

SNX9 is Required for Fumarate Hydratase-Regulated Mitochondrial RNA (mtRNA) Release and IFN- β production in Macrophages

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Abstract

Fumarate hydratase (FH) has been shown to regulate mitochondrial RNA (mtRNA) release in lipopolysaccharide (LPS)-activated macrophages. In the cytosol, mtRNA activates the nucleic acid-sensing pattern recognition receptors RIG-I, MDA5 and TLR7 inducing IFN- β production. However, the mechanism of mtRNA release is still unclear. Here, we have investigated the role of Sorting Nexin 9 (SNX9) in this process. Silencing SNX9 blocked both the increase in cytosolic mtRNA and IFN- β production in both prolonged LPS stimulation and acute FH inhibition. Mechanistically, FH inhibition drives SNX9 mitochondrial localization 60 minutes after LPS stimulation where it interacts with TMEM11, a mitochondrial protein involved in mitochondrial dynamics. These results provide evidence for a role for SNX9 in the release of mtRNA in response to FH targeting leading to the induction of the anti-viral and immunomodulatory cytokine IFN- β in response to LPS.

Introduction

Macrophages undergo extensive metabolic reprogramming after pathogen exposure to rapidly engage in the inflammatory response [1, 2]. A key feature is a shift towards increased glycolysis to support immediate energy demands. At the same time, the Tricarboxylic Acid (TCA) Cycle is rewired to produce metabolites such as succinate and itaconate which have immunomodulatory effects [3, 4]. Fumarate has been shown to accumulate in macrophages upon LPS stimulation, due to fumarate hydratase (FH) repression to regulate cytokine production [5]. Fumarate accumulation impairs c-Fos activation to suppress IL-10, which subsequently boosts TNF expression. In addition, induction of interferon- β (IFN- β) is particularly prominent following FH repression. Mechanistically, FH repression induces release of mitochondrial RNA (mtRNA) into the cytosol, resulting in activation of the RNA-sensing pattern recognition receptors (PRRs) RIG-I, MDA5, and TLR7. This triggers IFN- β expression. However, the precise mechanistic steps leading to mtRNA release are still not fully elucidated.

In the last decade, several studies have identified different mechanisms involved in release of mitochondrial nucleic acids into the cytosol [6–10]. Mitochondrial stress has been shown to induce release of mtDNA fragments through the voltage dependent anion channel (VDAC), which in turn activate in the cytosol cGAS-STING and the NLRP3 inflammasome [7, 10]. Furthermore, Gasdermins (GSDMs) have also been implicated in mitochondrial nucleic acid release [11, 12]. GSDMD translocates to mitochondria in response to LPS and nigericin treatment and allows mtDNA escape from mitochondria in macrophages before pyroptosis [11]. GSDME also induces mtDNA release in neurons which is linked to neurodegeneration [12]. Mitochondrial derived vesicles (MDVs) fuse with lysosome, and GSDME causes its rupture and mtDNA release into cytosol [13]. BAX and BAK oligomerization has also been shown to induce mitochondrial nucleic acids release [9, 14]. In this case, inhibition of BCL-2, which blocks BAX and BAK, promotes their oligomerization. Both mtDNA and mtRNA have been shown to release through BAX and BAK pore to drive IFN- β . Silencing BAX and BAK effectively reduces inflammatory cytokine expression in the senescence-associated secretory phenotype (SASP) induced by mtRNA [15]. However, our knowledge of how mtRNA is released into the cytosol is more limited.

SNX9 is a membrane binding protein that was initially found to be involved in endocytosis [16]. It facilitates vesicle scission before budding off with dynamin. Apart from endocytosis, SNX9 has also been linked to mitochondrial dynamics, especially with MDV formation. It was first identified from a genome-wide screen of mitochondrial morphology [17]. A later study found that SNX9 colocalized with mitochondria under oxidative stress [18]. *Snx9* deficient cells have significantly reduced MDV formation. Small incomplete mitochondrial membrane buds also accumulate in *Snx9* deficient cells, which indicates that SNX9 is required for the final vesicle scission in the MDV formation process. Recently, covalent modification of SNX9 by β -hydroxybutyrate has been shown to induce SNX9 localization and MDV formation [19]. Importantly for this study, SNX9 has also been implicated in mtDNA release in FH deficient kidney epithelial cells promoting interferon (IFN) expression [20].

Here, we report SNX9 translocation to mitochondria after FH repression in response to LPS, or via direct inhibition of FH using a small molecule inhibitor FHIN1, leading to mtRNA release through MDV formation. This promotes IFN production. Furthermore, SNX9 interacts with TMEM11, a known regulator of mitochondrial dynamics. Our results identify SNX9 as a key regulator of inflammatory macrophages required for Type I IFN production.

Results

SNX9 regulates mtRNA release and downstream IFN-dependent responses upon LPS stimulation.

LPS treatment was shown to suppress FH expression in bone marrow-derived macrophages (BMDMs) as previously described [5]. We measured FH expression upon LPS stimulation in bone marrow-derived macrophages (BMDMs). While we observed a slight but significant decrease in FH expression upon 24 hours of LPS stimulation, it was further downregulated after 48 hours (Fig. 1a, b). We then confirmed that LPS could cause mtRNA release by performing digitonin fractionation (Fig.S1a). The cytosolic fraction was enriched with mtRNA *D-Loop* upon 48h of LPS stimulation (Fig. 1c). Thus, prolonged LPS lowers FH expression and induces mtRNA release.

We next examined whether SNX9 is involved in LPS-induced mtRNA release in macrophages. SNX9 was silenced by small interfering RNA (siRNA) prior to digitonin fractionation (Fig.S1b-e). The SNX9 knockdown (KD) suppressed *Ifnb1* expression at both 24 and 48 hours upon LPS stimulation (Fig. 1d, e). SNX9 KD also decreased cytosolic mtRNA upon 48 hours of LPS stimulation (Fig. 1f). Thus, SNX9 KD reduced mtRNA release and interferon expression. We further measured different interferon-stimulated genes (ISGs). Expression of mtRNA sensing PRRs *Ddx58* and *Ifih1*, encoding for the RNA-sensing PRRs RIG-I and MDA5, respectively, were reduced upon both 24 and 48 hours of LPS stimulation in SNX9 KD BMDMs compared to control (Fig. 1g, h). Similarly, 48 hours of LPS treatment on SNX9 KD cells reduced the expression of the transcription factors Interferon Regulatory Factor 1 and 7 (IRF1 and IRF7) (Fig. 1i, j). Taken together, these results indicate that prolonged LPS stimulation induces sustained Type I IFN response most likely through release of mtRNA in macrophages with SNX9 having a key role in this process.

Fumarate hydratase inhibition induces IFN- β expression and mtRNA release via SNX9

Having established a role for SNX9 in inducing type-I IFN responses during prolonged LPS stimulation, we next asked whether SNX9 also contributes to the early IFN response following LPS exposure. As previously reported, FH inhibition with the small molecule inhibitor FHIN1 alters cytokine response upon LPS stimulation in macrophages, including IFN- β , IL-10, and TNF [5, 21]. We investigated whether SNX9 affects this response. SNX9 KD reduced the FHIN1-induced IFN- β expression upon 4 hours stimulation with LPS both transcriptionally and at the protein level (Fig. 2a, b, Fig.S2a, b). FH inhibition blocked IL-10 and boosted TNF as described previously, but these responses were not affected by SNX9 KD indicating specificity for IFN- β production and consistent with a mitochondrial role for SNX9 (Fig. 2c-f). FH inhibition also induced Growth Differentiation Factor 15 (GDF15) as shown previously, but this was also not affected by KD of SNX9 (Fig. 2g, h). These results indicate that SNX9 mediates the Type I IFN response triggered by FH inhibition specifically through a mtRNA dependent route without affecting cytokines (IL-10, TNF, and GDF15) induced via mtRNA-independent pathways.

We next performed digitonin fractionation with SNX9 KD and measured cytosolic mtRNA level following FH inhibition (Fig.S2c). Cytosolic mtRNA *D-Loop* and *Nd4* were increased in FH-inhibited macrophages upon LPS stimulation, and this increase was abrogated upon SNX9 KD (Fig. 2i, j). Collectively, these results indicate that SNX9 regulates FH inhibition-induced mtRNA and downstream IFN- β induction during LPS stimulation.

FH inhibition causes SNX9 localization on mitochondria

Studies have shown that SNX9 localizes to mitochondria following oxidative stress and post-translational modification [18, 19]. We therefore investigated whether FH inhibition drove SNX9 localization to mitochondria. A time course study of *Ifnb1* expression was performed to find optimal timing of SNX9 localization upon LPS stimulation and previous FH inhibition. Results indicated that the cytokine was increased after 120 minutes of LPS stimulation but not earlier (Fig. 3a-c). Therefore, we hypothesised that SNX9 would migrate to mitochondria within 120 minutes of LPS stimulation in concomitance with FH inhibition. Next, mitochondrial fractionation is performed, and western blotting of mitochondrial fractions illustrated that there is basal SNX9 localization with mitochondria in the absence of LPS stimulation (Fig. 3d lane 1). We then analyzed SNX9 localization upon FH inhibition with LPS stimulation. SNX9 mitochondrial localization was increased upon 1 hour (Fig. 3d lane 2 and 3, quantified in 3e), but not after 30 mins (Fig.S3a lane 2 and 3, quantified in s3b) of LPS stimulation in combination with FH inhibition compared to LPS stimulation alone. These results suggest that SNX9 increases its mitochondrial localization after 1 hour of LPS stimulation in FHIN1 treated cells.

In order to detect potential interactors of SNX9 involved in the mtRNA release, we performed SNX9 immunoprecipitation and mass spectrometry upon 1 hour of LPS stimulation in the presence of FHIN1.

Both western blot and mass spectrometry results showed that SNX9 was successfully pulled down (Fig. 3f, g). Following this, mass spectrometry analysis revealed that FHIN1 pretreatment, followed by LPS stimulation, increased SNX9 interaction with TMEM11, a mitochondrial outer membrane protein that is reported to regulate mitochondrial dynamics (Fig. 3h) [22].

Overall, these results show that FH inhibition induces SNX9 mitochondrial translocation, where it likely directly regulates the release of mtRNA leading to induction of IFN- β via RIG-I, MDA5 and TLR7 (Fig. 3i).

Discussion

In this study, we showed that LPS stimulation suppresses FH, leading to mtRNA release and downstream Type I IFN expression [5]. This finding positions FH as a potential metabolic checkpoint that restrains inappropriate induction of IFN during inflammation, with SNX9 as the key regulator of mtRNA release. Mechanistically, loss of SNX9 reduces cytosolic mtRNA accumulation and attenuates IFN- β production and downstream ISG expression. Consistent with this, acute FH inhibition promotes SNX9 translocation to mitochondria and enhances its interaction with the mitochondrial outer membrane protein TMEM11.

FH inhibition with acute LPS stimulation leads to SNX9 selectively regulating IFN- β without affecting other cytokines, notably IL-10 and TNF. This is because for IL-10, fumarate blocks its expression via inhibition of c-Fos, which in turn boosts TNF production. These events are not inhibited by SNX9 KD because they do not involve mtRNA release. During prolonged stimulation with LPS, SNX9-dependent mtRNA release and Type I IFN response may progressively reshape downstream inflammatory programs.

FH inhibition promotes SNX9 mitochondrial localization and interaction with TMEM11. Previous studies have shown that TMEM11 can bind and suppress the mitophagy receptors BNIP3 and BNIP3L, and also regulate mitochondrial reactive oxygen species (ROS) production [22, 23]. Although our study does not directly establish a mechanistic connection between these events, these observations raise the possibility that mitochondrial stress induced by FH deficiency may facilitate SNX9 recruitment through TMEM11-associated pathways [5]. In addition, SNX9 has been shown to interact with mitochondrial intermembrane protein OPA1 to promote MDV formation, which has been implicated in ageing and disorders including Parkinson's disease and alcohol-induced liver injury, further supporting a broader role for SNX9 in mitochondrial stress responses and disease [18, 19, 24]. FH deficiency has been reported in diseases such as systemic lupus erythematosus (SLE) and multiple sclerosis, and mtRNA release, as well as dysregulated Type I IFN signalling, are among the potential drivers for their pathology [5, 25–27]. Targeting SNX9 could represent a strategy to dampen mtRNA-driven IFN signalling.

Overall, our findings uncover a link between mitochondrial metabolism dysregulation and SNX9-induced mtRNA release. This work provides insight into how metabolic stress can shape innate immune responses and identifies SNX9 as a potential regulator of inflammation. The mechanisms of SNX9

recruitment to mitochondria by FH inhibition and how mtRNA escapes from mitochondrial compartments into the cytosol require further investigation.

Method

Animal details

6–18-week-old female C57BL/6J0laHsd Wild-type (WT) mice purchased from Harlan laboratories were used for all in vitro experiments. Mice are used to generate BMDMs for all the experiments.

Mouse BMDMs generation

Mice were euthanised in a CO₂ chamber, and cervical dislocation was used to confirm death. Bone marrow was subsequently harvested from the ilia, femora, and tibiae in a laminar flow hood. Cells were resuspended in 3mL of red cell lysis buffer for 5 minutes before again being supplemented with 7mL DMEM and centrifuged at 453 x g for 5 minutes. Supernatant was removed and cells were resuspended in 30mL DMEM containing 20% L929 supernatant, 10% FCS, and 1% penicillin/streptomycin. Cells were seeded in non-tissue culture-coated 10cm dishes at a volume of 10mL per dish. Cells were incubated for 6 days at 37°C and 5% CO₂. Following this incubation period, cells were counted and plated at either 0.5 or 2x10⁶ cells/well in DMEM containing 10% L929 supernatant, 10%FCS and 1% penicillin-streptomycin.

Reagents

LPS from Escherichia coli, serotype EH100 (ALX-581-010-L001), was purchased from Enzo Life Sciences. DMSO (D8418) was purchased from Sigma Aldrich. FHIN1 (HY-100004) was purchased from MedChemExpress.

Compound treatments

FHIN1 used DMSO as a vehicle. LPS was applied at a concentration of 100 ng ml⁻¹ for indicated time points (0.5, 1, 2, 4, 24 and 48 hours). FHIN1 (20 µM) was pre-treated for 3 h before the addition of LPS.

Antibodies

Working dilutions of primary antibodies were 1:1000 for western blot. Anti-rabbit Lamin B1 (12586), VDAC (12454) and α-tubulin (2144) antibodies were purchased from Cell Signaling. Anti-rabbit SNX9 (15721-1-AP) antibody was purchased from Proteintech. Anti-mouse β-actin antibody (A5316) was purchased from Sigma Aldrich. Horseradish peroxidase (HRP)-conjugated anti-mouse (115-035-003) and anti-rabbit (111-035-003) IgG antibodies (all used at 1:2000) were purchased from Jackson ImmunoResearch.

Working dilutions of primary antibodies were 1:500 for immunofluorescence. Anti-mouse TOM20 (ab283317) was purchased from Abcam. Anti-rabbit GRSF1 antibody (11H24L16) was purchased from Sigma Aldrich. Alexa Fluor 488 goat anti-mouse IgG1 antibody (A21121) and Alexa Fluor 647 goat anti-rabbit IgG antibody (A21244) were purchased from Invitrogen.

Details of antibody validation are given in Supplementary Table 1.

RNA interference

Pre-designed silencer select siRNAs for *Snx9* (s83576) and negative control (4390843) were ordered from Thermo Fisher. siRNA sequences are given in Supplementary Table 2. 50 nM siRNA with 5 μ L Lipofectamine RNAiMAX was used for cell transfection according to the manufacturer's instructions (Thermo Fisher). Cells were transfected in serum and antibiotics free DMEM for 8 hours, which was replaced with DMEM containing 10% L929 supernatant, 10% FCS and 1% penicillin-streptomycin. Cells were left to rest for at least a further 36 hours before treatment.

RT-qPCR

RNA extraction from cells was performed with the Purelink RNA kit (Invitrogen) according to the manufacturer's instructions. Following treatment of BMDMs, cells were lysed in 350 μ L RNA lysis buffer. The NanoDrop 2000 spectrophotometer is used to quantify mRNA concentration, which was then normalized to the lowest concentration across all samples with RNase-free water. Samples were DNase-treated after quantification using DNase I (Thermo Fisher) according to the manufacturer's instructions. Isolated RNA samples were converted into cDNA using a High-Capacity cDNA Reverse Transcription kit (Thermo Fisher) as 10 μ L of RNA (at a maximum concentration of 100 ng μ L⁻¹) was added to 10 μ L of reverse transcription master mix to complete the reaction mixture according to the manufacturer's instructions. Primers are designed in-house and ordered from Eurofins Genomics, as detailed in Supplementary Table 2. 96-well qPCR plates and the 7500 Fast Real-Time PCR machine (Thermo Fisher) are used to carry out the Real-time qPCR experiment. CT values for each sample and gene of interest were obtained to calculate relative expression ($2^{-\Delta\Delta CT}$).

Western blotting

Supernatant was collected from cells following stimulation, and lysates were collected in 50 μ L lysis buffer (0.125 M Tris pH 6.8, 10% glycerol, 0.02% SDS and 5% DTT) and heated to 95°C for 5 minutes to denature proteins. Protein was resolved by molecular weight using SDS-PAGE. The percentage resolving gel was depended on the molecular weight of the given protein using the Bio-Rad gel running system. For the electrophoretic transferring of proteins onto a PVDF membrane, the Bio-Rad wet transfer system was used. Following transfer, the membrane was blocked in 5% milk powder TBST for 1 hour and then incubated in primary antibody rolling overnight at 4°C. The membrane was washed for 2 times and incubated for 1 hour with secondary antibody in 5% milk powder TBST at room temperature. WesternBright ECL Spray (Advansta) or SuperSignal West Femto Maximum Sensitivity Substrate

(Thermo Fisher Scientific) is used to activate the membrane before imaging. Both chemiluminescent and white light images were acquired using a ChemiDoc MP imaging system (Bio-Rad)

Digitonin fractionation

BMDMs were plated at 2×10^6 cells per well and treated as desired. Cells were washed once with room temperature PBS before being scraped on ice into ice-cold PBS. 500g centrifugation for 5 min at 4°C is performed to pellet cells. Supernatant was removed and discarded. The pellet was resuspended in 400 μ L extraction buffer (150 mM NaCl, 50 mM HEPES pH 7.4, and 25 μ g mL⁻¹ digitonin). Samples were then placed in a rotating mixer at 4°C for 10 min before centrifugation at 2,000g at 4°C for 5 min. RNA in the half of resulting supernatant constituted the cytosolic fraction (200 μ L) is extracted with Purelink RNA kit (Invitrogen). The other half of cytosolic fraction (200 μ L) was concentrated using Strataclean resin (Agilent) and analyzed by western blotting. The membrane-bound organelles pellet fraction was lysed in western blot lysis buffer for western blotting. To determine the presence of mtRNA in the cytosol, qPCR was performed using primers specific for mitochondrial D-loop on cDNA, which had been reverse-transcribed from RNA isolated from the cytosolic fraction (mtRNA). Values were normalized using a housekeeping control gene (Rps18) amplified in cDNA, which had been reverse-transcribed from RNA isolated from the cytosolic fraction.

Mitochondrial Isolation

1×10^6 cells were seeded in 10 cm dishes and treated as desired. Cells were scraped and washed once in PBS on ice. 500g centrifugation for 5 min at 4°C was performed to pellet cells. 1 mL of mitochondrial isolation buffer (320 mM sucrose, 10 mM TRIS, 1 mM EDTA and 1xprotease inhibitor cocktail) was used to resuspend the sample. Samples were transferred to a glass Dounce and homogenised with a glass pestle for 30 strokes. Cell lysates were centrifuged at 700 g for 10 minutes at 4°C. The resulting supernatants were removed and centrifuged at 3000 g for 15 minutes at 4°C to isolate the mitochondrial fraction. Mitochondrial pellets were washed at least twice in 500 μ L mitochondrial isolation buffer at 3000 g for 15 minutes 4°C. The remaining supernatants as the cytosolic fraction were concentrated and harvested using Strataclean resin (Agilent). Both mitochondrial and cytosolic fraction were lysed in western blot lysis buffer for western blotting.

Co-Immunoprecipitation

BMDMs were seeded at 1×10^7 cells mL⁻¹ in 10 cm dishes and treated as required. Cells were washed with 3 mL PBS before lysis in 1 mL high stringency lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonident P40 (NP-40), 1 mM phenylmethylsulfonyl fluoride (PMSF), 11.5 μ g/mL aprotinin, 1 μ g/mL leupeptin and 1 mM sodium orthovanadate) on ice for 15 minutes. Cell lysate is scraped and transferred into microcentrifuge tubes. Tubes were agitated at 21380 x g for 10 min at 4°C. 25 μ L of resulting supernatant was mixed with 25 μ L sample lysis buffer and treated as the whole cell lysate (WCL) sample. The rest of the supernatant is moved to new tubes. 2mg SNX9 or the equivalent concentration of rabbit IgG antibody (Millipore, pp64), and 5 μ L Dynabeads™ (Thermo Fisher) was added to the remaining supernatant, which was subsequently incubated at 4°C in a rotator

for 2 hours. Supernatant of IP samples was subsequently removed using MagRack (Merck). And samples are washed three times with 1 mL high stringency lysis buffer. 40 µL sample lysis buffer were added to the immune complexes and boiled for 5 min and analyzed by western blotting.

ELISA

DuoSet ELISA kits for IL-1 β , TNF, IL-6, IL-10, IFN β and GDF15 were purchased from R&D Systems and were carried out according to the manufacturer's instructions with appropriately diluted cell supernatants added to each plate in duplicate or triplicate. The FLUOstar Optima plate reader is used to quantify absorbance at 450 nm, and cytokine concentrations were subsequently obtained by extrapolation from a standard curve plotted using GraphPad Prism.

Pulldown Proteomic analysis

BMDMs were seeded at 1×10^7 cells mL⁻¹ in 10 cm dishes and treated as required. Cells were washed with 3 mL PBS before lysis in 1 mL high stringency lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonident P40 (NP-40), 1 mM phenylmethylsulfonyl fluoride (PMSF), 11.5 µg/mL aprotinin, 1 µg/mL leupeptin and 1 mM sodium orthovanadate) on ice for 15 minutes. Cell lysate was scraped and transferred into microcentrifuge tubes. Tubes were agitated at 21380 x g for 10 min at 4°C. The rest of the supernatant was moved to new tubes. 2mg SNX9 or the equivalent concentration of rabbit IgG antibody (Millipore, pp64), and 5 µL DynabeadsTM (Thermo Fisher) was added to the remaining supernatant, which was subsequently incubated at 4°C in a rotator for 2 hours. Supernatant of IP samples was subsequently removed using MagRack (Merck). And samples are washed three times with 1 mL high stringency lysis buffer. The samples were added to a KingFisher deep well plate prepared for an 8 h digest protocol on the Kingfisher Flex. Lysate was added to Plate G with MagReSyn HILIC beads and ACN is added to 70% final concentration. Plates D, E, and F are filled with 95% ACN and Plates B and C are filled with 70% EtOH. The digest buffer (1 µg/mL MS grade trypsin in 50 mM triethylammonium bicarbonate) is added to Plate A. Alternatively, cell pellets were lysed, reduced and alkylated in 50 µl of 6 M Gu-HCl, 200 mM Tris-HCl pH 8.5, 10 mM TCEP, 15 mM chloroacetamide by probe sonication and heating to 95°C for 5 mins. Protein concentration was measured by a Bradford assay and initially digested with LysC (Wako) with an enzyme to substrate ratio of 1:200 for 4 h at 37°C. Subsequently, the samples were diluted 10-fold with water and digested with porcine trypsin (Promega) at 37°C overnight. Samples were acidified to 1% TFA, cleared by centrifugation (16,000 × g at RT) and ~ 20 µg of the sample was desalted using a Stage-tip. Eluted peptides were lyophilised, resuspended in 0.1% TFA/water, and the peptide concentration was measured by A280 on a nanodrop instrument (Thermo). The sample was diluted to 2 µg/ 5 µl for subsequent analysis.

Quantification and statistical analyses

Details of all statistical analyses performed are provided in the figure legends. Data were shown as the mean $\pm$ s.e.m. unless stated otherwise. Representative western blots were shown. For pulldown proteomics data, protein signal intensity was converted to a log2 scale, and biological replicates were grouped by experimental condition. The Bioconductor package limma was used to carry out the analysis

using LFQ-Analyst, an R based online tool [28]. Data were visualized using a enrichment plot and a volcano plot, which shows the $\log_2(\text{fold change})$ on the x axis and the $-\log_{10}(\text{adjusted P value})$ on the y axis and a protein enrichment plot. GraphPad Prism was used to calculate statistics in bar plots using appropriate statistical tests depending on the data, including two-way ANOVA, one-way ANOVA and two-tailed unpaired t-test. Appropriate correction methods, such as Tukey, Kruskal–Wallis and Holm–Sidak tests are used for Adjusted P values. Sample sizes were determined on the basis of previous experiments using similar methodologies. All depicted data points are biological replicates taken from distinct samples unless stated otherwise. Each figure consists of a minimum of three independent experiments from multiple biological replicates unless stated otherwise.

Declarations

Competing Interests Statement

The authors declare no competing interests.

Approval and Accordance Statement

All mouse maintenance and experimental procedures were approved by the Trinity College Dublin Comparative Medicine / Bioresource Research Ethics Committee and authorised by the Health Products Regulatory Authority (HPRA), Ireland, in accordance with applicable European Union regulations.

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Author Contribution

Yukun Min designed the study, performed the experiments, analysed the data, and wrote the manuscript. Vasile Mihai Sularea performed the experiments, analysed the data and revised the manuscript. Órlaith C. Henry performed the experiments, analysed the data. Jaiya Randhawa, Alessia Zotta, Victoria Evans, Thomas Pfefer, Joe Ganellin, Anna Hanga Bessenyei, Maureen Yin, Juliana Escher Toller-Kawahisa, Shane O'Carroll, Eoin O'Neill, Eloïse Marques, Alva M. Casey, Chinmayi Pednekar, Alex von Kriegsheim assisted with data collection and methodology. Cliona O'Farrelly, Dylan G. Ryan, Julien Prudent supervised the project. Luke A.J. O'Neill supervised the project and revised the manuscript. All authors reviewed and approved the final version.

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Data Availability

All data generated or analysed during this study are included in this published article and its supplementary information file.

References

1. Van den Bossche, J., O'Neill, L. A. & Menon, D. Macrophage Immunometabolism: Where Are We (Going)? *Trends Immunol.* **38** (6), 395–406 (2017).
2. Randhawa, J. & Palsson-McDermott, E. M. Beyond energy: how TCA cycle-derived metabolites regulate gene expression and inflammation in the nucleus. *J. Inflamm. (Lond).* **22** (1), 39 (2025).
3. Hooftman, A. et al. The Immunomodulatory Metabolite Itaconate Modifies NLRP3 and Inhibits Inflammasome Activation. *Cell. Metab.* **32** (3), 468–478e7 (2020).
4. Pfefer, T., Bessenyei, A. H. & O'Neill, L. A. Immunometabolites and cancer: the role of 2-hydroxyglutarate, succinate, fumarate and itaconate in tumour development and anti-tumour immunity. *Immunol. Lett.* **279**, 107153 (2026).
5. Hooftman, A. et al. Macrophage fumarate hydratase restrains mtRNA-mediated interferon production. *Nature* **615** (7952), 490–498 (2023).
6. Yukun Min, L. O. N. *Targeting mitochondrial metabolites and nucleic acids as an anti-inflammatory strategy.* *Front. Drug Discov.*, 3. (2023).
7. Xian, H. et al. Oxidized DNA fragments exit mitochondria via mPTP- and VDAC-dependent channels to activate NLRP3 inflammasome and interferon signaling. *Immunity* **55** (8), 1370–1385e8 (2022).
8. West, A. P. et al. Mitochondrial DNA stress primes the antiviral innate immune response. *Nature* **520** (7548), 553–557 (2015).
9. Tigano, M. et al. Nuclear sensing of breaks in mitochondrial DNA enhances immune surveillance. *Nature* **591** (7850), 477–481 (2021).
10. Kim, J. et al. VDAC oligomers form mitochondrial pores to release mtDNA fragments and promote lupus-like disease. *Science* **366** (6472), 1531–1536 (2019).
11. Miao, R. et al. Gasdermin D permeabilization of mitochondrial inner and outer membranes accelerates and enhances pyroptosis. *Immunity* **56** (11), 2523–2541e8 (2023).
12. Neel, D. V. et al. Gasdermin-E mediates mitochondrial damage in axons and neurodegeneration. *Neuron* **111** (8), 1222–1240e9 (2023).

13. Nguyen, M. et al. MAPL regulates gasdermin-mediated release of mtDNA from lysosomes to drive pyroptotic cell death. *Nat. Cell. Biol.* **27** (10), 1708–1724 (2025).
14. McArthur, K. et al. *BAK/BAX macropores facilitate mitochondrial herniation and mtDNA efflux during apoptosis.* *Science*, **359**(6378). (2018).
15. Victorelli, S. et al. Mitochondrial RNA cytosolic leakage drives the SASP. *Nat. Commun.* **16** (1), 10992 (2025).
16. Yarar, D., Waterman-Storer, C. M. & Schmid, S. L. SNX9 couples actin assembly to phosphoinositide signals and is required for membrane remodeling during endocytosis. *Dev. Cell.* **13** (1), 43–56 (2007).
17. Norton, M. et al. ROMO1 is an essential redox-dependent regulator of mitochondrial dynamics. *Sci. Signal.* **7** (310), ra10 (2014).
18. Matheoud, D. et al. Parkinson's Disease-Related Proteins PINK1 and Parkin Repress Mitochondrial Antigen Presentation. *Cell* **166** (2), 314–327 (2016).
19. Tang, M. et al. *beta-hydroxybutyrate facilitates mitochondrial-derived vesicle biogenesis and improves mitochondrial functions.* *Mol Cell*, 85(7): pp. 1395–1410 e5. (2025).
20. Zecchini, V. et al. Fumarate induces vesicular release of mtDNA to drive innate immunity. *Nature* **615** (7952), 499–506 (2023).
21. Takeuchi, T., Schumacker, P. T. & Kozmin, S. A. Identification of fumarate hydratase inhibitors with nutrient-dependent cytotoxicity. *J. Am. Chem. Soc.* **137** (2), 564–567 (2015).
22. Gok, M. O. et al. *The outer mitochondrial membrane protein TMEM11 demarcates spatially restricted BNIP3/BNIP3L-mediated mitophagy.* *J. Cell. Biol.*, **222**(4). (2023).
23. Wu, B. et al. Mitochondrial reactive oxygen species regulate acetyl-CoA flux between cytokine production and fatty acid synthesis in effector T cells. *Cell. Rep.* **44** (3), 115430 (2025).
24. Marzetti, E. et al. Mitochondrial quality in aging and neurodegeneration: The emerging role of mitochondria-derived vesicles. *Mech. Ageing Dev.* **231**, 112167 (2026).
25. Davis, P., Cunnington, P. & Hughes, G. R. Double-stranded RNA antibodies in systemic lupus erythematosus. *Ann. Rheum. Dis.* **34** (3), 239–243 (1975).
26. Sarkar, P. et al. Reduced expression of mitochondrial fumarate hydratase in progressive multiple sclerosis contributes to impaired in vitro mesenchymal stromal cell-mediated neuroprotection. *Mult Scler.* **28** (8), 1179–1188 (2022).
27. Steadman, T. & O'Reilly, S. Aberrant fumarate metabolism links interferon release in diffuse systemic sclerosis. *J. Dermatol. Sci.* **117** (2), 30–35 (2025).
28. Shah, A. D. et al. LFQ-Analyst: An Easy-To-Use Interactive Web Platform To Analyze and Visualize Label-Free Proteomics Data Preprocessed with MaxQuant. *J. Proteome Res.* **19** (1), 204–211 (2020).

Figures

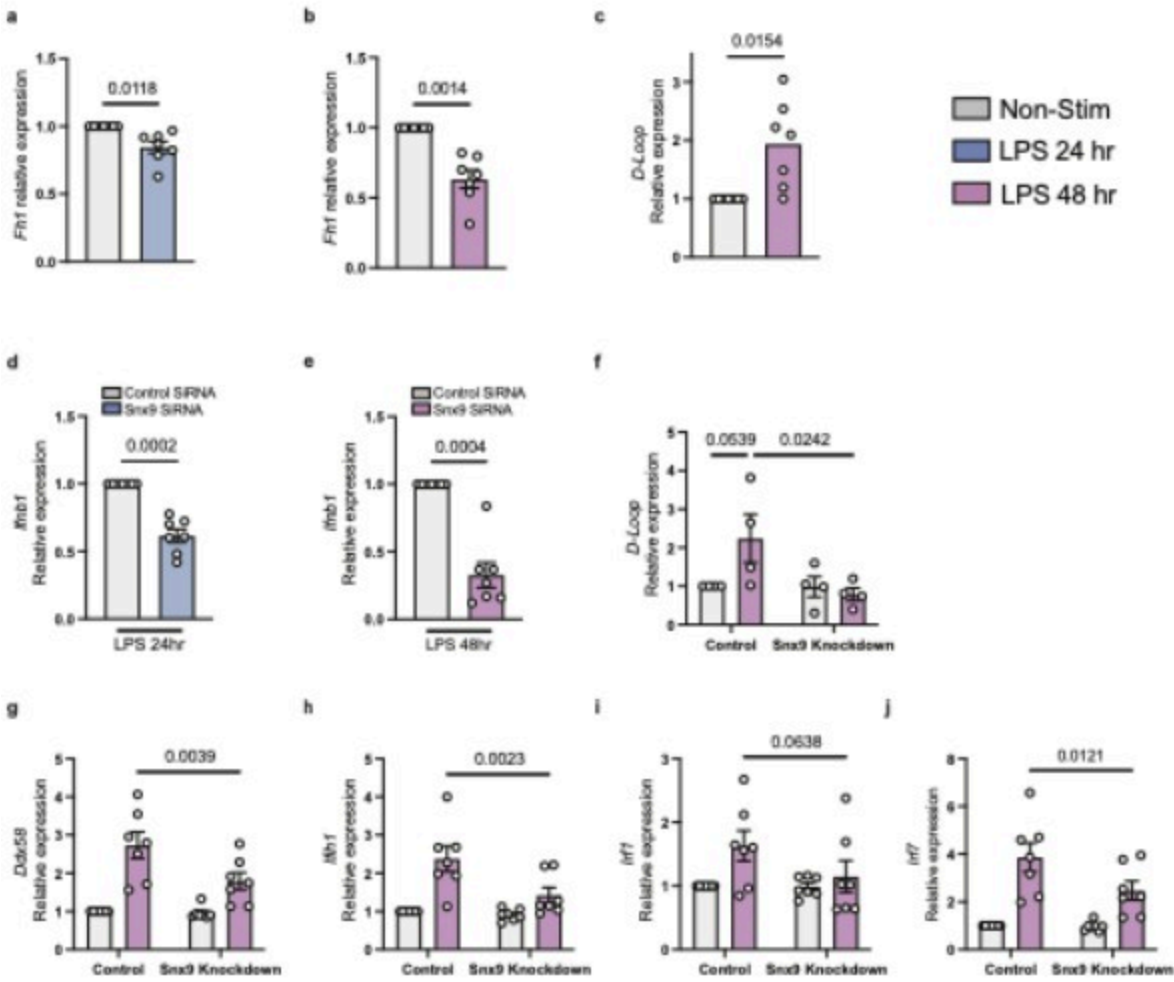


Figure 1

SNX9 regulates mtRNA release and downstream IFN- β and Interferon-stimulated gene expression upon prolonged LPS stimulation. **a, b**, *Fh* expression in lipopolysaccharide (LPS 100 ng mL⁻¹) stimulated bone marrow derived macrophages (BMDMs, plated at 0.5 million mL⁻¹) ($n = 7$; LPS 24 or 48 h). **c**, Cytosolic RNA *D-loop* expression in LPS stimulated BMDMs ($n = 3$; LPS 48 h). **d, e**, *Ifnb1* expression in LPS stimulated BMDMs with control or *Snx9* SiRNA ($n = 7$; LPS 24 or 48h). **f**, Cytosolic RNA *D-loop* expression in LPS stimulated BMDMs with control or *Snx9* SiRNA ($n = 4$; LPS 24 or 48h). **g**, *Ddx58* expression in LPS stimulated BMDMs with control or *Snx9* SiRNA ($n = 7$; LPS 48h). **h**, *Ifih1* expression in LPS stimulated BMDMs with control or *Snx9* SiRNA ($n = 7$; LPS 48h). **i**, *Irf1* expression in LPS stimulated BMDMs with control or *Snx9* SiRNA ($n = 7$; LPS 48h). **j**, *Irf7* expression in LPS stimulated BMDMs with control or *Snx9* SiRNA ($n = 7$; LPS 48h). Data are from three independent experiments. P values calculated using two-tailed Student's t-test for paired comparisons or two-way ANOVA for multiple comparisons.

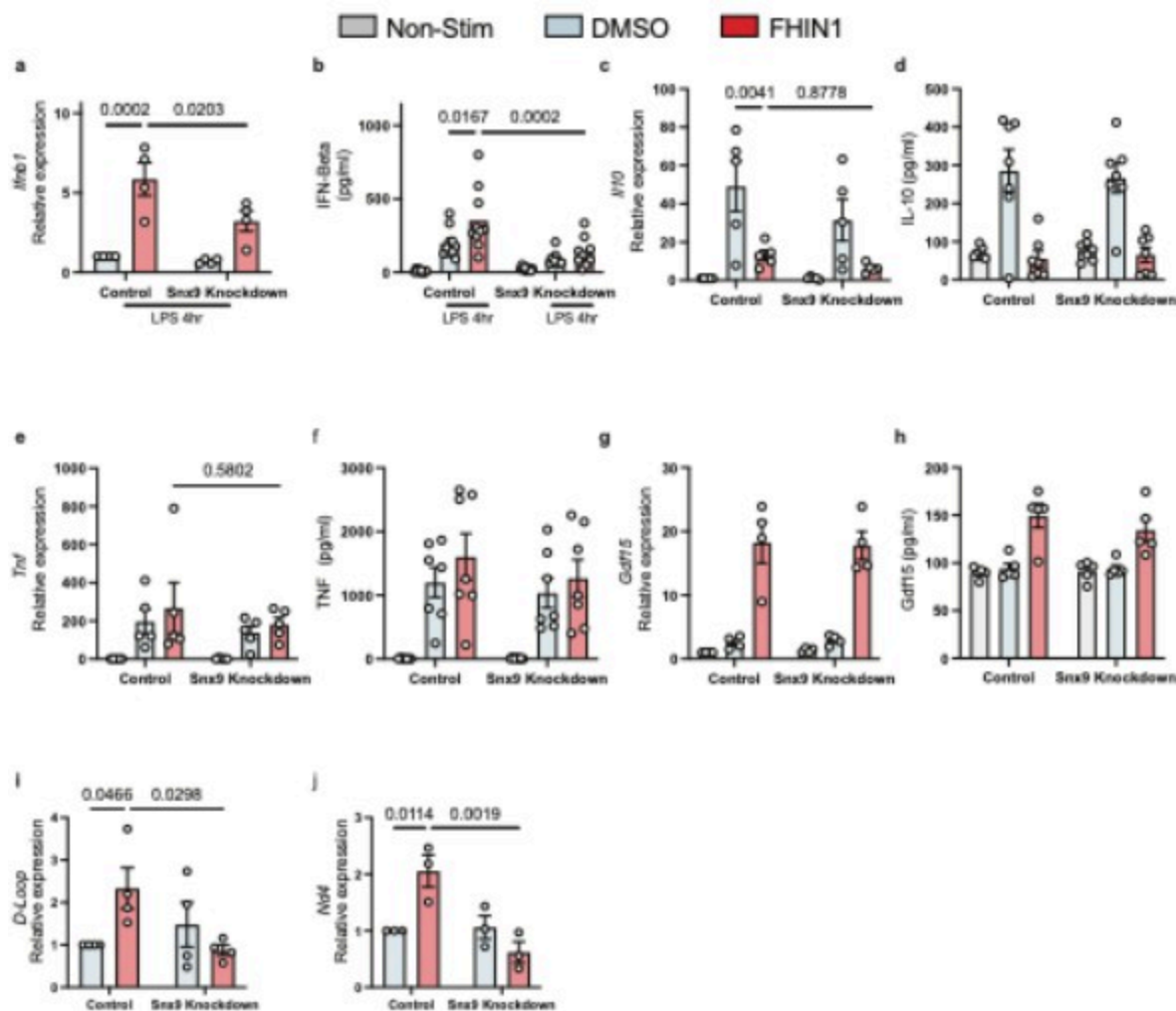


Figure 2

Acute fumarate hydratase inhibition induces IFN-β expression dependently on SNX9 **a**, *Ifnb1* expression in LPS (100 ng mL⁻¹) stimulated FHIN1 (3h) pre-treated BMDMs (Plated at 0.5 million mL⁻¹) with control or *Snx9* SiRNA (*n* = 4; LPS 4h). **b**, IFN-β release in LPS stimulated and FHIN1 (3h) pre-treated BMDMs with control or *Snx9* SiRNA (*n* = 9; LPS 4h). **c**, *Il10* expression in LPS stimulated FHIN1 (3h) pre-treated BMDMs with control or *Snx9* SiRNA (*n* = 5; LPS 4h). **d**, IL-10 release in LPS stimulated FHIN1 (3h) pre-treated BMDMs with control or *Snx9* SiRNA (*n* = 7; LPS 4h). **e**, *Tnf* expression in LPS stimulated FHIN1 (3h) pre-treated BMDMs with control or *Snx9* SiRNA (*n* = 5; LPS 4h). **f**, TNF expression in LPS stimulated FHIN1 (3h) pre-treated BMDMs with control or *Snx9* SiRNA (*n* = 7; LPS 4h). **g**, *Gdf15* expression in LPS stimulated FHIN1 (3h) pre-treated BMDMs with control or *Snx9* SiRNA (*n* = 4; LPS 4h). **h**, GDF15 release in LPS stimulated FHIN1 (3h) pre-treated BMDMs with control or *Snx9* SiRNA (*n* = 5; LPS 4h). Data are from three independent experiments. P values calculated using two-way ANOVA for multiple comparisons. **i**, **j**, Cytosolic *D-loop* and *ND4* expression in RNA in LPS stimulated and FHIN1 (3hr) pre-treated BMDMs with control or *Snx9* SiRNA (*n* = 4 and 3; LPS 2 h).

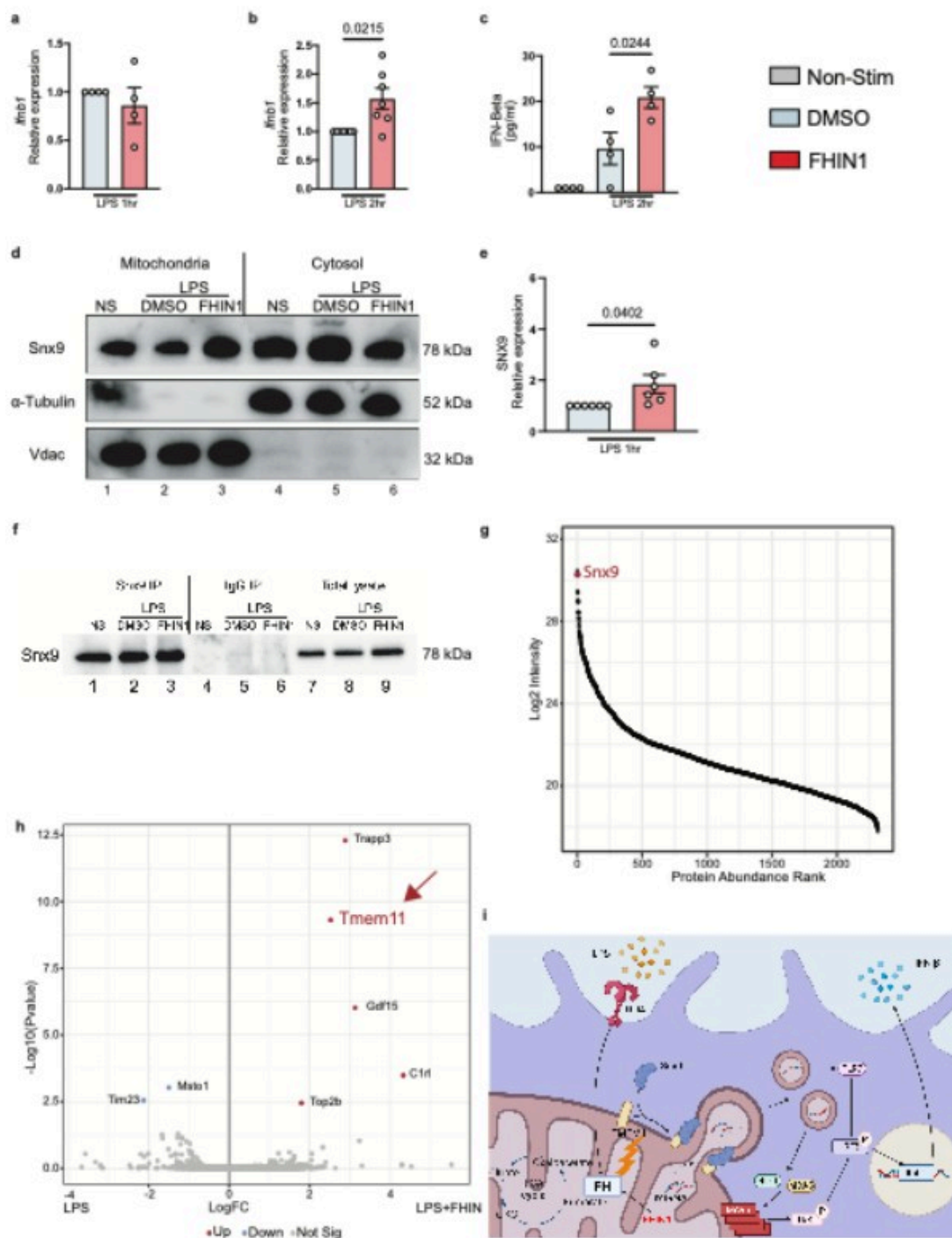


Figure 3

FH inhibition causes SNX9 localization on mitochondria. **a, b**, *fnb1* expression in LPS (100 ng mL⁻¹) stimulated FHIN1 (3h) pre-treated BMDMs (Plated at 0.5 million mL⁻¹) ($n = 4$ and 7 ; LPS 1h or 2h). **c**, IFN- β release in LPS stimulated and FHIN1 (3h) pre-treated BMDMs ($n = 4$; LPS 2h). **d**, Representative blot of SNX9, α -Tubulin and Vdac in mitochondrial and cytosolic fractions following mitochondria isolation in LPS stimulated and FHIN1 (3hr) pre-treated BMDMs. Original uncropped blots are provided in

Supplementary information file. **e**, Densitometry of SNX9 in LPS stimulated FHIN1 (3h) pre-treated BMDMs ($n = 6$; LPS 1h). **f**, Representative blot of SNX9 in SNX9 and IgG immunoprecipitation pulldown and total lysate LPS stimulated FHIN1 (3h) pre-treated BMDMs ($n=3$, LPS 1h). Original uncropped blots are provided in Supplementary information file. Blots shown in separate panels were obtained from independently run membranes processed in parallel from the same experiment. **g**, Abundance plot of differentially expressed proteins from SNX9 pulldown in LPS with or without FHIN1 treated BMDMs ($n = 3$; LPS 1h). **h**, Volcano plot of differentially expressed proteins from SNX9 pulldown in LPS with or without FHIN1 treated BMDMs ($n = 3$; LPS 1h). **i**, Schematic diagram showing the mechanism of SNX9 regulating mtRNA release. Data are from three independent experiments. P values calculated using two-tailed Student's t-test for paired comparisons or two-way ANOVA for multiple comparisons.

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