Mitochondria-Derived Vesicles prevent inflammation by controlling the inclusion of mitochondrial proteins into extracellular vesicles

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Abstract

Most cells constitutively secrete mitochondrial DNA and proteins in extracellular vesicles (EVs), small vesicles that allow the transfer of material between cells. While under pro-inflammatory conditions, this mitochondrial content can further enhance inflammation, its role in the absence of inflammation remains elusive. Here, we demonstrate that mitochondrial components found in normal EVs are not pro-inflammatory and that cells actively prevent the packaging of pro-inflammatory, damaged mitochondrial proteins into EVs. Importantly, the distinction between material to be included into EVs and damaged mitochondrial content to be excluded was dependent on their selective targeting to one of two distinct Mitochondria-Derived Vesicles (MDVs) pathways, MDVs being small vesicles carrying material between mitochondria and other organelles. Sorting nexin 9 (Snx9)-dependent MDVs were required to target mitochondrial proteins into EVs, while the Parkinson's disease related protein Parkin blocked this process by targeting damaged mitochondrial content to lysosomes. Our results thus provide important insight into the interplay between mitochondrial quality control mechanisms and mitochondria driven immune responses.

Introduction

Most cells secrete a range of extracellular vesicles (EVs) that act as communication devices by carrying proteins and nucleic acids between cells [1-3]. EVs can be broadly divided in two classes: exosomes, small vesicles arising from the fusion of a multivesicular body with the plasma membrane, and the larger microvesicles, which are thought to arise from the direct budding of vesicles from the plasma membrane [4]. Intriguingly, a number of studies have identified mitochondrial proteins as EV cargo [5-10].

Mitochondria are essential organelles that act as a central metabolic hub. As a consequence, mitochondria regulate a number of key cellular processes, ranging from the production of cellular energy to the induction of apoptosis and cellular differentiation [11-13]. Impaired mitochondrial function has thus major impacts on both cells and the organism, which are compounded by the production of toxic reactive oxygen species by damaged mitochondria [14,15]. Importantly, damaged mitochondria, or the release from mitochondria of N-formyl peptides and mitochondrial DNA can act as damage-associated molecular patterns (DAMPs) that activate the innate immune system [16-22]. In fact, mitochondrial cargo within EVs and free mitochondria released by some cell types following pro-inflammatory stimulation like exposure to lipopolysaccharide (LPS) have been shown to stimulate the production of proinflammatory cytokines, further enhancing LPS-induced inflammation [21,23-27].

While inflammation promotes the secretion of mitochondrial content, mitochondrial proteins are also clearly present in EVs under unstimulated conditions [9,28]. The mechanism by which they are secreted and whether they also participate in immune activation remain unknown. Here we show that mitochondrial EV content from unstimulated cells is not proinflammatory and that cells selectively target damaged mitochondrial components for lysosomal degradation to prevent their release into EVs and subsequent inflammation. This process is dependent on Mitochondria-Derived Vesicles (MDVs), small vesicles that carry mitochondrial proteins to other organelles. Specifically, this accurate sorting of mitochondrial components requires two distinct MDV pathways. First, delivery of mitochondrial proteins to EVs requires Snx9-dependent MDVs, a subset of MDVs that were previously shown to regulate mitochondrial antigen presentation [29]. Second, MDVs carrying damaged mitochondrial components are instead targeted for lysosomal degradation in a process that depends on the Parkinson's disease related protein Parkin. Altogether,

our results demonstrate that cells selectively regulate the packaging of mitochondrial protein into EVs to prevent the release of damaged components that would otherwise act as pro-inflammatory DAMPs.

Results

Mitochondrial proteins within EVs are not pro-inflammatory

Mitochondrial content, especially damaged or oxidized components, can act as DAMPs that activate an inflammatory response when present in the cytosol or released from cells [16-22]. This can be demonstrated by incubating mitochondria isolated from cells with the RAW264.7 macrophage cell line and monitoring two different pro-inflammatory pathways (irf3 using IP10, and NF-κB using IL6). Exposure of RAW cells to isolated mitochondria caused a dramatic increase in IL6 (Fig. 1A) and a more modest increase in IP10 (Fig. 1B), as measured at the mRNA level and in the cell culture media. Mitochondrial content could readily be found in EVs isolated from different cell lines under conditions where cell death was kept below 5% to avoid the presence of apoptotic bodies (Cell death in Fig. 1C; Protein yield in Fig. 1D; Mitochondrial content in Fig. 1E). Therefore, we then tested the ability of EVs to stimulate inflammation. Compared to mitochondria, EVs caused a similar increase in IP10 (Fig. 1B) but did not stimulate IL6 production (Fig. 1A). This suggests that while EVs can somewhat stimulate inflammation, they do not activate the strong NF-κB response associated with mitochondrial DAMPs.

We then determined whether inducing oxidative damage to mitochondria further stimulated the IP10 response. For this, MEFs were treated for 24 hours with Antimycin A (AA), a complex III inhibitor that stimulates the production of Reactive Oxygen Species (ROS) [30-32] but causes minimal cell death (Fig. 1F). RAW cells were then exposed to EVs or mitochondria collected from these cells. Consistent with damaged mitochondria being more inflammatory, mitochondria isolated from AA-treated cells induced a larger IP10 secretion than control mitochondria (Fig. 1G). In contrast EVs isolated from AA-treated cells did not further stimulate IP10 secretion (Fig. 1G), suggesting that these mitochondrial DAMPs are prevented from being incorporated into EVs. In fact, examination of mitochondrial content revealed that the Complex I subunit NDUFA9 and the Complex III subunit UQCRC2 were selectively degraded following AA treatment (Fig. 1H). In contrast the matrix proteins mtHSP70 and PHD were not affected (Fig. 1H). Nevertheless, mtHSP70 levels within EVs were significantly decreased (Fig. 1I), while PDH levels were very low in control EVs and further decreased following AA treatment (Fig. 1H). In contrast, the amount of the mitochondrial outer membrane protein TOM20 found in EVs was not affected by AA treatment (Fig. 1I), consistent with outer membrane proteins not being affected by AA

treatment [33]. Altogether, these results suggest that cells selectively regulate the incorporation of mitochondrial components within EVs to prevent the secretion of pro-inflammatory DAMPs.

Mitochondrial proteins are selectively enriched in EVs

To identify the mechanism through which cells regulate the inclusion of mitochondrial proteins into EVs, we first characterised MEFs EVs and their mitochondrial content. EV preparations were enriched in the exosomal markers Alix and CD9, but excluded the nuclear protein Lamin B1 and the recycling endosome protein Rab11 (Fig. 2A). Protein quantification by normalizing the amount present in EVs to the cellular content confirmed that the exosome marker Alix was enriched in our EV preparations, while different endosomal markers were not (Rab11, Rab9 (late endosome), Snx9 (endocytosis); Fig. 2B), consistent with the selective inclusion of proteins within EVs. Having demonstrated the selectivity of EV content isolated from MEFs, we quantified the presence of a number of mitochondrial proteins in these EVs and found striking differences in the enrichment of individual mitochondrial proteins (Fig 2B). Among the proteins tested, TOM20 was present at the highest levels in unstimulated EVs, while PDH and the intermembrane space protein Cytochrome c (Cyt C) were present at very low levels (Fig 2B). Proteins associated with the inner membrane (mtHSP70, NDUFA9 and the mitochondrial fusion protein OPA1) were present at intermediate levels (Fig 2B). In addition to mitochondrial proteins, mitochondrial DNA (mtDNA) has been reported to be released from different cell types [6,24,34]. Consistent with this, we found that mtDNA was enriched in unstimulated MEF EV preparations (Fig 2B).

Some proteins, such as actin or histones, are peripherally associated with EVs rather than being sequestered inside these vesicles [35]. We thus tested whether mitochondrial proteins found in EVs were sensitive to trypsin digestion. As shown in Figure 2C, mitochondrial proteins, but not actin, were protected from trypsin digestion in EV preparations, unless the membranes were solubilised with Triton X-100 (Fig. 2C; TX100), indicating that mitochondrial proteins are found inside EVs. Overall, the distinct pattern we observed for the inclusion of mitochondrial content within unstimulated EVs suggest that this is a selective process distinct from bulk mitochondrial export. To confirm this, we examined our EV preparations by electron microscopy (EM). The majority of the vesicles were small, electron translucent and often cup-shaped (Fig. 2D, Top Right panel, quantified in 2E), consistent with exosomes [28,35]. A second population of vesicles had a distinct structure, containing a dense core (Fig. 2D Bottom Right panel, quantified in 2E), while a small

amount of material seemed aggregated and did not have a clear ultrastructure (Fig. 2D-E). Quantification of vesicle size indicated that the majority of the isolated EVs were less than 150 nm in diameter, while the few larger vesicles were smaller than 600 nm (Fig. 2F). Importantly, we did not find vesicles with a structure or size consistent with whole mitochondria. Altogether, these results are consistent with the reported characteristics of isolated EVs (including exosomes and microvesicles) [28,35], not extracellular mitochondria.

Snx9 regulates the inclusion of mitochondrial IM/Matrix proteins in EVs

To determine if this process is associated with the presence of intracellular vesicles accumulating specific mitochondrial proteins, we stained U2OS cells stably expressing GFP (to mark cytosol) for TOM20 and mtHSP70, two mitochondrial proteins enriched in our EV preparations (Fig. 2B). Structures positive for one mitochondrial marker but not the other were present close to the plasma membrane of these cells (Fig. 2G-H). In addition, there were more TOM20-positive structures than mtHSP70-positive structures (Fig. 2G), correlating with the amount of each protein present in EVs (Fig. 2B). These results are thus consistent with the presence of an intracellular vesicular pathway regulating the selective inclusion of mitochondrial proteins into EVs. The cargo-selective structures we observed are highly reminiscent of MDVs, small cargo-selective vesicles budding off mitochondria that are used to transport specific mitochondrial proteins to other organelles, including lysosomes where the material is degraded [36,37]. As this suggests that MDVs could target mitochondrial proteins to EVs, we addressed their role in EV cargo selection of mitochondrial proteins (general hypothesis in Fig. 3A).

A recent study demonstrated that Snx9, a dynamin-binding protein essential for clathrin-mediated endocytosis, is required for the production of MDVs positive for the mitochondrial matrix protein PDH [29]. To confirm that Snx9 could be used to manipulate IM/Matrix MDV formation, we knocked down Snx9 using siRNA (Fig. 3B). Snx9 knockdown did not affect intracellular mitochondrial content or overall mitochondrial morphology (Fig. 3B-C). We thus measured the presence of MDVs in these cells. MDVs are defined by their cargo selectivity, and distinct mechanisms are thought to regulate the formation of MDVs containing outer membrane proteins and IM/matrix proteins [36,37]. We thus labelled cells with TOM20 (outer membrane) and mtHSP70 (matrix) and counted the total number of small vesicles positive for one marker but not the other (Fig 3C). Loss of Snx9 caused a significant reduction in MDVs positive for mtHSP70 and negative

for TOM20 (mtHSP70 MDVs), but did not affect MDVs positive for TOM20 and negative for mtHSP70 (TOM20 MDVs) (Fig. 3D). This indicates that Snx9 regulates the formation of IM/matrix MDVs but not outer membrane MDVs.

Having confirmed that Snx9 knockdown can be used as a tool to define the role of MDVs in the inclusion of IM/Matrix proteins into EVs, we isolated EVs from siCtrl and siSnx9 cells media and measured their mitochondrial EV content. Consistent with the hypothesis that MDVs are required to target mitochondrial proteins to EVs, mtHSP70 and NDUFA9 were dramatically reduced in siSnx9 EVs (Fig 3E). In contrast, but consistent with the MDV data (Fig. 3D), knockdown of Snx9 did not affect the cellular levels of TOM20 (Fig 3B) or its inclusion into EVs (Fig. 3E). The selective loss of MDV formation and EV incorporation of mitochondrial proteins upon Snx9 knockdown thus suggest that MDVs are required for the inclusion into EVs of mitochondrial proteins associated with the mitochondrial IM/matrix.

Loss of OPA1 inhibits MDV formation and inclusion of mitochondrial proteins in EVs

To confirm our hypothesis, we sought for an independent way to prevent MDV formation without directly affecting the endosomal compartment. OPA1 is a mitochondrial IM protein required for mitochondrial IM fusion and maintenance of cristae structure [38-41]. Given that OPA1 was one of the mitochondrial proteins enriched in our EV preparation (Fig. 2B) and that its Dynamin-like activity could promote the budding of IM/Matrix MDVs from the IM, we reasoned that genetic deletion of OPA1 should abrogate the formation of IM/Matrix-derived MDVs, but not of outer membrane MDVs. To determine whether this was the case, we measure MDV formation in WT and OPA1 KO MEFs.

We first measured PDH MDVs (PDH-positive, TOM20-negative), as these MDVs have been extensively studied [29,36,42,43]. As shown in Fig. 4A, the number of PDH MDVs was greatly reduced in OPA1 KO MEFs. NDUFA9 and mtHSP70 MDVs were similarly reduced in OPA1 KO MEFs (Fig. 4B-C), further supporting a role for OPA1 in the generation of IM/Matrix MDVs. Importantly, TOM20 MDVs were not affected by OPA1 deletion (Fig. 4D).

As these results demonstrate that OPA1 deletion selectively affects the formation of MDVs containing IM/Matrix proteins, we then used OPA1 KO cells to test our hypothesis that MDVs are required to package IM/Matrix proteins into EVs. Size and overall protein content were similar

between WT and OPA1 KO EVs (Fig. 5A-B), and these contained similar amounts of cytoplasmic proteins (Fig. 5C, ACC1 and DJ1 quantified in 5D). As these results indicate that overall EV formation is not disrupted by the loss of OPA1, we measured the incorporation of mitochondrial proteins in WT and OPA1 KO EVs. At the cellular level, none of the mitochondrial proteins examined were affected by OPA1 deletion (Fig. 5E). However, consistent with MDVs being required for the inclusion of IM/Matrix proteins into EVs, NDUFA9 and mtHSP70 were almost completely absent from OPA1 KO EVs (Fig. 5E-F). Other electron transport chain components (SDHA (Complex II), UQCRC2 (Complex III) were also affected (Fig. 5E). In contrast, TOM20 was still incorporated into EVs in OPA1 KO MEFs (Fig. 5E, quantification in 5F), further supporting the selectivity of this process. As mtDNA was present in WT EVs, we also determined the effect of OPA1 deletion on mtDNA incorporation into EVs. Similar to IM/Matrix proteins, mtDNA content was decreased in EVs isolated from OPA1 KO MEFs (Fig. 5G; relative to cellular levels to take into account mtDNA differences between WT and OPA1 KO MEFs [38]). Altogether, our results indicate that OPA1 is required for the formation of IM/matrix MDVs, which are then required for the selective inclusion of mitochondrial content into EVs.

OPA1 KO mitochondria, but not EVs enhance inflammation

Overall, our results indicate that MDVs are required to package IM/Matrix proteins into EVs. As we observed that mitochondrial damage (AA treatment) prevents the inclusion of mitochondrial proteins into EVs (Fig. 1I), our results suggest that this pathway is inhibited following mitochondrial damage. OPA1 deletion prevents MDV formation and packaging of mitochondrial proteins into EVs, but also leads to the accumulation of damaged mitochondria [30,44], allowing us to directly test if inhibiting MDV formation is sufficient to prevent the generation of pro-inflammatory EVs. Consistent with OPA KO mitochondria being damaged, exposure of RAW cells to mitochondria isolated from these cells induced a greater secretion of IL6 and IP10 than WT mitochondria (Fig. 6A-B). In contrast, exposure of RAW cells to OPA1 KO EVs did not stimulate cytokine secretion to a greater extent than WT EVs (Fig. 6A-B). In addition, mRNA expression of RSAD2 and mIFIT1, two IFN-dependent genes, was slightly but significantly lower in cells exposed to OPA1 KO EVs compared to those exposed to WT EVs (Fig. 6C). Altogether, these results indicate that inhibiting the release of damaged mitochondrial proteins is sufficient to prevent the generation of pro-inflammatory EVs.

Mitochondrial damage blocks the inclusion of mitochondrial proteins within EVs

We next determined how mitochondrial damage inhibits the constitutive, MDV-dependent secretion of mitochondrial proteins in EVs. Different classes of MDV have been identified based on cargo selectivity and destination. These include Snx9-dependent MDVs that deliver mitochondrial antigens to endosome for antigen presentation [29], and damaged-induced MDVs that deliver oxidized mitochondrial cargo to lysosomes for degradation [36]. Importantly, the Parkinson's Disease related protein Parkin, which stimulates the formation of lysosome-targeted MDVs, inhibits the Snx9-dependent pathway in the context of antigen presentation [29]. We thus determined whether activation of the Parkin-dependent, damage-induced pathway targeted mitochondrial content to lysosomes at the expense of the EV pathway (Fig. 3A). Treatment of WT MEFs with AA induced the formation of mtHSP70 and TOM20 MDVs (Fig. 7A), and GFP-Parkin transiently colocalized with mtHSP70 MDVs in AA-treated U2OS cells stably expressing GFP-Parkin (Fig. 7B). Consistent with MDVs containing damaged proteins being delivered to lysosomes for degradation, AA treatment also increased the proportion of mtHSP70 MDVs colocalizing with the lysosomal marker LAMP1 (in the presence of lysosomal inhibitors E64 and Pepstatin A to prevent cargo degradation; Fig.7C). Similarly, while the total number of LAMP1-positive mtHSP70 MDVs was similar between WT and OPA1 KO cells (Fig. 4D), they represented a much larger fraction of total mtHSP70 MDVs (Fig. 7D), indicating that the few IM/Matrix MDVs remaining in OPA1 KO MEFs are preferentially targeted to lysosomes for degradation. Overall, as mtHSP70 inclusion into EVs is blocked under these conditions (Fig. 1I, 5F), our results suggest that the EV-targeted MDV pool is inhibited by the activation of the damage-induced pathway.

To determine whether directly stimulating the lysosomal pathway in the absence of mitochondrial damage is sufficient to inhibit the inclusion of IM/Matrix proteins into EVs, we used U2OS cells stably expressing GFP-Parkin. Consistent with its role in the formation of MDVs destined for lysosomes, increased Parkin expression stimulated MDV formation but did not alter the intracellular levels of mitochondrial proteins (Fig. 7E-F). We then measured the inclusion of mitochondrial proteins into EVs. Consistent with Parkin stimulating the delivery of mitochondrial proteins to lysosomes at the expense of EVs, mtHSP70 and NDUFA9 were significantly decreased in EVs from GFP-Parkin compared to GFP-expressing cells (Fig. 7G). In contrast, TOM20 inclusion into EVs was not affected (Fig. 7G). Altogether, these results indicate that Parkin inhibits

the recruitment of mitochondrial proteins to EVs by promoting instead their lysosomal degradation.

Discussion

Cells secrete mitochondrial proteins and mtDNA into their environment. This has been proposed to serve many functions, including serving as a form of quality control, participating in long-range metabolic regulation or stimulating the immune system [10,18,21,25,27,34,45-47]. However, the underlying mechanisms have remained for the most part elusive. Here we show that mitochondria-derived vesicles are required for release of mitochondrial proteins in EVs. This is blocked upon mitochondrial damage, preventing the release of pro-inflammatory damaged mitochondrial content.

A number of mitochondria-secreted factors behave as DAMPs that are associated with inflammatory cell activation during various pathological conditions. In these conditions, mtDNA and other mitochondrial components (cardiolipin, formyl peptides) can be released in extracellular media and act as an activator for immune cells to stimulate clearance of damaged cells and activate proinflammatory responses [48,49]. Importantly, these studies used LPS stimulation to trigger the release of mitochondrial DAMPs. For example, Joshi et al showed that damaged extracellular mitochondria released from LPS-activated microglia acts as effectors of the innate immune system by targeting adjacent neurons and astrocytes [46]. Similarly, a number of studies have demonstrated that extracellular DAMPs act as a feedback loop for neutrophil activation [17,50,51]. On the other hand, several studies including this report have shown that mitochondrial proteins can be secreted in EVs in the absence of pro-inflammatory signals [9,28], but the role or mechanism of release remains poorly understood.

To study this question, we first quantified a number of mitochondrial proteins present in EVs. Consistent with a previous report showing selective enrichment of mitochondrial proteins in cancer cell lines [9], our results demonstrated that mitochondrial proteins are selectively incorporated into EVs under non-stimulated conditions. This process is dependent on MDVs, small cargo-selective vesicles that target mitochondrial proteins to other intracellular locations, including lysosomes where they are degraded [36,37]. Specifically, inhibition of IM/Matrix MDV formation by either knocking down Snx9 or deleting OPA1 inhibited the inclusion of these proteins in EVs.

While MDV cargo is selected based on target destination and the nature of mitochondrial stress, the mechanisms defining MDV mitochondrial cargo selection are still unclear. Our results show that the same protein cargo (mtHSP70) can be targeted to EVs or lysosomal degradation depending

on the stimulus. Specifically, we observed that upon mitochondrial damage, a greater number of MDVs get transported to lysosome which in turn blocks the secretion of mitochondrial proteins within EVs. Formation of MDVs containing damaged oxidized proteins is dependent on the PINK/Parkin pathway [52]. Consistent with this, Parkin inhibited the secretion of IM/Matrix protein in EVs, further demonstrating that damage-induced MDVs prevent the secretion of damaged mitochondrial proteins by targeting them to lysosomes for degradation.

This dual targeting mechanism requiring Snx9 but blocked by Parkin is similar to the mechanism by which professional antigen presenting cells present mitochondrial antigens (mitAP) [29]. In these studies, stimulation of immune cells (heat shock, LPS or bacteria) stimulates Snx9-dependent mitAP. This was exacerbated by the loss of the PINK1/Parkin pathway, leading to increased inflammation and Parkinsonian symptoms [53-55]. Here, we found that in cells that are not professional antigen presenting cells (fibroblasts, epithelial cells), Snx9 is required for the basal secretion of mitochondrial proteins in EVs, and that this is not associated with a strong inflammatory response. In this context, the role of the PINK1/Parkin pathway would be to prevent the release and pro-inflammatory role of damaged mitochondrial components. This could be particularly important given that damaged mitochondrial components are more immunogenic and trigger a larger IL6 response (AA, Fig. 1G; OPA1 KO, Fig. 6A-B) [21,56,57] and suggests that, as with bacterial infection [58], mutations in the PINK1/Parkin pathway could lead to increased inflammation following oxidative damage to mitochondria.

Altogether, our results demonstrate that cells constitutively incorporate mitochondrial DNA and proteins in their EVs in the absence of an inflammatory response. One remaining question concerns their physiological role. Previous work suggested that secretion of mitochondrial proteins could be a form of quality control where cells export their damaged mitochondria destined for degradation in distant cells [7,46,47]. However, given that the PINK1/Parkin pathway prevents the release of damaged mitochondrial components, this is unlikely to be a widespread phenomenon. On the other hand, an increased number of studies has demonstrated transfer of mitochondrial content (including mtDNA) between cells, affecting the metabolic output of the recipient cells and promoting tumour growth [59-61]. In this context, the PINK1/Parkin pathway would serve the dual role of preventing inflammation and ensuring that only functional mitochondrial components are transferred.

Materials and Methods

Cell culture and treatments

The following cell lines were used: WT and OPA1 KO MEFs (gift from Dr. Luca Scorrano, University of Padua), U2OS cells stably transfected with pcDNA3-GFP and GFP-Parkin (gift from Edward Fon) and Raw 264.7, mouse macrophage cell line. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wisent) supplemented with 10% fetal bovine serum. Where indicated, cells were treated with Antimycin A (AA) (50 μM) for 24 hours and samples were collected for Immunofluorescence and western blots. For lysosomal inhibition experiments, cells were treated with combination of E64 (10 μM) and Pepstatin-A (20 μM) with or without AA for 1 hour and processed for immunofluorescence. Cell viability was measured by trypan blue exclusion. For suppression of endogenous Snx9 levels in WT MEFs, Snx9 siRNA (Silencer® Select ID s83577) and negative control siRNA (*Silencer*™ Select Negative Control No. 2 siRNA) were used (Thermo life technology). Cells were allowed to adhere on plate/coverslips for 24 hours and then transfected with 20 nm of siRNA using siLentFect™ lipid reagent (Bio-Rad) for 24 hours.

EV collection

24 hours after seeding cells in 150mm plates, media was replaced with DMEM supplemented with FBS that had been heat-inactivated and ultracentrifuged at 100,000xg speed for 9hrs to remove extracellular vesicle content. Media and cells were collected 24 hours later for EV isolation. To isolate EVs, media was centrifuged at 400xg for 5 mins to remove dead cells. EVs were then pelleted by ultracentrifugation at 100,000xg for 1h (Rotor 70.1Ti). The pellet was washed once with PBS. For trypsin digestion, 5 μg of EVs were digested with trypsin (0.01μg/ μl) in HIM buffer (200 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.4, 1 mM EGTA) for 20 minutes on ice. The reaction was then stopped with soybean trypsin inhibitor.

Electron microscopy

EVs collected from ultracentrifugation were fixed in 2% paraformaldehyde at room temperature. The pellets were then shipped to Mount Sinai Hospital for processing. Images were acquired using EMS 208S (Philips). EVs size analysis was performed using ImageJ software.

Antibodies and immunoblots:

The following antibodies were used: mouse anti-actin (A-5316, Sigma-Aldrich); mouse anti-Cytochrome C (SC-13156), rat anti-LAMP1 (SC-19992), goat anti-AIF (SC-9416), rabbit anti-TOM20 (SC-11415), and mouse anti-SDHA (SC-390381) mouse anit-CD9 (SC-13118) (Santa Cruz Biotechnology, Inc.); mouse anti-NDUFA9 (ab-14713), rabbit anti-DJ1 (ab-18257), and mouse anti PDH1 (ab-110330), mouse anti-UQCRC2 (ab-14745); mouse anti-Alix (ab117600) (Abcam); rabbit anti-ACC (3676-P), rabbit anti-Rab7 (9367-S), and rabbit anti-Rab11 (5589-P) (Cell Signaling Technologies); mouse anti-mtHSP70 (MA3-028) (Thermo scientific); and mouse anti-OPA1 (612607) (BD Biosciences);; rabbit anti-SNX9 (15721-1-AP) (proteintech).

EVs and whole cell lysates were lysed in 10 mm Tris-HCl, pH 7.9, 150 mm NaCl, 1 mm EDTA, 1% Triton X-100, 50 mm sodium fluoride. Triton-insoluble material was then pelleted at 15890 x g for 10 min and protein concentration measured with the DC assay (Bio-rad). For immunoblot analysis, 5 µg EVs and 20 µg whole cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose membranes and blotted with the indicated antibodies. Membranes were then incubated with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch) and visualized by enhanced chemiluminescence (Thermo scientific) using a Bio-Rad imaging system. Samples were quantified using ImageJ (National Institutes of Health).

Immunofluorescence

Cells were seeded overnight onto glass coverslips (Fischer Scientific) in 24 well plates, then treated as indicated and fixed with 4% paraformaldehyde for 10 mins at room temperature (RT). Fixed cells were washed with PBS, permeabilized with 0.2% Triton X-100 in PBS and blocked with 1% BSA in 0.1% Triton X-100 in PBS. Coverslips were incubated with primary antibodies, followed by incubation with fluorescent conjugated secondary antibodies (Jackson Immunoresearch). Coverslips were then mounted on slides with Immu-mount (Thermo Scientific). Cells were examined under a Leica SP8 laser scanning fluorescence confocal microscope using 63x oil immersion objective. Image were quantified using ImageJ.

Mitochondrial DNA Quantification

Total DNA from cells and EVs was isolated using a silica-based column DNA purification kit (Purelink DNA isolation kit) following the manufacturer's instructions. Relative mitochondrial DNA levels was measured by quantitative PCR using PowerUp™ SYBR™ Green (Applied biosystems™). Independent reactions were performed for the mtDNA gene cytochrome *c* oxidase 1 (mouse forward, 5'-GCCCCAGATATAGCATTCCC-3'; mouse reverse, 5'-GTTCATCCTGTTCCTGCTCC-3' [24] and the nuclear gene coding for the 18S ribosomal RNA (18S forward, 5'-TAGAGGGACAAGTGGCGTTC-3'; 18S reverse, 5'-CGCTGAGCCAGTCAGTGT-3').

Quantification of inflammatory responses

The proinflammatory effects of extracellular mitochondrial components was tested by adding isolated EVs (EVs from 20 x 10^6 cells/1 x 10^6 cells) or whole mitochondria (120 µg/1 x 10^6 cells, isolated as previously described [62] to RAW264.7 cells for 16 hours. Media and cells were then collected. The presence of IP10 in the media was then determined by ELISA using the Mouse CXCL10/IP-10/CRG2 DuoSet ELISA from R&D system (cat. DY466) according to the manufacturer's instructions. The presence of IL-6 in the media was then determined by ELISA using the Mouse IL-6 OptEIA™ ELISA set from BD biosciences (cat. 555240) according to the manufacturer's instructions. For mRNA quantification, cellular RNA was extracted using the GENEzolTM TriRNA pure kit (Geneaid, Cat No. GZX100), from which cDNAs were generated using a high capacity cDNA reverse transcript kit (Applied biosystems, Cat No. LS4368814). qPCRs were done using the Sensifast SYBR No-ROX kit (Bioline Cat no. BIO-98005) using the following primers: mCXCL10 (Rev 5-CAGTGGGTAAAGGGGAGTGA-3; Fwd 5-GCTGCCGTCATTTTCTGC-3), RSAD2 (Rev 5-ATTCAGGCACCAAACAGGAC-3; Fwd 5-CTGTGCGCTGGAAGGTTT-3), Actin (Rev 5-AGCACTGTGTTGGCATAGAG-3; Fwd 5-GCCTTCCTTCTTGGGTATGG-3); IL-6 (Rev 5-GTCCTTAGCCACTCCTTCTG-3; Fwd 5-CAAAGCCAGAGTCCTTCAGAG); mIFit1 (Rev 5-TCTCACTTCCAAATCAGGTATGT-3, Fwd 5-GAGAGTCAAGGCAGGTTTCT-3).

Statistical analysis

For western blots and mtDNA quantification, EV content was calculated by measuring the ratio of EV content to cellular content normalized to the equivalent amount of total material for each fraction. For experiments where different treatments/cells were compared, the amount of each

tested protein/DNA was first normalized in each fraction (cells, EVs) to that of the control (Actin for proteins, 18S for DNA) before calculating the ratios. Individual experiments are shown along with the average and SD. For MDV quantification, quantification is shown for at least 20 individual cells within 3 experiments, along with the average and SD. Statistical significance was determined using Student's t test (between 2 groups) or one-way ANOVA with a tukey post hoc test (multiple comparisons).

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Author Contributions

Experiments were designed and data analysed by KT, GP and MG. KT, LC, VD and FEM performed experiments. KT and MG wrote the manuscript, which was critically reviewed by all authors.

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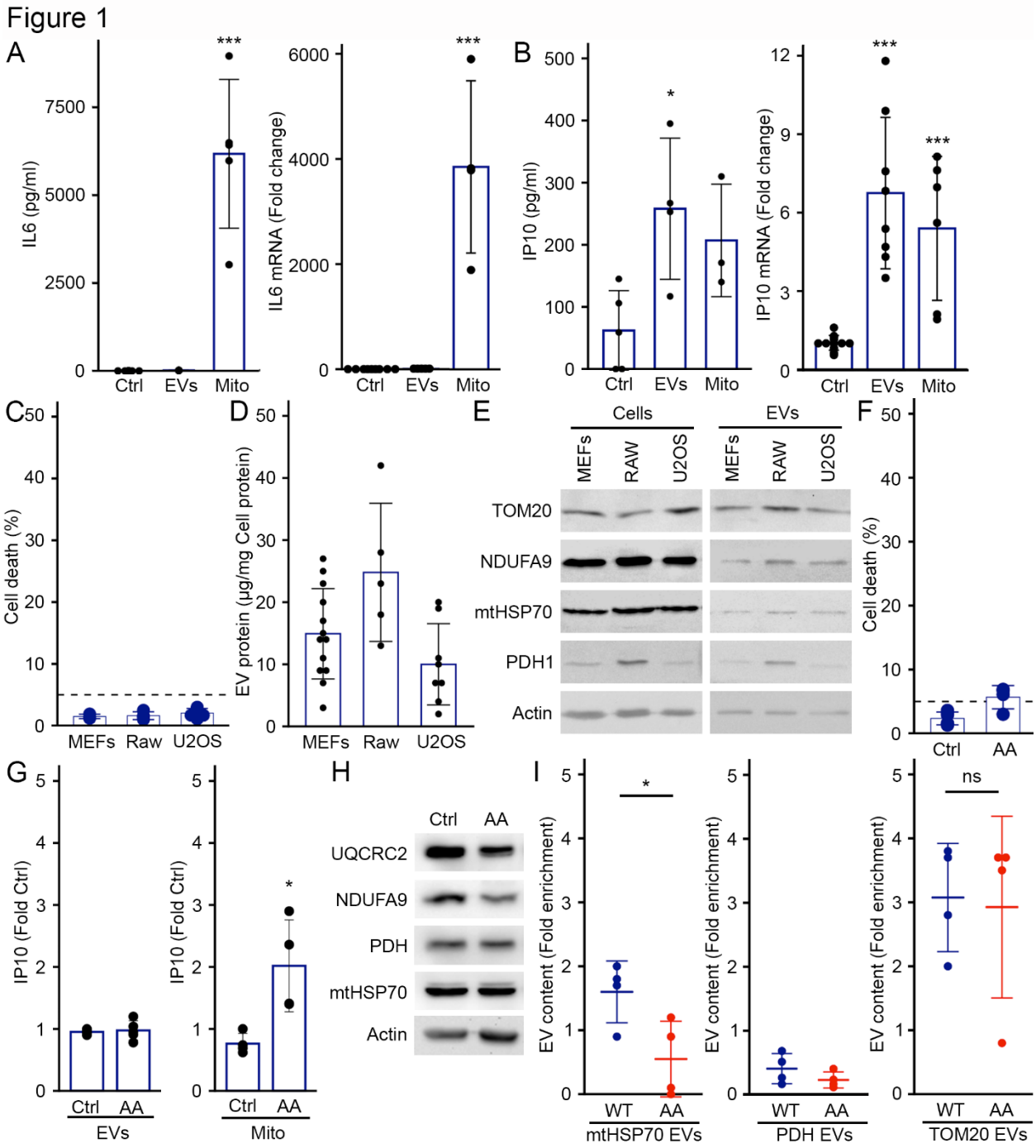

Figure 1. EV mitochondrial content is not pro-inflammatory. (A-B) Extracellular mitochondria induce the secretion of pro-inflammatory cytokines. Mitochondria (isolated from MEFs) or EVs (isolated by differential centrifugation from the media of MEFs grown for 24 hours in EV-depleted media) were added to RAW cells in their culture media. Release of IL6 (A) and IP10 (B) into culture media was measured by ELISA 24 hours (Left panels). mRNA levels of the two cytokines

was also measured in cells by qPCR (Right panels). Individual points represent independent experiments. Bars show the average ± SD. *** $p<0.001$, * $p<0.05$ (one-way ANOVA). (C) Cell viability was measured by Trypan Blue (dashed line represents 5% cell death). Individual points represent independent experiments. Bars show the average ± SD. (D) Quantification of EV protein yield relative to cellular protein content. Each point represents one experiment. Bars show the average ± SD. (E) Representative western blot showing the amount of the specified mitochondrial proteins in the indicated cell types (20 µg) vs their EVs (5 µg). TOM20, outer membrane protein; NDUFA9, Complex I subunit; mtHSP70 matrix protein associated with the IM; PDH, matrix protein. Actin is used as a control, as it has been shown to associate with EVs. (F) Cell viability (measured by Trypan Blue) following 24 hours treatment of WT MEFs with AA. Individual points represent independent experiments. The dashed line represents 5% death. Bars show the average ± SD. (G) AA-treated MEFs mitochondria but not AA-treated MEFs EVs stimulate IP10 production. RAW cells were treated as above with EVs and mitochondria isolated from Control or AA treated MEFs and IP10 release in culture media measured by ELISA. Individual points represent independent experiments normalised to the release caused by Control mitochondria or EV. Bars show the average ± SD. * $p<0.05$ (t-test). (H) AA treatment of WT MEFs causes the selective degradation of mitochondrial proteins. MEFs were treated with AA for 24 hours and the indicated mitochondrial proteins analysed by western blot. (I) Enrichment of the indicated mitochondrial proteins was measured by western blot in Control and AA-treated (24 hours) WT MEFs. Individual points represent independent experiments. Bars show the average ± SD. * $p<0.05$ (t-test)

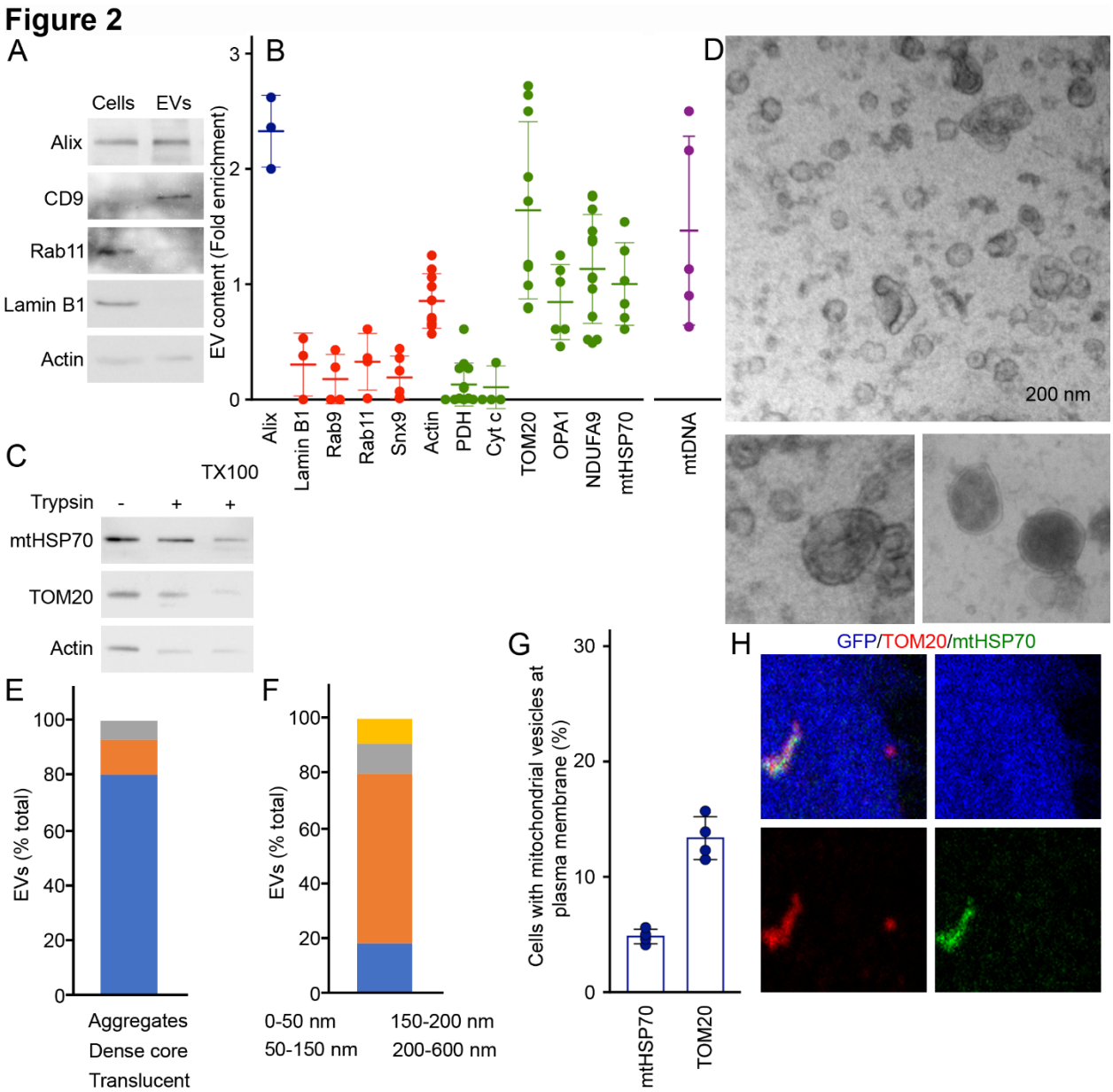

Figure 2. Selective inclusion of mitochondrial proteins in EVs. (A) EV markers were analysed by western blot in 20 µg cell extracts and 5 µg MEFs EVs isolated as in Figure 1. (B) Quantification of protein inclusion in MEF EVs. The amount of the indicated protein present in EVs was normalised to its cellular content. Individual points represent independent experiments. Bars show the average ± SD. Alix, Exosome marker; Lamin B1, nuclear protein; Rab9, Rab11, Snx9, endosomal proteins; Cyt c, IMS protein, OPA1, IM protein. (C) MEF EVs isolated as above were treated with Trypsin in the absence or the presence of detergent (TX100) and analysed by western blot for the presence of the indicated proteins. (D-F) MEF EV ultrastructure was analysed

by EM and quantified based on structure (E) and size (F). Representative images are shown in (D). Scale bars, 200 nm. (G-H) Vesicles containing selective mitochondrial cargo are found in proximity to the plasma membrane. The number of TOM20-positive and mtHSP70-positive vesicles are quantified in (G) with individual points representing the fraction of cells with plasma membrane associated vesicles. Points show independent experiments and bars show the average ± SD. A representative image is shown in (H) with GFP (Blue) used as a cytosolic marker to identify cellular boundaries. The arrowhead denotes a TOM20-positive vesicle close to the plasma membrane. Scale bar, 2 μm.

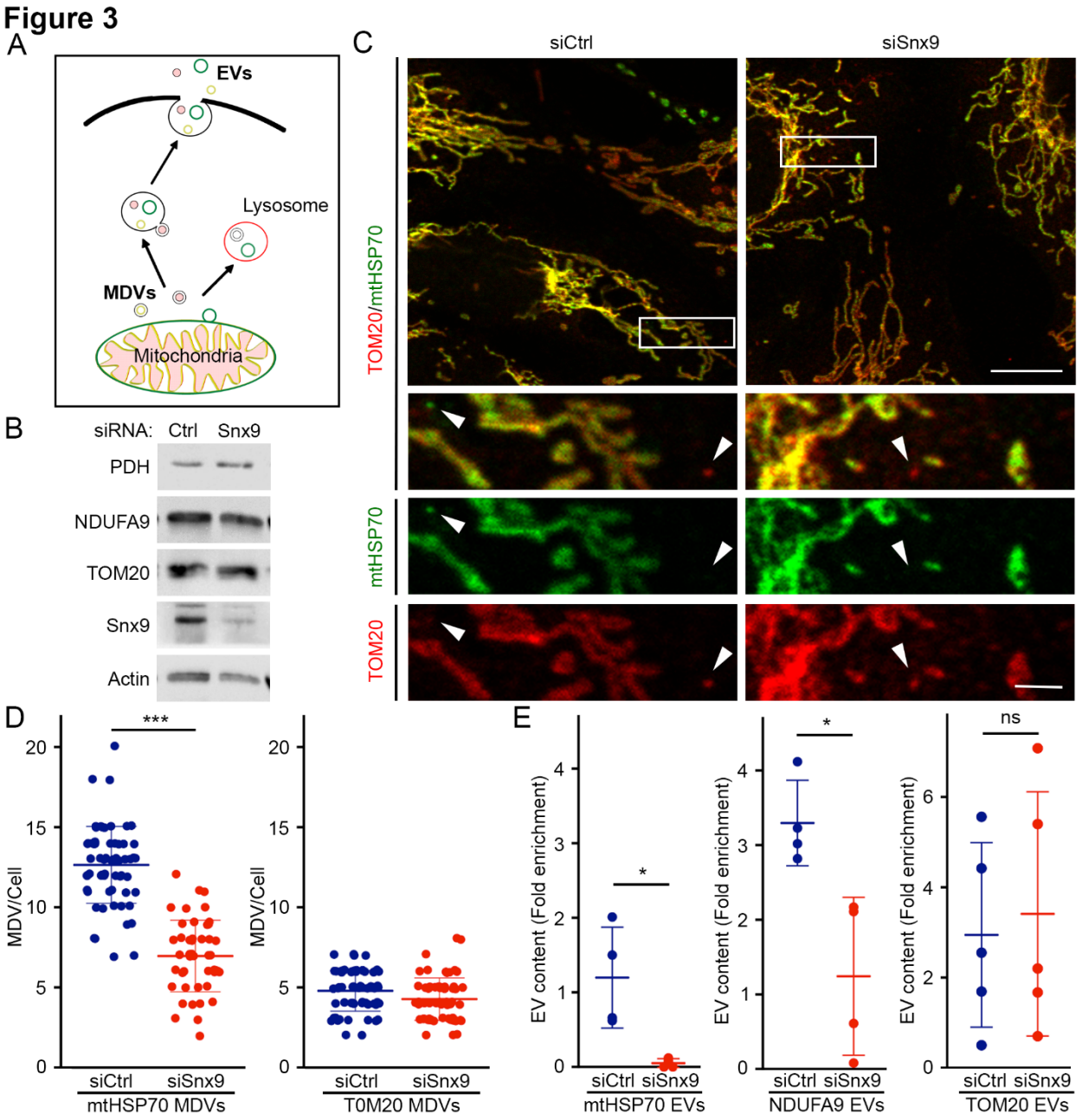

Figure 3. Snx9-dependent MDVs are required for the inclusion of IM/matrix proteins into EVs. (A) Schematic representation of the two distinct MDV pathways leading to EVs and lysosomes, respectively. (B) Knockdown of Snx9 does not affect mitochondrial protein content. MEFs were treated for 24 hours with a control siRNA (siCtrl) or a siRNA against Snx9 (siSnx9). Mitochondrial proteins were then measured by western blot, with Actin serving as a loading control. (C) Representative images showing TOM20 and mtHSP70 MDVs in MEFs treated with a control siRNA (siCtrl) or a siRNA against Snx9 (siSnx9). Arrowheads denote MDVs (positive

for one marker but negative for the other). Scale bar, 2 μm. (D) MDV quantification from images as in (C). Each data point represents one cell. Bars represent the average of at least 30 cells in a minimum of 3 independent experiments ± SD. *** $p<0.001$ (t-test). (E) Enrichment of the indicated mitochondrial proteins was measured by western blot as in Figure 1. Individual points represent independent experiments. Bars are shown as average ± SD. * $p<0.05$ (t-test)

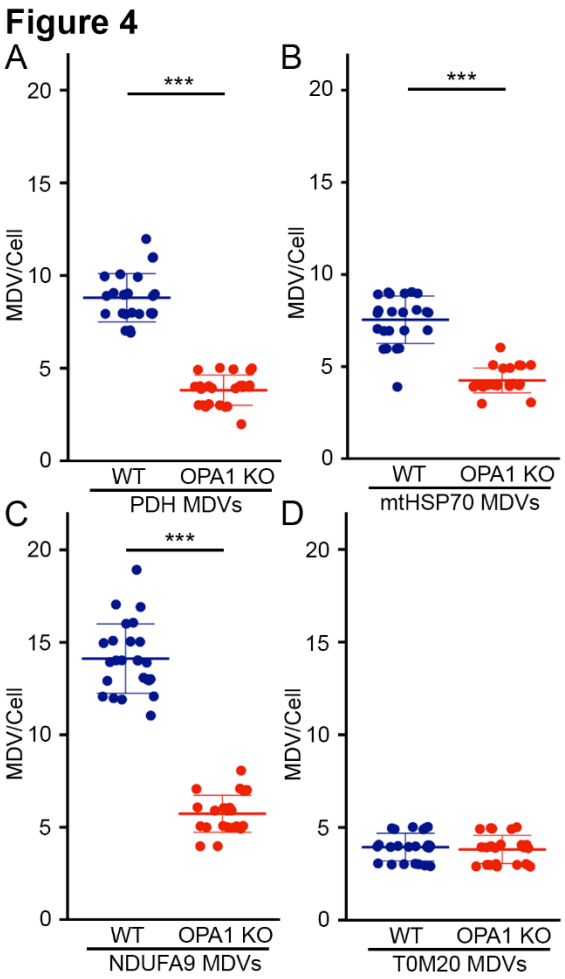

Figure 4. OPA1 deletion inhibits the formation of IM/matrix MDVs. Quantification of MDVs positive for PDH (A), mtHSP70 (B), NDUFA9 (TOM20-negative)(C), as well as TOM20-positive (mtHSP70-negative)(D) in WT and OPA1 KO MEFs. Each data point represents one cell. Bars represent the average of at least 20 cells in a minimum of 3 independent experiments ± SD. *** $p<0.001$ (t-test).

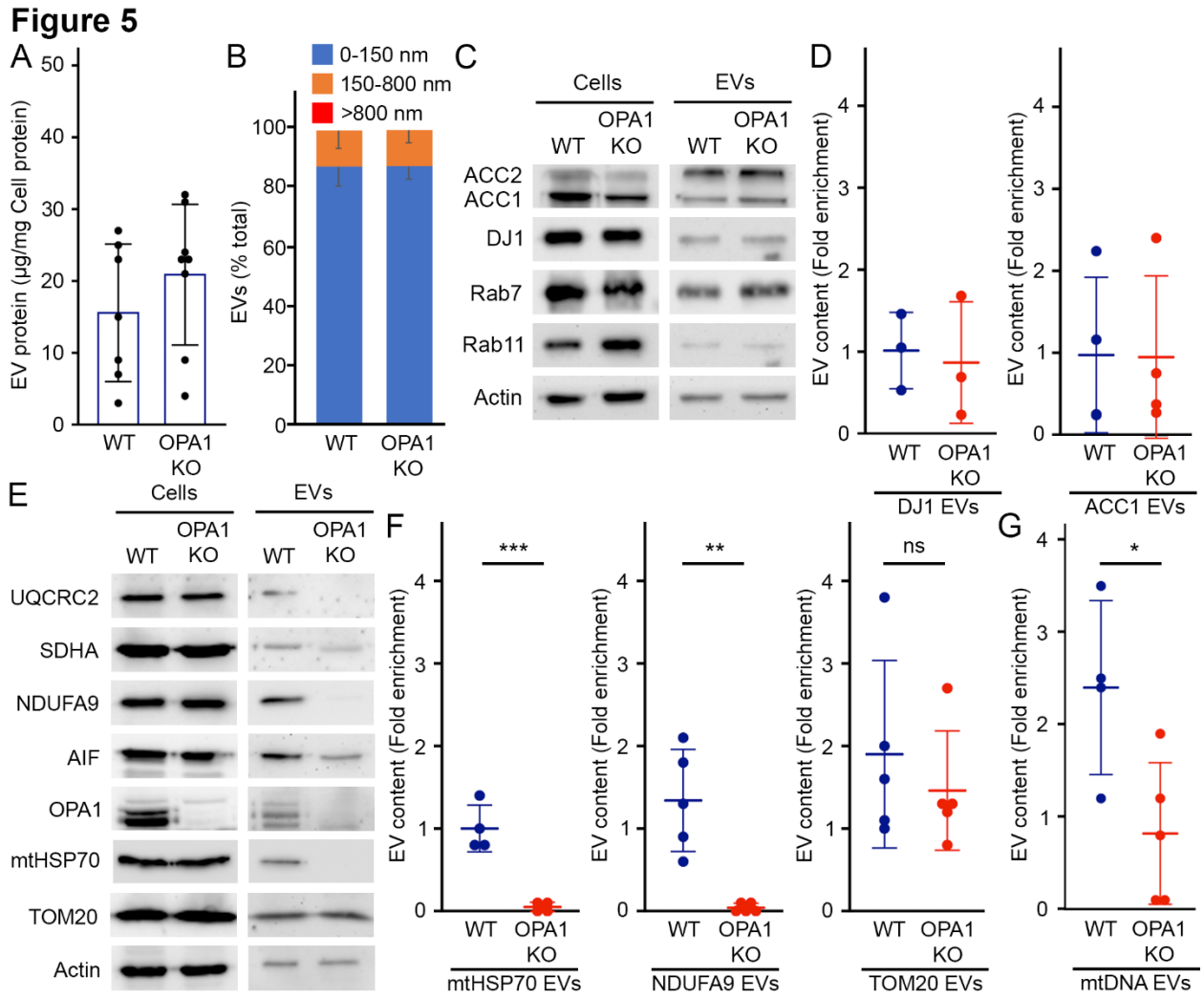

Figure. 5. OPA1 is required for the inclusion of IM/matrix proteins and mtDNA into EVs. (A) Quantification of EV protein yield relative to cellular protein content in WT and OPA1 KO EVs isolated as in Figure 1. Each point represents one experiment. Bars show the average ± SD. (B) OPA1 deletion does not alter overall EV size. WT and OPA1 KO EV size was analyzed by EM and binned as indicated. The average percent of EVs in each category ± SD is shown (n=3). No EVs larger than 800 nm were detected. (C-D) The inclusion of cytoplasmic proteins into EVs is not altered following OPA1 deletion. Representative western blot (C) and quantification (D) as in Figure 1. Individual points represent independent experiments. Bars show the average ± SD. (E-G) The inclusion of IM/matrix proteins, but not TOM20, into EVs is prevented by OPA1 deletion. Representative western (E) and quantification as in Figure 1 (F). mtDNA was quantified by PCR

(G) Individual points represent independent experiments. Bars show the average ± SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (t-test)

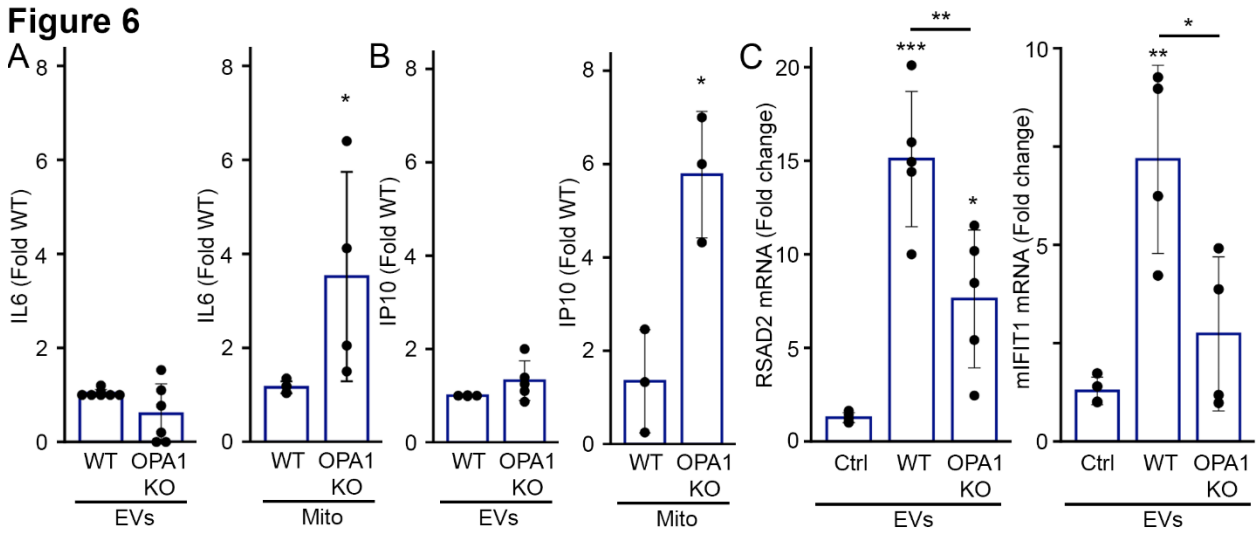

Figure 6. OPA1 KO mitochondria but not EVs isolated from the same cells induce a pro-inflammatory response. (A-B) RAW cells were treated as in Figure 1 with EVs and mitochondria isolated from WT or OPA1 KO MEFs and IL6 (A) or IP10 (B) release in culture media measured by ELISA. Individual points represent independent experiments normalised to the release caused by WT mitochondria or EVs. Bars show the average ± SD. * $p<0.05$ (t-test). (C) OPA1 KO EVs show reduced expression of IFN-dependent genes. RAW cells were treated as in (A) and the mRNA levels of the IFN-dependent RSAD2 (Left) and mIFIT1 (Right) measured by qPCR. Individual points represent independent experiments. Bars show the average ± SD. *** $p<0.001$, **, $p<0.01$, * $p<0.05$ (one-way ANOVA).

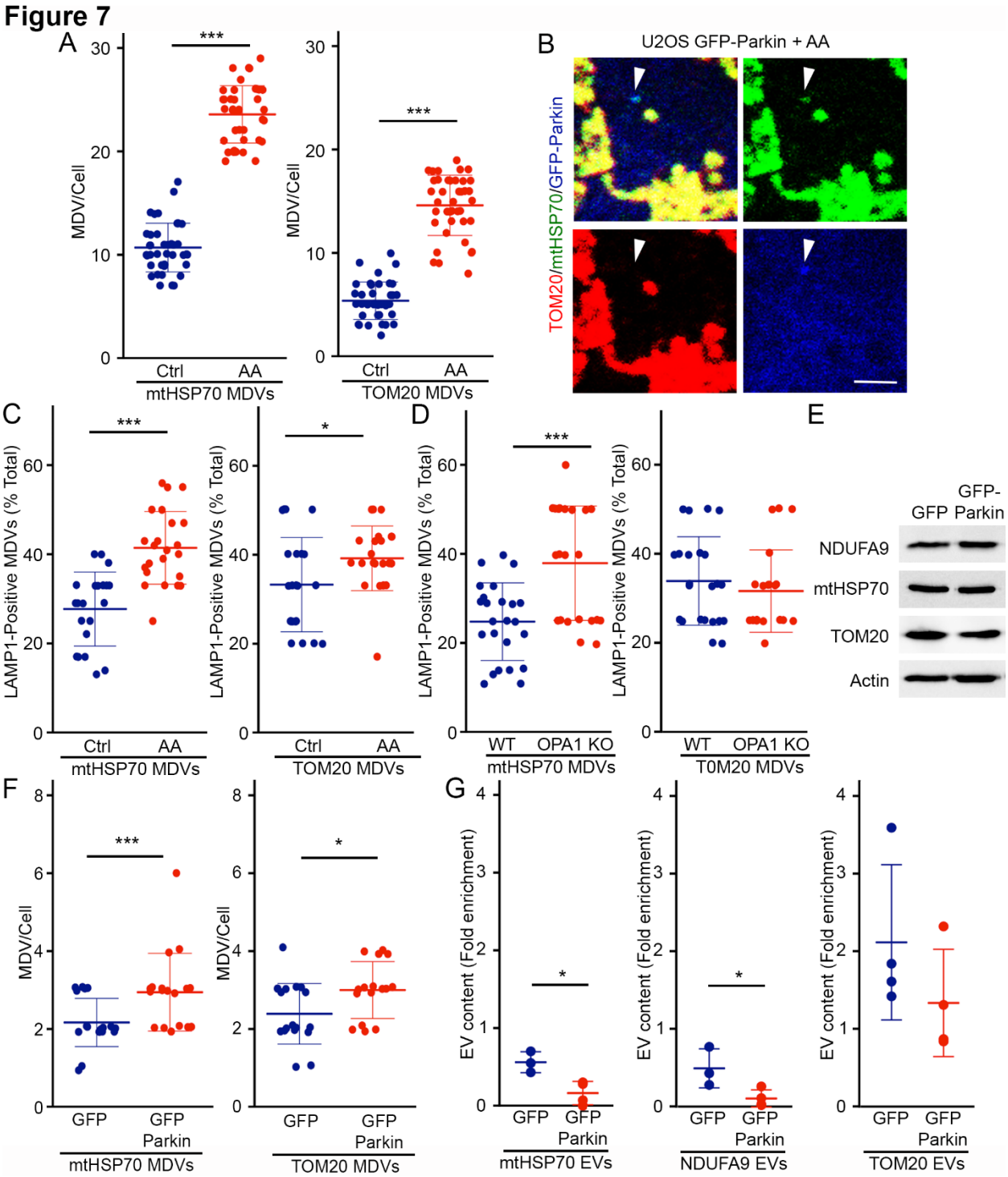

Figure 7. The Parkin MDV pathway regulates the inclusion of mitochondrial proteins within EVs. (A) AA treatment induces MDV formation. WT MEFs grown on coverslips were incubated in the absence or presence of AA for 1 hour. mtHSP70 and TOM20 MDVs were then quantified. Each data point represents one cell. Bars represent the average of at least 30 cells in a minimum

of 3 independent experiments ± SD. *** $p<0.001$ (t-test). (B) mtHSP70 MDVs colocalize with Parkin. U2OS cells stably transfected with GFP-Parkin were treated as in (A) and stained for TOM20 (Red) and mtHSP70 (Green). The arrowhead denotes a mtHSP70 MDV colocalizing with GFP-Parkin (Blue). Scale bar, 2 µm. (C-D) Damage-induced MDVs are targeted to lysosomes. WT MEFs were treated as in (A) in the presence of E64/Pepstatin and MDV colocalization with the lysosomal marker LAMP1 quantified as the fraction of total MDVs associated with LAMP1. Bars represent the average of at least 20 cells in a minimum of 3 independent experiments ± SD in AA-treated (C) and OPA1 KO (D) MEFs. *** $p<0.001$, * $p<0.05$ (t-test). (E-F) Stable expression of GFP-Parkin increase MDV formation. U2OS cells stably expressing GFP or GFP-Parkin were analysed for the presence of the indicated proteins by western blot (E) or mtHSP70 and TOM20 MDVs by immunofluorescence (F). Each data point represents one cell. Bars represent the average of a minimum of 20 cells in at least 3 independent experiments ± SD. *** $p<0.001$, * $p<0.05$ (t-test). (G) GFP-Parkin expression impairs the inclusion of IM/matrix proteins into EVs. EVs were isolated from U2OS cells stably expressing GFP or GFP-Parkin and the indicated mitochondrial proteins measured as in Figure 1. Individual points represent independent experiments. Bars show the average ± SD. * $p<0.05$ (t-test).