Mitochondrial DNA stress primes the antiviral innate immune response

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Mitochondrial DNA (mtDNA) is normally present at thousands of copies per cell and is packaged into several hundred higher-order structures termed nucleoids6. The abundant mtDNA-binding protein TFAM (transcription factor A, mitochondrial) regulates nucleoid architecture, abundance and segregation7. Complete mtDNA depletion profoundly impairs oxidative phosphorylation, triggering calcium-dependent stress signalling and adaptive metabolic responses8. However, the cellular responses to mtDNA instability, a physiologically relevant stress observed in many human diseases and ageing, remain poorly defined9. Here we show that moderate mtDNA stress elicited by TFAM deficiency engages cytosolic antiviral signalling to enhance the expression of a subset of interferon-stimulated genes. Mechanistically, we find that aberrant mtDNA packaging promotes escape of mtDNA into the cytosol, where it engages the DNA sensor cGAS (also known as MB21D1) and promotes STING (also known as TMEM173)-IRF3-dependent signalling to elevate interferon-stimulated gene expression, potentiate type I interferon responses and confer broad viral resistance. Furthermore, we demonstrate that herpesviruses induce mtDNA stress, which enhances antiviral signalling and type I interferon responses during infection. Our results further demonstrate that mitochondria are central participants in innate immunity, identify mtDNA stress as a cell-intrinsic trigger of antiviral signalling and suggest that cellular monitoring of mtDNA homeostasis cooperates with canonical virus sensing mechanisms to fully engage antiviral innate immunity.

To explore the cellular responses to mtDNA stress in the absence of oxidative phosphorylation deficiency, we employed a TFAM heterozygous knockout (Tfam+/−) mouse model. Cells and tissues from these animals exhibit modest or no significant differences in mtDNA-encoded transcripts and oxygen consumption rates, despite an approximately 50% depletion of mtDNA (Extended Data Fig. 1a–c)5,6. In addition to mtDNA depletion, Tfam+/− mouse embryonic fibroblasts (MEFs) have reduced oxidative mtDNA damage repair capacity and markedly altered mtDNA packaging, organization and distribution (Fig. 1a). Nucleoids in Tfam+/− MEFs were less numerous and exhibited a larger size distribution (Fig. 1a and Extended Data Fig. 1d). Thus, Tfam+/− cells provide a robust model to characterize cellular responses triggered by moderate mtDNA stress.

Gene expression profiling of Tfam+/− MEFs revealed an unexpected enrichment of interferon-stimulated genes (ISGs) and antiviral signalling factors (Fig. 1b). Of the 45 most overexpressed genes, 39 were ISGs, including many with direct antiviral activity (Ifi44, Ifit1, Ifit3, Oas1, Rtp4)5,6. We also observed increased expression of cytoplasmic RNA and DNA sensors, such as Ddx58 and Ifih1 and p200 family proteins Ifi203, Ifi204 and Ifi205, as well as transcription factors Ifr7, Stat1 and Stat2, ISGs that function to positively reinforce the antiviral response. Direct measurement of basal ISG mRNA and protein expression in Tfam+/− MEFs validated the microarray results (Fig. 1c, d). Finally, Tfam+/− MEFs expressed three- to fourfold more Ifnb and Ifna4 upon transfection with the IFIH1 agonist poly(I:C) (Fig. 1e), consistent with enhanced type I interferon responses.

Figure 1 | Tfam+/− cells exhibit mtDNA stress, elevated ISG expression and augmented type I interferon responses. a. Confocal microscopy images of MEFs stained with anti-DNA (DNA) and anti-HSP60 (Mito.) antibodies. b. Heat maps of microarray analyses. Genes in Tfam+/− MEFs exhibiting statistically significant (P < 0.05), twofold or greater increases over wild type (WT) are shown. c, d. Quantitative real-time-PCR (qRT–PCR) (c) and western blots (d) of basal ISG expression in two littermate wild-type and Tfam+/− MEF lines. e. qRT–PCR analysis of type I interferon expression in MEFs 9 h after cytosolic delivery of poly(I:C). Error bars indicate ±s.e.m. of triplicate technical replicates and are representative of three independent experiments. ***P < 0.001.
To ensure that the mtDNA stress and ISG expression phenotypes were not unique to Tfam<sup>+/−</sup> MEFs, we employed inducible TFAM depletion models (TFD). Analogous to Tfam<sup>+/−</sup> cells, TF<sup>D</sup> MEFs and bone-marrow-derived macrophages (BMDMs) displayed mtDNA stress phenotypes, augmented ISG expression, and heightened type I interferon responses to poly(I:C) (Extended Data Fig. 1d–i). Collectively, these data indicate that TFAM depletion induces mtDNA nucleoid stress that triggers antiviral ‘priming’, characterized by basally elevated ISG expression and potentiated type I interferon production.

Since mitochondrial stress can trigger the release of mtDNA into the cytosol to engage the NLRP3 inflammasome, we assayed for extramitochondrial mtDNA in TF<sup>D</sup> cells<sup>10</sup>. Analysis of pure cytosolic extracts revealed a three- to fourfold increase of specific mtDNA fragments from the D-loop regulatory region, indicating liberation of immunostimulatory mtDNA into the cytosol (Extended Data Fig. 2)<sup>11</sup>. Confocal and electron microscopy of TFD cells revealed significantly elongated, interconnected mitochondrial networks consistent with a hyperfused phenotype (Fig. 1a and Extended Data Figs 1c, g and 3a, b). Since mitochondrial fission facilitates proper nucleoid distribution and removal of damaged mtDNA, we examined whether mitochondrial hyperfusion in TF<sup>D</sup> cells governed mtDNA stress-induced ISG expression<sup>12,13</sup>. Knockdown of mitofusin 1 (Mfn1) induced fission and largely abrogated ISG expression in TF<sup>D</sup> MEFs (Extended Data Fig. 3c–e). Moreover, depletion of the mtDNA quality-control enzyme endo/exonuclease (5′–3′), endonuclease G-like (EXOG) exacerbated ISG expression in Tfam<sup>+/−</sup> MEFs (Extended Data Fig. 3f)<sup>14</sup>. Collectively, these data indicate that TFAM depletion promotes accumulation of aberrant mtDNA, which accesses the cytosol to engage innate immune signalling.

We next examined the involvement of the cytosolic DNA sensor cGAS in mtDNA stress signalling, as it mediates ISG expression in response to exogenous and endogenous immunostimulatory DNA species<sup>15–17</sup>. Knockdown of cGAS in Tfam<sup>+/−</sup> MEFs or TFAM depletion in cGas<sup>−/−</sup> MEFs largely abrogated ISG expression (Fig. 2a). Furthermore, ISG mRNAs in TF<sup>D</sup> cells were reduced 70–90% in the absence of STING, indicating cGAS–STING signalling is the predominant driver of mtDNA stress-induced ISG expression (Fig. 2b). STING signals via the TBK1–IRF3/7 axis to trigger antiviral gene expression, and knockdown of either TBK1 or IRF3 robustly blocked ISG expression in Tfam<sup>+/−</sup> MEFs (Fig. 2c, d)<sup>18,19</sup>. Consistent with IRF3 activating ISG transcription, we observed enhanced nuclear accumulation of IRF3 in TF<sup>D</sup> cells (Fig. 2e). Finally, using cGas<sup>−/−</sup> MEFs reconstituted with hemagglutinin (HA)-tagged, murine cGAS, we observed prominent re-localization of cGAS from nuclear and/or cytoplasmic pools to the vicinity of aberrant mtDNA nucleoids in TF<sup>D</sup> MEFs (Fig. 2f, g). Taken together, these results indicate that mtDNA stress facilitates cGAS-dependent sensing of cytoplasmic mtDNA, resulting in STING–TBK1–IRF3 signalling to trigger ISG expression.

To establish functional significance of mtDNA stress-induced antiviral priming, we challenged MEFs with herpes simplex virus 1 (HSV-1) or vesicular stomatitis virus (VSV) that express green fluorescent protein (GFP) for easy detection. In contrast to wild-type cells, which displayed robust viral GFP expression post-infection, Tfam<sup>+/−</sup> MEFs were markedly resistant to HSV-1 and VSV (Fig. 3a). In addition, Tfam<sup>+/−</sup> MEFs exhibited heightened type I interferon and ISG expression upon viral challenge, consistent with potentiated type I interferon responses in these cells (Extended Data Fig. 4a). Similar results were obtained upon challenge with the rodent gammaherpesvirus MHV-68 (Fig. 3b and Extended Data Fig. 4b). Furthermore, TF<sup>D</sup> BMDMs displayed augmented antiviral gene expression and markedly lower HSV-1 and VSV-encoded mRNA and protein 6–24 h post-infection (Extended Data Fig. 4c–d).

Figure 2 | mtDNA stress triggers ISG expression in a cGAS- and STING-dependent fashion.

**a, b,** ISG expression in Tfam<sup>−/−</sup> MEFs transfected with the indicated short interfering RNAs (siRNAs; top panels), or wild-type (WT), cGas<sup>−/−</sup> (a), and Sting<sup>−/−</sup> (b) MEFs transfected with TFAM siRNAs (bottom panels). Ctrl, control. **c, d,** ISG expression in Tfam<sup>+/−</sup> MEFs transfected with the indicated siRNAs for 96 h. **e,** Western blots of whole-cell and nuclear extracts of wild-type and Tfam<sup>−/−</sup> MEFs or Tfam<sup>+/−</sup> ER-cre<sup>−/−</sup> (cre<sup>−/−</sup>) BMDMs exposed to 4-hydroxytamoxifen (4OHET) for 96 h. **f, g,** cGas<sup>−/−</sup> MEFs reconstituted with cGAS–HA were transfected with the indicated siRNAs for 96 h, then stained with anti-DNA (DNA), anti-HSP60 (Mito.) and anti-HA (cGAS–HA) antibodies and imaged. cGAS co-localization scoring was performed as described in the Methods. Error bars indicate ±s.e.m. of triplicate technical (a–d) or biological (g) replicates and are representative of three independent experiments.

*P < 0.05; **P < 0.01; ***P < 0.001.
Finally, we found that Tfam+/− mice exhibit basally elevated ISG expression, which confers resistance to acute infection by lymphocytic choriomeningitis virus (LCMV) Armstrong (Extended Data Fig. 5a and Fig. 3c).

To probe a direct requirement for mtDNA stress in antiviral priming in TFAM-deficient cells, we used dideoxycytidine (ddC), a deoxyribonucleoside analogue that specifically inhibits mtDNA replication and decreases mtDNA nucleoid size. Treatment of wild-type MEFs with ddC resulted in reduced mtDNA copy number and decreased average nucleoid size without altering basal ISG expression (Extended Data Fig. 5b–d). In contrast, ddC drastically diminished mtDNA stress (that is, enlarged nucleoids measuring greater than 450 nm$^2$) in Tfam−/− and TFAM−/− MEFs (Fig. 3d and Extended Data Fig. 5e), which was accompanied by attenuation of antiviral priming and basal ISG expression (Fig. 3e and Extended Data Fig. 5f, g, h). Moreover, ddC ablated the viral resistance phenotype of Tfam−/− MEFs (Fig. 3f). We observed similar decreases in type I interferon production and a reduction in the viral resistance phenotype in ddC-treated TFAM−/− BMDMs (Extended Data Fig. 5g, h, blue bars). These results demonstrate that mtDNA stress directly potentiates antiviral innate immunity.

The observation that ddC-treated wild-type BMDMs displayed reduced Ifnb and increased viral gene expression upon challenge with HSV-1, despite normal responses to cytosolic nucleic acids (Extended Data Fig. 5h, i, grey bars), indicates that virus-induced mtDNA stress may boost host antiviral responses, consistent with reports linking viral infection to mtDNA dysregulation. The alphaherpesvirus protein UL12.5, encoded by HSV-1 and HSV-2, localizes to mitochondria and promotes rapid mtDNA depletion in human cells, which we confirmed in MEFs (Extended Data Fig. 6a). Since mtDNA depletion and nucleoid stress are often coupled, we explored nucleoid architecture and abundance kinetically during HSV-1 infection. Notably, 3 h after challenge with HSV-1, mtDNA stress was readily apparent, with nucleoids less evenly distributed and enlarged (Fig. 4a). After 6 h, ~10% of nucleoids measured larger than 450 nm$^2$, and there was a significant decrease in total nucleoid intensity (Fig. 4b). After 12 h, we observed pronounced mtDNA depletion. The mtDNA stress observed 3 to 6 h after HSV-1 challenge closely mirrored that of TFAM-deficient cells (Fig. 4b), as did TFAM protein levels (Fig. 4c). MHV-68 and HSV-2 triggered mtDNA stress similar to HSV-1, indicating that mtDNA stress is a common cellular perturbation during herpesvirus infection (Extended Data Fig. 6b, c). However, induction of mtDNA stress and TFAM depletion were not a general consequence of viral infection, as cells infected with VSV, influenza, LCMV or vaccinia possessed normal mtDNA architecture, TFAM expression and copy number (Fig. 4a–c and Extended Data Fig. 6c, d).

Finally, we sought to determine whether HSV-1-induced mtDNA dysregulation is necessary to fully engage antiviral signalling. Transduction of MEFs and BMDMs with replication-incompetent retroviruses encoding only the mitochondria-targeted HSV-1 UL12 M185 gene product was sufficient to cause mitochondrial hyperfusion, nucleoid enlargement and mtDNA loss, indicative of mtDNA stress (Fig. 4d). UL12 M185 expression was also sufficient to trigger TFAM depletion and antiviral priming (that is, increased ISG mRNA and protein expression) (Fig. 4e and Extended Data Fig. 7a). To explore the effect of HSV-1-induced mtDNA stress on innate antiviral responses, we employed a recombinant, UL12-deficient HSV-1 strain (ΔUL12 + UL98–FLAG) that is severely impaired in its ability to induce mtDNA stress but replicates similarly to a matched UL12-sufficient strain (Extended Data Fig. 7b, c). Infection with AUL12 HSV-1 resulted in attenuated TBK1 phosphorylation and type I interferon
and ISG expression between 3 to 6 h post-infection, despite comparable early HSV-1 gene expression (Fig. 4f, g). However, after 24 h, AUL12 HSV-1 genome abundance was roughly threefold higher compared to the UL12-sufficient control, consistent with impaired antiviral innate immunity (Fig. 4h). Finally, AUL12 HSV-1 elicited less robust antiviral innate immune responses in the vagina and more readily spread to dorsal root ganglia of wild-type mice due to a deficit in mtDNA stress-dependent antiviral priming (Extended Data Fig. 7d, e). These results reveal that herpesvirus-induced mtDNA stress is necessary to effectively engage ISG expression and antiviral priming, and suggest that cellular monitoring of mtDNA homeostasis represents an additional sensory mechanism to robustly engage antiviral innate immunity.

In closing, our work uncovers a novel cellular response to mtDNA stress that engages the antiviral innate immune response. Specifically, we show that mtDNA stress, induced by herpesvirus infection and mediated by loss of the mtDNA packaging protein TFAM, triggers a <span>GCAS–STING–IRF3</span>-dependent pathway to upregulate ISGs and potentiate type I interferon responses to viral infection (Extended Data Fig. 8). Our results support a model whereby viral-mediated disruption of mtDNA homeostasis serves as a cell-intrinsic indicator of infection that works in parallel with canonical virus sensing to enhance antiviral innate immunity. Conversely, pathologic type I interferon signatures promote autoimmune diseases such as systemic lupus erythematosus, and altered ISG expression correlates with radiation-resistant and

Figure 4 | HSV-1 induces mtDNA stress and TFAM depletion sufficient to trigger ISG expression and necessary to fully engage antiviral immunity. 

The results were consistently observed across multiple independent experiments. **p < 0.001; NS, not significant.
metastatic phenotypes in some cancers.\textsuperscript{26,27} Mitochondrial and mtDNA dysregulation have been noted in systemic lupus erythematosus, and perturbations in TFAM and/or mtDNA homeostasis are frequently observed in cancer.\textsuperscript{28–30} Therefore, further investigation into this pathway will not only expand our knowledge of innate antiviral defence, but may also broaden our understanding of how mitochondria contribute to the pathogenesis of human diseases and ageing beyond their well characterized roles in metabolism, apoptosis and reactive oxygen species production.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

**Received 4 July; accepted 15 December 2014.**

**Published online 2 February; corrected online 22 April 2015 (see full-text HTML version for details).**

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METHODS
Animal strains. Tjam1+/− and Tjam1fl/fl mice were previously described and maintained on a C57BL/6 background.1 Tjam1fl/fl mice were bred to Estrogen receptor (ER)−Cre transgenic mice from Jackson (stock no. 004682) for inducible, 4OHT-mediated deletion. All animal experiments were conducted in compliance with guidelines established by the Yale University Institutional Animal Care and Use Committee.

Antibodies and reagents. Rabbit anti-mouse TFAM polyclonal anti-sera was previously described,2 rabbit anti-VDV2 polyvalent anti-sera was a gift from J. Rose at Yale University, mouse anti-Viperin was a gift from P. Cresswell at Yale University, and rabbit anti-IFT3 was a gift from G. Sen at Cleveland Clinic. The following antibodies were obtained commercially: goat anti-HSP60 (N-20) and rabbit anti-calnexin (H-70) (Santa Cruz Biotechnology); mouse and rabbit anti-FLAG (F1804, F7425) (Sigma); mouse anti-human CRL186 (Millipore); mouse anti-GFP (I1, II) (BD Biosciences); rabbit anti-HSV-1/2 (ab9533) and anti-histone H3 (ab1791) (Abcam); rat anti-HA−FITC (I1998506001) (Roche); rabbit anti-NLRX1 (17215-1-AP) (Proteintech); mouse anti-α-tubulin (DM1A) (Neomarkers); mouse anti-GAPDH (6CS) (Ambion); and rabbit anti-DDX5 (D146E).−IIFH1 (D74E).−STAT1 (9172), IRF3 (D83B9), TBKI (D1B4) and anti-phospho-TBKI (D52C2) (Cell Signalling Technology). Mouse IFNα enzyme-linked immunosorbent assay (ELISA) and recombinant mouse IFNβ was from PBL Assay Science, and mouse IL-6 ELISA was from eBioscience. All primer sequences and siRNAs used are found in Extended Data Tables 1 and 2.

Cell culture. Primary wild-type, Tjam1−/−, Sting−/− and Gas−/− MEFS were generated from E12.5–14.5 embryos, maintained in DMEM (Invitrogen) supplemented with 10% FBS (Atlanta Biologicals), and sub-cultured no more than five passages before experiments. Sting−/− MEFS were provided by G. Barber at the University of Miami.3 L929 cells were obtained from ATCC and maintained in DMEM supplemented with 10% FBS. siRNA transfection of MEFS was performed with 25 nM siRNA duplexes (Integrated DNA Technologies) and Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions. ddC (Sigma) was resuspended in PBS, added to MEFS or BMDMs at a final concentration of 10–20 μM, and replenished every 48 h. BMDMs were generated from bone marrow of 8–12-week-old littermate Tjam1fl/fl−/− and Tjam1fl/fl−/− mice and cultured on Petri plates in DMEM containing 10% FBS plus 30% L929 culture media. To induce Cre-mediated deletion, 1 μM 4OHT dissolved in DMSO (Sigma) was added to BMDM cultures on day 6 and incubated for an additional 2–3 days. Cells were then lifted from plates by incubating in cold PBS containing 1 mm EDTA, re-plated in fresh media containing 10% L929 conditioned media, and allowed to rest overnight before experimentation (for a total of 72 or 96 h of 4OHT exposure). Transfection of interferon-stimulatory DNA (ISD)33 and poly(I:C) (Sigma) into transfection of cytosolic BMDMs was performed using Lipofectamine 2000 (Invitrogen). In brief, 1 × 10^6 BMDMs were seeded in 6-well dishes after 4OHT treatment, and transfected the next day with 4 μg ISD per well or 2.5 μg per well of poly(I:C) complexed at a ratio of 2:1 Lipofectamine 2000 to nucleic acid. Poly(I:C) transfection into the cytosol of MEFS was performed as described previously.4

Viral infections and infections. VSV−G−GFP, HSV−1−GFP, MHV−68−GFP, HSV−2, vaccinia virus (strain WR) expressing bacteriophage T7 RNA polymerase, influenza A PR8 NS1−GFP, HSV−1 (UL12−FLAG) and HSV−1 (UL12A + UL98−FLAG)5 were maintained as described previously.6−11 MEFS or BMDMs were infected at the indicated multiplicity of infection (MOI) in serum-free DMEM for 1 h, washed, and incubated for various times. Cells were then fixed and stained for microscopy, lysed for western blot, solubilized in buffer RLT Plus (Qiagen) for RNA extraction, or prepared for FACS analysis. FACS was performed by first trypanosmitizing MEFS, followed by labelling with LIVE/DEAD Fixable Red dead (Molecular Probes). Cells were then fixed with 4% paraformaldehyde, washed, and analysed on a FACScan (Becton Dickinson) flow cytometry machine (BD). FACS plots were first gated on live cells before analysing viral GFP fluorescence. Viral gene expression in BMDMs was determined using qRT−PCR as described below, except that after values were normalised against GAPDH cDNA using the 2^−ΔΔCt method. All data points were subtracted by one to centre on zero.

LCMV Armstrong infection of wild-type and Tjam1−/− mice was performed as described previously.12 In brief, 10-week-old female mice were infected with 2 × 10^3 plaque-forming units of virus intraperitoneally, and 4 days post-infection, mice were euthanized, tissues isolated, and total RNA prepared using RNeasy Plus kits (Qiagen). After generating complementary DNA, samples were subjected to qPCR analysis as described below using published methods.12,13

In vivo HSV−1 infection, dorsal root ganglia isolation and viral titration. Six-week-old female mice were purchased from Charles River Laboratories and inoculated with 10^5 plaque-forming units of virus intraperitoneally. Epidural, lumbar, and thoracic spinal cord sections were harvested 3 days post-infection. Reduced spinal cord sections were further dissected and homogenized using a Leica UltraCut UCT. 60−nm sections were collected on formvar/carbon-coated grids and contrast stained using 2% uranyl acetate and lead citrate. Samples were viewed on a FEI Tencai Biotwin TEM at 80 K. Images were taken using Morada CCD and iTEM (Olympus) software.

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For mitochondrial perimeter quantification, approximately 10–15 unique electron microscopy images of each genotype were captured at random. After incorporating scale information from iTEM software, the perimeter along the outer membrane of each mitochondrion was traced and quantified using ImageJ. Mitochondria were divided into the three size cutoffs: < 2 μm, 2–5 μm, and > 5 μm, and the percentage of mitochondria falling within each category was plotted.

**Oxygen consumption analysis.** Cells were plated in XF96 plates (SeaHorse Biosciences) at 10,000 cells per well and the next day cellular O2 consumption was determined in a SeaHorse Bioscience XF96 extracellular flux analyser according to the manufacturer’s instructions. Cells were maintained at 37 °C in normal growth medium without serum.

**Nuclear fractionation and western blotting.** Whole-cell extracts were solubilized in SDS lysis buffer (20 mM Tris-HCl pH 7.5, containing protease and phosphatase inhibitors), boiled for 5 min, and DNA was sheared by sonication. For nuclear extraction, PBS-washed cell pellets were resuspended in 10 pellet volumes of RSB buffer (10 mM NaCl, 1.5 mM CaCl2, 10 mM Tris-HCl pH 7.5), swollen on ice for 10 min, homogenized with a motorized Teflon pestle, and the homogenate was centrifuged at 980 × g for 10 min to pellet nuclei. Pellets were washed five times in PBS, SDS was then added to a final concentration of 1%, and extracts were boiled for 5 min before sonication to shear DNA and normalizing protein concentration. Western blotting was performed using standard protocols, and HSP60 was used as whole-cell extract loading controls, while histone H3 was probed as a nuclear loading control.

**Detection of mtDNA in cytosolic extracts.** Diginonin extracts from MEFs and BMDMs were generated largely as described previously. Wild-type and *Tjmn*−/− MEFs (7 × 10⁶) or *Tjmn*−/− ER-creMDMs exposed to 4OHT for 72 h (1 × 10⁶) were each divided into two equal aliquots, and one aliquot was resuspended in 500 μl of 50 mM NaOH and boiled for 30 min to solubilize DNA. 50 μl of 1 M Tris-HCl pH 8 was added to neutralize the pH, and these extracts served as normalization controls for total mtDNA. The second equal aliquots were resuspended in roughly 500 μl buffer containing 150 mM NaCl, 50 mM HEPES pH 7.4, and 15–25 μg ml⁻¹ diginonin (EMD Chemicals). The homogenates were incubated end over end for 10 min to allow selective plasma membrane permeabilization, then centrifuged at 980g for 3 min times three to pellet intact cells. The first pellet was saved as the ‘Pel’ fraction for western blotting. The cytosolic supernatants were transferred to fresh tubes and spun at 17000g for 10 min to pellet any remaining cellular debris, yielding cytosolic preparations free of nuclear, mitochondrial and endoplasmic reticulum contamination. DNA was then isolated from these pure cytosolic fractions using QIAQuick Nucleotide Removal Columns (QIAGEN). Quantitative PCR was performed on both whole-cell extracts and cytosolic fractions using nuclear DNA primers (Tert) and mtDNA primers (Dloop1-3, Cyrb, 165 and Nd4). The Ct values obtained for mtDNA abundance for whole-cell extracts served as normalization controls for the mtDNA values obtained from the cytosolic fractions. This allowed effective standardization among samples and controlled for any variations in the total amount of mtDNA in control and TFAM-deficient samples. Using this diginonin method, no nuclear Tert DNA was detected in the cytosolic fractions, indicating nuclear lysis did not occur.

**Bioinformatic analyses.** Total cellular RNA from wild-type and *Tjmn*−/− littermate MEFs was prepared using RNeasy Plus RNA extraction kits (QIAGEN) and used for the expression microarray procedure in conjunction with the Emory University Integrated Genomics Core. RNA integrity was first verified by an Agilent Bioanalyzer and then amplified, labelled, and hybridized onto Mouse Gene 1.0 ST arrays (Affymetrix) using standard protocols recommended by the manufacturer, starting from 2 μg of total RNA. Data were normalized by the DMA method using GeneSpring software (Agilent) for each biological sample in duplicate. Student’s *t*-test was used to determine statistically significant changes in expression in *Tjmn*−/− MEFs relative to wild type, with a cut-off *P* value of 0.05*. Heat maps were generated using MultiExperiment Viewer®.

**Statistical analyses.** Error bars displayed throughout the manuscript represent s.e.m. unless otherwise indicated, and were calculated from triplicate or quadruplicate technical replicates of each biological sample. For *in vivo* experiments, error bars were calculated from the average of triplicate technical replicates of 3–4 mice per point. Sample sizes were chosen by standard methods to ensure adequate power, and no randomization or blinding was used for animal studies. No statistical method was used to predetermine sample size. Statistical significance was determined using unpaired Student’s *t*-tests; *P* < 0.05; **P** < 0.01; ***P*** < 0.001; NS, not significant (*P* > 0.05). Data shown are representative of 2–3 independent experiments, including microscopy images, western blots and viral challenges.

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Extended Data Figure 1 | TFAM deficiency induces mtDNA depletion, nucleoid stress, elevated ISG expression and augmented type I interferon responses, but does not drastically alter oxygen consumption and mitochondrial transcription rates. a, Quantitative PCR analysis of relative mtDNA copy number from wild-type (WT) and Tfam<sup>−/+</sup> MEFs. b, Basal oxygen consumption rate of wild-type and Tfam<sup>−/+</sup> MEFs as determined by Seahorse Bioscience XF96 Extracellular Flux assay. c, qRT–PCR of mtDNA-encoded rRNA (16s) and mRNA (ND6, Cytb, Cox1) transcripts in wild-type and Tfam<sup>−/+</sup> MEFs. d–f, Untransfected Tfam<sup>−/+</sup> (d) or wild-type MEFs transfected with control (siCtrl) or Tfam (siTfam) siRNAs (d–f) were stained with anti-HSP60 (Mito.) and anti-DNA (DNA) antibodies. Nucleoid area from multiple independent images was calculated, stratified into groups, and graphed as percentage of the total number of nucleoids counted for each sample (d). Inset panels are ×3 magnification to enhance viewing of mitochondrial network and nucleoid architecture (e). TFAM and ISG mRNA expression were measured by qRT–PCR (f). g–i, Tfam<sup>fl/fl</sup> ER-cre<sup>−</sup> or Tfam<sup>fl/fl</sup> ER-cre<sup>+</sup> BMDMs were incubated in 4OHT for 96 h to induce TFAM depletion. Immunofluorescence staining was performed as described above (g). ISG mRNA and protein expression was monitored by qRT–PCR and western blotting (h). qRT–PCR analysis of type I interferon and Il6 expression in 4OHT-treated Tfam<sup>fl/fl</sup> ER-cre<sup>−</sup> BMDMs 2 h post-cytosolic delivery of interferon-stimulatory DNA (ISD) or poly(I:C) (PIC) (i). Error bars
Extended Data Figure 2 | TFAM deficiency promotes accumulation of cytosolic mtDNA. a, Wild-type (WT) or Tfam+/− MEFs were subjected to digitonin fractionation as described in the Methods and whole-cell extracts (WCE), pellets (Pel) or cytosolic extracts (Cyt) were blotted using the indicated antibodies. b, DNA was extracted from digitonin extracts of wild-type and Tfam+/− MEFs or Tfamfl/fl ER-cre− or Tfamfl/fl ER-cre+ BMDMs incubated in 4OHT for 72 h. Cytosolic mtDNA was quantitated via qPCR using a mitochondrial Dloop primer set (mt-Dloop3). Normalization was performed as described in the Methods. c, Samples were prepared as described in b, and cytosolic mtDNA was quantitated via qPCR using the indicated primer sets. Error bars indicate ±s.e.m. of triplicates and data are representative of three independent experiments. **P < 0.01, ***P < 0.001.
Extended Data Figure 3 | Mitochondrial hyperfusion regulates the accumulation of mtDNA nucleoid stress in Tfam MEFs. a, b, Wild-type (WT) MEFs were transfected with control or Tfam siRNAs for 96 h. Cells were fixed and processed for electron microscopy analysis (a). Mitochondrial perimeter measurements were obtained from multiple independent images, stratified into groups, and graphed as a percentage of the total number of mitochondria counted for each sample (b). c–e, Wild-type MEFs were transfected with control, Mfn1 and/or Tfam siRNAs for 96 h. Cells were fixed and stained with an anti-HSP60 antibody (Mito.) and an anti-DNA antibody (DNA) for confocal microscopy (c). Nucleoid area from multiple independent images was calculated as previously described (d). RNA was extracted for ISG expression analysis by qRT–PCR (e). f, Wild-type and Tfam MEFs were transfected with the indicated siRNAs for 96 h and ISG expression analysed by qRT–PCR. Error bars indicate ± s.e.m. of triplicates and data are representative of two independent experiments. **P < 0.01; ***P < 0.001.
Extended Data Figure 4 | mtDNA stress in TFD MEFs and BMDMs potentiates type I interferon responses to viral infection and enhances viral clearance. a, b, Wild-type (WT) and Tfam−/− MEFs were infected with VSV-GFP (a) or MHV68-GFP (b) and, after the indicated times, cytokine and ISG mRNA expression was determined by qRT–PCR, or cytokine secretion was determined by ELISA. c–f, Tfamfl/fl ER-cre or Tfamfl/fl ER-cre BMDMs were incubated in 4OHT for 96 h to induce TFAM depletion. Cells were infected with HSV-1-GFP (c, e, f) or VSV-GFP (d, e), incubated for the indicated times, and viral gene expression was determined by qRT–PCR (c, d) and western blotting (e), or cytokine and ISG mRNA expression was determined by qRT–PCR (f). Error bars indicate ± s.e.m. of triplicates and data are representative of two independent experiments. **P < 0.01; ***P < 0.001; A.U., arbitrary units; ND, not detected; NS, not significant.
Extended Data Figure 5 | Tissues from Tfam<sup>−/−</sup> mice display elevated ISG expression, and ddC abrogates mtDNA stress, ISG expression and viral resistance phenotypes of TFD cells. a, RNA was extracted from the liver and kidneys of 8-week-old wild-type (WT) and Tfam<sup>−/−</sup> mice (n = 2 each) and subjected to qRT–PCR analysis for basal ISG expression. b-d, Relative mtDNA copy number (b), mtDNA nucleoid area (c) and ISG expression (d) of wild-type and Tfam<sup>−/−</sup> MEFs exposed to ddC for 96 h. e, f, mtDNA nucleoid area (e) and ISG expression (f) of wild-type MEFs transfected with control or Tfam siRNAs for 96 h in the presence or absence of ddC. g-i, Tfam<sup>fl/fl</sup> ER-cre<sup>−</sup> or Tfam<sup>fl/fl</sup> ER-cre<sup>+</sup> BMDMs were incubated in 4OHT for 96 h to induce TFAM depletion in the presence of ddC. ddC was washed out and cells allowed to recover overnight before infection. Cells were infected with VSV-GFP (g) or HSV-1-GFP (h) at MOI 1, or wild-type BMDMs were transfected with poly(I:C) or interferon-stimulatory DNA (ISD) (i), and incubated for the indicated times. Ifnb expression or viral gene expression was determined by qRT–PCR. Error bars indicate ± s.e.m. of triplicates and data are representative of two independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant.
**Extended Data Figure 6** | Alpha- and gammaherpesviruses induce mtDNA stress, but influenza, LCMV, and vaccinia do not. 

**a.** Relative mtDNA copy number of wild-type (WT) MEFs 24 h post-infection with VSV-GFP, HSV-1-GFP or mock infection at the indicated MOIs. 

**b.** Wild-type MEFs were infected with MHV68-GFP at MOI 0.5. After the indicated times cells were stained and subjected to confocal microscopy or the relative mtDNA copy number was determined. 

**c.** Wild-type MEFs were infected with HSV-2, influenza-GFP or LCMV-GFP at MOI 10. After 6 h, cells were stained and subjected to confocal microscopy. 

**d.** Wild-type MEFs were infected with vaccinia virus at MOI 10 (for microscopy) or 1. After the indicated times cells were stained and subjected to confocal microscopy or the relative mtDNA copy number was determined. Error bars indicate ± s.e.m. of triplicates and data are representative of two independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; A.U., arbitrary units; ND, not detected; NS, not significant.
Extended Data Figure 7 | HSV-1 UL12 M185 expression is sufficient to trigger mtDNA stress, TFAM depletion and antiviral priming in BMDMs; infection with UL12-deficient HSV-1 fails to induce mtDNA stress, elicits lower vaginal type I interferon responses and spreads more readily to dorsal root ganglia. a, Wild-type (WT) BMDMs were transduced with HSV-1-UL12-M185-expressing- or empty retroviruses (RV) and relative mtDNA abundance, protein expression, and ISG mRNA expression determined. b, Wild-type MEFs were infected with HSV-1 (UL12–FLAG) or UL12-deficient HSV-1 (ΔUL12–UL98–FLAG) at MOI 10 for 3 h and analysed by confocal microscopy. c, Wild-type MEFs were infected with HSV-1 (UL12–FLAG) or UL12-deficient HSV-1 (ΔUL12 + UL98–FLAG) at MOI 2 for 24 h and mtDNA abundance was determined by qPCR. d, The vaginas of wild-type mice (n = 3 per condition) were inoculated with 10^6 plaque-forming units of HSV-1 (UL12–FLAG) or UL12-deficient HSV-1 (ΔUL12 + UL98–FLAG) and 24 h post-infection, vaginal RNA was extracted and gene expression analysed by qRT–PCR. e, Mice (n = 3 per condition) were infected as previously described and 6 days post-infection, DNA from dorsal root ganglia was isolated for mtDNA and HSV-1 genome abundance measurements by qPCR. Error bars indicate ± s.e.m. of triplicates and data are representative of two independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant.
Extended Data Figure 8 | Model illustrating mtDNA stress-dependent antiviral priming. TFAM depletion, induced genetically or during herpesvirus infection, triggers mtDNA stress, characterized by nucleoid loss and enlargement. This results in the release of fragmented mtDNA that recruits and activates peri-mitochondrial cGAS to generate the second messenger cyclic GMP-AMP (cGAMP) and activate endoplasmic-reticulum-resident STING. STING then activates TBK1, which phosphorylates IRF3 to induce dimerization and nuclear translocation. Active IRF3 elevates basal gene expression of ISGs with antiviral signalling and effector functions. Signalling molecules encoded by ISGs, such as IRF7, ISG15, STAT1 and STAT2, cooperate with IRF3 to potentiate the RIG-I-like receptor (RLR), interferon-stimulatory DNA (ISD) and type I interferon (IFN-I) responses, while effector molecules encoded by ISGs, such as IFI44, IFIT1, IFIT3 and OASL2, augment viral resistance. Both outcomes collectively and robustly boost innate antiviral defences to dampen viral replication.
## Extended Data Table 1 | Oligonucleotides used in qPCR

| Gene name       | Forward and reverse oligo. sequences                                      |
|-----------------|--------------------------------------------------------------------------|
| mGapdh          | GACTCTCAACACGCAACTCCCAAC                                                 |
|                 | TCCACCACCCTGGTTCTGTA                                                     |
| mTfam           | AAGGATGATTCGCCTCAAGG                                                    |
|                 | GGCTTTGAGACCTAATCGG                                                     |
| mmt-16S         | GTTACCCTAGGGAATAACAGCGC                                                  |
|                 | GATCCACATCGAGGATGTGAAAC                                                  |
| mmt-ND6         | TTAGCATTAAGGCCCTTCACC                                                   |
|                 | CCACAAGACCCACTAACAAT                                                   |
| mmt-Cytb        | AGTAGAAGAACACGACCTTTGA                                                  |
|                 | CCGCGATAATCAATGTGGA                                                     |
| mmt-Cox1        | GCCGCCGATATAAGCTTCC                                                    |
|                 | GTCATCTGTTTCCTGCCTCC                                                   |
| m1hna4          | CTTTCCTCTATGACTCGTGTTATGAT                                              |
|                 | AATCCAAATACCTCTCCTGCTCCTCC                                              |
| mifo              | CCACTGAGATGCAAGGGA                                                     |
| ml6             | CCGCATGCTGGGAATAGTGT                                                    |
| mlf1             | AACCTGTCATCGAAAGCTG                                                    |
| mlf3             | TCCACGAGCAGAACAA                                                       |
| mlf44            | AAATCCAGGGTAGGAAAGG                                                      |
| mlg15            | AGGCCAATCCCAAAGACCTCA                                                  |
| mlUsp18          | AGTAGGCGATGCAAGAAGGA                                                    |
| mlr7             | TAAACCAACAGACCACTGAG                                                  |
| mCox10           | CAAATGCTGCAGAGGATTTG                                                   |
| mStat1           | CGGCCATGCAACTGCGTATCAAATACCTC                                           |
| mStat2           | ATGCATCTGGTTCCACGATGAGACCTT                                            |
| mMda5            | CCGAGATCTTGGAGATCAGGA                                                  |
| mRig-I           | GAGTACCACCACTTAAGGCAAGAG                                               |
| HSV1 ICP27 RNA   | TTTCTCCAGTGCTACCTGGAAGG                                                |
| HSV1 UL30 RNA    | TCAATCCTGACAGACAGACCTG                                                  |
| VSV G            | GGGCGTTGGGAGGTATAAACAT                                                  |
| VSV M            | TTTCTCCAGTGCTACCTGGAAGG                                                |
| LCMV GP          | TTTCGCCACCATAATAGGTGATT                                               |
| LCMV NP          | TGCTGTGTGTCCGGAAAACACT                                                 |
| MHV68 gDNA ORF40 | TAGGCCACACCTTCCCAAGG                                                   |
| Vaccinia E9L     | ATTTACAGAGCTGCAATGAGATGTC                                               |
| HSV1 gDNA TK     | ATACCGACAGATCGGCTGGAATCCT                                              |
| HSV1 gDNA UL30   | TTATGCGTCATAGCGGCGG                                                   |
| m.milDNA Dloop 1 | CAGAAATGTGGATGTCGGAGCTCG                                                 |
| m.milDNA Dloop 2 | TCGGCGTCGCTCAAGGGAACC                                                  |
| m.milDNA Dloop 3 | CACTCATCTCGGGTTAAGACC                                                  |
| m.milDNA CytB    | GCTTTCTCAGCTCTCCATCTCCATTTACCATTAC                                      |
| m.milDNA 16S     | TGGTGATGGTGGTGGATCCTG                                                   |
| m.milDNA ND4     | AAAGCTCGAGAGGCGG                                                      |
| m.mucDNA Tert    | CTAGTCAGTGTTGCAAAGCCACCCTCCTT                                          |
|                 | GCCAGACGTGTTTCTGGTT                                                     |
Extended Data Table 2 | Dicer substrate siRNAs used

| Gene name            | IDT Duplex name        |
|----------------------|------------------------|
| mTfam                | MMC.RNAI.N009360.12.1  |
| mExog                | MMC.RNAI.N172456.12.1  |
| mSting               | MMC.RNAI.N025261.12.1  |
| mcGas                | MMC.RNAI.N173386.12.1  |
| mTbk1                | MMC.RNAI.N019786.12.1  |
| mItf3                | MMC.RNAI.N018849.12.1  |
| mMfn1                | MMC.RNAI.N024230.12.1  |
| Firefly luciferase (si-Ctrl) | FLuc-S1                 |

All siRNAs were predesigned by Integrated DNA Technologies and transfected at 25 nM final concentration.