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RESEARCH ARTICLE

AMBRA1 promotes dsRNA- and virus-induced apoptosis through interacting with and stabilizing MAVS

Yuxia Lin[1,2,*], Changbai Huang[1,2,*], Huixin Gao[1,2], Xiaobo Li[1,2], Quanshi Lin[1,2], Shili Zhou[1,2], Zhiting Huo[1,2], Yanxia Huang[1,2], Chao Liu[1,3,‡] and Ping Zhang[1,2,‡]

ABSTRACT

Apoptosis is an important cellular response to viral infection. In this study, we identified activating molecule in Beclin1-regulated autophagy protein 1 (AMBRA1) as a positive regulator of apoptosis triggered by double-stranded (ds)RNA. Depletion of AMBRA1 by gene editing significantly reduced dsRNA-induced apoptosis, which was largely restored by trans-complementation of AMBRA1. Mechanistically, AMBRA1 interacts with mitochondrial antiviral-signaling protein (MAVS), a key mitochondrial adaptor in the apoptosis pathway induced by dsRNA and viral infection. Further co-immunoprecipitation analysis demonstrated that the mitochondrial localization of MAVS was essential for their interaction. The impact of AMBRA1 on dsRNA-induced apoptosis relied on the presence of MAVS and caspase-8. AMBRA1 was involved in the stabilization of MAVS through preventing its dsRNA-induced proteasomal degradation. Consistently, AMBRA1 upregulated the apoptosis induced by Semliki Forest virus infection. Taken together, our work illustrated a role for AMBRA1 in virus-induced apoptosis through interacting with and stabilizing MAVS.

KEY WORDS: Apoptosis, Virus, dsRNA, AMBRA1, MAVS

INTRODUCTION

In response to viral infection, cells rapidly elicit multiple signaling pathways to mount different responses, including the formation of stress granules, protein translation inhibition (McCormick and Khaperskyy, 2017), endoplasmic reticulum stress, unfolded protein response, autophagy (Chiramel et al., 2013), innate immunity (Takeuchi and Akira, 2009) and programmed cell death (PCD) (Imre, 2020). Among these responses, autophagy is a highly conserved pathway that maintains cellular homeostasis by the removal of damaged or excess cellular components, thereby achieving cell survival. The autophagy pathway activated by virus may play either an antiviral or proviral role (Chiramel et al., 2013; Jordan and Randall, 2012). PCD includes apoptosis, necroptosis and pyroptosis (Imre, 2020; Nagata and Tanaka, 2017). Apoptosis is the most extensively studied type of cell death in the context of viral infection. Apoptosis occurs through two distinct pathways, the caspase 8-dependent extrinsic pathway initiated by death receptors of the tumor necrosis factor (TNF) family, and the caspase 9-dependent intrinsic pathway mediated by mitochondrial BCL-2 family members (Nagata and Tanaka, 2017). Necroptosis is a type of programmed necrosis mediated by receptor-interacting protein kinases (RIPKs) and its substrate mixed lineage kinase domain-like protein (MLKL) (Pasparakis and Vandenabeele, 2015). Pyroptosis is another type of programmed necrosis regulated by activated caspases, which cleave and activate substrates [interleukin (IL)-1β, IL-18, and gasdermin D (GSDMD)]. The N terminus of GSDMD forms holes in the plasma membrane, leading to pyroptotic cell death (Sanjo et al., 2019; Shi et al., 2015).

In terms of viral infection, a variety of factors are responsible for triggering cell death pathways, such as viral proteins, viral genomic nucleotides and double-stranded RNA (dsRNA), which is a common byproduct of viral replication (Son et al., 2015). To date, a few cellular proteins have been reported to participate in the cell death triggered by viral infection. These proteins are mainly involved in the innate immune pathway, including pattern recognition receptors (PRRs), such as melanoma differentiation-associated gene 5 (MDA5, also known as IFIH1), retinoic acid inducible gene I (RIG-I, also known as DDX58) and toll like receptor 3 (TLR3) (Amarante-Mendes et al., 2018), mitochondrial antiviral-signaling protein (MAVS, also known as IPS-1/VISA/Cardif) (Kawai et al., 2005), transcriptional factors, such as interferon regulatory factor 3 (IRF3) (Chattopadhyay and Sen, 2017), and interferon-stimulated genes (ISGs), such as dsRNA-activated protein kinase (PKR) (Barber, 2005), 2′-5′ oligoadenylate synthetase (OAS) and ribonuclease L (RNaseL) (Castelli et al., 1997; Chawla-Sarkar et al., 2003). To identify more regulators of cell death, we carried out a genome-wide CRISPR/Cas9-based screen using poly(I:C), a synthetic dsRNA, as a death stimulator. We found that a new gene, *AMBRA1*, which encodes activating molecule in Beclin1-regulated autophagy protein 1 (AMBRA1) (Fimia et al., 2007), was among the top hits (data not shown).

AMBRA1 is highly conserved in vertebrates, and promotes autophagy either by forming a complex with Beclin1 and Vps34 (also known as PIK3C3), and then favoring the formation of autophagosomes (Di Bartolomeo et al., 2010; Fimia et al., 2007, 2011), or by acting as an E3 ligase to ubiquitylate Beclin1 and finally promoting Beclin1-mediated Vps34 activity (Xia et al., 2013). Apart from autophagy, AMBRA1 positively or negatively regulates apoptosis, depending on physiological and pathological conditions. The C-terminal part of AMBRA1 binds the anti-apoptotic protein BCL2 and inhibits its function, thus contributing to the onset of apoptosis induced by staurosporine (STS) (Strappazzon et al., 2016). In contrast, functional deficiency of AMBRA1 causes severe apoptosis in mouse embryos (Fimia et al., 2007), and downregulation of AMBRA1 sensitizes cells to apoptotic stimuli, such as STS and etoposide (Pagliarini et al., 2012), implying that

[1]Key Laboratory of Tropical Diseases Control (Sun Yat-sen University), Ministry of Education, Guangzhou 510080, China. [2]Department of Immunology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, China. [3]Department of Microbiology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, China.
*These authors contributed equally to this work

‡Authors for correspondence (zhangp36@mail.sysu.edu.cn; liuchao9@mail.sysu.edu.cn)

P.Z., 0000-0002-5400-8767

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AMBRA1 also has an inhibitory effect on apoptosis. Whether AMBRA1 plays a pro-apoptosis or an anti-apoptosis role is probably directed by its control of the conversion between autophagy and apoptosis. For example, a pivotal role of AMBRA1 in positively regulating autophagy is often associated with its anti-apoptosis function (Gu et al., 2014; Li et al., 2016; Liu et al., 2019; Sun et al., 2018, 2019). Although AMBRA1 has been shown to regulate apoptosis under various physiological circumstances, the underlying mechanisms have been rarely investigated. Particularly, whether AMBRA1 acts in virus-induced cell death has not been investigated.

In this study, we examined the role of AMBRA1 in cell death induced by dsRNA or Semliki Forest virus (SFV) infection. We found that AMBRA1 confers a pro-apoptotic activity. In addition, our work illustrated the underlying mechanism by which AMBRA1 interacts with MAVS and prevents its proteasomal degradation. Finally, we demonstrated that the pro-apoptotic effect of AMRBA1 relies on the presence of MAVS.

RESULTS

AMBRA1 is involved in dsRNA-induced cell death

To validate the CRISPR/Cas9 screen data showing AMBRA1 may have a potential function in dsRNA-induced cell death, we used the CRISPR/Cas9 gene editing technique to generate AMBRA1 knockout (KO) cells. Single guide (sg)RNA against the *AMBRA1* gene was selected from the pooled library used for CRISPR/Cas9-based screens (Fig. 1A). Two independent AMBRA1^{KO} cell clones were isolated and designated as AMBRA1^{KO}-#1 and AMBRA1^{KO}-#2. The disruption of the *AMBRA1* gene was confirmed by genomic DNA sequencing. One or more nucleotides were deleted in two AMBRA1^{KO} cell clones (Fig. 1B), indicating the effective disruption of *AMBRA1*. In our study, the endogenous AMBRA1 in A549 cells could not be directly detected by anti-AMBRA1 antibodies from two different companies, possibly due to their short half-life as reported previously (Pagliarini et al., 2012). Then, we conducted a co-immunoprecipitation (co-IP) assay to enrich endogenous AMBRA1 protein, followed by western blot. As shown in Fig. 1C, AMBRA1 protein was readily detected in the control cells but not in two AMBRA1^{KO} cell clones, confirming the disruption of the *AMBRA1* gene. In addition, qRT-PCR data showed that the mRNA levels of *AMBRA1* in both AMBRA1^{KO} cell clones were remarkably decreased (~90%, Fig. 1D). To rule out potential off-target effects of *AMBRA1*-specific sgRNA, we introduced the *AMBRA1* gene back to #2 KO cell clone by lentivirus-mediated transduction. The AMBRA1-complemented cells, designated as AMBRA1^{RES}, were sorted by flow cytometry. The qRT-PCR data revealed that the mRNA levels of *AMBRA1* were successfully restored in AMBRA1^{RES} cells (Fig. 1D).

To examine the role of AMBRA1 in dsRNA-induced cell death, we compared the cell viabilities of AMBRA1 sufficient and deficient cells. We first optimized the dose of poly(I:C) transfected

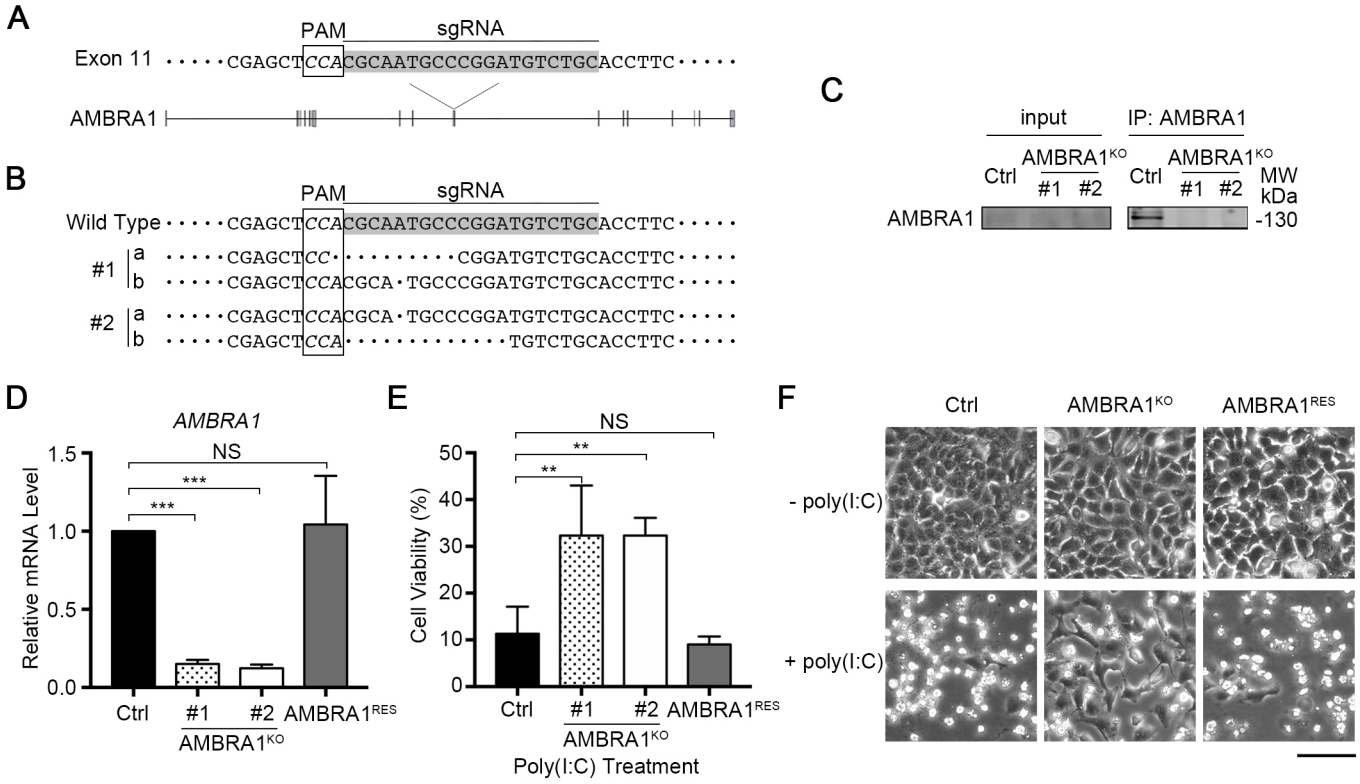

Fig. 1. AMBRA1 promotes dsRNA-induced cell death. (A) The sequence of sgRNA against the CDS region of *AMBRA1* gene. (B) The sequences of two AMBRA1^{KO} cell clones. Genomic DNA of two cell clones was extracted and then the region surrounding the sgRNA targeting sequence was amplified and sequenced. (C) Co-IP assay. The cell lysates of the control (Ctrl) and two cell clones were incubated with anti-AMBRA1 antibody. The levels of AMBRA1 were detected by western blotting. (D) qRT-PCR analysis of *AMBRA1* mRNA levels. Total RNAs of the control, AMBRA1^{KO} and AMBRA1^{RES} A549 cells were extracted for qRT-PCR analysis. (E) Cell viabilities measured by MTT assay. The control, AMBRA1^{KO} and AMBRA1^{RES} A549 cells were transfected with 1 µg/ml poly(I:C). The MTT assay was performed at 24 h post-transfection. (F) Cell images. The control, AMBRA1^{KO} and AMBRA1^{RES} A549 cells were transfected with 1 µg/ml poly(I:C). Then, cell images were taken at 24 h post-transfection. Representative images of three independent experiments are shown. Data are mean±s.d. from three experiments. ***P*<0.01; ****P*<0.001; NS, not significant (ANOVA with Dunnett's multiple comparison test). Scale bar: 50 µm. IP, immunoprecipitation; MW, molecular weight; PAM, protospacer adjacent motif.

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into cells. The control and AMBRA1^{KO} cells were transfected with different doses of poly(I:C) (0.5, 1 and 1.5 µg/ml), and at 24 h post-transfection, the cell viabilities were measured. Control cells underwent cell death upon poly(I:C) treatment, and the extents of cell death were correlated with the doses of poly(I:C). The cell viabilities of AMBRA1^{KO} were about 1.4-, 2-, and 1.8-fold higher than control cells at 0.5, 1 and 1.5 µg/ml poly(I:C), respectively (Fig. S1A). Therefore, 1 µg/ml of poly(I:C) were used in the following assays unless indicated. At 24 h post-transfection, the viability of control cells was ∼10% (black columns, Fig. 1E), and the viabilities of two KO cell clones were ∼30% (Fig. 1E). As expected, complementation of AMBRA1 resulted in a comparable level of cell viability as control cells (Fig. 1E). Under microscopy, characterizations of cell death, including membrane blebbing and cell shrinkage were observed in poly(I:C)-treated control and AMBRA1^{RES} cells, and the degree of cell death in the AMBRA1^{KO} cells was significantly reduced (Fig. 1F).

In addition, we generated two AMBRA1^{KO} cell clones in HeLa cells and validated the successful editing by genomic DNA sequencing (Fig. S1B) and qRT-PCR (Fig. S1C). In HeLa cells, depletion of AMBRA1 also led to a reduction of cell death induced by dsRNA (Fig. S1D,E). Altogether, these observations indicated that AMBRA1 positively regulates dsRNA-induced cell death.

AMBRA1 is involved in the apoptosis induced by dsRNA

As dsRNA can induce three types of PCD, including apoptosis (Zhao et al., 2012), necroptosis (Takaki et al., 2017; Yu et al., 2017) and pyroptosis (Sanjo et al., 2019), we explored which type of cell death AMBRA1 mediates. First, we evaluated whether the cell death is affected by inhibitors of apoptosis or RIPK1, a key mediator of necroptosis. The control and AMBRA1^{KO} cells were transfected with poly(I:C) in the presence of vehicle (DMSO), the apoptosis inhibitor z-VAD(OMe)-FMK (zVAD) or RIPK1 inhibitor Necrostatin-1 (Necro-1) (Gao et al., 2019). Cell viabilities were measured by MTT assay at 24 h post-transfection. In the control cells, zVAD treatment resulted in a dramatic increase of cell viability, reaching a similar level as AMBRA1^{KO} cells (Fig. S2A), implying that AMBRA1 is involved in apoptosis. In contrast, the cell viabilities of control or AMBRA1^{KO} cells were not altered by Necro-1 treatment, indicating an exception of RIPK1-mediated necroptosis (Wang et al., 2019). To further probe whether RIPK1-independent necroptosis and pyroptosis occur in poly(I:C)-triggered A549 cells, we used necrosulfonamide (NSA), an inhibitor that blocks both necroptosis (Sun et al., 2012) and pyroptosis (Rathkey et al., 2018). A549 cells were stimulated with poly(I:C) in the presence of vehicle (DMSO) and different concentrations of NSA. The NSA treatment alone did not alter the cell viabilities (Fig. S2B), suggesting that necroptosis and pyroptosis are not the main types of cell death in poly(I:C)-stimulated A549 cells.

To confirm that AMBRA1 regulates poly(I:C)-induced apoptosis, we compared the apoptosis extents by western blotting, immunofluorescence microscopy (IFM) and flow cytometry. Levels of caspase-3, an executor caspase of apoptosis, cleaved caspase-3 (c-caspase-3) and poly(ADP-ribose) polymerase (PARP), an apoptosis indicator, were detected by western blotting. The ratio of c-caspase-3/caspase-3 in the AMBRA1^{KO} cells decreased compared to control or AMBRA1^{RES} cells at 9 h and 12 h post-transfection, respectively (Fig. 2A). Likewise, the cleavage of PARP in the AMBRA1^{KO} cells was decreased compared to control or AMBRA1^{RES} cells (Fig. 2A). Flow cytometry analysis showed that the percentage of apoptotic cells was significantly reduced in AMBRA1^{KO} cells (∼41%) compared to control cells (∼60%) and AMBRA1^{RES} cells (∼68%) (Fig. 2B,C). Finally, we monitored the extent of cell karyopyknosis, another indicator of apoptosis, by IFM. In the mock-treated cells, the nuclei were intact, and nuclear condensation was clearly visualized in poly(I:C)-treated cells (Fig. 2D). The percentage of cells with nuclear condensation was lower in the AMBRA1^{KO} cells (∼22%) compared to the control (∼50%) and AMBRA1^{RES} cells (∼48%). Similarly, in HeLa cells, the cleavage of PARP and karyopyknosis was remarkably decreased in AMBRA1^{KO} cells compared to control cells (Fig. S2C,D). Collectively, our results demonstrated that AMBRA1 promotes dsRNA-induced apoptosis.

AMBRA1 interacts with MAVS

Caspases play a vital role in the initiation and execution of apoptosis (Creagh et al., 2003). To distinguish the role of caspase-8 and caspase-9, two key initiator caspases for extrinsic and intrinsic pathways, respectively, in poly(I:C)-induced apoptosis (Budihardjo et al., 1999; Van Opdenbosch and Lamkanfi, 2019), we applied an RNAi strategy. A549 cells were transfected with a negative control siRNA (siNC), *caspase-8*-specific siRNA (si-cas8), or *caspase-9*-specific siRNA (si-cas9) for 48 h, followed by poly(I:C) stimulation. At 9 h post-transfection of poly(I:C), the cells were harvested for western blotting. The caspase-8 or caspase-9 protein levels in si-cas8-transfected or si-cas9-transfected cells were significantly lower than those in the siNC-transfected cells (Fig. 3A), indicating that knockdown of caspase-8 or caspase-9 was effective. The ratio of c-caspase-3/caspase-3 and the cleavage extents of PARP in mock-treated (Fig. 3A, lane 2) and siNC-treated cells (Fig. 3A, lane 4) were comparable. Knockdown of caspase-8 (Fig. 3A, lane 6), but not caspase-9 (Fig. 3A, lane 8), dramatically decreased the ratio of c-caspase-3/caspase-3 and the cleavage of PARP compared to mock-treated (Fig. 3A, lane 2) or siNC-treated cells (Fig. 3A, lane 4). Similarly, significant reductions of caspase-3 cleavage and PARP cleavage were also observed in caspase-8 knockdown HeLa cells compared to mock-treated or siNC-treated cells (Fig. 3B). These data suggest that caspase-8, rather than caspase-9, plays a dominant role in dsRNA-triggered apoptosis.

Next, we tested whether AMBRA1 affects the activation of caspase-8 in poly(I:C)-treated A549 and HeLa cells. Upon poly(I:C) stimulation, the cleavage of caspase-8, caspase-3 and PARP in AMBRA1^{KO} A549 cells was dramatically decreased compared with control cells (Fig. 3C). Similar results were observed in AMBRA1^{KO} HeLa cells (Fig. 3D), implying that AMBRA1 plays a role in caspase-8 activation.

In the dsRNA-triggered apoptosis pathway, the activation of caspase-8 is mediated by the mitochondrial adaptor MAVS (El Maadidi et al., 2014; Li et al., 2009), which receives the signal from RIG-I-like receptor (RLR) sensors (Kawai et al., 2005). As AMBRA1 is involved in the activation of caspase-8, and partially locates in mitochondria (Strappazzon et al., 2011), we proposed that AMBRA1 probably functions through interacting with RLR sensors or their adaptor. To test this hypothesis, we constructed plasmids expressing these sensors or the adaptor, including RIG-I, MDA5 and MAVS. The 293T cells were co-transfected with plasmid expressing AMBRA1-HA with plasmids expressing RIG-I-FLAG, MDA5-FLAG, MAVS-FLAG or GFP-FLAG. At 24 h post-transfection, whole-cell lysates were collected for co-IP assay. The data showed that AMBRA1-HA co-precipitated with MAVS-FLAG but not RIG-I-FLAG and MDA5-FLAG (Fig. 3E). Interestingly, although two forms of MAVS, namely full-length (∼72 kDa) and mini-MAVS (∼50 kDa) (Brubaker et al., 2014) were present in the cell extracts (Fig. 3E, left panel), only the full-length

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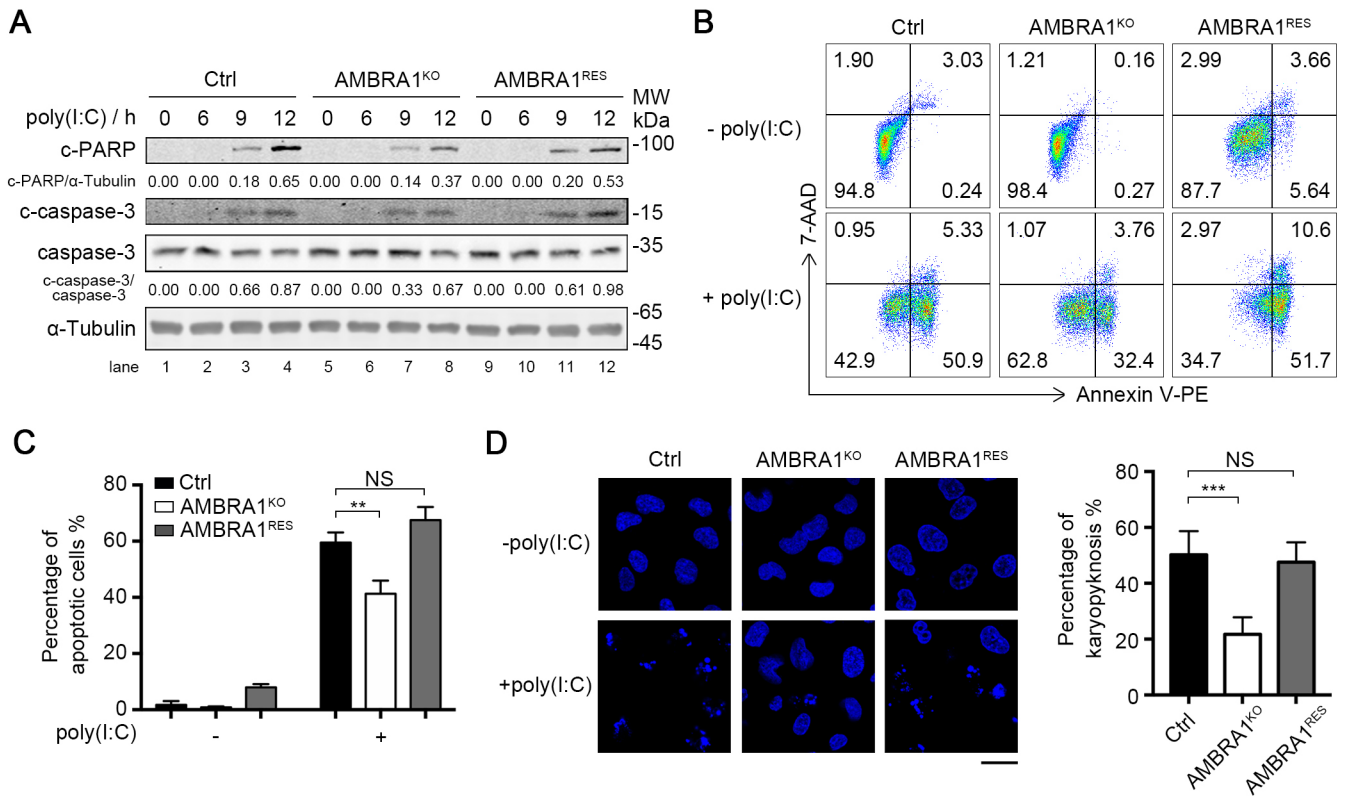

Fig. 2. AMBRA1 is involved in dsRNA-induced apoptosis. (A) Western blot analysis. The control (Ctrl), AMBRA1^{KO} and AMBRA1^{RES} A549 cells were transfected with 1 µg/ml poly(I:C) for the indicated times. The cells were collected for measurement of caspase-3, c-caspase-3 and c-PARP by western blotting. α-Tubulin was probed as an internal control. Representative blots of three independent experiments are shown. (B) Flow cytometry analysis. The control, AMBRA1^{KO} and AMBRA1^{RES} A549 cells were treated with 1 µg/ml poly(I:C) for 12 h. The whole cells were collected for flow cytometry analysis by staining with Annexin V-PE and 7-AAD. Representative data of three independent experiments are shown. (C) Statistical analysis was carried out to reveal the proportion of apoptotic cells (Annexin V-PE^{+}). (D) IFM analysis. The control, AMBRA1^{KO} and AMBRA1^{RES} A549 cells were stimulated with 1 µg/ml poly(I:C). At 12 h post-transfection, the cells were stained with Hoechst 33342. Images were captured to reveal karyopyknosis. Representative images of three independent experiments are shown. Data are mean±s.d. from three experiments. $^{**}P<0.01$; $^{***}P<0.001$; NS, not significant (ANOVA with Dunnett's multiple comparison test). Scale bar: 20 µm. MW, molecular weight.

MAVS (~72 kDa) was precipitated with AMBRA1-HA (Fig. 3E, right panel).

To confirm that endogenous AMBRA1 interacts with MAVS, we further performed co-IP assay using AMBRA1 antibody or MAVS antibody. As expected, the AMBRA1 antibody immunoprecipitated with AMBRA1 and MAVS; and reciprocally, the MAVS antibody immunoprecipitated with MAVS and AMBRA1 in A549 cells (Fig. 3F). Next, we examined the localization of AMBRA1 and MAVS by IFM. The 293T and HeLa cells were co-transfected with plasmids expressing AMBRA1-HA and MAVS-FLAG, and were collected at 24 h for IFM assay using FLAG and HA antibodies. As shown in Fig. 3G, AMBRA1 (magenta) and MAVS (green) substantially colocalized in the cytoplasm. The intensity plot profile indicated a colocalization between AMBRA1 and MAVS (Fig. 3H) in both cells. These data demonstrate that AMBRA1 interacts with MAVS.

The transmembrane domain of MAVS is indispensable for MAVS to interact with AMBRA1

MAVS protein is composed of three domains, including the CARD-like domain (CARD), the proline-rich domain (Pro) and the transmembrane domain (TM) (Ren et al., 2020). To map which domain of MAVS mediates the MAVS/AMBRA1 interaction, we generated a series of constructs expressing MAVS truncates: 1-173-FLAG expressing 1-173 amino acids containing the CARD and Pro domains; Pro-FLAG expressing 78-173 amino acids containing the Pro domain; and TM-FLAG expressing 174-540 amino acids containing the TM domain (Fig. 4A). The 293T cells were co-transfected with plasmids expressing AMBRA1-HA and MAVS truncates, and were harvested for co-IP assay. Surprisingly, none of the MAVS truncates interacted with AMBRA1 (Fig. 4B). As a positive control, the full length MAVS was detected in the co-precipitated complex.

To further determine which domain of MAVS mediates the MAVS/AMBRA1 interaction, we used another panel of vectors expressing MAVS mutants fused to FLAG (Zhang et al., 2014). These vectors expressed full-length MAVS (MAVS-FLAG), MAVS lacking the CARD domain (ΔCARD-FLAG), MAVS lacking the Pro domain (ΔPro-FLAG) and MAVS lacking the TM domain (ΔTM-FLAG) (Fig. 4C). The 293T cells were co-transfected with plasmids expressing AMBRA1-HA and MAVS truncates, and were harvested for co-IP assay. Full-length MAVS-FLAG, ΔCARD-FLAG and ΔPro-FLAG but not ΔTM-FLAG co-precipitated with AMBRA1-HA (Fig. 4D), indicating that the TM domain is critical for protein interaction.

MAVS is crucial for AMBRA1 promotion of dsRNA-triggered apoptosis

As AMBRA1 interacts with MAVS, and MAVS is the key mediator in dsRNA-induced apoptosis (Yu et al., 2010), we hypothesized that

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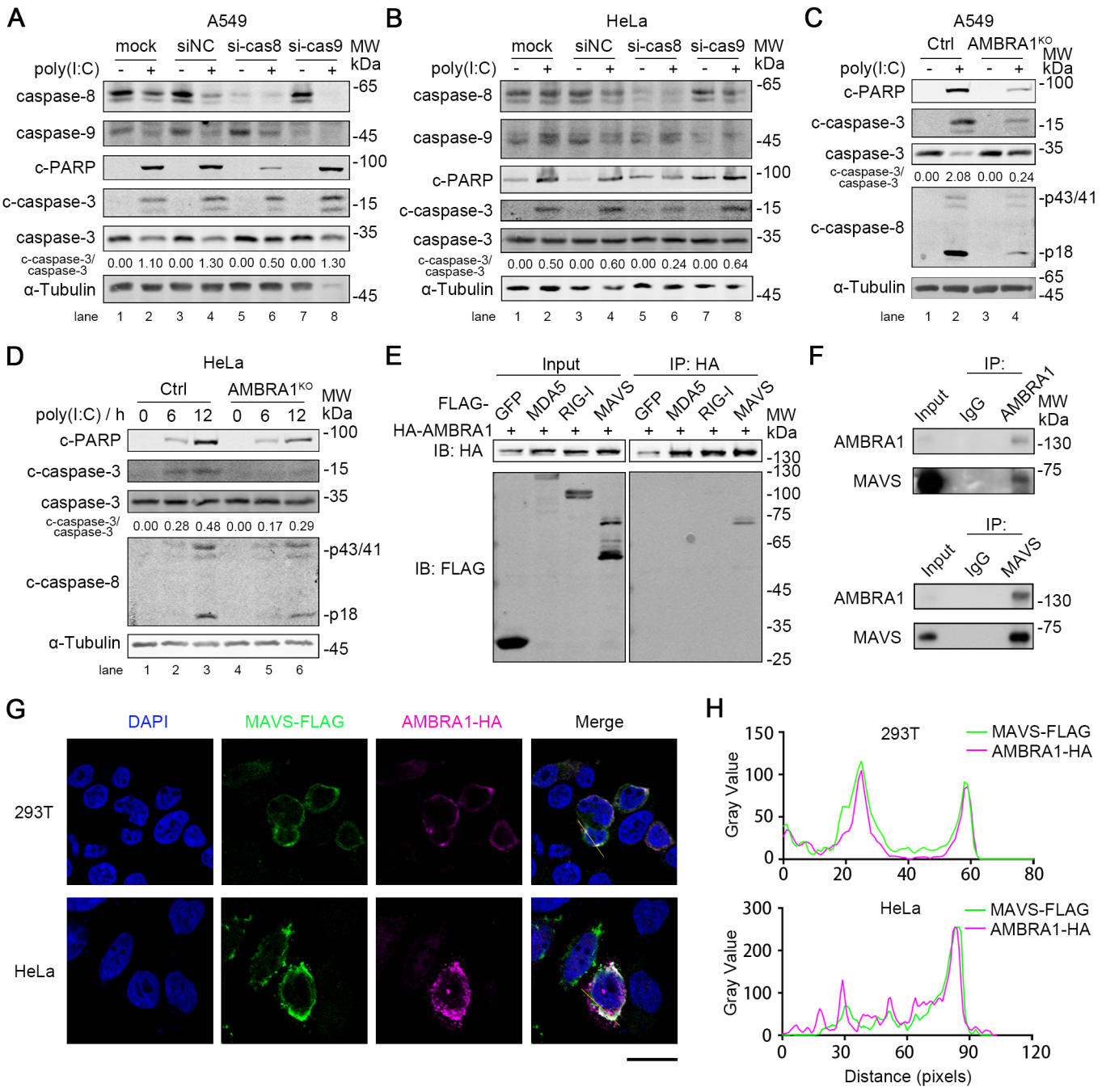

Fig. 3. AMBRA1 interacts with MAVS. (A,B) PARP and caspase-3 cleavage in A549 (A) and HeLa (B) cells. A549 or HeLa cells were transfected with siNC, si-caspase-8 (si-cas8) or si-caspase-9 (si-cas9). At 48 h post-transfection, A549 or HeLa cells were transfected with 1 µg/ml or 2 µg/ml poly(I:C) for 9 h, respectively. Then, the whole-cell lysates were harvested for the measurement of caspase-8, caspase-9, c-PARP, c-caspase-3 and caspase-3 levels. (C,D) C-PARP and c-caspase-8 levels. Control (Ctrl) and AMBRA1^{KO} A549 and HeLa cells were transfected with 1 µg/ml or 2 µg/ml poly(I:C), respectively. A549 cells were harvested at 12 h post-transfection (C) and HeLa cells were harvested at 6 h and 12 h post-transfection (D) for the measurement of c-PARP, c-caspase-3, caspase-3 and c-caspase-8 levels. (E) Co-IP assay in which 293T cells were co-transfected with plasmid expressing AMBRA1-HA and plasmids expressing FLAG-tagged GFP, MDA5, RIG-I and MAVS. At 24 h post-transfection, the whole-cell lysates were harvested and then prepared for co-IP assay using anti-HA antibody. Western blotting was performed to detect the interaction. (F) Endogenous AMBRA1 and MAVS interaction in A549 cells. Cell lysates of A549 cells were collected for immunoprecipitation using anti-AMBRA1 antibody or anti-MAVS antibody. (G) Subcellular localization of AMBRA1 and MAVS. The 293T and HeLa cells were co-transfected with plasmids expressing AMBRA1-HA and MAVS-FLAG. Anti-FLAG and anti-HA antibodies were used to indicate the subcellular localization of MAVS (green) and AMBRA1 (magenta). Nuclei were stained with DAPI (blue). Scale bar: 20 µm. (H) Colocalization analysis using Image J software is shown. α-Tubulin was probed as an internal control (A-D). Representative blots of three independent experiments are shown. IB, immunoblot; IP, immunoprecipitation; MW, molecular weight.

the pro-apoptotic effect of AMBRA1 might rely on MAVS. To test this hypothesis, we generated MAVS^{KO} A549 cells using CRISPR/Cas9. Depletion of MAVS was confirmed by western blotting (Fig. 5A, lanes 5-8). The apoptosis extents were measured by western blotting and flow cytometry. As expected, in response to poly(I:C), the cleavage of caspase-3 and PARP in MAVS^{KO} was

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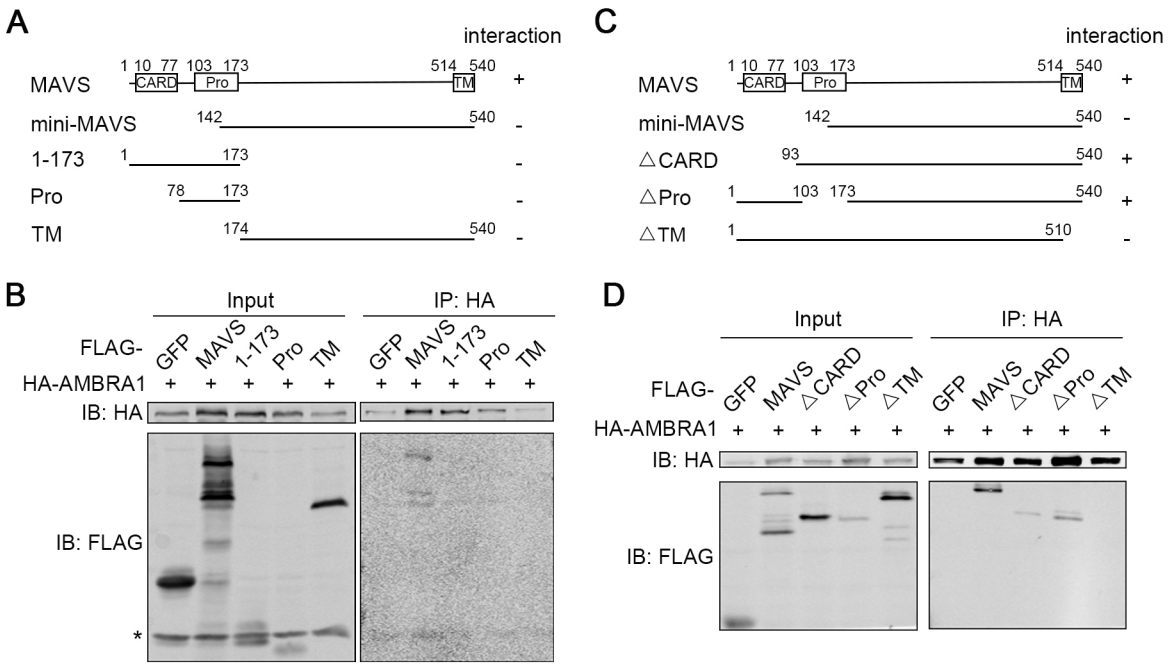

Fig. 4. The TM domain of MAVS is indispensable for MAVS to interact with AMBRA1. (A,C) Schematic illustration of constructs expressing truncated MAVS. (B,D) Co-IP assay to map the AMBRA1-interacting domain of MAVS. The 293T cells were co-transfected with plasmid expressing AMBRA1-HA and different truncates of MAVS. The cell lysates were harvested for co-IP assay using anti-HA antibody. Representative blots of at least three independent experiments are presented. The asterisk indicates non-specific bands. IB, immunoblot; IP, immunoprecipitation.

significantly lower than the control cells at the indicated time points (Fig. 5A). The flow cytometry analysis revealed that the apoptosis percentage of MAVS^{KO} cells (~17%) was significantly reduced compared to control cells (~54%) (Fig. 5B), confirming a pro-apoptotic role for MAVS in dsRNA-treated A549 cells.

Next, we investigated whether MAVS depletion affects the regulatory effect of AMBRA1 on apoptosis. Cells were transduced with lentivirus expressing AMBRA1-HA into control, AMBRA1^{KO} and MAVS^{KO} cells, followed by blasticidin selection. The ectopic expression of AMBRA1-HA in selected cells was clearly detected by western blotting using anti-AMBRA1 or anti-HA antibodies (Fig. 5C). Then, we compared the apoptosis extent induced by poly(I:C) in these cells by western blotting. As shown in Fig. 5D, the cleavage of PARP, caspase-3 and caspase-8 was readily detected in the vector-transduced control cells upon poly(I:C) stimulation (Fig. 5D, lane 2), and was enhanced in the AMBRA1-HA-expressing cells (Fig. 5D, lane 4). In the poly(I:C)-treated AMBRA1^{KO} cells, the cleavage of PARP, caspase-3 and caspase-8 in the AMBRA1-HA-expressing cells (Fig. 5D, lane 8) was also increased compared to the vector-transduced cells (Fig. 5D, lane 6). The depletion of MAVS led to a significant reduction of PARP, caspase-3 and caspase-8 cleavage, regardless of whether AMBRA1-HA was absent (Fig. 5D, lane 10) or present (Fig. 5D, lane 12). The pro-apoptotic effect of AMBRA1 was abolished in the absence of MAVS, suggesting that the regulatory effect of AMBRA1 on dsRNA-triggered apoptosis is mediated through MAVS.

AMBRA1 maintains the stability of MAVS

The observation that the pro-apoptotic effect of AMBRA1 relies on MAVS prompted us to examine whether their interaction is affected by dsRNA stimulation. A549 cells were transfected with mock or poly(I:C), and were harvested for co-IP assay. Fig. 6A shows that in the whole-cell extract, poly(I:C) led to a reduction of MAVS levels, as a result of proteasomal degradation (Zhou et al., 2012). Still, more AMBRA1 was associated with MAVS upon poly(I:C)-stimulation (Fig. 6A). Based on this observation, we proposed that AMBRA1-MAVS interaction might have an influence on MAVS stability. To test this hypothesis, we compared the protein levels of MAVS in control and AMBRA1^{KO} cells. Cells were transfected with mock or poly(I:C), and harvested at the indicated time points for western blotting. In the control cells, the MAVS levels gradually decreased, consistent with previous reports (Zhou et al., 2012). In the absence of AMBRA1, the MAVS levels were downregulated at higher extents (Fig. 6B,C), suggesting that AMBRA1 plays a role in maintaining the stability of MAVS. Of note, the mRNA levels of *MAVS* were comparable in the control and AMBRA1^{KO} cells (data not shown).

To further confirm the role of AMBRA1 in stabilizing MAVS, we tested whether MAVS expression was correlated with AMBRA1. The 293T cells were co-transfected with a fixed amount of plasmid expressing MAVS-FLAG, together with different doses of plasmid expressing AMBRA1-HA. At 24 h post-transfection, cells were collected for western blotting or an IFM assay. The AMBRA1-HA levels increased when transfected with higher doses of plasmid. Interestingly, the MAVS-FLAG levels were also correlated with the dosages of plasmid expressing AMBRA1-HA (Fig. 6D). Consistently, the cell images showed that the percentages of MAVS^{+} cells (green) were correlated with the percentages of AMBRA1^{+} cells (magenta) (Fig. 6E). These data suggest that AMBRA1 might function in preventing MAVS degradation through interacting it.

As the degradation of MAVS upon poly(I:C) treatment is mediated by proteasome (Castanier et al., 2012; Zhou et al., 2012), we sought to explore whether AMBRA1 is involved in the proteasomal degradation of MAVS. Cells were pretreated with MG132, an inhibitor of proteasome pathway, for 1 h, followed by poly(I:C) stimulation for 9 h. The degradation of MAVS in the control cells was abolished in the presence of MG132 (Fig. 6F, lanes

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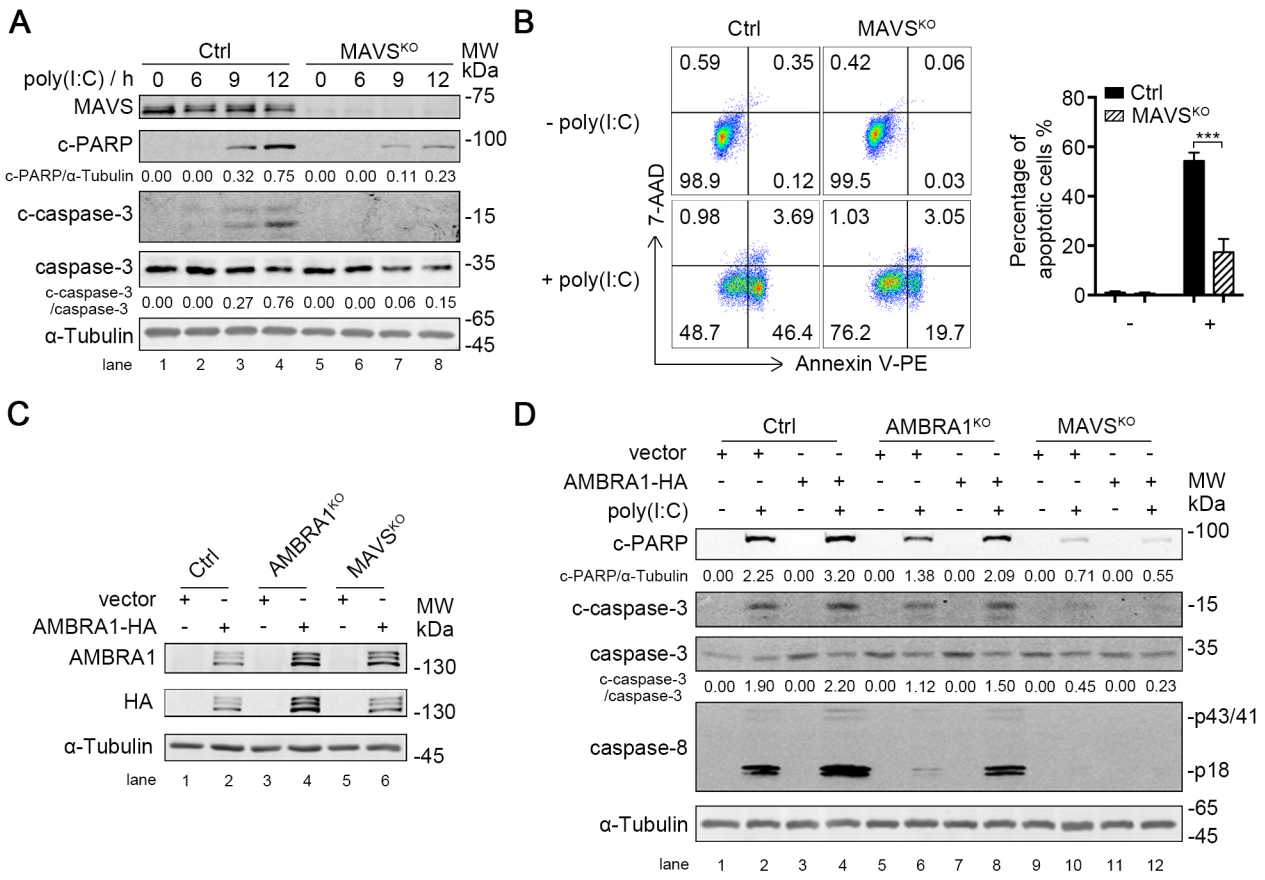

Fig. 5. The pro-apoptotic role of AMBRA1 is dependent on MAVS. (A) Caspase-3, c-caspase-3 and c-PARP, as measured by western blotting. The control (Ctrl) and MAVS^{KO} A549 cells were transfected with 1 µg/ml poly(I:C). At the indicated timepoints post-transfection, the cells were harvested for measurement of caspase-3, c-caspase-3 and c-PARP. The depletion of MAVS in MAVS^{KO} A549 cells was also confirmed. (B) Flow cytometry analysis. The control and MAVS^{KO} cells were transfected with 1 µg/ml poly(I:C). At 12 h post-transfection, the cells were collected for flow cytometry analysis by staining with Annexin V-PE and 7-AAD. Representative data of three independent experiments are shown. The statistical results of apoptosis (Annexin V-PE^{+}) are shown. Data are mean±s.d. from three experiments. ***P<0.001 (unpaired two-tailed Student's t-test). (C,D) Western blot analysis. (C) The control, AMBRA1^{KO} and MAVS^{KO} A549 cells were transduced with lentivirus expressing AMBRA1-HA and vector serving as a negative control, and selected using blasticidin. Selected cells were harvested for western blotting to analyze the ectopic expression of AMBRA1. (D) Cells were transfected with 1 µg/ml poly(I:C) for 9 h. The whole-cell lysates were collected for western blotting to measure c-PARP, c-caspase-3, caspase-3 and c-caspase-8. α-Tubulin was probed as an internal control (A,C,D). Representative blots of three independent experiments are presented. MW, molecular weight.

2 and 6; Fig. 6G, black columns), indicating that poly(I:C)-induced degradation of MAVS occurs through the proteasome pathway in A549 cells. Consistent with Fig. 6B, the MAVS levels in AMBRA1^{KO} cells were lower than the control cells after poly(I:C) stimulation (Fig. 6F, lanes 2 and 4). Intriguingly, the MAVS levels in AMBRA1^{KO} cells were restored to levels similar to that of the control cells in the presence of MG132 (Fig. 6F, lanes 6 and 8). The levels of MAVS in the control and AMBRA1^{KO} cells were comparable (MG132-treated group, Fig. 6G). These data indicate that AMBRA1 probably stabilizes MAVS by blocking the poly(I:C)-mediated proteasomal degradation of MAVS.

AMBRA1 promotes apoptosis induced by SFV infection

To explore whether AMBRA1 plays a role in virus-induced apoptosis, we used SFV as an infection model. SFV is an enveloped single-stranded positive RNA virus, and it leads to apoptosis through producing dsRNA during replication (El Maadidi et al., 2014). The control, AMBRA1^{KO}, AMBRA1^{RES} and MAVS^{KO} A549 cells were infected with SFV at a multiplicity of infection (MOI) of 1. At 6 h and 12 h post-infection, the cells were harvested for SFV RNA levels analysis by qRT-PCR. The RNA levels of SFV in all tested cells were comparable at the indicated time points (Fig. 7A), indicating that AMBRA1 or MAVS depletion does not alter the viral RNA replication prior to 12 h post-infection. At 48 h post-infection, typical apoptosis characteristics, including severe membrane blebbing and cell shrinkage were visualized in control A549 cells (Fig. S3A). As expected, the morphological changes of two AMBRA1^{KO} clones or MAVS^{KO} cells were less severe than the control and AMBRA1^{RES} cells upon SFV infection (Fig. S3A), indicating that AMBRA1 and MAVS are involved in SFV-induced apoptosis.

Next, we evaluated the role of AMBRA1 or MAVS in SFV-induced apoptosis using western blotting and flow cytometry. In the SFV-infected AMBRA1^{KO} and MAVS^{KO} cells, the ratio of c-caspase-3/caspase-3 and the cleavage of PARP and caspase-8 were decreased compared to control or AMBRA1^{RES} cells (Fig. 7B). The flow cytometry analysis showed that the percentages of apoptotic cells were significantly reduced in two AMBRA1^{KO} cell clones compared with control and AMBRA1^{RES} cells at 24 h and 48 h post-infection (Fig. 7C,D). As expected, the apoptosis extents in MAVS^{KO} were also dramatically reduced (Fig. 7C,D). These data suggest that AMBRA1 and MAVS positively regulate apoptosis induced by SFV. Then, we tested whether AMBRA1 or MAVS depletion has an impact on the late stage replication level of SFV. The control,

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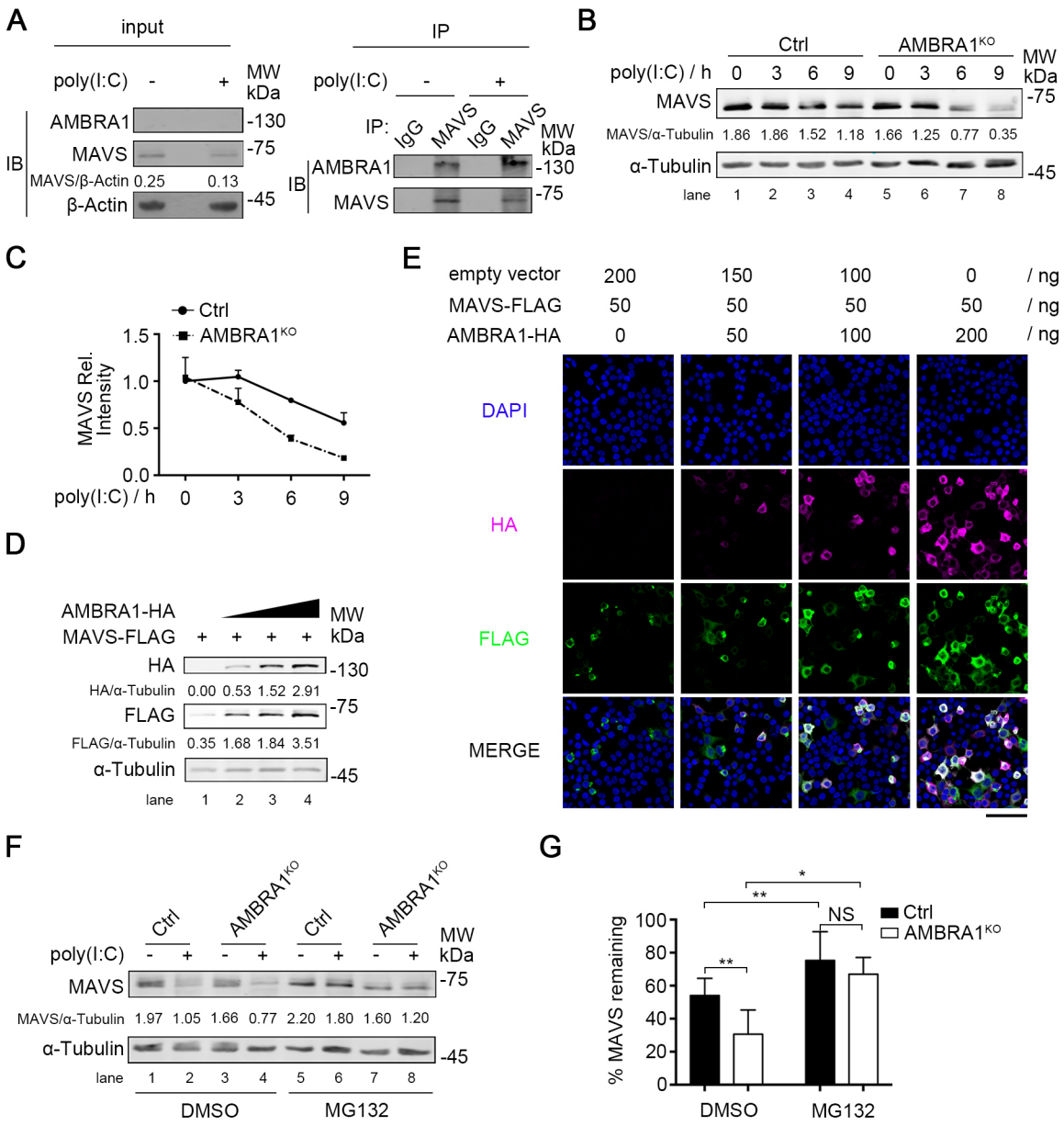

Fig. 6. AMBRA1 maintains the stability of MAVS. (A) Co-IP assay to detect the AMBRA1-MAVS interaction. A549 cells were treated with 1 µg/ml poly(I:C) for 9 h, and the cell lysates were prepared for co-IP assay using anti-MAVS antibody. Western blotting was performed to detect the interaction. Representative blots of three independent experiments are shown. β-Actin was probed as an internal control. (B) Western blot showing MAVS levels. The control (Ctrl) and AMBRA1^{KO} A549 cells were treated with 1 µg/ml poly(I:C) for the indicated times. The protein levels of MAVS were measured by western blotting, and α-Tubulin was probed as an internal control. Representative blots of three independent experiments are shown. (C) The relative intensities of MAVS were determined by normalizing the intensities of MAVS by the respective intensities of α-Tubulin. (D) Western blot analysis. The 293T cells were co-transfected with 100 ng MAVS-FLAG and 0, 200, 400 or 800 ng AMBRA1-HA in 12-well plate. Empty vectors were used to fill up the whole dose of plasmids. Anti-FLAG and anti-HA antibodies were used to show the protein levels of MAVS-FLAG and AMBRA1-HA, and α-Tubulin was probed as an internal control. Representative blots of three independent experiments are shown. (E) Immunofluorescence assay. The 293T cells were co-transfected with 50 ng MAVS-FLAG and 0, 50, 100 or 200 ng AMBRA1-HA in a 24-well plate. Empty vectors were used to fill up the whole dose of plasmids. Anti-FLAG and anti-HA antibodies were used to indicate the subcellular localization of MAVS (green) and AMBRA1 (magenta). Nuclei were stained with DAPI (blue). Scale bar: 50 µm. (F) Western blot showing MAVS levels. The control (Ctrl) and AMBRA1^{KO} A549 cells were pretreated with 10 µM MG132, followed by treatment with 1 µg/ml poly(I:C). Transfected cells were harvested for western blotting to detect MAVS protein at 9 h post-transfection, and α-Tubulin was probed as an internal control. Representative blots of three independent experiments are shown. (G) Statistical analysis of the percentages of remaining MAVS in the mock or poly(I:C)-treated cells of panel F. Data are mean±s.d. from three experiments. *P<0.05; **P<0.01; NS, not significant (unpaired two-tailed Student's t-test). IB, immunoblot; IP, immunoprecipitation; MW, molecular weight.

AMBRA1^{KO}, AMBRA1^{RES} and MAVS^{KO} cells were infected with SFV and harvested at 24 h post-infection for plaque assay. The viral yields in the AMBRA1^{KO} and MAVS^{KO} cells were enhanced by ∼10-fold and ∼4.5-fold, respectively, compared to the control cells (Fig. S3B). These data imply that AMBRA1 and MAVS promote SFV-induced apoptosis, which might help to block viral production.

DISCUSSION

AMBRA1 has been implicated in the modulation of the autophagic pathway and apoptosis induced by various stimuli (Di Bartolomeo et al., 2010; Fimia et al., 2007; Gu et al., 2014; Li et al., 2016; Liu et al., 2019; Pagliarini et al., 2012; Strappazzon et al., 2016; Sun et al., 2018, 2019). Nonetheless, whether AMBRA1 plays a role in

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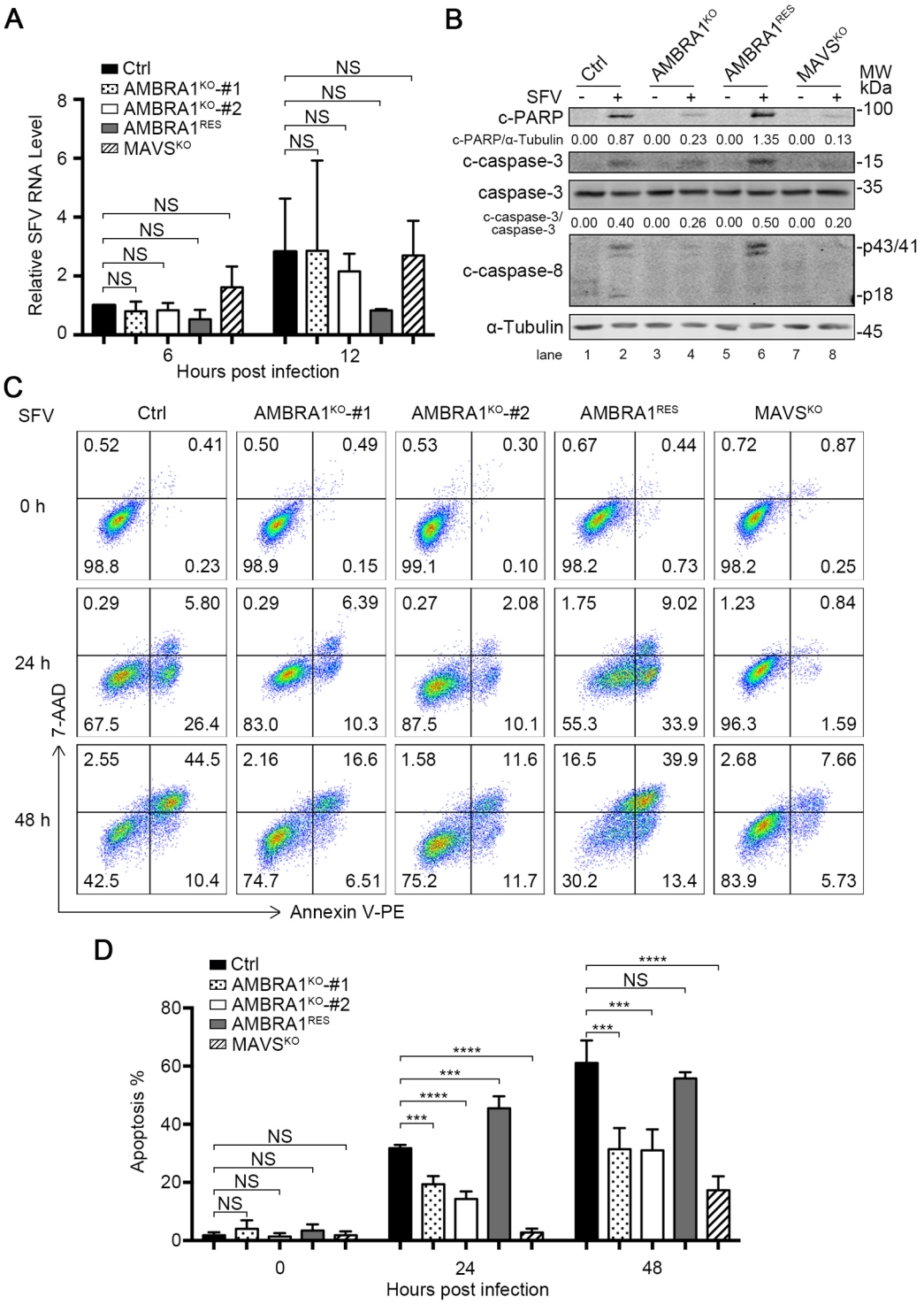

Fig. 7. AMBRA1 promotes apoptosis induced by SFV infection. (A) SFV RNA levels. The control (Ctrl), AMBRA1^{KO}, AMBRA1^{RES} and MAVS^{KO} A549 cells were infected with SFV at an MOI of 1. At 6 h and 12 h post-infection, the cells were harvested for qRT-PCR to determine the SFV RNA levels. (B) C-PARP, caspase-3, c-caspase-3 and c-caspase-8 levels measured by western blotting. The cells were infected with SFV at an MOI of 1. At 24 h post-infection, the cells were collected for the measurement of c-PARP, caspase-3, c-caspase-3 and c-caspase-8. α-Tubulin was probed as an internal control. Representative blots of three independent experiments are shown. (C) Flow cytometry analysis. The cells were infected with SFV at an MOI of 1 for 24 h and 48 h. Then, the whole cells were collected for flow cytometry analysis by staining with Annexin V-PE and 7-AAD. Representative data of three independent experiments are shown. (D) Statistical analysis was carried out to reveal the proportion of apoptotic cells (Annexin V-PE^{+}). Data are mean±s.d. from three experiments ***P<0.001; ****P<0.0001; NS, not significant (ANOVA with Dunnett's multiple comparison test). MW, molecular weight.

the cellular responses to virus remains uninvestigated. This study provides a first view of AMBRA1 regulation in apoptosis induced by dsRNA or virus. Several important findings emerged from our study.

First, we found that AMBRA1 positively regulates dsRNA-induced apoptosis. The KO of AMBRA1 resulted in less cell death induced by poly(I:C) or SFV, a virus that triggers apoptosis through

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the production of dsRNA (El Maadidi et al., 2014), and complementation of AMBRA1 restored the extent of cell death. Furthermore, we determined that AMBRA1 is a positive regulator of apoptosis induced by dsRNA or SFV, supported by data obtained from the apoptosis inhibitor (zVAD) assay, as well as apoptosis measurements (caspase-3 and PARP cleavage, karyopyknosis and the percentage of apoptotic cells). Although AMBRA1 has been reported to regulate apoptosis in different contexts, our work first established an association of AMBRA1 and apoptosis induced by virus or dsRNA that mimics viral byproduct. Intriguingly, AMBRA1 depletion does not affect the cell death induced by H_2O_2 or UV (data not shown), suggesting that AMBRA1-mediated apoptosis is stimuli dependent. AMBRA1 is also a key regulator of autophagy. Interestingly, we found that the extent of autophagy in the AMBRA1^{KO} cells was more significant compared to control cells by detecting the protein levels of two indicators of autophagy, p62 and LC3 II converted from LC3 I (Fig. S2E), suggesting that AMBRA1 downregulates dsRNA-induced autophagy, in accordance with the fact that AMBRA1 promotes dsRNA-induced apoptosis.

Mechanistically, our study associated AMBRA1 with MAVS, a key regulator of apoptosis induced by virus (Kawai et al., 2005). First, our study demonstrated an interaction between AMBRA1 and MAVS, and further mapped the region of MAVS required for their interaction. The co-IP and IFM data demonstrated that AMBRA1 interacts and colocalizes with mitochondrial MAVS. Importantly, we found that the TM domain of MAVS is indispensable for their interaction, suggesting that the mitochondria localization of MAVS is crucial. Moreover, most individual regions of MAVS except for the TM domain, including the N-terminal domain (1-173 amino acids), the Pro domain (78-173 amino acids) and the C-terminal domain (174-540 amino acids) were not sufficient to mediate their interaction. In combination with ΔCARD lacking 1-92 amino acids and ΔPro lacking 104-172 amino acids binding to AMBRA1, we propose that the 93-103 amino acid fragment of MAVS might be the key region mediating their interaction. Particularly, the interaction between MAVS and AMBRA1 was strengthened by poly(I:C) stimulation despite less MAVS present in the stimulated cells, illustrating a biological significance of their interaction.

Then, we found that the pro-apoptotic effect of AMBRA1 relies on the presence of MAVS. MAVS plays an essential role in apoptosis stimulated by dsRNA or SFV in A549 cells, consistent with previous reports (El Maadidi et al., 2014; Kumar et al., 2015; McAllister and Samuel, 2009). Interestingly, the pro-apoptotic effect of AMBRA1 is largely abolished in poly(I:C)-treated MAVS^{KO} cells, indicating that the pro-apoptotic effect of AMBRA1 depends on MAVS. As the mitochondrial localization of MAVS is critical for apoptosis induction (Okazaki et al., 2013) and for its interaction with AMBRA1, the function of AMBRA1 might be executed through mitochondrial MAVS.

We demonstrated that AMBRA1 prevents MAVS from proteasomal degradation. In the AMBRA1^{KO} cells, the MAVS protein levels but not its mRNA levels decreased more rapidly in the context of dsRNA stimulation, and MAVS protein was expressed at higher levels along with higher doses of AMBRA1, strongly suggesting that AMBRA1 is important for maintaining MAVS stability. When treated with the proteasomal inhibitor MG132, the MAVS levels in poly(I:C)-treated control cells were similarly increased as in poly(I:C)-treated AMBRA1^{KO} cells, indicating that AMBRA1 helps to prevent the proteasome-mediated degradation of MAVS, which is similar to its role in stabilizing ULK1 (Nazio et al., 2013). Therefore, we illustrated a new mechanism whereby AMBRA1 upregulates apoptosis through stabilizing IPS-1 (MAVS), which differs from a report showing AMBRA1 promoting apoptosis through binding and inactivating anti-apoptosis BCL2 (Strappazzon et al., 2016).

Furthermore, our data revealed that AMBRA1 is involved in the activation of caspase-8 induced by dsRNA. The pan-caspase inhibitor zVAD abolished poly(I:C)-induced apoptosis, consistent with a previous study showing caspase is essential in this pathway (Salaun et al., 2006). Further RNAi study demonstrated that caspase-8 and caspase-9, two main initiators in the apoptosis pathway, played differential roles in the dsRNA-induced apoptosis pathway. Caspase-8 knockdown in A549 and HeLa cells led to less apoptosis, in keeping with the observation that inhibition of caspase-8 efficiently blocks the cleavage of PARP in dsRNA-treated HeLa cells (Iordanov et al., 2005), whereas knockdown of caspase-9 did not alter the cleavage of PARP in poly(I:C)-treated A549 and HeLa cells. Intriguingly, some studies reported that caspase-9 is required for apoptosis triggered in poly(I:C)-treated SK-N-AS cells, AfMNPV-treated Sl-1 cells and silica- and poly(I:C)-induced NHBE cells (Chuang et al., 2012; Liu et al., 2012; Unno et al., 2014). These discrepancies suggested that the role of caspase-9 might be stimuli and cell specific, and in some cell types an alternative pathway might exist, so caspase-9 knockdown alone does not show an alteration of apoptosis. In addition, depletion of either AMBRA1 or MAVS significantly affected the cleavage of caspase-8, demonstrating that they function upstream of caspase-8 activation. As poly(I:C) triggers production of TNFα in dendritic cells (Kobayashi et al., 2013), which might elicit caspase-8-dependent apoptosis, we also tested whether the pro-apoptotic role of AMBRA1 is mediated through regulating TNFα. We found the TNFα level in A549 cells was only enhanced by 2-fold upon poly(I:C) stimulation, and treatment of TNFα did not elicit apoptosis in A549 cells (data not shown). Therefore, an involvement of a paracrine effect of TNFα in AMBRA1-regulated apoptosis was ruled out.

Given the role of AMBRA1 in stabilizing MAVS and activating caspase-8, we proposed that AMBRA1 mediates apoptosis through regulating the recruitment and activation of caspase-8 by MAVS. MAVS has been shown to regulate apoptosis through various mechanisms, one of which is by recruiting and activating caspase-8 (El Maadidi et al., 2014; Li et al., 2009). As AMBRA1 interacts with MAVS, and AMBRA1 promotes the activation of caspase-8, we deduced that AMBRA1 has an impact on the interaction between MAVS and caspase-8, which benefits the cleavage and activation of caspase-8 (El Maadidi et al., 2014; Li et al., 2009).

In conclusion, our work identified AMBRA1 as a new regulator involved in apoptosis induced by poly(I:C) and SFV. Furthermore, we illustrated that AMBRA1 interacts with MAVS and prevents its proteasomal degradation, thereby facilitating the activation of caspase-8 (Fig. 8). Therefore, we have established a relationship between AMBRA1 and MAVS, a central molecule in the cellular response to viral infection, and revealed a new mechanism of AMBRA1 in apoptosis.

MATERIALS AND METHODS

Cells and reagents

Human lung carcinoma epithelial cells [A549, American Type Culture Collection (ATCC), CCL-185], human cervical cancer cells (HeLa, ATCC, CCL-2) and human embryonic kidney cells (293 T, ATCC, CRL-3216) were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37°C in an incubator with 5% CO_2. African green monkey kidney cells (Vero, ATCC, CCL-81) were maintained in DMEM supplemented with 5% FBS at 37°C in

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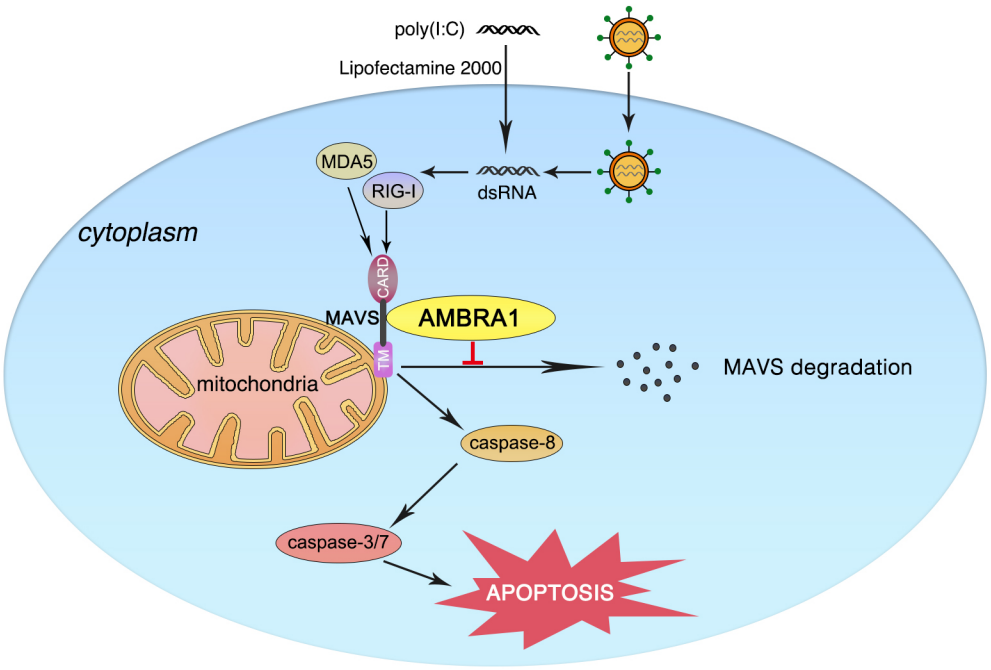

Fig. 8. A proposed model to illustrate the mechanism by which AMBRA1 promotes dsRNA- and virus-induced apoptosis. In response to dsRNA or viral infection, MDA5 and/or RIG-I recognize dsRNA and signal to their adaptor, MAVS. MAVS recruits and activates the initiator caspase-8, and subsequently activates executor caspase-3/7, resulting in apoptosis. AMBRA1 interacts with and stabilizes MAVS through preventing the proteasomal degradation of MAVS, therefore promoting the induction of apoptosis.

an incubator with 5% CO_2. *Aedes albopictus* cells (C6/36, ATCC, CRL-1660) were maintained in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Gibco) and non-essential amino acids (Gibco) at 28°C in an incubator with 5% CO_2. The media were added with 100 units/ml of streptomycin and penicillin (Invitrogen). Poly(I:C) and puromycin was purchased from Sigma-Aldrich; z-VAD, Necrostatin-1, NSA, and MG132 were purchased from MCE; and Lipofectamine 2000 Reagent was purchased from Invitrogen. Blasticidin was purchased from InvivoGen.

Virus, virus propagation, titration and virus infection

SFV was kindly provided by Dr Zhou Xi (Qian et al., 2020). It was propagated by infecting the C6/36 cells with an MOI of 20. After culturing at 28°C for 24 h, the supernatant was harvested and centrifuged at 350 ***g*** for 5 min to remove cell debris. The supernatant was stored at −80°C. Virus titers of SFV were determined by a standard plaque assay. In the plaque assay, Vero cells in 12-well plates were infected with a 10-fold serial dilution of viruses. The cells were cultured at 37°C for 2 h to allow the adsorption of all viruses. The supernatant was then replaced with a mixture of 2× DMEM containing 20% FBS, 2% penicillin-streptomycin and isopycnic 2% low melting point agarose (Sigma-Aldrich) (1:1). After incubation at 37°C for 24 h, the cells were fixed with 10% formaldehyde and stained with 0.5% Crystal Violet.

Cells were infected with SFV at an MOI of 1 in DMEM without FBS. Cells were harvested at the indicated time points for qRT-PCR, western blotting or flow cytometry detection.

Antibodies

Primary antibodies used included the following: anti-p62 (Santa Cruz Biotechnology sc-28359; 1:1000); anti-β-actin (Sigma-Aldrich A1928; 1:5000); anti-cleaved PARP (Cell Signaling Technology 5625; 1:1000); anti-caspase-3 (Cell Signaling Technology 14220; 1:1000); anti-LC3 I/II (MBL PM036; 1:500); anti-α-tubulin (BBI Life Science RM2007; 1:5000); anti-AMBRA1 (Santa Cruz Biotechnology sc-398204; 1:500); anti-MAVS (Santa Cruz Biotechnology sc-166583; 1:1000); anti-HA (MBL M180-3; 1:1000); anti-FLAG (MBL PM020; 1:1000); anti-caspase-8 (Proteintech 13423-1-AP; 1:1000); anti-caspase-9 (Proteintech 10380-1-AP; 1:1000); and anti-c-caspase-8 (Cell Signaling Technology 9496; 1:1000). Secondary antibodies used included the following: IRDye 800 CW-conjugated anti-rabbit IgG; IRDye 680 CW-conjugated anti-mouse IgG (LI-COR); horseradish peroxidase-conjugated anti-mouse IgG (Cell Signaling Technology); and anti-rabbit IgG (Bio-Rad). Secondary antibodies used for immunofluorescence assay included goat anti-rabbit IgG secondary antibody (Alexa Fluor 488) and goat anti-mouse IgG secondary antibody (Alexa Fluor 647) from Invitrogen.

Generation of AMBRA1 and MAVS KO cell clones

AMBRA1 and MAVS KO cell clones were generated using CRISPR/Cas9 (Ran et al., 2013). sgRNAs targeting *AMBRA1* and *MAVS* were cloned into lentiCRISPR v2 (Addgene, 52961). Sequences of sgRNAs are listed in Table S1. Lentiviruses were packaged in 293T cells. LentiCRISPR v2 containing single sgRNA, along with pSPAX2 (Addgene, 12260) and pVSVG (Addgene, 12259), were introduced into 293T cells using FuGENE HD Reagent (Promega). After 2 days, culture supernatants were passed through a 0.45 µm filter, and used for gene transduction. A549 and HeLa cells were transduced with lentiviruses expressing single sgRNA and selected by puromycin (1 µg/ml), and single clones were isolated. AMBRA1 KO cell clones were confirmed by Sanger sequencing, and mRNA levels of *AMBRA1* were measured by qRT-PCR. Genomic DNA was extracted using a genomic DNA extraction kit (Bioteke). Regions surrounding sgRNA target sequence were amplified by PCR and then PCR products were cloned into pMD-18T (TaKaRa) for Sanger sequencing. Primer sequences are listed in Table S2.

Generation of AMBRA1^{RES} cells and stable expression of exogenous AMBRA1 in AMBRA1^{KO} and MAVS^{KO} cells

The *AMBRA1* gene fragment was amplified by PCR and cloned into the lentivirus vector CSII-EF-MCS-IRES2-Venus (Riken, RDB04384) or pLV-EF1α-IRES-Blast (Addgene #85133). The primer sequences used are listed in Table S2. Lentiviruses were packaged in 293T cells. pLV-EF1α-IRES-Blast-AMBRA1 or CSII-EF-MCS-IRES2-Venus-AMBRA1, along with pSPAX2 and pVSVG, were introduced into 293T cells using FuGENE HD Reagent. After 2 days, culture supernatants were collected and passed through a 0.45 µm filter, and used for gene transduction. AMBRA1^{KO} cells were transduced with lentivirus carrying the *AMBRA1* gene (CSII-EF-MCS-IRES2-Venus), and Venus^{+} cells were sorted as AMBRA1^{RES} cells by flow cytometry (BD). AMBRA1^{KO} or MAVS^{KO} cells were transduced with lentivirus carrying the *AMBRA1* gene (pLV-EF1α-IRES-Blast), and then selected by Blasticidin (15 µg/ml) for 1 week.

qRT-PCR

Total RNAs were reverse transcribed using HI Script Q RT SuperMix (Vazyme). The cDNA was used as the template for qRT-PCR. The qRT-PCR

was performed using SYBR Select Master Mix for CFX (Applied Biosystems) and a Bio-Rad CFX96 machine. The PCR data were analyzed using SDS software (Applied Biosystems). The mRNA level of *β-actin* was measured as an internal control. The primers used for qRT-PCR are listed in Table S3.

Plasmid construction and transfection

To amplify full-length *AMBRA1*, *MDA5* and *RIG-I*, cDNA prepared from A549 cells was used as template. The PCR primers used are listed in Table S2. The 1-173-FLAG construct expressing amino acids 1-173 containing the CARD and Pro domains was amplified using full-length MAVS. Pro-FLAG and TM-FLAG were gifts from Dr Zhu Xun (School of Medicine, Sun Yat-Sen University, Guangzhou, China) (Hu et al., 2019). The amplified fragments were purified and cloned into pSG5 vector. All plasmids were transfected into cells using Lipofectamine 2000 Reagent following the manufacturer's instructions.

Treatment of poly(I:C) and cell imaging

Cells were seeded in 12-well plates. Poly(I:C) (1 µg/ml) was transfected into cells using Lipofectamine 2000 Reagent in all experiments according to the protocols provided by the manufacturer. At 24 h post-transfection, photographs were taken using a Leica DMi8 microscope.

MTT assay

MTT (Genview) was dissolved in PBS at a concentration of 5 mg/ml before use. The cells were incubated in the presence of MTT for 4 h. After centrifugation at 350 ***g*** for 5 min, the supernatant was discarded and DMSO was added to the plate, followed by 10 min of gentle shaking. The OD value was measured at 490 nm using a BioTek Instrument (BioTek).

CCK8 assay

Cell Counting Kit-8 (CCK8) was purchased from MCE. Reagent (80 µl) was added to cell culture (12-well plate) and incubated at 37°C and 5% CO_2. One hour later, the OD value was measured at 450 nm using a BioTek Instrument (BioTek).

Western blotting

Cells were lysed in RIPA lysis buffer [pH 7.4; 50 mM Tris-HCl, 0.5% (v/v) NP-40, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% protease inhibitor cocktails (Sigma-Aldrich), 1 mM Na_3VO_4 and 1 mM NaF]. Proteins were separated on SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked in PBS plus Tween 20 with 5% bovine serum albumin (BSA; New England Biolabs) and incubated with the indicated primary antibodies at 4°C overnight. IRDye 800 CW-conjugated anti-rabbit IgG (LI-COR), IRDye 680 CW-conjugated anti-mouse IgG (LI-COR) or horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) served as secondary antibodies. Detection was performed according to the manufacturer's protocols.

Inhibitor assay

zVAD and Necrostatin-1 (Necro-1) were dissolved in DMSO at a stock concentration of 100 mM. NSA and MG132 were dissolved in DMSO at a stock concentration of 5 mM and 40 mM, respectively. Cells were pretreated with 50 µM zVAD, 100 µM Necro-1, their combination or indicated concentration of NSA for 1 h. Cells were transfected with poly(I:C) along with DMSO or these inhibitors. Cells were pretreated with DMSO or 10 µM of MG132 for 1 h, followed by poly(I:C) transfection.

RNAi

The sequences of the siRNAs targeting human *caspase-8* mRNA and *caspase-9* mRNA were 5′-GUCAUGCUCUAUCAGAUUU-3′ and 5′-GUCGAAGCCAACCCUAGAA-3′, respectively. A control siRNA with scrambled sequence was used as a negative control (siNC). Transfection was carried out with 16 nmol of siRNAs by using Lipofectamine 2000 Reagent according to the manufacturer's instructions. At 48 h post-transfection, cells were harvested for further analysis.

Flow cytometry

Apoptotic cells were assessed by Annexin V-PE/7-AAD detection. Cells were seeded into 12-well plates, and then transfected with poly(I:C) or infected with SFV at an MOI of 1. At 12 h post-transfection or indicated time points of SFV infection, cells were collected by centrifugation at 600 ***g*** for 5 min, washed with ice-cold PBS, and subsequently resuspended in 100 µl of 1× binding buffer prior to incubation with 2.5 µl Annexin V-PE and 2.5 µl 7-AAD (BD). The specimens were incubated on ice for 15 min, and then 300 µl of 1× binding buffer was added to dilute the cell suspension. Subsequently the portions of apoptotic cells were detected using a flow cytometer (Beckman CytoFLEX, USA).

Confocal assay

Cells were washed with PBS, fixed with 4% (v/v) paraformaldehyde, permeabilized with 0.02% Triton X-100, and then blocked in PBS with 5% BSA for 1 h. Cells were incubated with primary antibodies at 4°C overnight. Cells were incubated for 1 h with Alexa Fluor 647-conjugated goat anti-mouse-IgG (Invitrogen) and Alexa Fluor 488-conjugated anti-rabbit-IgG (Thermo Fisher Scientific) at room temperature. Cells were stained with DAPI (Invitrogen, CA, USA). Fluorescent images were captured using a Nikon Eclipse Ni-E microscope.

Co-immunoprecipitation

The 293T cells were co-transfected with AMBRA1-HA and the indicated plasmids tagged with FLAG. At 24 h post-transfection, whole-cell lysates were harvested with RIPA lysis buffer. Lysates were incubated on ice for 30 min and then spun for 15 min at 4°C. Anti-HA beads (20 µl; Sigma-Aldrich) were added to the cell lysate and incubated overnight. The beads were washed with wash buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA and 0.5% NP40), and then the precipitated proteins were eluted by boiling in the loading buffer, and detected by western blot.

To detect the physical interaction between endogenous AMBRA1 and MAVS, A549 cell lysates were prepared with RIPA lysis buffer. The co-IP assay was performed using protein A/G agarose (Calbiochem) along with antibodies for AMBRA1 or MAVS, with IgG serving as a negative control.

Statistical analysis

All the data are means±s.d. from at least three independent experiments. Data were analyzed with GraphPad Prism 7.0 software. The statistical analysis was performed by ANOVA with Dunnett's multiple comparison test or with an unpaired two-tailed Student's *t*-test, and the differences were considered statistically significant when $P<0.05$.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Y.L., C.H., H.G., S.Z., C.L., P.Z.; Methodology: Y.L., C.H., H.G., X.L., S.Z., Z.H., Y.H., P.Z.; Software: Y.L., C.H., X.L., Q.L., S.Z., Z.H., Y.H.; Validation: Y.L., H.G., Q.L., S.Z.; Formal analysis: Y.L., C.H., X.L., Q.L., Z.H.; Investigation: Y.L., C.H., H.G., C.L.; Data curation: Y.L., Y.H.; Writing - original draft: Y.L., C.H., P.Z.; Writing - review & editing: C.L., P.Z.; Visualization: S.Z., C.L., P.Z.; Supervision: C.L., P.Z.; Project administration: C.L., P.Z.; Funding acquisition: C.L., P.Z.

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