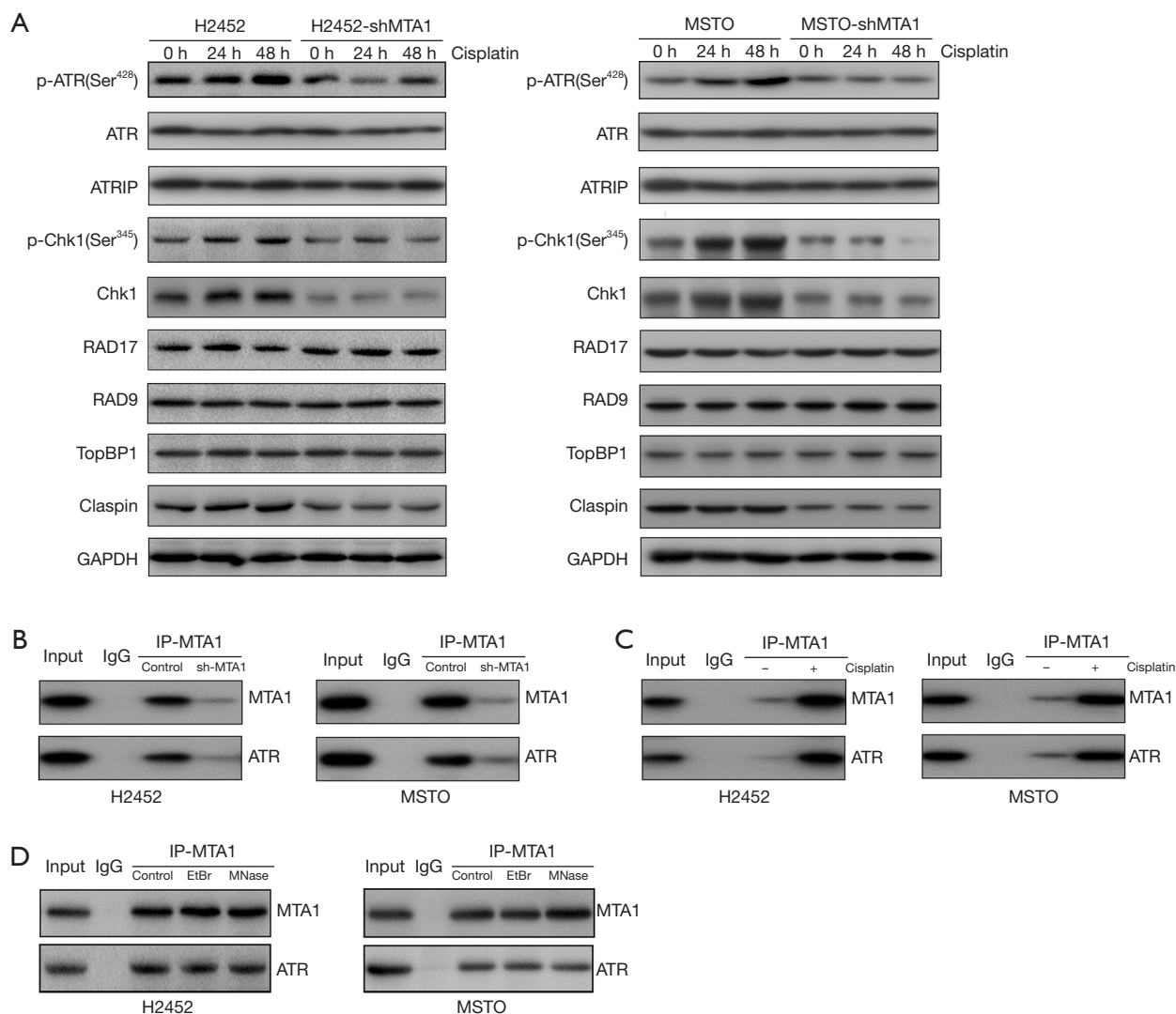
Figure 5 Metastasis-associated gene 1 (*MTA1*) regulates the phosphorylation of the ataxia telangiectasia mutated and rad3 related-checkpoint kinase 1 pathway, protecting tumor genomes against damage caused by chemotherapeutic agents. (A) Malignant pleural mesothelioma (MPM) wild-type cells transduced with *MTA1*-shRNA cells were treated with 2 μM cisplatin at indicated time points for Western blot analysis with antibodies. (B) Protein extracted from MPM wild-type cells transfected with pLL3.7-sh*MTA1* cells were subjected to immunoprecipitation (IP) analysis with an anti-*MTA1* antibody or immunoglobulin G (IgG) control, followed by immunoblotting with the indicated antibodies. (C) Protein extracted from MPM wild-type cells treated with or without cisplatin was subjected to IP analysis with an anti-*MTA1* antibody or IgG control, followed by immunoblotting with the indicated antibodies. (D) Protein extracted from MPM wild-type cells incubated with ethidium bromide and Micrococcal nuclease was subjected to IP analysis with an anti-*MTA1* antibody or IgG control, followed by immunoblotting with the indicated antibodies.

MTA1 has been supposed to be closely correlated with carcinogenesis and the progression of various types of human cancers, such as breast cancer (30), gastrointestinal cancer (31-33), non-small cell lung cancer (34), and ovarian cancer (35). Our previous study also showed that MTA1 could promote the metastasis by direct regulation of E-cadherin in MPM patients (20). In addition, increasing evidence has shown that *MTA1* overexpression can induce cisplatin resistance in various tumor types, including cervical cancer and nasopharyngeal carcinoma (36,37). Therefore,

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we conducted further research on the role of *MTA1* in MPM. Consistent with the above research, we found that the *MTA1* protein was upregulated in cisplatin-resistant MPM cells and was correlated with cisplatin resistance. Of importance, we reported that MPM patients who received cisplatin chemotherapy with a high expression of *MTA1* had significantly shorter PFS.

It is well known that DNA damage response (DDR) is critical to protect against genomic instability, and *ATR* kinase has key roles in DDR (17,38). When cancer cells are treated with radiation therapy or chemotherapeutic drugs, such as cisplatin, the cell cycle checkpoint protein, *ATR*, and its key downstream regulator, *Chk1*, halt their pace into mitosis, leading to treatment failure and tumor progression (39). Therefore, a series of agents targeting the *ATR-Chk1* pathway have been designed to achieve more ideal chemotherapeutic effectiveness (40-42). Li *et al.* demonstrated that *MTA1* participates in the UV-induced DNA damage checkpoint pathway, which is mediated by the *ATR-Chk1* pathway (21), indicating the potential relationship between *MTA1* and the *ATR-Chk1* pathway. In the present study, we verified the interaction between *MTA1* and the *ATR-Chk1* pathway in MPM cells treated with cisplatin.

In summary, we demonstrate a vital role for *MTA1* in chemotherapy resistance, induced, at least in part, by the *ATR*-mediated DNA checkpoint pathway. Nevertheless, compelling evidence on the clinical efficacy of *MTA1* inhibitors are still lacking and further studies on the clinical implementation of the drug are warranted. The development of relevant pharmacological inhibitors is also of importance.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Ethical Committee of the First Affiliated Hospital of Soochow University (Suzhou, China), and written informed consent was obtained from each patient. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013).

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