Assessing Mitochondrial DNA Release into the Cytosol and Subsequent Activation of Innate Immune-related Pathways in Mammalian Cells

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Mitochondria have emerged as key drivers of mammalian innate immune responses, functioning as signaling hubs to trigger inflammation and orchestrating metabolic switches required for phagocyte activation. Mitochondria also contain damage-associated molecular patterns (DAMPs), molecules that share similarity with pathogen-associated molecular patterns (PAMPs) and can engage innate immune sensors to drive inflammation. The aberrant release of mitochondrial DAMPs during cellular stress and injury is an increasingly recognized trigger of inflammatory responses in human diseases. Mitochondrial DNA (mtDNA) is a particularly potent DAMP that engages multiple innate immune sensors, although mounting evidence suggests that cytosolic mtDNA is primarily detected via the cyclic GMP-AMP synthase–stimulator of interferon genes (cGAS-STING) pathway. cGAS and STING are widely expressed in mammalian cells and serve as key regulators of type I interferon and cytokine expression in both infectious and inflammatory diseases. Despite growing roles for the mtDNA-cGAS-STING axis in human disease, assays to quantify mtDNA release into the cytosol and approaches to link mtDNA to cGAS-STING signaling are not standardized, which increases the possibility for experimental artifacts and misinterpretation of data. Here, we present a series of protocols for assaying the release of mtDNA into the cytosol and subsequent activation of innate immune signaling in mammalian cells. We highlight genetic and pharmacological approaches to induce and inhibit mtDNA release from mitochondria. We also describe immunofluorescence microscopy and cellular fractionation assays to visualize morphological changes in mtDNA and quantify mtDNA accumulation in the cytosol. Finally, we include protocols to examine mtDNA-dependent cGAS-STING activation by RT-qPCR and western blotting. These methods can be performed with standard laboratory equipment and are highly adaptable to a wide range of mammalian cell types. They will permit researchers working across the spectrum of biological and biomedical sciences to accurately and reproducibly measure cytosolic mtDNA release and resulting innate immune responses.

Basic Protocol 1: siRNA-mediated knockdown of TFAM to induce mtDNA instability, cytosolic release, and activation of the cGAS-STING pathway
Alternate Protocol: Pharmacological induction of mtDNA release and cGAS-STING activation using ABT-737 and Q-VD-OPH
Basic Protocol 2: Isolation and quantitation of DNA from cytosolic, mitochondrial, and nuclear fractions
Basic Protocol 3: Pharmacological inhibition of mtDNA replication and release

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INTRODUCTION

The innate immune system is the first line of defense against bacterial and viral infections. Detection of pathogens is carried out by a variety of pattern recognition receptors (PRRs) that sense specific components of invading organisms known as pathogen-associated molecular patterns (PAMPs) (Janeway & Medzhitov, 2002; West, Koblansky, & Ghosh, 2006). In order to discriminate between self and non-self, PRRs recognize distinctive features of invading organisms such as lipopolysaccharides, peptidoglycans, flagellar proteins, or nucleic acids, including double-stranded RNA or DNA with hypomethylated CpG motifs (Brubaker, Bonham, Zanoni, & Kagan, 2015; Janeway & Medzhitov, 2002; West et al., 2006). PRRs are broadly expressed in innate and adaptive immune cells such as macrophages, dendritic cells, and lymphocytes, but are also found in epithelial and stromal cells throughout body tissues. PRRs are broadly categorized into five main classes: Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)–like receptors (NLRs), C-type lectin receptors (CLRs), retinoic acid–inducible gene I (RIG-I)–like receptors (RLRs), and cytosolic DNA sensors (CDS) (Brubaker et al., 2015; Janeway & Medzhitov, 2002; Paludan & Bowie, 2013; West et al., 2006). Upon PAMP recognition, PRRs initiate signaling cascades that lead to the production of pro-inflammatory cytokines, chemokines, type I interferons (IFN-I), and interferon-stimulated genes (ISGs). These responses collectively mitigate the proliferation and survival of the invading microorganism and engage the adaptive immune system to mount robust and durable immunity (Brubaker et al., 2015; Janeway & Medzhitov, 2002; West et al., 2006).

Although PRRs are generally segregated away from host-specific agonists that may activate them, the innate immune system can also detect signatures of tissue injury and cellular damage. For example, TLR9, which binds hypomethylated CpG DNA common to bacterial and viral genomes (Akira & Takeda, 2004; O'Neill, Golenbock, & Bowie, 2013), is localized to endolysosomal compartments and does not ordinally encounter self-DNA from healthy cells (Nakahira, Hisata, & Choi, 2015; O'Neill et al., 2013). However, under conditions of cellular stress, injury, or death, nuclear or mitochondrial self-DNA can be released into the cytoplasm or extracellular space and be detected by TLR9 or other PRRs in phagocytes and stromal cells to trigger pro-inflammatory and IFN-I responses. Although PRR detection of such cellular damage-associated molecular patterns (DAMPs) is important for driving inflammatory responses that spur tissue repair and regeneration, persistent activation of the innate immune system by DAMPs may be a significant contributor to chronic inflammatory diseases (Gong, Liu, Jiang, & Zhou, 2020; Nakahira et al., 2015). Indeed, DAMP sensing and the resulting pro-inflammatory cascades have been linked to numerous inflammatory diseases such as atherosclerosis, lupus, and rheumatoid arthritis.

Likely owing to their evolutionary origin as protobacteria (Roger, Muñoz-Gómez, & Kamikawa, 2017), mitochondria contain many components that act as DAMPs (mt-DAMPs) when leaked or released from the organelle, including N-formylated peptides,

hypomethylated mtDNA, mitochondrial RNA-DNA hybrids, and ATP (Riley & Tait, 2020; West, 2017; West & Shadel, 2017). Mitochondria play important roles in numerous cellular processes including metabolism, calcium homeostasis, signaling, and cell death; under conditions of stress or mitochondrial dysfunction, mitochondria can release mtDAMPs that trigger pro-inflammatory responses. One such mtDAMP is the mitochondrial genome or mtDNA. Although the majority of the ∼1000 proteins found in mitochondria are encoded in the nucleus (Fox, 2012), the mitochondrial genome encodes 13 proteins involved in oxidative phosphorylation as well as 2 ribosomal RNAs and 22 transfer RNAs (Shadel & Clayton, 1997). Under conditions of cellular stress and mitochondrial dysfunction, mtDNA can be released into the cytosol, where it is sensed by the cyclic GMP-AMP synthase–stimulator of interferon genes (cGAS-STING) pathway and other PRRs (Lei et al., 2021; Sun et al., 2017; Torres-Odio et al., 2021; West et al., 2015). cGAS then synthesizes the second messenger cyclic GMP-AMP (cGAMP), which activates STING on endoplasmic reticulum (ER) membranes (Ablasser et al., 2013; Diner et al., 2013). STING then activates TANK-binding kinase 1 (TBK1), which phosphorylates Interferon Regulatory Factor 3 (IRF3), initiating its dimerization and translocation into the nucleus, where it induces the expression of IFN-I and ISGs (Collins et al., 2015; Gao et al., 2013; Schoggins et al., 2014). Cytosolic mtDNA can also be sensed by Absent in Melanoma 2 (AIM2) and NLR family pyrin domain–containing 3 (NLRP3) inflammasomes, as well as TLR9 if trafficked to the endolysosomal compartment (West & Shadel, 2017). Engagement of inflammasomes leads to processing and secretion of pro-inflammatory cytokines interleukin-1β (IL-1β) and interleukin-18, while TLR9 can induce IFN-I and pro-inflammatory cytokines.

Although significant progress has been made in this field, there are many lingering questions that have not been fully addressed. For example, the physiological and pathological contexts of mtDNA release, as well as the molecular mechanisms governing its transit across mitochondrial membranes, are still unclear. Furthermore, how the cytosolic presence of mtDNA contributes to beneficial innate immune signaling versus damaging inflammation remains under investigation. In this context, our intention is to provide a collection of adaptable protocols to guide researchers in diverse fields interested in studying the cytosolic release of mtDNA and its role as a DAMP in infectious and sterile inflammatory diseases. The general workflow involves inducing mtDNA instability to trigger its release into the cytosol and measuring the resulting IFN-I signaling and ISG expression by RT-qPCR and western blotting; measuring cytosolic mtDNA and visualizing morphological changes to mitochondria and mtDNA; and probing the contribution of mtDNA to innate immune responses by inhibiting its release from mitochondria (Fig. 1). Each of these protocols may be used independently or in combination depending on the model system being used. We provide two different methods of inducing mtDNA instability and release from the mitochondria. The first (Basic Protocol 1) is genetic and involves depletion of the mtDNA packaging protein Transcription Factor A, mitochondrial (TFAM), resulting in instability and cytosolic release of fragmented mtDNA. The second (Alternate Protocol) is pharmacological, relying on the inhibitors ABT-737 and Q-VD-OPH to induce release of mtDNA via Bax/Bak pores on the outer mitochondrial membrane. Basic Protocol 2 outlines methods for fractionating cells into cytosolic, mitochondrial, and nuclear compartments and detecting the presence of mtDNA by real-time qPCR. Finally, Basic Protocol 3 provides methods to block mtDNA synthesis and inhibit release into the cytosol, which are useful to evaluate whether mtDNA is involved in triggering innate immune activation.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly. Work should be performed in a biosafety cabinet (NuAire, BSC Class II, Type A2 or equivalent) and sterile filter tips are recommended.

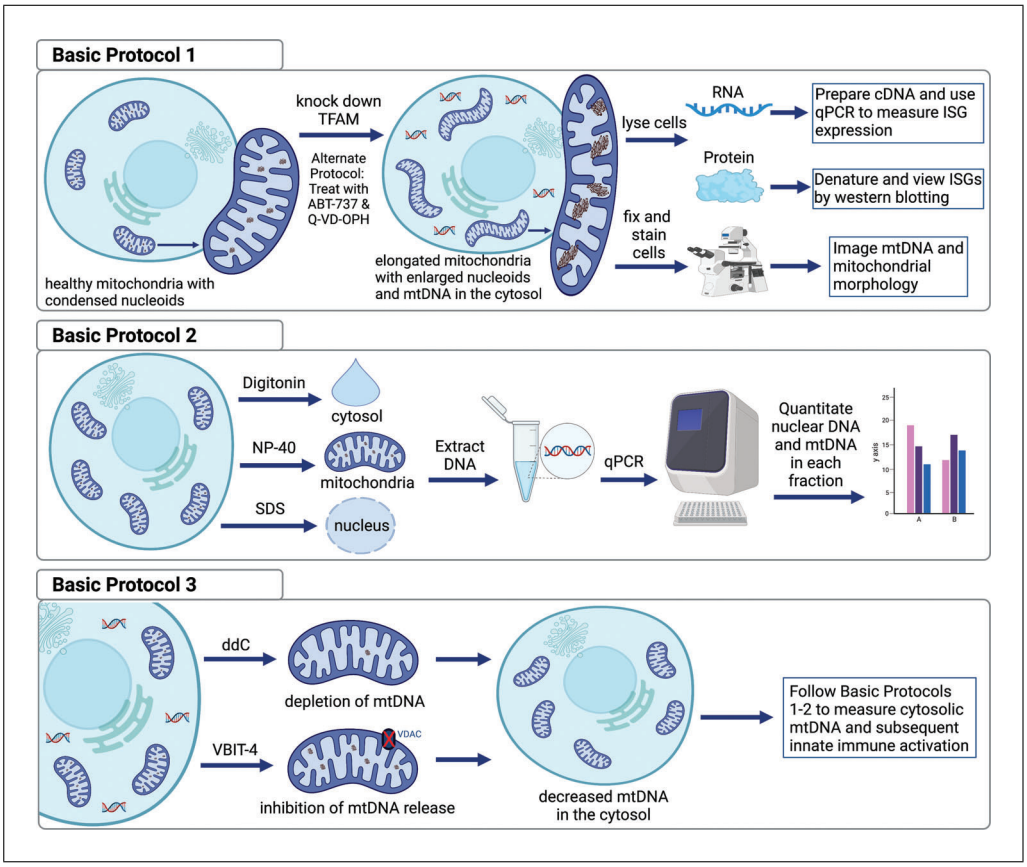


Figure 1 Overview of protocols and experimental workflow. Definitions: cGAS, cyclic GMP-AMP synthase; ISG, interferon-stimulated gene; mtDNA, mitochondrial DNA; STING, stimulator or interferon genes; ABT-737, Bcl-2 inhibitor; Q-VD-OPH, pan-caspase inhibitor; NP-40, Nonidet P-40; SDS, sodium dodecyl sulfate; ddC, 2′,3′-dideoxycytidine; VBIT-4, voltage-dependent anion channel inhibitor.

BASIC PROTOCOL 1

siRNA-MEDIATED KNOCKDOWN OF TFAM TO INDUCE mtDNA INSTABILITY, CYTOSOLIC RELEASE, AND ACTIVATION OF THE cGAS-STING PATHWAY

The following protocol provides a method to induce mtDNA instability in mouse and human fibroblasts through siRNA-mediated knockdown of TFAM. TFAM is a mitochondrial transcription factor and mtDNA packaging protein that is essential for mtDNA replication and fidelity. Reduced expression of TFAM induces morphological alterations to mtDNA nucleoids, the functional packaging unit of mtDNA, while also causing mitochondrial network hyperfusion and liberation of mtDNA from mitochondria into the cytoplasm. Cytosolic mtDNA is detected by cGAS or other DNA sensors, leading to intracellular signaling cascades that result in activation of IFN-I and/or pro-inflammatory cytokine responses (West & Shadel, 2017; West et al., 2015). This protocol can serve as a positive control to induce mtDNA instability and cytosolic release, or to probe additive effects of mtDNA stability and innate immune activation in a cell culture model of interest. We detail RT-qPCR-based methods to assess TFAM knockdown by siRNA as well as RT-qPCR and western blotting approaches to quantitate subsequent cGAS-STING activation and ISG expression. In addition, we describe cell fixation and antibody staining techniques for visualizing alterations in the mitochondrial network and mtDNA nucleoid morphology by immunofluorescence microscopy. These techniques may be used in conjunction with the TFAM knockdown protocols described, or may be used to visualize mitochondria and mtDNA across various conditions and cell types of interest.

Materials

70% (v/v) ethanol (Koptec, V1016) in sterile water
Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich, D8537)
Cells: primary mouse embryonic fibroblasts (MEFs) from day 13.5-15.5 embryos, isolated as described (*Current Protocols*, Xu, 2005) *or* human foreskin fibroblasts (HFF-1, ATCC, SCRC-1041)
DMEM-FBS: Dulbecco's Modified Eagle Medium, high glucose (Sigma-Aldrich, D5796) with 10% fetal bovine serum (Avantor Seradigm, 97068-085, or other extremely low-endotoxin, high-quality option)
Trypsin/EDTA (Sigma-Aldrich, T3924)
Opti-MEM I Reduced-Serum Medium (Thermo Fisher Scientific, 31985062)
20 μM TFAM and control DsiRNAs (see Table 1)
Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific, 13778150)
Quick-RNA Microprep Kit (Zymo Research, R1050)
qScript cDNA Master Mix (Quantabio, 95047-100)
Nuclease-free water (Sigma-Aldrich, W4502)
PerfeCTa SYBR Green SuperMix (Quantabio, 95054-500)
Primers for RT-qPCR (see Table 2)
NP-40 lysis buffer (see recipe)
Micro BCA Protein Assay Kit (Thermo Fisher Scientific, 23235)
SDS polyacrylamide mini gels
Antibodies for western blotting (see Table 3)
4% (v/v) paraformaldehyde diluted in DPBS from 32% stock (Electron Microscopy Sciences, 15714)
Permeabilization buffer: DPBS with 0.1% (v/v) Triton X-100 (Sigma-Aldrich, X100)
Blocking buffer (DPBS with 10% (v/v) FBS)
Primary and secondary antibodies for immunofluorescence (see Table 4)
ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific, P36962)
Clear fingernail polish

High precision no. 1.5H 18-mm round coverslips (Azer Scientific, ES0117580)
Fine-tip forceps (Fisher Scientific, 16-100-113)
12-well cell culture plates (USA Scientific, 5666-5180)
10-cm cell culture dishes (USA Scientific, 5666-4160)
37°C, 5% CO_2 culture incubator
Tissue culture microscope (e.g., Nikon Eclipse Ts2)
Clear fingernail polish
15-ml conical tube (USA Scientific, 5618-8271)
Hemocytometer (e.g., Hausser Scientific Bright-Line)
1.5-ml microcentrifuge tubes (USA Scientific, 1615-5500)
BioTek Epoch plate reader with Take3 microplate (or equivalent absorbance plate reader/spectrophotometer)
96-well PCR plates (USA Scientific, 1402-8120)
E1-ClipTip Electronic Adjustable Tip Spacing Multichannel Equalizer pipette (0.5-12 μl, Thermo Fisher Scientific, 4672010BT)
384-well white PCR plates (USA Scientific, 1438-4790)
Optically clear real-time PCR plate sealing film (USA Scientific, 2921-7800)
Bio-Rad CFX384 Touch Real-time PCR Detection System (or comparable 384-well real-time PCR machine)
1-ml syringes (Becton Dickinson, 309628)
Parafilm (Santa Cruz Biotechnology, sc-200311)

21-G × 1-in. hypodermic needle (Santa Cruz Biotechnology, 4710008025)
25 × 75–mm microscope slides (Globe Scientific, 1384-50W)
Widefield fluorescence or confocal microscope with a 60× or higher objective

Additional reagents and equipment for western blotting (e.g., *Current Protocols*, Gallagher, 2010)

Sterilize coverslips (for immunofluorescence staining only)

This procedure may be performed immediately prior to plating cells or up to a week in advance. If performing in advance, keep the plate covered to ensure that the coverslips remain sterile.

1. In a laminar flow hood, add two or more 18-mm coverslips to separate wells of a 12-well tissue culture plate using sterile forceps.

   At least two coverslips are required: one for the TFAM siRNA (siTFAM) knockdown and one for the scrambled siRNA control (siControl). More will be required if additional time-points and/or biological replicates are needed.

   Forceps may be sterilized by immersing in 70% EtOH for several minutes, then allowed to air dry in the laminar flow hood.

   For ease of handling and to ensure that only one coverslip is placed in each well, spread the coverslips in a sterile 10-cm tissue culture dish before transferring them one-by-one to the 12-well plate using forceps.

2. Soak coverslips in 500 µl of 70% EtOH for 30 min to sterilize.
3. Aspirate off the EtOH and leave the plate partially open in the laminar flow hood until the wells dry completely (~20 min).
4. Wash coverslips with 500 µl DPBS and store (covered) until cells are ready for plating.

Plate cells

5. Seed ~1×10^6 MEFs or HFFs onto a 10-cm dish in 10 ml DMEM-FBS. Incubate at 37°C, 5% CO_2 until confluent (usually 2-4 days).

   Low-passage MEFs (i.e., P1-P4) should be used, as later-passage MEFs undergo replicative senescence and downregulate innate immune pathways.

6. Aspirate medium and wash plate with 5 ml DPBS.
7. Aspirate DPBS and add 1 ml trypsin/EDTA. Incubate at 37°C for 5 min or until cells have detached.

   It may be necessary to tilt the plate back and forth periodically to ensure complete coverage with trypsin/EDTA and efficient detachment of the cells. Be sure to visualize cell detachment under a tissue culture microscope.

8. Quench trypsin/EDTA by adding 3 ml DMEM-FBS, then transfer cells to a 15-ml tube.
9. Centrifuge at 200 × *g* for 5 min, room temperature. Decant medium.
10. Resuspend cells in 5 ml DMEM-FBS and count using a hemocytometer or other preferred method.
11. Dilute to 4×10^4 cells/ml in DMEM-FBS.
12. Plate cells in 12-well dishes, adding 1 ml (4×10^4 cells/well) to at least two wells with coverslips (step 4) and at least four wells in a new 12-well plate. Incubate at 37°C, 5% CO_2 overnight.

Table 1 DsiRNA Sequences for TFAM and Control Knockdown

Target	Sequence
Human TFAM[a]	Sense = 5′-rGrCrArGrArArCrUrCrArUrCrUrArGrGrUrArArArUrUrACA-3′
	Antisense = 5′-rUrGrUrArArUrUrUrArCrCrUrArGrArUrGrArGrUrUrCrUrGrCrCrU-3′
Mouse TFAM[a]	Sense = 5′-rGrCrUrArUrCrCrArArArGrArArArCrCrUrArUrGrArGrUTC-3′
	Antisense = 5′-rGrArArCrUrCrArUrArGrGrUrUrUrCrUrUrUrGrGrArUrArArGrCrUrA-3′
Control siRNA[b]	Sense = rCrGrUrUrArArUrCrGrCrGrUrArUrArCrGrCrGrUAT
	Antisense = rArUrArCrGrCrGrUrArUrUrArUrArCrGrCrGrArUrUrArArCrGrArC

[a] 27-mers synthesized by Integrated DNA Technologies (IDT), 10 nmol, standard desalting. For working solution, diluted to 20 μM in nuclease-free duplex buffer (IDT, 11-05-01-12).
[b] DsiRNA NC1, IDT, 51-01-14-03.

The siTFAM and siControl groups each require a coverslip for staining plus two plate wells for RT-qPCR and western blotting. More will be required if additional timepoints and/or biological replicates are needed. Wells should be set up and labeled accordingly.

Transfect cells with siRNA

13. Prepare transfection mix as follows (volumes are per well of cells):
    a. In separate 1.5-ml microcentrifuge tubes, mix 50 μl Opti-Mem and 1.25 μl of 20 μM control or TFAM siRNA. See Table 1 for DsiRNA sequences and details.
    b. In a second microcentrifuge tube for each siRNA, mix 50 μl Opti-Mem and 3 μl RNAiMAX.
    c. Add each diluted siRNA to a tube of diluted RNAiMax and mix by pipetting several times.
    d. Incubate at room temperature for 10 min.
14. Add 100 μl transfection mix dropwise to the appropriate wells and rock plates back and forth to mix.
15. Incubate plates for 72 hr.

    This is the optimal timepoint for knocking down TFAM in MEFs and HFFs. For other cell lines, additional timepoints ranging from 24 to 96 hr may be tested.

16. Proceed to RNA extraction, protein extraction, and preparation of cells for immunofluorescence.

Extract RNA and perform RT-qPCR analysis

17. Remove plate from the incubator, aspirate medium from the wells labeled for RNA extraction, and wash cells twice with 1 ml DPBS.
18. Aspirate DPBS and add 300 μl RNA lysis buffer from the Quick-RNA Microprep kit, pipetting up and down to mix.
19. Transfer lysate to a 1.5-ml tube and continue with RNA isolation according to manufacturer's instructions.

    RNA may be stored in RNA lysis buffer at –80°C if necessary. We have stored RNA in this buffer for more than a month without any noticeable loss in RNA quality.

20. Determine the 260/280 nm absorbance ratio (e.g., using an Epoch plate reader equipped with a Take3 microplate) to determine RNA purity and concentration.

    A ratio >1.9 is considered acceptable.

21. Use 100-1000 ng total RNA to generate cDNA using the qScript cDNA Mastermix kit according to manufacturer's instructions.

Table 2 Optimized Primer Sequences for Assessing ISG Expression in Human and Mouse Cells by RT-qPCR

Name	Species	RefSeq ID	Use	Forward (5′-3′)	Reverse (5′-3′)
Cmpk2	Mouse	NM_020557.4	ISG	AAAGAATCAACCAACTTT	GGCCTCCACTCACCTCAGTA
Ifi44	Mouse	NM_133871.3	ISG	CTGATTACAAAAGAAGAC ATGACAGAC	AGGCAAAACCAAAGACTCCA
Ifit1	Mouse	NM_008331.3	ISG	CAAGGCAGGTTTCTGAGG AG	GACCTGGTCACCATCAGCAT
Ifit3	Mouse	NM_010501.2	ISG	TTCCCAGCAGCACAGAA AC	AAATTCCAGGTGAAATGGCA
Rsad2	Mouse	NM_021384.4	ISG	ATAGTGAGCAATGGCAGG CCT	AACCTGCTGATGCAAGCTGT
Zbp1	Mouse	NM_021394.2	ISG	TCAAAGGGTGAAGTCAT GGA	GTGGAGTGGCTTCAGAGCTT
Gapdh	Mouse	NM_008084.3	Control	GACTTCAACAGCAACTCC CAC	TCCACCACCCTGTTGCTGTA
Rpl37	Mouse	NM_026069.3	Control	CATCCTTTGGTAAGCGTC GCA	TGGCACTCCAGTTATACTTCCT
CMPK2	Human	NM_207315.4	ISG	ACCCAGTCAGTGGCAGAT TC	TGAGCAGCAGGATAAGGTCA
IFIT44l	Human	NM_006820.4	ISG	CAATTTAAGCCTGATCTA ACCCC	CAGTTGCGCAGATGATTTTC
IFIT1	Human	NM_001548.5	ISG	GCAGCCAAGTTTTACCGA AG	GCCCTATCTGGTGATGCAGT
IFIT3	Human	NM_001549.6	ISG	TGGGAACAGCAGAGACA CAG	AAGTTCCAGGTGAAATGGCA
RSAD2	Human	NM_080657.5	ISG	GCCAAAACATCCTTTGTG CT	TGGCTCTCCACCTGAAAAGT
ZBP1	Human	NM_030776.3	ISG	TGGACACGGGAACATCAT TA	GAATCACCTGGTGCCATTG
GAPDH	Human	NM_002046.7	Control	AGCCACATCGCTCAGAC AC	GCCCAATACGACCAAATCC
RPL37	Human	NM_000997.5	Control	AGTGCCTTCTCTTCCGGT CT	TTCCAAACGATGACGTTCC

22. Dilute cDNA 1:10 in nuclease-free water and add an appropriate amount to wells of a clean 96-well plate.

    When analyzing two genes in technical triplicate for a given sample, the total amount of 1:10 diluted cDNA per sample needed is 13.5 μl (2.25 μl × 2 × 3). It is useful to prepare 5%-10% extra to account for pipetting errors.

23. Prepare PerfeCTa SYBR Green and primer master mixes for each ISG or reference control primer set in 1.5-ml microcentrifuge tubes. See Table 2 for ISG and reference primer sequences.

    For each PCR reaction, use 2.5 μl SYBR mix plus 0.125 μl each of 10 μM forward and reverse primers, for a total volume of 2.75 μl. The total volume of master mix needed is 2.75 × # samples × # replicates. Prepare accordingly and pipette into a 96-well plate. Prepare 5%-10% extra to account for pipetting errors.

24. Using a E1-ClipTip Electronic Adjustable Tip Spacing Multichannel Equalizer pipette, add 2.25 μl diluted cDNA to each well of a 384-well plate.

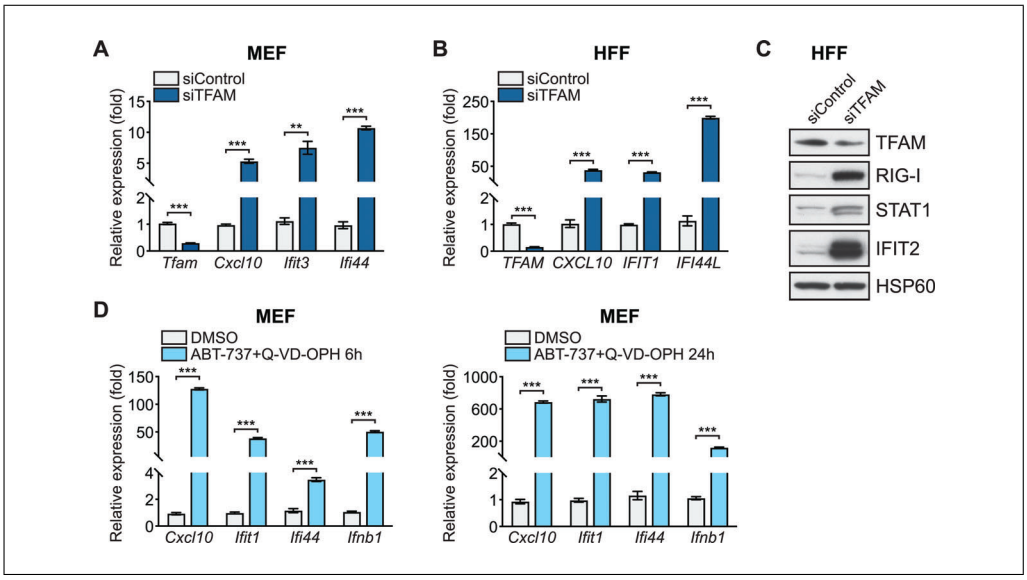


Figure 2 Triggering mtDNA release into the cytosol induces IFN-I and ISG expression in mouse and human fibroblasts. (**A,B**) MEFs or HFFs were transfected with control or TFAM siRNAs for 72 hr. RNA was extracted and reverse-transcribed into cDNA, which was subjected to SYBR Green–based qPCR analysis to profile *TFAM* and ISG (e.g., *Cxcl10, Ifit1, Ifit3, Ifi44*) expression. (**C**) HFFs were transfected with siRNAs for 72 hr. Proteins were extracted and subjected to western blotting using antibodies against TFAM, heat shock protein 60 (HSP60), and ISGs (RIG-I, STAT1, IFIT2). (**D**) MEFs were treated with 10 μM ABT-737 (Bcl-2 inhibitor) and 10 μM Q-VD-OPH (pan-caspase inhibitor) for 6 (left) or 24 (right) hr. RNA was extracted and reverse-transcribed into cDNA, which was subjected to SYBR Green–based qPCR analysis to profile ISG (e.g., *Cxcl10, Ifit1, Ifi44, Ifnb1*) expression. Data in **A**, **B**, and **D** are mean fold change ± SEM ($n = 3$); unpaired Student's *t* test after Shapiro-Wilk normality test, $^{**}p < 0.01$, $^{***}p < 0.001$.

To add the DNA, gently touch the pipette tip to one side of the well. Take note of which side of the well the tip touches, and then add the SYBR master mix to the well using the opposite side.

25. Centrifuge for 1 min at 2000 × *g*, room temperature.
26. Add 2.75 μl SYBR Green and primer master mix to each well in the 384-well plate.
27. Centrifuge for 1 min at 2000 × *g*, room temperature.
28. Seal all edges of the plate tightly with optical film. Tap plate gently to mix the cDNA, SYBR Green, and primers.
29. Centrifuge for 1 min at 2000 × *g*, room temperature.
30. Run a program suggested for PerfeCTa SYBR Green FastMix using a CFX384 Real-Time PCR Detection System.

    Example program:

    Step 1: 95°C for 30 s
    Step 2: 95°C for 5 s, then 60°C for 30 s, repeated 40 times
    Step 3: 65°C for 5 s
    Step 4: 60° to 95°C at increments of 0.5°C for 5 s
31. Calculate ISG abundance relative to a reference gene using the ΔΔCq method. Set ISG transcript abundance in siControl samples to 1 and calculate ISG fold changes in siTFAM samples relative to siControl samples.

Figure 2A,B shows representative TFAM and ISG expression in MEFs and HFFs after RT-qPCR. See Understanding Results for a detailed description.

Table 3 Suggested Primary Antibodies to Assess cGAS-STING Pathway Activation, ISG Expression, and Cellular Fraction Purity by Western Blotting

Name	Use	Company	Catalog number	Dilution	Species reactivity
cGAS	ISG	Cell Signaling Technology	31659	1:1000	Mouse
cGAS	ISG	Cell Signaling Technology	15102	1:1000	Human
GAPDH	Cytosolic loading control	Proteintech	60004-1-Ig	1:1000	Human, mouse
IFIT2	ISG	Proteintech	12604-1	1:1000	Human, mouse
IFITM1	ISG	Proteintech	60074-1-Ig	1:5000	Human
IRGM1	ISG	Cell Signaling Technology	14979	1:1000	Mouse
ISG15	ISG	Cell Signaling Technology	2743	1:1000	Human, mouse
LAMIN B1	Nuclear loading control	Proteintech	66095-1-Ig	1:1000	Human, mouse
RIG-I	ISG	Cell Signaling Technology	4200	1:1000	Human, mouse
STAT1	ISG	Cell Signaling Technology	9172	1:1000	Human, mouse
STAT2	ISG	Cell Signaling Technology	72604	1:1000	Human, mouse
TFAM	Mitochondrial loading control	Proteintech	22586-1-AP	1:2000	Human
TFAM	Mitochondrial loading control	Millipore	ABE483	1:1000	Mouse
ZBP1	ISG	Adipogen	AG-20B-0010	1:1000	Mouse
β-Actin	Cytosolic loading control	Proteintech	66009-1-Ig	1:5000	Human, mouse
Calnexin	ER loading control	Proteintech	10427-2-AP	1:1000	Human, mouse
HSP60	Mitochondrial loading control	Santa Cruz Biotechnology	sc-1052	1:5000	Human, mouse
VDAC1	Mitochondrial loading control	Proteintech	55259-1-AP	1:2000	Human, mouse

Extract protein and perform western blotting

32. Take plate of cells (step 15), aspirate medium from wells labeled for protein extraction, and wash cells twice with 1 ml DPBS.
33. Add 50 µl ice-cold NP-40 lysis buffer to each well and scrape the wells using the rubber plunger from a 1-ml syringe to lyse the cells.
34. Place two 1.5-ml microcentrifuge tubes on ice and then transfer lysate to the chilled tubes.
35. Centrifuge for 10 min at 21,000 × *g*, 4°C, to pellet cellular debris.
36. Transfer supernatant to a new 1.5-ml tube and keep on ice.

    The pellet may be discarded.

37. Quantify protein concentration using the Micro BCA Protein Assay kit per manufacturer's instructions.
38. Run ∼20-30 µg denatured and reduced protein extract on SDS polyacrylamide mini gels and perform western blotting using a preferred method (e.g., Gallagher, 2010). See Table 3 for validated antibodies.

Table 4 Suggested Primary and Secondary Antibodies for Immunofluorescence Staining of Mitochondria and mtDNA

Primary antibodies					
	Host species	Species reactivity	Dilution	Supplier	Catalog number
Anti-DNA	Mouse	Human, mouse, rat	1:300	Millipore	CBL186
Anti-HSP60	Goat	Human, mouse, rat	1:300	Santa Cruz Biotechnology	sc-1052
Anti-TFAM	Rabbit	Mouse	1:800	Millipore	ABE483
Anti-TFAM	Rabbit	Human	1:800	Proteintech	22586-1-AP
Secondary antibodies					
	Host species	Conjugate	Dilution	Supplier	Catalog number
Anti-mouse IgM (μ chain specific)	Donkey	Rhodamine Red-X	1:600	Jackson Immuno Research Labs	715-295-020
Anti-goat IgG (heavy + light chains)	Bovine	Alexa 647	1:600	Jackson Immuno Research Labs	805-605-180
Anti-rabbit IgG (heavy + light chains)	Donkey	Alexa 488	1:800	Jackson Immuno Research Labs	711-545-152

Figure 2C shows representative TFAM and ISG protein expression in HFFs after western blotting. See Understanding Results for a detailed description.

Fix and stain cells

39. Take plate of coverslips (step 15), aspirate medium, and wash coverslips twice with 1 ml DPBS.
40. Fix cells with 600 μl of 4% PFA for 20 min at room temperature.
41. Wash twice for 5 min each with 1 ml DPBS.
42. Store in DPBS at 4°C until ready to stain.

    For best quality, analyze stored cells within 3 days.
43. Wash coverslips with 1 ml DPBS.
44. Permeabilize cells with 1 ml permeabilization buffer for 5 min at room temperature.
45. Aspirate buffer and wash wells twice for 5 min each with 1 ml DPBS.
46. Add 1 ml blocking buffer and leave 30 min at room temperature.
47. Dilute primary antibodies in blocking buffer to give 80 μl per coverslip. See Table 4 for suggested antibodies and dilutions.
48. Spray a benchtop (or other flat surface) with 70% EtOH and then place a sheet of Parafilm on top to adhere. Ensure that the Parafilm is flat and use a marker to divide it into sections as needed.

    Use a large enough sheet of Parafilm so that all coverslips fit with around a half inch of space on all sides. This will ensure that the antibody droplets do not run into each other on the Parafilm.
49. Bend the tip of a 21-G, 1-in. hypodermic needle to form a small (∼1 mm) hook by pressing the bevel gently against a hard surface (Fig. 3B).
50. Spot one 60-μl droplet of primary antibody per coverslip onto the Parafilm, being sure that there is enough space between droplets to prevent coverslips from touching each other.

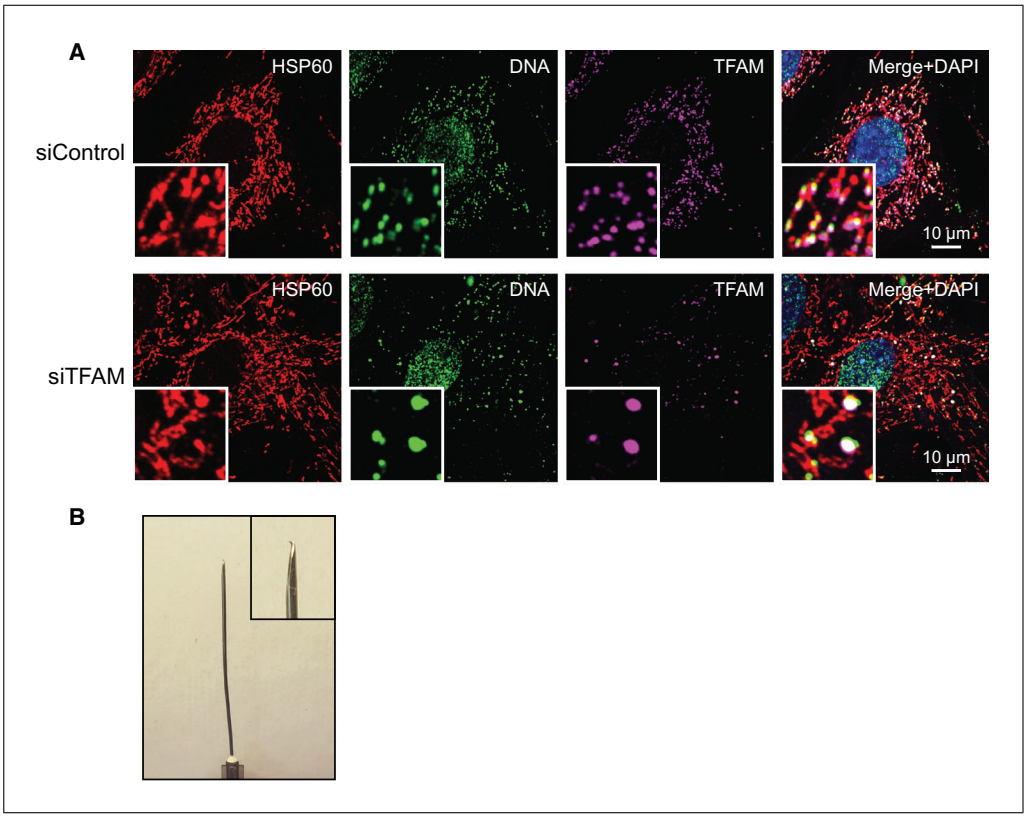


Figure 3 Knockdown of TFAM results in enlargement of mtDNA nucleoids and elongation of the mitochondrial network in mouse fibroblasts. (**A**) MEFs were mounted on coverslips and transfected with control or TFAM siRNAs for 72 hr. Cells were fixed and stained with primary antibodies against DNA, TFAM, and heat shock protein 60 (HSP60). After counterstaining with fluorescently conjugated secondary antibodies, coverslips were mounted on slides and imaged on a confocal microscope equipped with a 60× oil-immersion objective. Insets represent 3× digital zoom. (**B**) Example of a bent 21-G needle tip for picking up coverslips.

51. Remove a coverslip from the plate by raising one side carefully with the hooked needle and then lifting the coverslip with fine-tip forceps. Remove excess liquid by gently tapping the edge of the coverslip on a Kimwipe.
52. Place the coverslip cell-side down on an antibody dilution droplet using the hooked needle and forceps.
53. Repeat until all coverslips have been placed on a drop of antibody solution. Cover to protect from light and incubate for 1 hr at room temperature.
54. Return coverslips cell-side up to the plate.
55. Wash twice with 1 ml blocking buffer for 5 min each.
56. Repeat steps 50-58 with secondary antibody diluted in blocking buffer. See Table 4 for suggested antibodies and dilutions.
57. Wash twice with 1 ml DPBS for 5 min at room temperature.
58. Carefully apply a drop (~100 μl) of ProLong Antifade Mountant with DAPI to a microscope slide for each coverslip.

    Two 18-mm round coverslips can fit on one slide.

59. Lift each coverslip out of its well, remove DPBS by gently touching the edge to a Kimwipe, and place cell-side down on a drop of mountant.

To avoid creating bubbles, lower the coverslip slowly using the forceps and hooked needle.

60. When all coverslips have been mounted, allow to dry overnight in the dark.

61. Seal coverslip by painting the edges with a generous amount of clear fingernail polish.

    Mounted slides can be stored at 4°C in the dark for several months.

62. Image slides on a widefield fluorescence or confocal microscope with a 60× or higher objective.

    Figure 3A shows MEFs stained using primary antibodies against DNA, TFAM, and the mitochondrial matrix chaperone HSP60. See Understanding Results for a detailed description.

PHARMACOLOGICAL INDUCTION OF mtDNA RELEASE AND cGAS-STING ACTIVATION USING ABT-737 AND Q-VD-OPH

ALTERNATE PROTOCOL

This protocol provides an alternative to Basic Protocol 1 for inducing mtDNA release into the cytosol. It relies on co-administration of two different inhibitors. The first, ABT-737, is a pan-Bcl-2 inhibitor which triggers herniation of the inner mitochondrial membrane and Bax/Bak-dependent mitochondrial outer membrane permeabilization (MOMP) (McArthur et al., 2018). Increased MOM permeability mediates the release of cytochrome c as well as mtDNA. When released from the intermembrane space, cytochrome c can trigger a caspase cascade and lead to apoptosis, which is generally immunologically silent. When cells are co-exposed to the pan-caspase inhibitor Q-VD-OPH, apoptosis is inhibited and cytosolic mtDNA triggers robust IFN-I responses via cGAS-STING signaling (Rongvaux et al., 2014). Similar to the TFAM knockdown approach, cells treated with ABT-737 and Q-VD-OPH will exhibit significant mtDNA accumulation in the cytosol, which can be measured using the steps described in Basic Protocol 2. Moreover, cells treated with these inhibitors will display a significant upregulation of ISG expression at both the RNA and protein levels, which can be measured by the RT-qPCR and western blotting techniques described in Basic Protocol 1, respectively. In contrast to Basic Protocol 1, however, cells treated with these inhibitors will display mitochondrial network fragmentation, as opposed to network hyperfusion, as observed in TFAM depleted cells. Thus, this method induces the rapid liberation of mtDNA into the cytosol via a different route, which may be preferable in cases where TFAM knockdown by siRNA transfection is not possible or more acute timepoints are desired.

Additional Materials *(also see Basic Protocol 1)*

Dimethyl sulfoxide (DMSO, Corning, 25-950-CQC)
10 mM ABT-737 (EMD Millipore, 197333) in DMSO, 0.2-μm filter sterilized
10 mM Q-VD-OPH (EMD Millipore, 551476) in DMSO, 0.2-μm filter sterilized
0.2-μm PES syringe filters (Corning, 431229)
10-ml syringe (BD Biosciences, 302995)

1. Grow and isolate cells as described (see Basic Protocol 1, steps 5-10).

2. Dilute cells to 6×10^4 cells/ml in DMEM-FBS.

3. Plate cells in 12-well dishes, adding 1 ml (6×10^4 cells/well) to at least four wells. Incubate at 37°C, 5% CO_2 overnight.

   At least two wells are required for both the treatment and DMSO control groups: one for RT-qPCR and one for western blotting. If immunofluorescence staining is desired, two sterile 18-mm coverslips in a culture plate will also be needed (see Basic Protocol 1, steps 1-4).

4. Add 1 μl each of 10 mM ABT-737 and 10 mM Q-VD-OPH to the treatment wells (final concentration 10 μM each).

   The indicated concentrations have been tested in primary MEFs, B16F10 murine melanoma cells, NOR10 murine fibroblasts, and LMTK-murine fibroblasts. Drug concentration and cell plating density may need to be optimized for other cell lines.

5. Add an equal volume (2 μl) of DMSO to the control wells.

6. Incubate 6-24 hr at 37°C, 5% CO_2.

7. Proceed with analysis by RT-qPCR, western blotting, and/or immunofluorescence (see Basic Protocol 1).

   Figure 2D shows representative ISG expression in MEFs and HFFs by RT-qPCR after treatment with ABT-737 and Q-VD-OPH. See Understanding Results for a detailed description.

BASIC PROTOCOL 2

ISOLATION AND QUANTITATION OF DNA FROM CYTOSOLIC, MITOCHONDRIAL, AND NUCLEAR FRACTIONS

In order to understand the role of mtDNA as a DAMP in disease endpoints, and to characterize the molecular mechanisms governing cytosolic release and sensing by the innate immune system, standardized methods are needed. Efforts to visualize mtDNA release using fluorescence in-situ hybridization or other microscopy-based methods are limited by the low abundance and fragmented nature of cytosolic mtDNA. Moreover, the ability to measure free mtDNA in the cytosol is often confounded by the unintentional lysis of mitochondria during mechanical or detergent-based cellular fractionation. This protocol utilizes three detergent-containing buffers and differential centrifugation to gently, yet cleanly, extract DNA from cytosolic, mitochondrial, and nuclear pools. Fractions are then immunoblotted to assess purity, and DNA is precipitated and quantified by a sensitive SYBR Green-based qPCR approach. This protocol includes validated human and mouse primer sets, thus allowing the relative quantitation of nuclear and mitochondrial DNA across many primary cells and cell lines from various genetic, biological, and pathophysiological contexts.

Materials

Cells: primary mouse embryonic fibroblasts (MEFs) from day 13.5-15.5 embryos, isolated as described (*Current Protocols*, Xu, 2005) *or* human foreskin fibroblasts (HFF-1, ATCC, SCRC-1041)
DMEM-FBS: Dulbecco's Modified Eagle Medium, high glucose (Sigma-Aldrich, D5796) with 10% fetal bovine serum (Avantor Seradigm, 97068-085, or other extremely low-endotoxin, high-quality option)
Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich, D8537)
Trypsin/EDTA (Sigma-Aldrich, T3924)
SDS lysis buffer (see recipe)
Digitonin lysis buffer (see recipe)
NP-40 lysis buffer (see recipe)
Nuclease-free water for molecular biology (Sigma-Aldrich, W4502)
5 mg/ml RNase A (Worthington, LS005650) in nuclease-free water
20 mg/ml protease K (Worthington, LS004240) in nuclease-free water
Phenol/chloroform/isoamyl alcohol (Acros Organics, 327155000)
Chloroform/isoamyl alcohol (Acros Organics, 327111000)
7.5 M ammonium acetate (NH_4OAc, Sigma-Aldrich, A2706)
Glycogen (Thermo Scientific, R0561)
100% and 95% (v/v) ethanol (Koptec, V1016 and V1101)
Micro BCA Protein Assay Kit (Thermo Fisher Scientific, 23235)

SDS polyacrylamide mini gels
Antibodies for western blotting (see Table 3)
PerfeCTa SYBR Green SuperMix (Quantabio, 95054-500)
Primers for qPCR (see Table 5)

37°C, 5% CO_2 culture incubator
15-cm cell culture dishes (USA Scientific, 5663-9160)
Tissue culture microscope (Nikon Eclipse Ts2 or equivalent)
50-ml conical tube (USA Scientific, 5622-7261)
1.5-ml microcentrifuge tubes (USA Scientific, 1615-5500)
95°C Thermo Scientific Drybath or equivalent
End-over-end tube rotator (e.g., Thermo Scientific, 88881001)
Sonication system (Diagenode, UCD-200)
BioTek Epoch plate reader with Take3 microplate (or equivalent absorbance plate reader/spectrophotometer)
E1-ClipTip Electronic Adjustable Tip Spacing Multichannel Equalizer pipette (0.5-12 µl, Thermo Fisher Scientific, 4672010BT)
384-well white PCR plates (USA Scientific, 1438-4790)
Optically clear real-time PCR plate sealing film (USA Scientific, 2921-7800)
Bio-Rad CFX384 Touch Real-time PCR Detection System (or comparable 384-well real-time PCR machine)

Additional reagents and equipment for western blotting (e.g., *Current Protocols*, Gallagher, 2010)

Prepare cells

1. Seed ~3 × 10^6 primary MEFs or HFFs onto each of two 15-cm dishes in 20 ml DMEM-FBS. Incubate at 37°C, 5% CO_2 for 2 days.

   Low-passage MEFs (i.e., P1-P4) should be used, as later passage MEFs undergo replicative senescence and downregulate innate immune pathways.

2. Aspirate medium and wash plates with 10 ml DPBS.

3. Aspirate DPBS and add 5 ml trypsin/EDTA per plate. Incubate at 37°C for 5 min or until cells have detached.

   It may be necessary to tilt the plate back and forth periodically to ensure complete coverage with trypsin/EDTA and efficient detachment of the cells. Be sure to visualize cell detachment under a tissue culture microscope.

4. Quench trypsin/EDTA by adding 9 ml DMEM-FBS, then pool the cell suspensions into a single 50-ml tube.

5. Centrifuge at 200 × *g* for 5 min, room temperature. Decant medium.

Fractionate cells

6. Resuspend pellet in 1 ml DPBS by pipetting and then divide into two 1.5-ml tubes labeled A and B. See Figure 4 for workflow diagram.

   Tube A will be used for a whole-cell extract (WCE) to serve as a normalization control for the subcellular fractions isolated from tube B.

7. Centrifuge tubes at 200 × *g* for 5 min, room temperature. Aspirate off the supernatant.

8. Resuspend the pellet in tube A with 500 µl SDS lysis buffer and heat to 95°C for 15 min to fully lyse the cells.

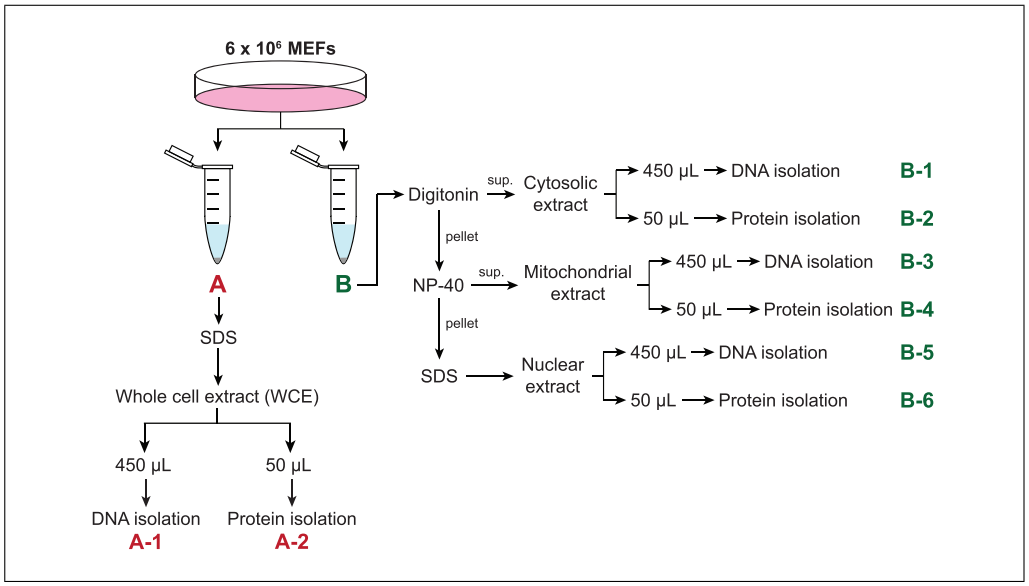


Figure 4 Overview of experimental workflow for cellular fractionation and analysis.

9. Transfer 450 µl lysate to a new 1.5-ml tube labeled as fraction A-1. Keep at room temperature until cell fractionation is completed.
10. Transfer the remaining 50 µl lysate to a new 1.5-ml tube labeled as fraction A-2.
11. Heat tube A-2 for 5 min at 95°C to denature proteins and inactivate cellular proteases.
12. Sonicate tube A-2 in a water bath sonicator for 5 min at room temperature and high intensity (alternating 30 s on/30 s off).
13. Centrifuge for 5 min at max speed (~21,000 × *g*), room temperature, and save the supernatant in a new 1.5-ml tube labeled B-2. Keep tube at room temperature until DNA extraction is completed.
14. Resuspend the pellet in tube B with 500 µl digitonin lysis buffer by pipetting gently up and down.

    The digitonin-based lysis buffer used here gently lyses plasma membranes without disrupting mitochondrial membranes or the nuclear envelope. For MEFs, a suitable concentration of digitonin is 18 µg/ml. This may need to be adjusted for other cell lines and treatment conditions. A good starting range is 10-30 µg/ml digitonin.

15. Incubate on an end-over-end tube rotator for 10 min at 4°C to allow selective plasma membrane permeabilization.
16. Centrifuge at 950 × *g* for 5 min, 4°C.
17. Gently transfer the supernatant to a new 1.5-ml tube, being careful not to disturb the pellet.
18. Centrifuge at 17,000 × *g* for 5 min, 4°C, to pellet any remaining cellular debris.

    The resulting supernatant should be a pure cytosolic fraction, free of organelle contamination.

19. Transfer supernatant to a new 1.5-ml tube and save as the cytosolic extract (fraction B-1). Keep on ice until cell fractionation is completed.
20. Transfer 50 µl of B-1 to a second 1.5-ml tube for western blotting (fraction B-2). Keep on ice until DNA extraction is completed.

21. Wash the pellet from step 18 three times with 1 ml ice-cold DPBS. Centrifuge each time at 950 × *g* for 3 min, 4°C.
22. Resuspend pellet in 500 μl NP-40 lysis buffer and incubate on ice for 10 min.

    NP-40 is a nonionic detergent that will break open the mitochondria and release mtDNA while leaving the nucleus intact. Other organelles, including ER and peroxisomes, will also be lysed.

23. Centrifuge at max speed (~21,000 × *g*) for 10 min, 4°C.
24. Gently transfer the supernatant to a new 1.5-ml tube without disturbing the pellet, and save as the mitochondrial extract (fraction B-3). Keep on ice until cell fractionation is completed.
25. Transfer 50 μl of B-3 to a second 1.5-ml tube for western blotting (fraction B-4). Keep on ice until DNA extraction is completed.
26. Wash the nuclear pellet from step 23 three times with 1 ml ice-cold DPBS. Centrifuge each time at max speed for 3 min, 4°C.
27. Resuspend pellet in 500 μl SDS lysis buffer and boil at 95°C for 15 min to lyse the nucleus.

    This lysate is the nuclear extract (fraction B-5) that will be used in step 28 for DNA isolation.

    SDS is an ionic detergent that can disrupt the nuclear envelope to extract nuclear DNA.

28. Transfer 50 μl of B-5 to a separate 1.5-ml tube for western blotting (fraction B-6).
29. Sonicate tube B-6 in a water bath sonicator for 5 min at room temperature and high intensity (alternating 30 s on/30 s off).
30. Centrifuge for 5 min at max speed (~21,000 × *g*), room temperature, and save the supernatant in a new 1.5-ml tube labeled B-6. Keep at room temperature until DNA extraction is completed.

Extract DNA

31. Beginning with 400 μl, treat fractions A-1, B-1, B-3, and B-5 with 4 μl of 5 mg/ml RNase A for 1.5 hr at 37°C.
32. Add 4 μl of 20 mg/ml proteinase K to each sample and incubate at 55°C for 1 hr.
33. Add 400 μl phenol/chloroform/isoamyl alcohol and vortex vigorously for 1 min.
34. Centrifuge at max speed (~21,000 × *g*) for 5 min, room temperature.
35. Transfer 320 μl of the top aqueous phase to a new tube, being careful to avoid any of the lower organic phase.
36. Add an equal volume of chloroform/isoamyl alcohol to the aqueous phase and vortex vigorously for 1 min.
37. Centrifuge at max speed for 5 min, room temperature.
38. Remove as much of the top aqueous phase as possible without aspirating any of the lower phase, and transfer to a new tube.
39. Add 1/10th volume of 7.5 M NH_4OAc to give a final concentration of 0.75 M.
40. Add 1 μl of glycogen (20 μg) and mix well.

    Glycogen is insoluble in ethanol and can trap nucleic acids, which increases the efficiency of the DNA precipitation.

Table 5 Optimized Primer Sequences to Assess mtDNA Abundance in Mouse and Human Cells by qPCR

Name	Species	Gene ID	Use	Forward sequence 5′-3′	Reverse sequence 5′-3′
TERT	Mouse	21752	Nuclear DNA control	CTAGCTCATGTGT CAAGACCCTCTT	GCCAGCACGTTTCT CTCGTT
MT-D-Loop	Mouse	mtDNA control region, non-coding	mtDNA	AATCTACCATCCT CCGTGAAACC	TCAGTTTAGCTACC CCCAAGTTTAA
MT-CYTB	Mouse	17711	mtDNA	GCTTTCCACTTCA TCTTACCATTTA	TGTTGGGTTGTTTG ATCCTG
MT-RNR2	Mouse	17725	mtDNA	CTAGAAACCCCG AAACCAAA	CCAGCTATCACCAA GCTCGT
KCNJ10	Human	3766	Nuclear DNA control	GCGCAAAAGCCT CCTCATT	CCTTCCTTGGTTTG GTGGG
MT-ND1	Human	4535	mtDNA	GAACTAGTCTCAG GCTTCAACATCG	CTAGGAAGATTGTA GTGGTGAGGGTG
MT-D-Loop	Human	mtDNA control region, non-coding	mtDNA	CATAAAGCCTAAA TAGCCCACACG	CCGTGAGTGGTTAA TAGGGTGATA

41. Add 2.5× volumes of 100% ethanol and mix well.
42. Incubate at –80°C for 1 hr (or –20°C overnight) to precipitate DNA.
43. Centrifuge 20 min at max speed, 4°C, to pellet DNA precipitate.
44. Decant supernatant carefully without disturbing the pellet.
45. Wash pellet with 300 µl of 95% EtOH and vortex by pulsing three times.
46. Centrifuge 15 min at max speed, 4°C.
47. Decant supernatant carefully without disturbing the pellet.
48. Repeat steps 45-47 for a second 95% EtOH wash.
49. After decanting the last wash, spin briefly to draw residual EtOH to the bottom.
50. Remove residual EtOH with a P20 pipetter, being careful not to disturb the pellet.
51. Air dry for 2-5 min.
52. Resuspend in 20-100 µl nuclease-free water.

    Tris-EDTA buffer may also be used, depending on the downstream analysis.

53. Measure DNA concentration with a Biotek Epoch plate reader and Take3 plate or appropriate spectrophotometer. Store at –80°C until the purity of fractions is verified.

    From 3 × 10^6 MEFs, one can expect 1-2 µg cytosolic DNA, 13-15 µg mitochondrial DNA, and 30-35 µg nuclear DNA. The 260/280 absorbance ratio should be between 1.8 and 1.9.

Determine fraction purity by immunoblotting

54. Quantify protein concentration from fractions A-2, B-2, B-4, and B-6 using a Micro BCA Protein Assay kit per manufacturer's instructions.
55. Run ∼10-30 µg denatured and reduced protein extract on SDS polyacrylamide mini gels and perform western blotting using a preferred method. Antibodies against

A

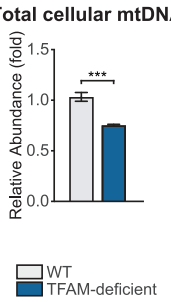
	Whole-Cell Extract Cq values		Cytosolic Extract Cq values
	TERT	D-Loop	D-Loop
WT	21.67	14.10	25.65
	21.93	14.20	25.30
	21.84	14.30	24.96
TFAM-deficient	21.82	14.69	23.69
	21.84	14.69	24.03
	21.76	14.56	23.81

B

Whole-Cell Extracts

	TERT	D-Loop	ΔCq	ΔΔCq	Rel. abun. mtDNA (fold)
WT	21.67	14.10	**–7.57**	0.00	1.00
	21.93	14.20	–7.73	–0.16	1.12
	21.84	14.30	–7.54	0.03	0.98
TFAM-deficient	21.82	14.69	–7.13	0.44	0.74
	21.84	14.69	–7.15	0.42	0.75
	21.76	14.56	–7.20	0.37	0.77

ΔCq = Cq (D–Loop) – Cq (TERT)
ΔΔCq = ΔCq – **Median ΔCq WT**
Relative abundance of WCE mtDNA = 2^(–ΔΔCq)



C

Cytosolic Extracts

	Median ΔCq WCE	D-Loop	ΔCq	ΔΔCq	Rel. abun. mtDNA (fold)
WT	–7.57	25.65	33.22	0.19	0.88
	–7.73	25.30	**33.03**	0.00	1.00
	–7.54	24.96	32.49	–0.54	1.45
TFAM-deficient	–7.13	23.69	30.82	–2.21	4.63
	–7.15	24.03	31.18	–1.85	3.60
	–7.20	23.81	31.01	–2.02	4.05

ΔCq = Cq (D–Loop) – Cq (ΔCq WCE)
ΔΔCq = ΔCq – **Median ΔCq WT**
Relative abundance of Cytosolic mtDNA = 2^(–ΔΔCq)

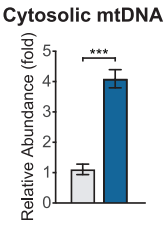


Figure 5 TFAM-deficient cells exhibit elevated levels of mtDNA in the cytosol. (**A-C**) TFAM knockdown MEFs were fractionated as described in Basic Protocol 2. DNA isolated from whole-cell and cytosolic extracts was subjected to SYBR Green–based qPCR to quantitate nuclear (TERT) and mitochondrial (D-loop) DNA using specific primers. (**A**) Raw Cq values. (**B,C**) Raw values and formulas for calculating total cellular mtDNA abundance are tabulated (left) and plotted as mean values (right). Plots show mean ± SEM ($n = 3$); unpaired Student's t test after Shapiro-Wilk normality test, $^{**}p < 0.01$, $^{***}p < 0.001$.

GAPDH (cytosolic extract), TFAM (mitochondrial extract), and Lamin (nuclear extract) can be used to assess purity (Table 3).

Fractions should be free of cross-contamination. If cross-contamination is observed, especially with mitochondrial or nuclear proteins in the cytosolic extract, the digitonin concentrations may need to be adjusted down to prevent inadvertent lysis of these organelles.

Detect DNA in fractions by qPCR

56. Dilute purified DNA from fractions A-1, B-1, B-3, and B-5 (step 53) to 2 ng/µl in nuclease-free water.

57. Prepare PerfeCTa SYBR Green and primer master mixes for each mtDNA or nuclear control primer set in 1.5-ml microcentrifuge tubes. See Table 5 for optimized primers for qPCR.

For each PCR reaction, use 2.5 µl SYBR mix plus 0.125 µl each of 10 µM forward and reverse primers, for a total volume of 2.75 µl. The total volume of master mix needed is 2.75 × # samples × # replicates. Prepare accordingly and pipette into a 96-well plate. It is useful to make 5%-10% extra to account for pipetting errors.

58. Using the E1-ClipTip Electronic Adjustable Tip Spacing Multichannel Equalizer pipette, add 2.25 μl of diluted DNA to each well of a 384-well plate.

    To add the DNA, gently touch the pipette tip to one side of the well. Take note of which side of the well the tip touches, and then add the SYBR master mix to the well using the opposite side.

59. Centrifuge for 1 min at 2000 × *g*, room temperature.

60. Add 2.75 μl SYBR green and primer master mix to each well in the 384-well plate.

61. Centrifuge for 1 min at 2000 × *g*, room temperature.

62. Seal all edges of the plate tightly with optical film. Tap plate gently to mix the cDNA, SYBR Green, and primers.

63. Centrifuge for 1 min at 2000 × *g*, room temperature.

64. Run a program suggested for PerfeCTa SYBR Green FastMix using a CFX384 Real-Time PCR Detection System.

    Example program:

    Step 1: 95°C for 30 s
    Step 2: 95°C for 5 s, then 60°C for 30 s, repeated 40 times
    Step 3: 65°C for 5 s
    Step 4: 60° to 95°C at increments of 0.5°C for 5 s

65. Calculate mtDNA abundance relative to nuclear DNA using the ΔΔCq method.

    Sample results are shown in Figure 5, including sample Cq values from raw data, formulas for calculating mtDNA abundance from whole-cell and cytosolic extracts, and representative graphs. See Understanding Results for a detailed description.

    The post-digitonin cytosolic (B-1) and post-NP-40 mitochondrial (B-3) extracts should contain little, if any, nuclear TERT or KCNJ10 DNA amplification. High amplification of nuclear DNA in either fraction (Cq values <30) indicates nuclear contamination.

BASIC PROTOCOL 3

PHARMACOLOGICAL INHIBITION OF mtDNA REPLICATION AND RELEASE

This protocol describes two pharmacological methods to inhibit the replication and release of mtDNA. These methods may be used in combination with Basic Protocols 1 and 2 to assess the contribution of mtDNA to any observed phenotypes, including cytosolic mtDNA accumulation and increased ISG expression induced via the cGAS-STING axis or other innate immune signaling pathways. The first method utilizes 2′,3′-dideoxcytidine (ddC), a chain-terminating nucleoside analog that inhibits mtDNA replication, to deplete mtDNA from mitochondria. As ddC is only incorporated by the mitochondrial DNA polymerase and therefore does not inhibit nuclear DNA replication or gene expression, ddC specifically reduces cellular mtDNA content. Although prolonged culture in ddC will dramatically reduce cellular mtDNA content, most primary cells and cell lines can tolerate the concentration used here for up to 5 days without dramatic loss of respiratory chain function and/or increased cell death. Following the protocol below, one is able to deplete mtDNA from cells to determine whether an observed innate immune response or phenotype (i.e., cGAS signaling and ISG expression) is reduced and therefore dependent on mtDNA. ddC has been used to dampen mtDNA-driven innate immune signaling in a variety of genetic and environmental conditions where mitochondrial integrity is compromised and mtDNA accumulates in the cytoplasm. The second method uses the voltage-dependent anion channel (VDAC) inhibitor VBIT-4, which blocks mtDNA release through VDAC-1 pores in the outer mitochondrial membrane (Ben-Hail et al., 2016). VBIT-4 has been utilized by many researchers to lower the amount of mtDNA

that enters the cytoplasm, thereby reducing ISG or pro-inflammatory gene expression mediated by cGAS-STING or another innate immune pathway. VBIT-4 is well-tolerated by most cells for up to 5 days.

Additional Materials *(also see Basic Protocol 1)*

Dimethyl sulfoxide (DMSO) (Corning, 25-950-CQC)
47 mM (10 mg/ml) 2′,3′-dideoxcytidine (ddC, Sigma-Aldrich, D5782) in DPBS, 0.2-μm filter-sterilized
10 mM VBIT-4 (Selleckchem, S3544) in DMSO, 0.2-μm filter-sterilized
0.2-μm PES syringe filters (Corning, 431229)
10-ml syringe (BD Biosciences, 302995)

1. Grow and isolate cells as described (see Basic Protocol 1, steps 5-10).
2. Dilute cells to 6×10^4 cells/ml in DMEM-FBS.
3. Plate cells in 12-well dishes, adding 1 ml (6×10^4 cells/well) to at least four wells per inhibitor. Incubate at 37°C, 5% CO_2 for 6 hr to allow the cells to attach.

   At least two sets of paired wells (control and treated) are required per condition: one for RT-qPCR and one for western blotting. Thus, four wells are needed for each inhibitor (ddC or VBIT-4). If immunofluorescence staining is desired, two sterile 18-mm coverslips in a culture plate will also be needed for each inhibitor (see Basic Protocol 1, steps 1-4).

   To examine mtDNA abundance in cellular fractions as in Basic Protocol 2, the numbers of cells plated and vessels should be scaled up appropriately, while keeping ddC and VBIT-4 concentrations and times as described below.

4. Add 3.2 μl of 47 mM ddC to the appropriate wells (final 150 μM). Add an equal volume of DPBS to the control wells.

   Primary MEFs are relatively resistant to ddC, so a high concentration is required. We have successfully used 10-20 μM ddC in HFFs and 40-60 μM ddC in SV40 T antigen-immortalized MEFs. A lower ddC concentration may be sufficient for other cell types, and this should be optimized for each cell line.

5. Add 1 μl of 10 mM VBIT-4 to the appropriate wells (final 10 μM). Add an equal volume of DMSO to the control wells.
6. Incubate for 2-4 days at 37°C, 5% CO_2.

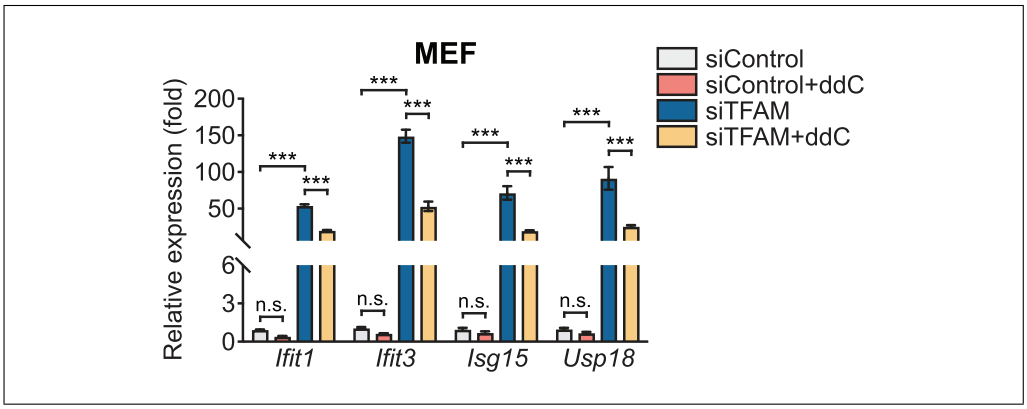


Figure 6 Depletion of mitochondrial DNA reduces ISG expression induced by cytosolic mtDNA. MEFs were transfected with control or TFAM siRNAs for 72 hr in the presence or absence of 150 μM ddC. RNA was extracted and reverse-transcribed into cDNA, which was subjected to SYBR Green–based qPCR to profile ISG (*Ifit1, Ifit3, Isg15, Usp18*) expression. Plot shows mean fold change ± SEM ($n = 3$); unpaired Student's t test after Shapiro-Wilk normality test, $^{**}p < 0.01$, $^{***}p < 0.001$.

7. If incubating longer than 48 hr, aspirate medium from ddC and matched control wells and add fresh DMEM-FBS containing 150 μM ddC every 2 days to maintain the active ddC concentration. No further manipulation is needed for VBIT-4 treatment.

   For ddC treatment, the optimal ddC concentration and incubation time must be determined empirically for each cell line. To identify the optimal conditions, total mtDNA content can be determined by microscopy (see Basic Protocol 1) or mtDNA abundance can be determined in whole-cell extracts by qPCR (see Basic Protocol 2).

   For VBIT-4 treatment, it may not be necessary to treat cells for the full 4 days to see an effect. Often, 1-2 days is enough to observe a decrease in cytosolic mtDNA and any coordinate downregulation in ISG transcript. For additional information and protocols on VBIT-4, see Kim et al. (2019), Sprenger et al. (2021), and Torres-Odio et al. (2021).

8. Proceed to analysis by RT-qPCR, western blotting, and/or immunofluorescence (see Basic Protocol 1).

   Figure 6 shows representative ISG expression in MEFs as measured by RT-qPCR after transfection with siTFAM to induce cytosolic DNA release in the presence or absence of ddC. See Understanding Results for a detailed description.

REAGENTS AND SOLUTIONS

Digitonin lysis buffer

8 ml nuclease-free water (Sigma-Aldrich, W4502)
500 μl 1 M HEPES, pH 7.4 (Sigma-Aldrich, H0887)
87.7 mg NaCl (Dot scientific, DSS23020-5000)
3.6 μl 50 mg/ml digitonin stock solution (see note)
Bring volume to 10 ml with nuclease-free water
Filter sterilize with a 0.2-μm PES syringe filter (Corning, 431229)
Prepare fresh each time
Before use, add protease inhibitors (Sigma-Aldrich, 11836170001)

The digitonin stock solution is prepared fresh by adding 50 mg digitonin (EMD Chemicals, 300410) to 1 ml nuclease-free water and heating to 95°C for 15 min or until dissolved.

Final composition: 50 mM HEPES, pH 7.4, 150 mM NaCl, 18 μg/ml digitonin, protease inhibitors.

NP-40 lysis buffer

350 ml nuclease-free water (Sigma-Aldrich, W4502)
3.03 g Tris base (Dot Scientific, DST60040-5000)
4.38 g NaCl (Dot Scientific, DSS23020-5000)
1 ml 500 mM EDTA (Sigma-Aldrich, E7889)
5 ml Nonidet P-40 Substitute (NP-40, Sigma-Aldrich, 74385)
50 ml glycerol (Dot Scientific, DSG22020-4000)
Adjust pH to 7.5 with conc. HCl
Bring volume to 500 ml with nuclease-free water
Filter sterilize with a 0.2-μm PES bottle-top filter (Thermo Fisher, 569-0020)
Store up to 6 months at 4°C
Before use, add protease inhibitors (Sigma-Aldrich, 11836170001)

Final composition: 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 10% (v/v) glycerol, protease inhibitors.

SDS lysis buffer

86 ml nuclease-free water (Sigma-Aldrich, W4502)
2 ml 1 M Tris, pH 8 (Dot Scientific, DST60040-5000)
10 ml 10% SDS solution (Sigma-Aldrich, 71736)

Adjust pH to 8.0 with conc. HCl
Bring volume to 100 ml with nuclease-free water
Filter sterilize with a 0.2-μm PES bottle-top filter (Thermo Fisher, 569-0020)
Store up to 6 months at room temperature
Before use, add protease inhibitors (Sigma-Aldrich, 11836170001)

Final composition: 20 mM Tris, pH 8, 1% (v/v) SDS, protease inhibitors.

COMMENTARY

Background Information

Over the last decade, interest in the intersection of mitochondria and innate immunity has grown considerably. Mitochondrial DNA has garnered significant interest for its role as a DAMP in numerous inflammatory processes and human diseases (Bai & Liu, 2019; Hopfner & Hornung, 2020; Riley & Tait, 2020; West, 2017). Despite growing roles for the mtDNA-innate immune axis in human disease, assays to quantify mtDNA release to the cytosol are not standardized. Here, we describe a series of protocols for assaying the release of mtDNA into the cytosol and the subsequent activation of innate immune signaling in mammalian cells. These methods can be performed with standard laboratory equipment and reagents, and are highly adaptable to a wide range of mammalian cell types. Our desire is to provide researchers working in various biological and biomedical fields the ability to accurately and reproducibly measure cytosolic mtDNA release and downstream innate immune responses.

The effects of mtDNA on the innate immune system are pleiotropic, and mtDNA may engage one of several PRRs depending on the physiological or pathological context. TLR9 specifically recognizes hypomethylated CpG motifs and was first described as a sensor for bacterial DNA (Hemmi et al., 2000). Nearly a decade later, Zhang and colleagues found that mtDNA released into the circulation during systemic inflammatory response syndrome could engage TLR9 on neutrophils, activating IL-8 secretion and chemotaxis (Zhang, Itagaki, & Hauser, 2010; Zhang, Raoof, et al., 2010). While TLR9 seems to be an important PRR for the detection of extracellular mtDNA, the cGAS-STING signaling axis is a key sensor of cytosolic mtDNA. Under conditions of mitochondrial dysfunction and loss of mitochondrial quality control, mtDNA is released into the cytosol, where it is sensed by cGAS, triggering downstream IFN-I responses (Lei et al., 2021; Torres-Odio et al., 2021; West et al., 2015). Cytosolic mtDNA may play important roles during viral and bacterial infections. For example, *Tfam*$^{+/-}$ MEFs, which exhibit constitutive release of mtDNA into the cytosol, have elevated ISG expression and are markedly resistant to infection by herpes simplex virus 1 (HSV-1) and vesicular stomatitis virus (VSV), suggesting that mtDNA release may be a factor in initiating the antiviral response to infection. In addition, there is growing evidence that cytosolic mtDNA may enhance inflammasome activation. The NLRP3 and AIM2 inflammasomes can directly bind mtDNA (Shimada et al., 2012, 3), and mtDNA accumulation in the cytosol enhances IL-1β and IL-18 secretion upon stimulation with inflammasome activators ATP or nigericin (Nakahira et al., 2010). Overall, the aberrant release of mtDNA and its sensing by TLR9, cGAS, NLRP3, and/or AIM2 is increasingly implicated in disease-promoting IFN-I and pro-inflammatory cascades in systemic lupus erythematosus, nonalcoholic steatohepatitis, hypertension, myocarditis, atherosclerosis, and neurodegeneration (Caielli et al., 2016; Garcia-Martinez et al., 2016; McCarthy et al., 2015; Oka et al., 2012; Zhang et al., 2015).

Basic Protocol 1 describes a method to induce mtDNA instability via depletion of TFAM. Experimental approaches that deplete TFAM modestly have the advantage of inducing mtDNA release while minimizing off-target effects to other mitochondrial functions. Depletion of TFAM by 50%-70% has only modest effects on mitochondrial RNA expression and does not significantly impair mitochondrial respiration in most cultured cells (West et al., 2015). In addition to serving as a mitochondrial transcription factor, TFAM binds mtDNA tightly and compacts it into a higher-order protein/DNA structure known as the nucleoid (Kasashima, Sumitani, & Endo, 2011). A nucleoid typically consists of a single copy of the mitochondrial genome, but may contain up to ten mtDNA genomes depending on cell type and condition (Kukat et al., 2011). TFAM expression level is directly correlated with mtDNA abundance (Ekstrand et al., 2004), and the complete loss of TFAM results in embryonic lethality

in mice (Larsson et al., 1998). Depletion of TFAM results in loss of mtDNA abundance, changes in nucleoid morphology, and instability of mtDNA (West et al., 2015). The resulting mtDNA instability leads to an efflux of mtDNA into the cytosol, which engages the cGAS-STING signaling axis to stimulate expression of IFN-I and ISGs (West et al., 2015). In addition to TFAM, disruptions to other mitochondrial proteins can lead to mtDNA instability that triggers IFN-I responses. A mutation in the proofreading domain of the gamma subunit of the mitochondrial DNA polymerase (POLG) leads to an accumulation of point mutations and deletions as well as fragmentation of the mitochondrial genome (Kujoth et al., 2005; Nissanka, Bacman, Plastini, & Moraes, 2018; Trifunovic et al., 2004), which is also coupled to the activation of cGAS and downstream IFN-I signaling (Lei et al., 2021; Guerra Martinez, Lei, & West, 2018). Alternatively, deletion of caseinolytic mitochondrial matrix peptidase proteolytic subunit (CLPP), a component of the mitochondrial unfolded protein response (Shpilka & Haynes, 2018), can also alter mtDNA packaging and stability, which induces IFN-I responses via cGAS-STING (Torres-Odio et al., 2021).

Release of mtDNA into the cytosol may also be induced pharmacologically by co-administration of ABT-737 and Q-VD-OPH. This method induces rapid liberation of mtDNA into the cytosol via a different route that may be preferable in cases where TFAM knockdown by siRNA transfection is not possible or more acute timepoints are desired. ABT-737 and Q-VD-OPH act synergistically to permeabilize mitochondria, allowing release of mtDNA and simultaneous inhibition of caspase activation and apoptosis (Rongvaux et al., 2014). ABT-737 is a pan-Bcl-2 inhibitor (Oltersdorf et al., 2005, 2) that triggers Bax/Bak-dependent mitochondrial outer membrane permeabilization (MOMP), which in turn allows release of mtDNA and cytochrome c into the cytosol. Unchecked, cytochrome c will initiate a caspase cascade leading to apoptosis-mediated cell death (Jiang & Wang, 2004). Caspase inhibition with Q-VD-OPH likely serves several functions in inducing IFN-I responses. In addition to inhibiting the caspase cascade that leads to cell death, this compound inhibits caspase cleavage of cGAS, IRF3, and MAVS, which could lead to inhibition of downstream IFN-I responses (Ning et al., 2019). Q-VD-OPH may also aid in mtDNA release from mitochondria. While Bax/Bak activation alone can lead to MOMP, Bax/Bak pore formation in the context of caspase inhibition dramatically increases pore size, allowing herniation of the inner mitochondrial membrane and extrusion of mtDNA into the cytosol (Ader et al., 2019; McArthur et al., 2018; Riley & Tait, 2020).

There may be situations in which mtDNA-dependent stimulation of innate immune signaling is suspected but not confirmed. In these cases, it can be useful to deplete cytosolic mtDNA to test the contribution of mtDNA to a given inflammatory or IFN-I phenotype. One method to accomplish this is depletion of mtDNA using the chain terminator ddC. This nucleoside analog was first developed as an antiviral therapy to treat HIV, but resulted in high levels of toxicity to patients (Lewis & Dalakas, 1995). It was determined that the mitochondrial DNA polymerase POLG efficiently incorporates ddC into the mitochondrial genome, resulting in depletion of mtDNA (Dalakas, Semino-Mora, & Leon-Monzon, 2001; Johnson et al., 2001; Lee, Hanes, & Johnson, 2003). The high affinity of POLG for ddC makes it an ideal drug for depleting mtDNA in a laboratory setting with minimal detrimental effects to nuclear DNA. Other nucleoside analogs such as AZT or PMPA are effective at depleting mtDNA but are incorporated by POLG with lower efficiency (Johnson et al., 2001). Mitochondrial DNA has also been depleted effectively with ethidium bromide (Chandel & Schumacker, 1999; King, 1996; Seidel-Rogol & Shadel, 2002), but ethidium may exhibit toxicity to nuclear DNA at higher concentrations, and therefore has a greater propensity for non-mitochondrial off-target effects (Turner & Denny, 1996).

The inhibition of mtDNA release into the cytosol is somewhat complicated because multiple mechanisms are involved. The first mechanism by which mtDNA may exit mitochondria is through Bax/Bak MOMP (McArthur et al., 2018; Riley et al., 2018; Tait & Green, 2010), which we have exploited in the Alternate Protocol. Inhibiting the release of mtDNA through this pathway may be problematic, however. Several inhibitors of Bax/Bak pore formation have been described (Jensen, WuWong, Wong, Matsuyama, & Matsuyama, 2019; Niu et al., 2017; Parone et al., 2006; Spitz, Zacharioudakis, Reyna, Garner, & Gavathiotis, 2021), but they have not proven universally effective at limiting mtDNA release. Another potential route

for mtDNA release is through the mitochondrial permeability transition pore (mPTP), a nonspecific pore which typically only allows passage of components smaller than 1.5 kDa (Bernardi, 1999; Halestrap, McStay, & Clarke, 2002). The mPTP has been shown to form in response to changes in cellular calcium homeostasis or irradiation, and to allow release of fragmented mtDNA (Patrushev et al., 2004, 2006). An additional mechanism of mtDNA release is through voltage-dependent anion channels (VDACs). There is evidence that VDACs participate in MOMP and mtDNA release during apoptosis (Ben-Hail et al., 2016; McCommis & Baines, 2012), but VDACs also mediate mtDNA release and cGAS-STING-mediated IFN-I responses independently of apoptosis, as shown in *EndoG*$^{-/-}$ mice (Kim et al., 2019). *EndoG*$^{-/-}$ mice exhibit cGAS-STING activation that is inhibited by treatment with the VDAC inhibitor VBIT-4 (Ben-Hail et al., 2016; Kim et al., 2019). We have since shown that VBIT-4 also abrogates mtDNA-induced IFN-I responses in cells lacking CLPP (Torres-Odio et al., 2021). Thus, although VBIT-4 appears to be an effective tool for pharmacologically inhibiting mtDNA release, it may not be appropriate in all situations due to the varied mechanisms governing mtDNA release *in cellulo*.

Critical Parameters

mtDNA abundance and ISG expression in cultured cells are highly sensitive to culture conditions. If cell culture conditions are not properly controlled, some experimental results may be difficult to reproduce. Conditions such as confluency, passage number, choice of serum, and incubator oxygen concentration can all impact the results obtained from these protocols. We have found that cells grown to high confluency show increased basal ISG expression; therefore, cell confluency should be kept consistent between experimental groups. ISG expression (baseline and after stimulation) can also be influenced by the type of serum used. FBS contains a large array of components, including growth factors, cytokines, and endotoxins, that can vary widely depending on the source, manufacturer, and even lot used. It is advisable to screen sera from multiple vendors to choose a lot that displays minimal negative effects on cell growth, morphology, and ISG expression before and after innate immune stimulation.

Although we have provided optimized concentrations for siRNAs, ABT-737, Q-VD-OPH, ddC, and VBIT-4, it is important to titrate these reagents depending on the cell source, cell number, and timeline. This is especially critical when using cells other than MEFs and HFFs. Similarly, the suggested cell numbers and antibody dilutions may need to be optimized for immunofluorescence microscopy.

Robust cellular subfractionation into cytosolic, mitochondrial, and nuclear extracts without cross-contamination requires precise control of timing and buffer/detergent conditions. The timing of membrane solubilization steps and detergent concentrations described here have been used successfully for MEFs and HFFs, but other cell types may require additional optimization. A proper concentration of digitonin that will gently permeabilize plasma membranes while keeping mitochondrial and nuclear membranes intact is critical for the extraction and analysis of cytosolic DNA. A good starting concentration ranges between 10 and 30 μg/ml digitonin for plasma membrane permeabilization. It is critical that the digitonin lysis buffer be prepared fresh before use, as digitonin has limited solubility in aqueous solutions and will begin to precipitate within several hours of buffer preparation (Kun, Kirsten, & Piper, 1979).

The post-digitonin cytosolic and post-NP-40 mitochondrial extracts should contain little, if any, nuclear TERT or KCNJ10 DNA amplification. High amplification of nuclear DNA in either fraction (Cq values <30) indicates nuclear contamination. If contamination between cellular compartments is found by western blotting, adjusting the solubilization times and detergent concentrations may yield cleaner results. Finally, the pH of the phenol/chloroform/isoamyl alcohol solution used in DNA precipitation is critical. In order for DNA to remain soluble in the aqueous phase, the phenol solution must have a basic pH (∼8). If the phenol/chloroform/isoamyl alcohol solution is acidic, DNA will remain dissolved in the lower organic phase and be lost during separation (McKiernan & Danielson, 2017).

Another important consideration is the choice of reference genes for normalization of RT-qPCR data. In our experience, commonly used reference genes such as *Gapdh* and *β-actin* frequently change in expression in response to innate immune activation. For this reason, we use *Rpl37*, which encodes a ribosomal protein that we have found is expressed stably in multiple conditions of innate immune signaling (e.g., TLR, RLR, and cGAS stimulation). It is recommended to independently

determine the most appropriate reference gene for the cells and conditions being tested using a tool such as GeNorm (Vandesompele et al., 2002) or Normfinder (Andersen, Jensen, & Ørntoft, 2004).

One final consideration is that not all cell types express a functional cGAS-STING signaling pathway. Many transformed cell lines, including HeLa, HEK293T, and other cells immortalized using SV40 large T antigen, downregulate expression of cGAS and/or STING. These cells may still exhibit changes to mtDNA morphology in response to mtDNA stress, but ISG expression will most likely remain unchanged. Primary cells such as MEFs, primary macrophages, skin fibroblasts, or cells immortalized with human telomerase reverse transcriptase (hTERT) work best for the analysis of innate immune signaling.

Troubleshooting

See Table 6 for a list of common problems, causes, and potential solutions.

Understanding Results

Knockdown of TFAM expression by siRNA will result in reduced TFAM expression. This will trigger a coordinate increase in ISGs at the RNA and protein levels in both MEFs (Fig. 2A) and HFFs (Fig. 2B,C). If TFAM knockdown by siRNA is not possible, co-treatment with ABT-737 and Q-VD-OPH is another effective method to promote mtDNA release into the cytosol, which will result in increased ISG transcript expression (Fig. 2D). Depletion of TFAM will also lead to a decrease in overall mtDNA abundance, as well as generalized mitochondrial stress, characterized by mtDNA nucleoid alterations and mitochondrial network hyperfusion. This can be visualized by immunofluorescence (Fig. 3A). In control cells, mtDNA nucleoids are expected to be of consistent size and evenly distributed throughout the mitochondrial network. After transfection with siTFAM, mtDNA will aggregate to form enlarged and fused nucleoids that are more variable in size and distribution (Fig. 3A). Reduction in TFAM levels or exposure of cells to ABT-737 and Q-VD-OPH will cause mtDNA leakage into the cytosol, which can be measured by qPCR after DNA fractionation (Fig. 5). Finally, depletion of mtDNA with ddC or inhibition of its release via VDAC pores will decrease ISG expression at baseline and after treatment with siTFAM or ABT-737 and Q-VD-OPH (Fig. 6).

Time Considerations

The minimum time to complete all procedures in Basic Protocol 1 is 6 days when beginning with a confluent 10-cm plate of cells. Cells may take 2-3 days to reach confluency, depending on cell type. Preparation of coverslips and cell plating on day 1 will take ∼1 hr to complete. Transfection with siRNA on day 2 will take ∼30 min. After the 3-day incubation following transfection, collection of RNA/protein and cell fixation/staining can be completed on the same day. Fixation and staining of cells usually take 5-6 hr. RNA and protein isolation can easily be completed during the 1-hr antibody incubations. At that point, RNA and protein can be stored at –80°C (RNA) or –20°C (protein) for future analysis. After mounting coverslips and drying overnight, sealing with fingernail polish takes less than 10 min, and coverslips can then be stored in the dark at 4°C for a month without a significant loss in quality.

Treatment with ABT-737 and Q-VD-OPH in the Alternate Protocol can be completed in 2-3 days. Plating of cells on day 1 will take less than 1 hr. ABT-737 and Q-VD-OPH can be added in the morning of day 2, allowing the 6-hr timepoint to be collected later in the day and the 24-hr timepoint to be collected the following day.

Cellular fractionation and isolation of DNA from the fractions in Basic Protocol 2 can be completed in a single day once cells have reached confluency. Cellular fractionation will take 2 hr and DNA extraction will take 5 hr.

Treatment of cells with ddC or VBIT-4 for Basic Protocol 3 will take a total of 5 days. Plating cells on the morning of day 1 will take less than 1 hr. Addition of ddC or VBIT-4 in the afternoon of day 1 will take 10 min. For ddC treatment, changing the medium containing ddC will take 10 min. Collection of cells on day 5 for RNA or protein analysis will take ∼1 hr.

For all procedures listed here, gel electrophoresis and western blotting will take 1-2 days, although they may take longer depending on the number of proteins being examined. qPCR setup and analysis can be completed in 4-5 hr.

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Table 6 Troubleshooting

Problem	Possible cause	Solution
High baseline ISG expression	Cells overly confluent at time of collection	Plate fewer cells or reduce incubation time
	Poor-quality serum	Purchase high-quality serum with low levels of endotoxins; screen serum from multiple vendors for effects on baseline ISG expression
Dead cells after siTFAM transfection	Toxicity from transfection reagent	Decrease ratio of RNAiMAX/siTFAM
	Excessive TFAM knockdown	Decrease amount of siTFAM transfected
Poor TFAM knockdown	Inefficient siRNA transfection	Increase ratio of RNAiMAX/siTFAM
		Increase amount of siTFAM transfected
Poor ISG induction	Insufficient TFAM knockdown	See above
	Cells do not express cGAS and/or STING	Use primary cells (e.g., MEFs, macrophages, human skin fibroblasts) or cells immortalized with hTERT; do not use cell lines immortalized with SV40 large T antigen
Weak fluorescence signal	Incomplete cell permeabilization	Prepare fresh Triton X-100 permeabilization buffer
Cytosolic contamination in mitochondrial or nuclear fractions	Incomplete cell lysis	Prepare fresh digitonin lysis buffer using a freshly made digitonin stock
		Increase incubation time with digitonin lysis buffer or increase digitonin concentration
	Incomplete washing	Complete all washes before proceeding to next step; agitate tubes gently by inversion to increase stringency of washes
Mitochondrial contamination in cytosolic fraction	Digitonin lysis step too stringent, leading to ruptured mitochondria	Decrease incubation time with digitonin lysis buffer or decrease digitonin concentration
	Centrifugation speed too high, leading to ruptured mitochondria	Reduce centrifugation speed
	Incomplete clearing of mitochondria from cytosolic fraction	Repeat centrifugation step, making sure not to transfer any of the pellet to the new tube
Mitochondrial contamination in nuclear fraction	Incomplete washing	Complete all washes before proceeding to next step; agitate tubes gently by inversion to increase stringency of washes
Nuclear contamination in mitochondrial fraction	Centrifugation speed too high, leading to ruptured nuclei	Reduce centrifugation speed
ddC treatment does not reduce ISG expression	Incomplete depletion of mtDNA	Increase ddC concentration
		Replace medium/ddC with fresh every day instead of every other day
VBIT-4 treatment does not reduce ISG expression	Cells may have had time to compensate for VDAC-1 inhibition	Collect RNA/protein at earlier timepoint
		Increase starting VBIT-4 concentration or spike in additional VBIT

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Author Contributions

Joshua Bryant: Data curation, methodology, visualization, writing – original draft, writing – review and editing; **Yuanjiu Lei**: Data curation, methodology, visualization, writing – original draft, writing – review and editing; **Jordyn VanPortfliet**: Methodology, visualization, writing – original draft, writing – review and editing; **Ashley Winters**: Methodology, writing – original draft; **Phillip West**: Conceptualization, data curation, funding acquisition, methodology, project administration, supervision, visualization, writing – original draft, writing – review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data, tools, and materials (or their source) that support the protocol are available from the corresponding author upon request.

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