AREL1 E3 ubiquitin ligase inhibits TNF-induced necroptosis via the ubiquitination of MTX2

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Abstract. Previously, we reported on a novel anti-apoptotic E3 ubiquitin ligase, apoptosis-resistant E3 ubiquitin protein ligase 1 (AREL1), that ubiquitinates inhibitors of apoptosis proteins antagonists. The present study demonstrated that AREL1 ubiquitinated Metaxin 2 (MTX2), which was involved in TNF-induced necroptosis. MTX2 has been identified as a protein that belongs to the Metaxin family. It interacts with another Metaxin protein, Metaxin 1 (MTX1), which is localized in the outer membrane of mitochondria, and is involved in TNF-induced necroptosis. This study found that AREL1 interacted with MTX2, but not MTX1, while the amino-terminal domain of MTX2 interacted with MTX1, AREL1 interacted with the carboxyl-terminal domain of MTX2. Furthermore, AREL1 expression led to a decrease in the protein expression of MTX2, but not MTX1. However, a mutant form of AREL1, AREL1C790A, which is deficient for E3 activity, did not cause MTX2 degradation. Moreover, the protein levels of MTX2 were increased by AREL1 knockdown. Therefore, these results implied that AREL1 ubiquitinates and promotes the degradation of MTX2. The expression of MTX2, together with MTX1, enhanced TNF-induced necroptosis. However, AREL1 inhibited necroptosis even in cells expressing Metaxin proteins. Therefore, these results suggested that the inhibition of AREL1-dependent ubiquitination of MTX2 could be beneficial to sensitize tumor cells to TNF-induced necroptosis.

Introduction

Apoptosis and necroptosis are two common forms of programmed cell death that play essential roles in development and maintaining tissue homeostasis (1). Defects in apoptosis and necroptosis are strictly connected to the pathogenesis of various human diseases (2). Apoptosis can be induced by proteolytic activation of the caspase protease family (3). Upon caspase activation and subsequent cleavage of intracellular substrates lead cells to break into small membrane-wrapped vesicles known as apoptotic bodies (4). On the other hand, necroptosis is a caspase-independent form of cell death. Like apoptosis, it is programmed one and induced by TNF and Fas ligand but results in organelle swelling and cytoplasmic membrane breakdown (5,6).

Apoptosis-resistant E3 ubiquitin protein ligase 1 (AREL1) was initially identified as a suppressor of p53-induced apoptosis (7). However, AREL1 functions as a general inhibitor of apoptosis in p53-positive and deficient tumor cells because it inhibited apoptosis induced by various stimuli such as staurosporine, etoposide, and doxorubicin (7). AREL1 did not affect the cytosolic release of mitochondrial pro-apoptotic proteins such as cytochrome *c* but inhibited caspase-3 activation (7). AREL1 encodes HECT-family E3 ubiquitin ligase and ubiquitinates IAP antagonists, such as SMAC, HtrA2, and ARTS, released into the cytosol from mitochondria upon apoptotic stimulation (7).

This study reports a new target protein of AREL1 E3 ubiquitin ligase and necroptosis-inhibitory function of AREL1. We found that AREL1 ubiquitinates and promotes ubiquitin-dependent degradation of Metaxin 2 (MTX2), localized in mitochondria's outer membrane. However, it has been reported that Metaxin family proteins are involved in TNF-induced necroptosis. These led us to find the necroptosis inhibitory function of AREL1 in association with MTX2.

Materials and methods

Yeast two-hybrid screen. The yeast cell expressing LexA-HECT (Homologous to E6-AP Carboxyl Terminus, aa. 454-823 of AREL1) was transformed with the HeLa cell cDNA library fused to the GAL4-AD. Positive clones were initially selected by their ability to grow on His-deficient media and produce β-galactosidase activity, as previously demonstrated (8).

Chemical reagents and Plasmid construction. Cycloheximide (C-7698), MG132 (Z-Leu-Leu-Leu-al, C-2211), human TNFα (T-0157), and mouse TNFα (T-7539) were purchased

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from Sigma. Blasticidin (R210-01) was purchased from Invitrogen. Human Metaxin 1 (MTX1) cDNA was cloned by RT-PCR using total RNA from HeLa cells with a pair of primers: the sense primer was 5'-CGGAATTCAACATGCTG CTCGG-3' (the *Eco*RI site is underlined), and the antisense primer was 5'-CCGCTCGAGAAATCATTCCTCTTCATC-3' (the *Xho*I site is underlined). The cDNA of MTX1 was confirmed by DNA sequencing and cloned into the N-terminally Flag-tagged vector at *Eco*RI/*Xho*I sites. Flag-, GFP-, and GST-tagged MTX2 were generated by subcloning the full-length cDNA of MTX2 into *Eco*RI/*Xho*I sites of pCMV-Tag2B, *Eco*RI/*Apa*I sites of the pEGFP-C2, and *Eco*R/*Xho*I sites of the pGEX-5X-1, respectively. The MTX2 deletion constructs (1-112, 113-264 aa) were made by PCR (Forward primer: 5'-GTCGAATTCATGTCTCTAGTGGC GGAAG-3', F-internal primer: 5'-CGCGAATTCAAAGCTTA CATGGAATTAG-3', Reverse primer: 5'-ACAGTCGACCTAT GACAGCCTGCCTTTAC-3', R-internal primer: 5'-CGCGTC GACGTAAGCTTTCATTTCTGC-3') and cloned into the *Eco*RI/*Sal*I sites of pCMV-Tag2B. AREL1 knockdown was applied as previously described (7). siRNA oligonucleotides corresponding to the sequences of AREL1 (5'-AATTGGTC CCTGAGAACCTTT-3') were generated and used for transfection with Lipofectamine RNAiMAX (Invitrogen). Scrambled siRNA was obtained from Proligo LLC. Flag-HtrA2 was previously described (7).

Cell culture and transfection. Human 293T (KCLB, 21573), DLD1 (KCLB, 10221), H1299 (KCLB, 91299), and mouse L929 cells (KCLB, 10001) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Gibco) at 37°C in an atmosphere containing 5% CO_2. Transfection was performed using the Lipofectamine™ 2000 (Invitrogen) for 90-95% density and the Optifect™ (Invitrogen) for low density as previously reported (9).

Co-immunoprecipitation. For co-immunoprecipitation experiments, 293T cells were lysed in NP40 buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Nonidet P40, 10 μM Na_3VO_4, 1 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM DTT) and lysates were incubated with primary antibodies at 4°C for 5 h and then with protein A (for anti-V5, -AREL1 and -MTX2 antibodies) or G (for anti-Flag antibody) agarose for 1.5 h. The immunoprecipitates were analyzed as described previously (9).

In vivo ubiquitination assay. 293T cells were transfected with indicated plasmids involving MTX2 or AREL1 or both. At 8 h following tsransfection, cells were treated with or not 4 μM MG132 for 12 h to inhibit protease activity. Cell lysates were prepared in NP40 lysis buffer and incubated overnight with an anti-Flag antibody or for 5 h with an anti-MTX2 antibody. The precipitates were submitted to western blotting with an anti-Ub antibody.

Immunoblot analysis. For the immunoblotting, cell lysates were obtained using RIPA buffer (50 mM Tris-HCl (pH 7.4), 5 mM NaCl, 1 μM EGTA, 1% Triton X-100, 50 μM NaF, 10 μM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml

pepstatin A, 0.1 mM PMSF, 1 mM DTT), and the soluble protein concentrations were determined according to a Bradford assay (Bio-Rad Laboratories). Extracted proteins were separated by SDS-PAGE, electrotransferred, and probed with the primary antibody. The following antibodies were used in this study - AREL1, MTX1 (611768, BD Biosciences), MTX2, GST (554805, Pharmingen), Flag (F-3165, Sigma), V5 (46-0705, Invitrogen), Actin (SC-1616, Santa Cruz Biotechnology Inc.), gamma-Tubulin (SC-7396, Santa Cruz Biotechnology Inc.), Ub (SC-8017, Santa Cruz Biotechnology Inc.). The signal was detected by the chemiluminescent ECL or ECL-plus system (Amersham Biosciences).

Necroptosis assay. Mouse L929 cells were used for the necroptosis assay. After transfection, cells were challenged with TNF, zVAD (10 μM) for 4 h. 10 μM of MG132 was treated to test its effects on cell death. The trypan blue exclusion assay was performed to detect cell death following 30 h of treatment. Data are expressed as the percentage of dead and viable cells by repeated experiments more than three times. Necroptosis of TNF-treated cells was confirmed by microscopic observation of necrosis-like morphologic features such as condensed nuclei and swelled cytoplasm with no apoptotic blebbing.

Statistical analysis. Data was entered in Microsoft excel spread sheet and analyzed using SPSS software (version 17). Numerical data were presented as mean and standard deviation values.

Results

AREL1 interacts with MTX2. For isolating target proteins of AREL1 E3 ubiquitin ligase involved in cell death, a yeast two-hybrid screen was carried out with the HeLa cell cDNA library to isolate AREL1-interacting proteins and identified MTX2 as a candidate of AREL1 target. Immunoprecipitation experiments were performed using anti-V5 and anti-Flag antibodies. This confirmed the molecular interaction of AREL1 with MTX2, but not MTX1 (Fig. 1A). Endogenous AREL1 was detected from immunoprecipitates collected from extracts of cells transfected with Flag-MTX2 using an anti-Flag antibody (Fig. 1B), however, endogenous MTX2 was not detected in cells expressing AREL1. This observation may correlate with AREL-1 directed degradation of MTX2 (Fig. 2A). In addition to the immunoprecipitation experiments, we confirmed co-localization of AREL1 and MTX2 in the cytosol (Fig. S1).

It has been shown that MTX1 interacts with MTX2, tethering it into the outer membrane of mitochondria (10), which results in MTX2 bound to the cytosolic face of the mitochondrial outer membrane. Since AREL1 protein is localized to the cytosol of cells (7), we examined whether AREL1 and MTX1 compete with each other to interact with MTX2 or interact to different sites in MTX2, MTX2 with deletion of either N-terminal 112 amino acids or C-terminal 152 amino acids were transfected with AREL1 or MTX1. Immunoprecipitation and western blotting showed that the C-terminus of MTX2 interacted with AREL1, whereas it's N-terminus for MTX1, indicating that AREL1 and MTX1 interact with distinct domains in MTX2 (Fig. 1C). These results suggest that MTX1
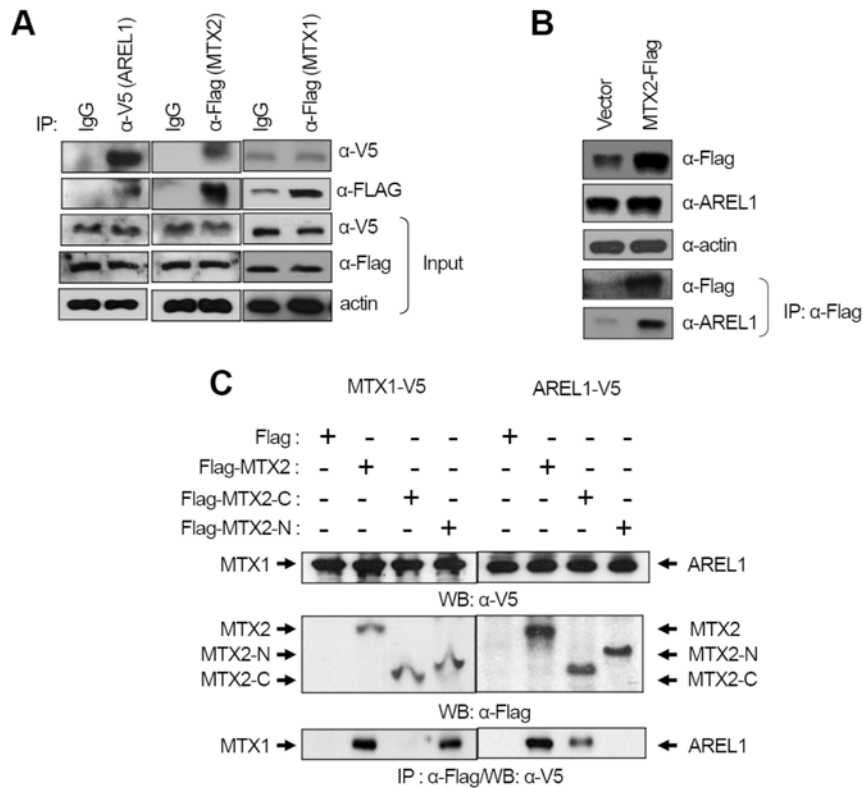
Figure 1. AREL1 interacts with MTX2. (A) AREL1-MTX2 interaction. 293T cells were transfected with V5-tagged AREL1-H and Flag-tagged MTX2 (left and middle panel). IPs were performed with either anti-V5 (AREL1) or anti-Flag (MTX2) antibodies. Flag-tagged MTX1 was used as a negative control (right panel). IPs were analyzed via WB with the indicated antibodies. (B) Interaction between endogenous AREL1 and Flag-MTX2. 293T cells were extracted after Flag-MTX2 transfection and subjected to IP with an anti-Flag antibody. Endogenous AREL1 was detected by WB with an anti-AREL1 antibody. (C) AREL1 and MTX1 interacted with the distinct sites of MTX2. 293T cells were transfected with either MTX2 mutants, which carry either the C- or N-terminal domain of MTX2, with AREL1 or MTX1. Whole cell extracts were analyzed via WB with either anti-V5 or anti-Flag antibodies. IPs were performed with anti-V5 antibodies for either AREL1 or MTX1 and followed up by WB with an anti-Flag antibody to detect MTX2 mutant proteins. AREL1, apoptosis-resistant E3 ubiquitin protein ligase 1; MTX2, Metaxin 2; MTX1, Metaxin 1; WB, western blotting; IP, immunoprecipitation.
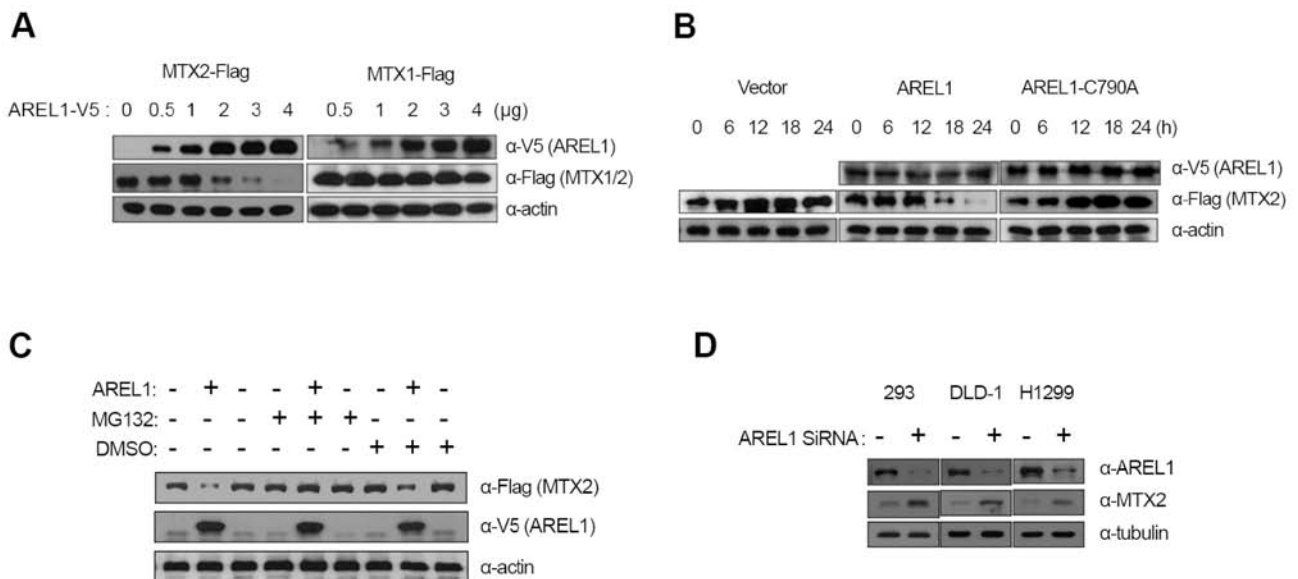

Figure 2. Degradation of MTX2 by AREL1. (A) MTX2 degradation by AREL1. Various amounts of V5-tagged AREL1-expressing plasmids were transfected into 293T cells with either Flag-tagged MTX1 or MTX2. WB of the 293T cell extracts was performed with the indicated antibodies. (B) E3 activity of AREL1 was found to be essential for MTX2 degradation. 293T cells were transfected with V5-tagged wild-type AREL1 or its E3-deficient mutant form, AREL1-C790A, with Flag-tagged MTX2. MTX2 protein was detected via WB of the cell extracts, which were prepared at the indicated times after transfection, with the indicated antibodies. (C) Proteasome-dependent degradation of AREL1-mediated MTX2 degradation. 293T cells were transfected with V5-tagged AREL1 and cultured with MG132 or DMSO. Endogenous MTX2 proteins were detected by WB of the cell extracts with the indicated antibodies. (D) siRNA-mediated knockdown of AREL1. 293T, DLD-1 and H1299 cells were treated for 48 h with AREL1-directed RNA interference. Next, cell extracts were generated and WB with anti-AREL1 and anti-MTX2 antibodies was conducted. AREL1, apoptosis-resistant E3 ubiquitin protein ligase 1; MTX2, Metaxin 2; MTX1, Metaxin 1; siRNA, small interfering RNA; WB, western blotting
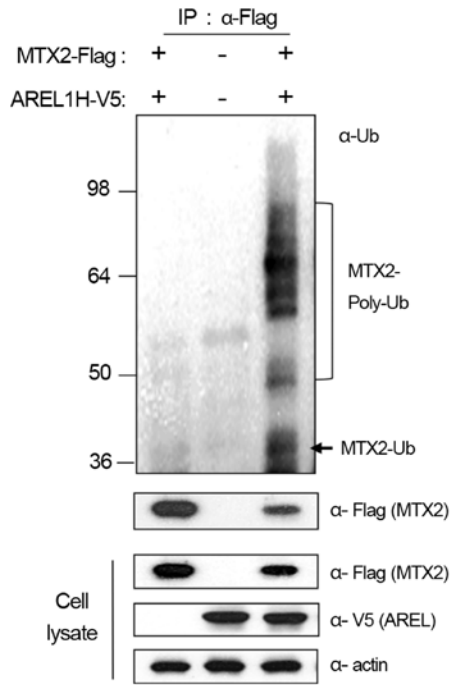
Figure 3. Ubiquitination of MTX2 by AREL1. 293T cells were transfected with V5-tagged AREL1 and Flag-tagged MTX2. IP with anti-MTX2 antibody and western blotting with anti-ubiquitin antibody showed a ubiquitinated MTX2 protein ladder. AREL1, apoptosis-resistant E3 ubiquitin protein ligase 1; MTX2, Metaxin 2; IP, immunoprecipitation.

does not bother the interaction between AREL1 and MTX2 proteins.

Ubiquitination and degradation of MTX2 by AREL1. To examine whether MTX2 is a target of AREL1 E3 ubiquitin ligase, we co-transfected MTX2 with an increasing amount of AREL1-encoding plasmid into 293T cells and analyzed the protein levels of both proteins. Transfection of AREL1 results in a significant reduction in MTX2 proteins in a dose-dependent manner (Fig. 2A). However, the protein levels of MTX1 were not affected by AREL1 expression (Fig. 2A).

A role of E3 ubiquitin ligase activity of AREL1 in MTX2 degradation was examined using a mutant form of AREL1, AREL1-C790A, in which 790th amino acid cysteine, a highly conserved residue among HECT family E3 ubiquitin ligase and essential for forming ubiquitin thioester complex was replaced with alanine (7). While AREL1 expression led to decrease in MTX2 proteins, AREL1-C790A did not (Fig. 2B), indicating that E3 ubiquitin ligase activity of AREL1 is essential for MTX2 degradation. Furthermore, the effects of MG132, a potent inhibitor of the proteasome, was also examined (Fig. 2C). MTX2 degradation in cells expressing AREL1 was inhibited by treatment with MG132, but not DMSO (Fig. 2C). We also examined the effects of AREL1 knockdown on endogenous MTX2 protein levels (Fig. 2D). SiRNA-mediated knockdown of AREL1 was applied to three different cell lines such as 293T, DLD-1, and H1299. Protein levels of MTX2 were significantly increased in all cell lines tested following knockdown of AREL1 (Fig. 2D). Together, these results suggest that the E3 ubiquitin ligase activity of AREL1 is essential for MTX2 degradation.

Based on these results, we further examined whether AREL1 increases the ubiquitination of MTX2 proteins in 293T cells with AREL1-V5 and MTX2-Flag (Fig. 3). Ubiquitinated MTX2 proteins were barely detected in 293T cells expressing AREL1 or MTX2 alone but significantly enhanced in cells expressing both AREL1 and MTX2 (Fig. 3). These results suggest that AREL1 ubiquitinates and promotes the proteasome-dependent degradation of MTX2.

Inhibition of TNF-induced necroptosis by AREL1. It was previously reported that Metaxin proteins are required for TNF-induced cell death (11,12). Therefore, we examined whether AREL1 also inhibits TNF-induced necroptosis as well as apoptosis. To investigate AREL1's function in TNF-induced necroptosis, we treated AREL1-expressing L929 cells with TNF and caspase inhibitor, zVAD, to induce necroptotic cell death. Necroptotic cell death was significantly suppressed in AREL1-expressing cells compared to control L929 cells (Figs. 4A and S2). However, the toxic effects of MG132 were not specific to AREL1-expressing cells (Fig. 4A). It may be due to broad effects of MG132 in necroptotic cells (13). AREL1 expression did not affect protein levels of RIP3 (Fig. 4B), which is increased and an essential effector during necroptotic cell death (14), implying that the anti-necroptosis functions of AREL1 is mediated by down-stream regulators.

We further examined the effects of AREL1 in TNF-induced cell death of L929 cells expressing MTX1 and MTX2 (Fig. 4C). Expression of both MTX1 and MTX2 increased susceptibility to TNF-induced necroptotic death (Fig. 4C), as previously reported (12). However, this susceptibility in L929 cells expressing MTX1 and MTX2 was suppressed by AREL1 expression (Fig. 4C and D). Therefore, these results suggest that the necroptosis-inhibitory function of AREL1 may relate to its ability to ubiquitinates MTX2 protein.

Discussion

This study demonstrated the AREL1-dependent ubiquitination of MTX2 and its involvement in necroptosis inhibitory functions. AREL1 interacted with the N-terminal domain of MTX2, whereas MTX1 did the C-terminal part (Fig. 2). We previously reported that AREL1 binds to and ubiquitinates IAP antagonists when released into the cytosol, but not when they reside in mitochondrial inter-membrane space (7). MTX2 binds to the mitochondrial outer membrane's cytosolic face through its interaction with MTX1, embedded in the mitochondria's outer membrane by its anchor domain (10,15). Therefore, MTX1-bound MTX2 and the cytosolic form of MTX2 could be ubiquitinated by AREL1 E3 ubiquitin ligase, which is mainly localized in the cytosol (Fig. S1) (7). Third member of Metaxin family protein, Metaxin 3, was lately identified from Zebrafish and *Xenopus* (16,17). Although it has been shown that Metaxin 3 is distinct from MTX1 and MTX2 based on amino acid sequence homology, only limited information is available for its cellular function.

Metaxin proteins have been reported to be involved in TNF-induced cell death (11,12). MTX1 deficiency results in resistance to TNF-induced cell death (12). Since MTX1 tethers MTX2 into the cytosolic face of the mitochondrial outer membrane (10), expression of MetaΔTM/C, which does not
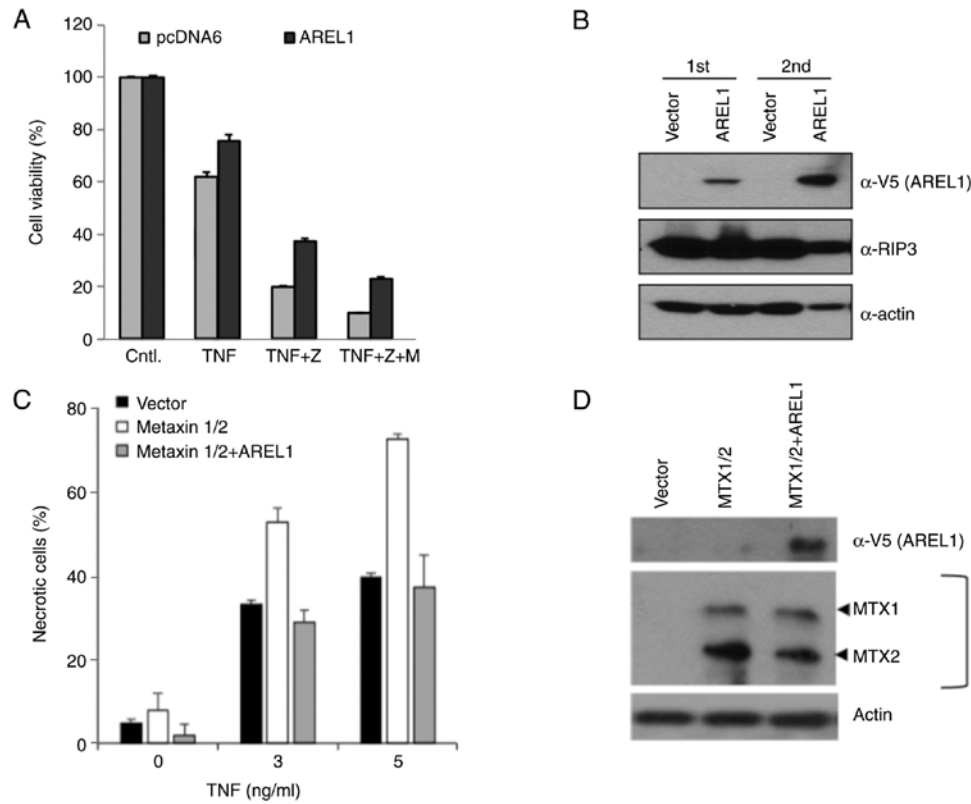
Figure 4. Inhibition of TNF-induced necroptosis by AREL1. (A) AREL1 expression resulted in resistance to TNF-induced necroptosis. L929 cells were transfected with AREL1, and then treated with TNF, Z and M for 4 h. A trypan blue exclusion assay was performed for cell viability. Experiments were conducted in triplicate and data are presented as the average ± SD. (B) Western blotting was performed with the indicated antibodies for detecting the expression of transfected AREL1. (C) TNF-induced necroptotic death of L929 cells transfected with MTX1/2 and AREL1. L929 cells were transfected with AREL1 and MTX1/2 and then treated with TNF and Z for 2 h. Necrotic cell fractions were obtained. (D) Western blotting was performed with the indicated antibodies for detecting the expression of transfected AREL1 and MTX1/2. AREL1, apoptosis-resistant E3 ubiquitin protein ligase 1; MTX2, Metaxin 2; MTX1, Metaxin 1; Cntl, control; Z, zVAD-fmk; M, MG132.

contain mitochondrial membrane anchor domain, may also result in MTX2 deficiency at the outer membrane of mitochondria. However, the effects of Metaxin-family proteins in TNF-induced apoptosis and necroptosis is controversial; Chen *et al* reported that knockdown of MTX1 and MTX2 had no noted effect on TNF-induced apoptosis (18). TNF induces apoptosis in many cancer cells, but necroptosis in certain cell lines including L929. In our hand, MTX2 knockdown was not successful by low transfection efficiency of L929 cells and failed to confirm that MTX2 depletion results in resistance to TNF-induced necroptosis.

Here, we focused on roles of AREL1 and MTX2 in TNF-induced necroptosis using L929 cells (Fig. 4). It is noteworthy that AREL1 has anti-necroptosis effects in addition to the anti-apoptosis effects that have been previously reported (7). AREL1 seems to function as a downstream regulator of cell death because it inhibits apoptosis induced by various stimuli including p53, staurosoporine, and DNA damaging agents (7). Necroptosis can be induced by not only TNF but also various stimuli such as ceramide, lonidamine, and sodium nitroprusside (19,20). Since AREL1 did not affect RIP3, upstream regulator of necroptosis, it seems to be a downstream regulator of necroptosis induced by various necroptosis stimuli.

Although it is still unclear how Metaxin deficiency in the outer membrane affects TNF-induced necroptosis, the role of Metaxin proteins has been implicated to serve in the

pre-protein import of mitochondria (15). Metaxin proteins have been identified recently as components of the sorting and assembly machinery (SAM)/translocase of the outer-membrane β-barrel protein (TOB) complex (SAM/TOB complex) in the mitochondrial outer membrane (21,22). It is recently reported that SAM/TOB complex is a component of the mitochondrial intermembrane space bridging (MIB) complex that maintains cristae morphology (23). MIB complex likely contains third member of Metaxin family protein, Metaxin 3 (23). The depletion of MTX2 leads to the reduction of MTX1 and inhibits the import and assembly of a voltage-dependent anion-selective channel (VDAC) located in the outer mitochondrial membrane (22,24); however, the role of these proteins in import has yet to be clearly defined. VDAC mediates metabolite exchange between the cytosol and mitochondria and is involved in apoptosis (25-28). These results imply that MTX2 depletion will affect cell death via the integrity of the outer membrane of mitochondria.

Although necroptosis involves the active destruction of mitochondrial and plasma membranes, our current knowledge has been limited in plasma membrane-proximal protein complex, including TNFR and its associated proteins (29,30). Therefore, it is interesting to identify proteins located in the cytosol and outer membrane of mitochondria and involved in necroptosis. AREL1 did not affect RIP3, an essential component of the membrane-proximal necroptosis complex (Fig. 4A).

Therefore, mitochondrial MTX2 protein will be a key target to elucidate the necroptosis-inhibitory function of AREL1 and the cytosolic process of necroptosis including mitochondrial membrane disintegration.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DS made substantial contributions to the conception and design of the present study, performed data interpretation, was involved in drafting and revising the manuscript, and also gave final approval of the version to be published. YJ and BK were involved in designing the experiments, and contributed to the acquisition, analysis and interpretation of data. DS, YJ and BK confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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