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Assessing mitochondrial DNA release into the cytosol and the subsequent activation of innate immune-related pathways in mammalian cells

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Abstract

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), which can engage innate immune sensors to drive inflammation. The aberrant release of these 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 (IFN-I) 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 data misinterpretation. Here, we present a series of protocols for assaying the release of mtDNA into the cytosol and the 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. This protocol will permit researchers working across the spectrum of biological and biomedical

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CONFLICT OF INTEREST STATEMENT:
The authors declare that they have no competing interests.

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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sciences to accurately and reproducibly measure cytosolic mtDNA release and resulting innate immune responses.

Basic Protocol 1: siRNA-mediated knowdown of Targeting Transcription Factor A, mitochondrial (TFAM) to induce mtDNA instability and cytosolic release, and activation of the cGAS-STING pathway

Alternate Protocol 1: Pharmacological induction of mtDNA release and cGAS-STING activation with ABT-737 and Q-VD-OPH

Basic Protocol 2: Cellular fractionation and isolation of DNA from the cytosol, nucleus, and mitochondria for quantitation by qPCR

Basic Protocol 3: Pharmacological depletion of mtDNA and inhibition of release from mitochondria

Keywords

Mitochondria; mitochondrial DNA; innate immunity; cGAS; STING

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 these invading organisms, known as pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002; West et al., 2006). In order to discriminate between self and non-self, PRRs recognize distinctive features of invading organisms such as lipopolysaccharide, peptidoglycans, flagellar proteins, or nucleic acids, including double-stranded RNA or DNA with hypomethylated CpG motifs (Brubaker et al., 2015; West et al., 2006; Janeway and Medzhitov, 2002). 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 and Medzhitov, 2002; West et al., 2006; Paludan and Bowie, 2013). 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 and 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 and Takeda, 2004; O'Neill et al., 2013), is localized to endolysosomal compartments and does not ordinally encounter self-DNA from healthy

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cells (O'Neill et al., 2013; Nakahira et al., 2015). However, under conditions of cellular stress, injury, or death, self-DNA from the nucleus or mitochondria may be released into the cytoplasm or extracellular space and can 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 to spur tissue repair and regeneration, persistent activation of the innate immune system by DAMPs may be a significant contributor to chronic inflammatory diseases (Nakahira et al., 2015; Gong et al., 2020). 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 a protobacterium (Roger et al., 2017), mitochondria contain many components that act as DAMPs (mtDAMPs) when leaked or released from the organelle, including N-formylated peptides, hypomethylated mtDNA, mitochondrial RNA-DNA hybrids, and ATP (West, 2017; Riley and Tait, 2020; West and Shadel, 2017). Mitochondria play important roles in numerous cellular processes, including metabolism, calcium homeostasis, signaling, and cell death, but 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. While the majority of the approximately 1,000 proteins found in mitochondria are encoded in the nucleus (Fox, 2012), the mitochondrial genome encodes 13 proteins involved in oxidative phosphorylation, 2 ribosomal RNAs, and 22 transfer RNAs (Shadel and Clayton, 1997). Under conditions of cellular stress and mitochondrial dysfunction, mtDNA can be released into the cytosol, where it is sensed by the cGAS-STING pathway and other PRRs (Lei et al., 2021; West et al., 2015; Torres-Odio et al., 2021; Sun et al., 2017). cGAS then synthesizes the second messenger cyclic GMP-AMP (cGAMP), which activates STING on 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 and Shadel, 2017). Engagement of inflammasomes leads to processing and secretion of pro-inflammatory cytokines interleukin-1 beta (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 various aspects of 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 the release of mtDNA into the cytosol and measuring the resulting IFN-I

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signaling and ISG expression by RT-qPCR and western blot; 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 (Figure 1). Each of these protocols may be used independently or in combination depending on the model system in which they are used. We provide two different methods of inducing mtDNA instability and release from the mitochondria. The first method (Basic Protocol 1) is genetic and involves depletion of the mtDNA packaging protein Transcription Factor A, mitochondrial (TFAM), resulting in instability and the cytosolic release of fragmented mtDNA. The alternative method (Alternate Protocol 1) 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.

BASIC PROTOCOL 1: siRNA-mediated knockdown of Targeting Transcription Factor A, mitochondrial (TFAM) to induce mtDNA instability and 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 then detected by cGAS or other DNA sensors, leading to intracellular signaling cascades that result in the activation of IFN-I and/or pro-inflammatory cytokine responses (West et al., 2015; West and Shadel, 2017). 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 one’s cell culture model of interest. We detail RT-qPCR-based methods to assess TFAM knock down by siRNA and provide 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 mitochondrial network and mtDNA nucleoid morphology by immunofluorescence microscopy. These techniques may be used in conjunction with the TFAM knockdown protocols described, but they may also be used to visualize mitochondria and mtDNA across various conditions and cell types of interest.

Materials:

Primary mouse embryonic fibroblasts (MEFs) from day 13.5–15.5 embryos, isolated as previously described (Xu, 2005) or Human foreskin fibroblasts (ATCC, HFF-1, SCRC-1041)

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Complete media (referred to as “DMEM-FBS”): Dulbecco’s Modified Eagle’s Medium - high glucose (Sigma-Aldrich, D5796) with 10% Fetal bovine serum (Avantor Seradigm, 97068-085, or another extremely low endotoxin, high-quality fetal bovine serum)

Dulbecco’s Phosphate Buffered Saline (PBS, Sigma-Aldrich, D8537)

Opti-MEM I Reduced Serum Medium (ThermoFisher Scientific, 31985062)

Trypsin/EDTA (Sigma-Aldrich, T3924)

Lipofectamine™ RNAiMAX Transfection Reagent (ThermoFisher Scientific, 13778150)

Nuclease-free duplex buffer (IDT, 11-05-01-12)

DsiRNA oligonucleotides (DsiRNA 27-mers synthesized by Integrated DNA Technologies, 10 nmol, standard desalting, diluted to 20 μM working stock in nuclease-free duplex buffer):

Human TFAM:

Sense = 5′-rGrCrArGrArArCrUrCrArUrCrUrArGrGrUrArArArUrUrACA-3′

Antisense = 5′-rUrGrUrArArUrUrUrArCrCrUrArGrArUrGrArGrUrUrCrUrGrCrCrU-3′

Mouse TFAM:

Sense = 5′-rGrCrUrArUrCrCrArArArGrArArArCrCrUrArUrGrArGrUTC-3′

Antisense = 5′-rGrArArCrUrCrArUrArGrGrUrUrUrCrUrUrUrGrGrArUrArGrCrUrA-3′

Control siRNA (IDT DsiRNA NC1, 51-01-14-03):

Sense: rCrGrUrUrArArUrCrGrCrGrUrArUrArCrGrCrGrUAT

Antisense: rArUrArCrGrCrGrUrArUrUrArUrArCrGrCrGrArUrUrArArCrGrArC

100% ethanol (KOPTEC, V1016), diluted to 70% (v/v) in sterile water

32% paraformaldehyde solution (Electron Microscopy Sciences, 15714), diluted to 4% (v/v) in PBS

Permeabilization buffer (PBS with 0.1% (v/v) Triton X-100 (Sigma-Aldrich, X100-500mL))

Blocking buffer (PBS with 10% (v/v) FBS)

ProLong Diamond Antifade Mountant with DAPI (ThermoFisher Scientific, P36962)

High precision No. 1.5H 18 mm circle coverslips (Azer Scientific, ES0117580)

Hypodermic needle 21G X 1” with a bent tip (Santa Cruz Biotechnology, 4710008025)

Fine tip forceps (Fisher Scientific, 16-100-113)

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Parafilm® M Laboratory Sealing Film (Santa Cruz Biotechnology, sc-200311)

Microscope slides 25 × 75mm (Globe Scientific, 1384-50W, or equivalent)

Clear fingernail polish

Kimwipes or other low-lint laboratory wipes

Quick-RNA Microprep Kit (Zymo Research, R1050)

qScript cDNA Master Mix (Quantabio, 95047-100)

PerfeCTa SYBR Green SuperMix (Quantabio, 95054-500)

Nuclease-free water (Sigma-Aldrich, W4502)

0.5 mL syringes (Becton Dickinson, 309628)

NP-40 lysis buffer (See Reagents and Solutions)

Micro BCA Protein Assay Kit (ThermoFisher Scientific, 23235)

Biosafety cabinet (NuAire, BSC Class II, Type A2 or equivalent)

Benchtop microcentrifuge (Eppendorf 5424 or equivalent)

Swinging bucket rotor centrifuge (Eppendorf 5702 or equivalent)

Tissue culture microscope (Nikon Eclipse Ts2 or equivalent)

Hausser Scientific Bright-Line hemocytometer or equivalent

Thermo Scientific Drybath or equivalent

Single channel pipettes (1000, 200, 20, and 10 μL)

Sterile filter tips (1000, 200, 20, and 10 μL)

Sterile serological pipettes (10 and 5 mL)

Motorized pipet controller (Corning Stripettor or equivalent)

10-cm cell culture dishes (USA Scientific, 5666-4160)

12-well or 24-well cell culture plates (USA Scientific, 5666-5180 and 5666-2160)

15-mL conical tubes (USA Scientific, 5618-8271)

1.5-mL microcentrifuge tubes (USA Scientific, 1615-5500)

Corning LSE vortex mixer or equivalent

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E1-ClipTip Electronic Adjustable Tip Spacing Multichannel Equalizer pipette (0.5 to 12 μL volume) (ThermoFisher Scientific, 4672010BT, or equivalent)

384-well white PCR plates (USA Scientific, 1438-4790, or equivalent)

Optically clear real-time PCR plate sealing film (USA Scientific, 2921-7800, or equivalent)

96-well PCR plates (USA Scientific, 1402-8120)

BioTek Epoch plate reader with Take3 microplate for performing DNA, RNA, and protein quantitation, or equivalent absorbance plate reader/spectrophotometer

Bio-Rad CFX384 Touch Real-time PCR Detection System, or other comparable 384-well real-time PCR machine

Protocol Steps

Sterilize coverslips for plating cells for immunofluorescence microscopy only

—This procedure may be performed immediately prior to plating cells on coverslips or up to a week in advance. If performing procedure in advance, keep 12-well plate lidded to ensure coverslips remain sterile.

1. In the laminar flow hood, add 2 or more 18-mm coverslips to separate wells of a 12-well tissue culture plate using sterile forceps.

   At least 2 coverslips are required for this protocol: one for immunofluorescence for the scrambled siRNA control group (siControl) and one for the TFAM siRNA knockdown group (siTFAM).

   Forceps may be sterilized by immersing in 70% EtOH for several minutes then allowing to air dry in 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 adding them one-by-one to the 12-well plate using sterile forceps.

2. Soak coverslips in 500 μL of 70% EtOH for 30 minutes to sterilize.

3. Aspirate off the 70% EtOH and let the wells dry completely.

   Leave the plate partially open in the laminar flow hood. Drying should take about 20 minutes.

4. Wash the coverslips in the plate with 500 μL of 1X PBS. Store the plates containing coverslips until cells are ready for plating.

Plate cells for RNA and protein extraction

5. Seed approximately 1×10^6 primary mouse embryonic fibroblasts (MEFs) or human foreskin fibroblasts (HFFs) onto a 10-cm dish in 10 mL of DMEM-FBS. Incubate at 37 °C, 5% CO_2 until confluent.

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Low passage MEFs (i.e. P1-P4) should be used, as later passage MEFs undergo replicative senescence and downregulate innate immune pathways. It usually takes 2–4 days for plates to reach confluency.

6. Aspirate media and wash plate with 5 mL of PBS.

7. Aspirate PBS and add 1 mL of trypsin/EDTA. Incubate at 37 °C for 5 minutes or until cells have detached.

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

8. Quench trypsin/EDTA by adding 3 mL of DMEM-FBS, and then transfer the cells to a 15-mL tube, centrifuge at 200 x g for 5 minutes at room temperature, and decant media.

9. Resuspend the cells in 5 mL of DMEM-FBS and count using hemocytometer or preferred method.

10. Dilute cells to 4×10^4 cells/mL in DMEM-FBS.

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

    At least six total wells are required for subsequent steps: three for the scrambled siRNA control group (siControl) and three for the TFAM siRNA knockdown group (siTFAM). A set of these paired wells will be used for RNA and the other, for protein extraction, as described below. In addition, a third pair of wells will be used for immunofluorescence staining.

    For immunofluorescence staining, plate the cells in wells with coverslips (from step 4)

siRNA transfection

12. The following day, prepare an RNAiMAX transfection as follows (for each well in a 12-well dish):

    a. In a 1.5-mL microcentrifuge tube labeled “tube A”, mix 50 μL of OptiMEM and 1.25 μL of 20 μM stock Control or TFAM siRNA.

    b. In a 1.5-mL microcentrifuge tube labeled “tube B”, mix 50 μL of OptiMEM and 3 μL of RNAiMAX.

    Users will need a total of three siControl transfections and 3 siTFAM transfections each for cells plated, for RNA extraction for RT-qPCR, protein extraction for western blotting, and immunofluorescence microscopy analysis. More will be required if additional timepoints and/or biological replicates are needed. Set up and label wells accordingly.

13. Add diluted siRNA (tube A) to diluted RNAiMax (tube B) and mix by pipetting a few times.

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14. Incubate the mixture at room temperature for 10 mins.

15. Add 100 μL of the transfection mix dropwise to the corresponding wells (siControl or siTFAM).

16. Rock plates back and forth to mix.

17. Incubate plates for 72 hours from time of siRNA transfection.

    Users can now proceed to isolate RNA (Steps 18–32) and proteins (Steps 33–39), and fix cells for microscopic analysis (Steps 40–66).

    Although 72 hours is the optimal timepoint to knock down TFAM in MEFs and HFFs, users may incorporate additional timepoints ranging from 24–96 hours after transfection, depending on the cell line utilized.

RNA collection and RT-qPCR analysis of ISGs

18. Remove plate from the incubator and aspirate media from the wells labeled for RNA extraction, then wash cells 2X with 1 mL of PBS.

19. Aspirate PBS and add 300 μL of RNA lysis buffer from the Quick-RNA Microprep kit.

20. Pipet up and down to mix, then transfer cell lysate to a 1.5-mL tube and continue with RNA isolation following the manufacturer's instructions.

    The 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.

21. Determine the 260/280 nm absorbance ratio with an Epoch plate reader equipped with a Take3 microplate, or other spectrophotometer, to determine RNA purity and concentration.

    A 260/280 ratio above 1.9 is considered acceptable.

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

23. Dilute the cDNA 1:10 in Nuclease-free water and add 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 x 2 × 3). It is useful to prepare 5–10% extra to account for pipetting errors.

24. Prepare PerfeCTa SYBR Green and primer master mixes for each ISG or reference control primer set in 1.5-mL microcentrifuge tubes. See Table 1 for ISG and reference primer sequences.

    For each PCR reaction, use 2.5 μL of SYBR mix plus 0.125 μL each of 10 μM forward and reverse primers, for a total volume of 2.75 μL.

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Volume of master mix needed = 2.75 x # samples x # replicates. Prepare accordingly and pipette into a 96-well plate. Prepare 5–10% extra to account for pipetting errors.

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

When adding cDNA to a well, gently touch the pipette tip to one side of the well before adding cDNA. Take note of which side of the well the tip touches and add the SYBR master mix to the well using the opposite side of the well.

26. Spin down the 384-well plate for 1 min at 2,000 x g at room temperature.

27. Add 2.75 μL of the SYBR green and primer master mix (from Step 24) to other side of each well in the 384-well plate.

28. Spin down the plate for 1 min at 2,000 x g at room temperature.

29. Seal the plate with optical film making sure that all the edges are tightly sealed.

30. Tap the plate gently to mix the cDNA, SYBR Green, and primers. Spin down the plate for 1 min at 2,000 x g at room temperature.

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

Example program: Step 1– 95°C for 30 sec, Step 2– 95°C for 5 sec then 60°C for 30 sec repeated 40 times, Step 3– 65°C for 5 sec, Step 4– 60°C to 95°C increasing at increments of 0.5°C for 5 sec.

32. For SYBR Green-based qPCR analysis, calculate ISG abundance relative to a reference gene using the delta delta Cq (ΔΔCq) method. ISG transcript abundance in siControl samples should be set to 1, and ISG fold changes in siTFAM samples should calculated relative to siControl samples.

Figure 2A-B shows representative TFAM and ISG expression result in MEFs and HFFs by RT-qPCR after completion of these steps. See Understanding Results for a detailed description.

Protein collection and western blotting of ISGs

33. Take plate from step 17 and aspirate media from the wells labeled for protein extraction, then wash cells twice with 1 mL of PBS.

34. Add 50 μL of ice-cold NP-40 lysis buffer to each well.

35. Scrape well using the rubber plunger from a 1-mL syringe to lyse cells. Place two 1.5-mL tubes on ice.

36. Transfer lysate to chilled 1.5-mL tubes and centrifuge at 21,000 x g for 10 minutes at 4°C to pellet cellular debris.

37. Transfer supernatant to a new 1.5-mL tube and keep on ice. The pellet may be discarded.

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38. Quantify protein concentration using the Micro BCA Protein Assay kit per the manufacturer's instructions.

39. Run approximately 20–30 μg of denatured and reduced protein extract on SDS polyacrylamide mini gels and perform western blotting using preferred method (see, for instance, (Gallagher, 2010). Validated antibodies can be found in Table 2.

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

Cell fixation and immunostaining

Fixation:

40. Take plate from step 17 and aspirate media from the wells containing coverslips, then wash cells twice with 1 mL of PBS.

41. Fix the cells with 600 μL of 4% PFA at room temperature for 20 minutes.

42. Wash twice with 1 mL of PBS, letting stand 5 minutes for each wash.

43. Store in PBS at 4°C until staining.

    For best quality, analyze stored cells within3 days.

Staining:

44. Wash the coverslips in the plate with 1 mL of 1X PBS.

45. Permeabilize the cells with 1 mL of Permeabilization Buffer for 5 minutes at room temperature.

46. Aspirate Permeabilization Buffer and wash wells twice with 1 mL of PBS, letting stand 5 minutes for each wash.

47. Add 1 mL of Blocking buffer to each well and leave at room temperature for 30 minutes.

48. Dilute primary antibodies in Blocking buffer, making enough to have 80 μL per coverslip (see Table 3 for suggested primary antibodies and dilutions).

49. Place a piece of parafilm on a flat surface such as a benchtop.

    Spray benchtop with 70% EtOH and then place the parafilm sheet on top, to adhere. Ensure that the surface is flat and use a marker to divide into sections as needed. Use a large enough sheet of parafilm so that all the 18-mm coverslips will fit on the sheet with around a half inch of extra space on all sides. This will ensure antibody droplet solutions do not run into one another on the parafilm sheet.

50. Bend the tip of a 21G X 1" hypodermic needle such that it forms a small hook.

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Gently press the bevel of the needle on a hard surface so that it forms a small ~ 1 mm hook (see Figure 3B).

51. Spot a 60-µL droplet of the primary antibody cocktail per coverslip onto the parafilm sheet.

Ensure there is enough space between droplets to prevent coverslips from contacting each other.

52. Remove coverslips from the 12-well plate and remove excess liquid by gently tapping the edge of the coverslip on a Kimwipe.

To remove from the plate, carefully raise one side of the coverslip using the hooked needle, and then use the forceps to gently lift the coverslip from the plate.

53. Place the coverslip cell-side down on the antibody dilution droplets using the hooked needle and fine-tip forceps. Cover to protect from light and incubate for 1 hour at room temperature.

54. Lift the coverslips using the hooked needle and forceps. Return the coverslip cell-side up to the plate.

55. Wash twice with 1 mL of Blocking buffer for 5 minutes each.

56. Prepare a new Parafilm sheet as in step 49.

57. Dilute secondary antibodies in Blocking buffer (suggested antibodies and dilutions can be found in Table 4), making enough for 80 µL/coverslip.

58. Spot a 60-µL droplet of the secondary antibody cocktail per coverslip onto the parafilm sheet.

59. Lift the coverslips using the hooked needle and forceps, remove excess liquid by tapping the edge on a Kimwipe, and place them cell-side down on the corresponding secondary antibody droplet. Incubate for 1 hour at room temperature in the dark.

60. Return the coverslips cell-side up to the plate and wash twice with 1 mL of Blocking buffer for 5 minutes each at room temperature.

61. Next, wash twice with 1 mL of PBS for 5 minutes at room temperature.

62. Carefully apply 1 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.

63. Lift the coverslips out of the well and remove PBS by gently touching the edge of a Kimwipe to wick away excess.

64. Mount coverslip cell side down onto the drop of ProLong Antifade Mountant with DAPI. Allow to dry overnight in the dark.

Slowly lower the coverslip down using the forceps and hooked needle to avoid creating air bubbles.

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65. The following day, seal the coverslip to the slide by painting the edges with a generous amount of clear fingernail polish.

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

66. Image slides on a widefield fluorescent or confocal microscope with a 60X or higher power objective.

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

ALTERNATE PROTOCOL 1: Pharmacological induction of mtDNA release and cGAS-STING activation with ABT-737 and Q-VD-OPH

This protocol provides an alternative to Basic Protocol 1 for inducing mtDNA release into the cytosol. It relies on the coadministration 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 type I interferon (IFN-I) responses via cGAS-STING signaling (Rongvaux et al., 2014). Similar to the TFAM knockdown approach described in Basic Protocol 1, 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 to those in Basic Protocol 1:

Dimethyl sulfoxide (DMSO) (Corning, 25-950-CQC)

0.2 μm PES syringe filters (Corning, 431229)

10 mL syringe (BD Biosciences, 302995)

ABT-737 (EMD Millipore, 197333, 10 mM in DMSO, 0.2 μm filter sterilized)

Q-VD-OPH (EMD Millipore, 551476, 10 mM in DMSO, 0.2 μm filter sterilized)

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Protocol steps:

1. Seed approximately 1×10^6 primary MEFs or HFFs onto a 10-cm dish in 10 mL of DMEM-FBS. Incubate at 37 °C, 5% CO_2 until confluent.
2. Aspirate media and wash plate with 5 mL of PBS.
3. Aspirate PBS and add 1 mL of trypsin/EDTA. Incubate at 37 °C for 5 minutes or until cells have detached.
4. Quench trypsin/EDTA by adding 3 mL of DMEM-FBS, and then transfer the cells to a 15-mL tube, centrifuge at 200 x g for 5 minutes at room temperature, and decant media.
5. Resuspend the cells in 5 mL of DMEM-FBS and count using hemocytometer or preferred method.
6. Dilute cells to 6×10^4 cells/mL in DMEM-FBS.
7. Plate cells in 12-well dishes, adding 1 mL each to at least 4 wells (6×10^4 cells/well). Incubate at 37 °C, 5% CO_2 overnight.

   At least four wells are required for subsequent steps: two for the DMSO control group and two for the ABT-737 Q-VD-OPH treatment group. A set of two paired wells will be used for RNA and the other, for protein extraction, as described in Basic Protocol 1.

   If immunofluorescence staining is also desired, sterilize two 18-mm coverslips and plate cells at 6×10^4 cells/coverslip.
8. The following morning, add both ABT-737 and Q-VD-OPH to appropriate wells at a final concentration of 10 μM (1000X dilution of 10 mM stock). Add an equivalent amount of DMSO to control wells (e.g, add 1 μL of DMSO to 1 mL media in control wells and 1 μL of each inhibitor to 1 mL media in treatment wells).

   The indicated concentrations for ATB-737 and Q-VD-OPH 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.
9. Incubate for between 6 to 24 hours at 37 °C, 5% CO_2.
10. At the end of incubation, lyse cells for downstream RNA and protein analyses as described in Steps 18–32 and Steps 33–39 of Basic Protocol 1, respectively, or fix cells for analysis by immunofluorescence, as described in Basic Protocol 1, Steps 40–66.

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

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BASIC PROTOCOL 2: Isolation of DNA from cytosolic, mitochondrial, and nuclear fractions for quantitation by qPCR

Mitochondrial DNA can be released from mitochondria to trigger multiple PRRs in the context of infection, but also in inflammatory diseases, cancer, and aging. 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. However, efforts to visualize mtDNA release with fluorescence in-situ hybridization (FISH) 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. The herein described 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 sensitive SYBR Green-based qPCR approaches. This protocol includes validated human and mouse primer sets, thus allowing the relative quantitation of nuclear DNA and mtDNA across many primary cells and cell lines from various genetic, biological, and pathophysiological contexts.

Materials:

Complete media (referred to as “DMEM”): Dulbecco’s Modified Eagle’s Medium - high glucose (Sigma-Aldrich, cat. no. D5796) with 10% Fetal bovine serum (Avantor Seradigm, cat. no. 97068-085)

Dulbecco’s Phosphate Buffered Saline (PBS, Sigma-Aldrich, D8537)

Trypsin/EDTA (Sigma-Aldrich, T3924)

NaCl (Dot scientific, cat. no. DSS23020-5000)

1M HEPES (Sigma-Aldrich, H0887)

Digitonin (EMD Chemicals, 300410).

Tris base (Dot Scientific, DST60040-5000)

Ethylenediaminetetraacetic acid disodium salt solution (EDTA) (Sigma-Aldrich, E7889)

Nonidet P 40 Substitute (NP-40) (Sigma-Aldrich, 74385)

Glycerol (Dot Scientific, DSG22020-4000)

Sodium dodecyl sulfate (SDS) 20% in aqueous solution (VWR, 97062-440)

Nuclease-Free Water, for Molecular Biology (Sigma-Aldrich, W4502)

RNase A (Worthington, LS005650); 5 mg/mL stock in nuclease-free water

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Protease K (Worthington, LS004240), 20 mg/mL stock in nuclease-free water

phenol/chloroform/isoamyl alcohol (ACROS ORGANICS, 327155000)

chloroform/isoamyl alcohol (ACROS ORGANICS, 327111000)

Ammonium acetate solution (NH4OAc), 7.5M (Sigma-Aldrich, A2706)

Glycogen (Thermo Scientific, R0561)

100% ethanol (KOPTEC, V1016)

95% ethanol (KOPTEC, V1101)

PerfeCTa SYBR Green SuperMix (Quantabio, 95054-500)

Laminar flow hood (NuAire, BSC Class II, Type A2 or equivalent)

Benchtop microcentrifuge (Eppendorf 5424 or equivalent)

Swinging bucket rotor centrifuge (Eppendorf 5702 or equivalent)

Mini 1.5-mL centrifuge (Corning 6765/c1501 or equivalent)

Tube revolver rotator (Thermo Scientific, 88881001)

Sonication system (Diagenode, UCD-200)

Tissue culture microscope (Nikon Eclipse Ts2 or equivalent)

Hausser Scientific Bright-Line hemocytometer or equivalent

Thermo Scientific Drybath or equivalent

E1-ClipTip Electronic Adjustable Tip Spacing Multichannel Equalizer pipette (0.5 to 12 μL volume) (ThermoFisher Scientific, 4672010BT, or equivalent)

Single channel pipettes (1000, 200, and 20 μL)

Sterile filter tips (1000, 200, and 20 μL)

Sterile serological pipettes (10 and 5 mL)

Motorized pipet controller (Corning Stripettor or equivalent)

10-cm cell culture dishes (USA Scientific, 5666-4160)

12-well or 24-well cell culture plates (USA Scientific, 5666-5180 and 5666-2160)

384-well white PCR plates (USA Scientific, 1438-4790, or equivalent)

96-well PCR plates (USA Scientific, 1402-8120)

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Optically clear real-time PCR plate sealing film (USA Scientific, 2921-7800, or equivalent)

15-mL conical tubes (USA Scientific, 5618-8271)

1.5-mL microcentrifuge tubes (USA Scientific, 1615-5500)

Corning LSE vortex mixer or equivalent

BioTek Epoch plate reader with Take3 microplate for performing DNA, RNA, and protein quantitation, or equivalent absorbance plate reader/spectrophotometer

Bio-Rad CFX384 Touch Real-time PCR Detection System, or other comparable 384-well real-time PCR machine

Protocol Steps

Cell plating

1. Prepare two 15-cm dishes with 20 mL of DMEM-FBS each. Seed approximately 3×10^6 primary mouse embryonic fibroblasts (MEFs) or human foreskin fibroblasts (HFFs) per dish. 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 media and wash each plate with 10 mL of PBS.

3. Aspirate PBS and add 5 mL of trypsin/EDTA per plate. Incubate at 37 °C for 5 minutes or until cells have detached.

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

4. Quench trypsin/EDTA by adding 9 mL of DMEM-FBS, pool the cell suspensions from each plate into a 50-mL tube, centrifuge at 200 x g for 5 minutes at room temperature, and decant media.

Detergent-based cellular fractionation

5. Resuspend the pellet from Step 4 in 1 mL of PBS by pipetting. Divide into two 1.5-mL tubes labeled “A” and “B”. See Figure 4 for workflow diagram.

   Tube A will comprise the “whole cell extract (WCE)” fraction and will be used as a normalization control for the subcellular fractions isolated from Tube B.

6. Centrifuge both tubes at 200 x g for 5 minutes at room temperature to pellet the cells, then aspirate the PBS.

7. Resuspend Tube A in 500 μL of SDS Lysis Buffer and heat to 95°C for 15 minutes to fully lyse cells.

8. Transfer 450 μL of the lysed cell suspension into a new 1.5-mL tube (Fraction A-1). Keep at room temperature until cell fractionation is completed.

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9. Transfer the remaining 50 μL of lysed cell suspension into a new 1.5-mL tube (Fraction A-2). Keep tube at room temperature until DNA extraction is completed.

10. Resuspend the cell pellet in Tube B in 500 μL of Digitonin Lysis Buffer by gently pipetting up and down.

    The digitonin-based cell 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 in the buffer; however, this may need to be adjusted based on cell line and treatment condition of interest. A good starting range is between 10–30 μg/mL digitonin for plasma membrane permeabilization.

11. Incubate the suspension end-over-end on a tube revolver rotator for 10 minutes at 4˚C to allow selective plasma membrane permeabilization.

12. Centrifuge the permeabilized cells in Tube B at 950 x g for 5 minutes at 4˚C.

13. Without disturbing the pellet, gently transfer the supernatant into a new 1.5-mL tube. Centrifuge at 17,000 x g for 5 min at 4˚C to pellet any remaining cellular debris.

    The supernatant should yield a pure cytosolic fraction free of organelle contamination.

14. Transfer the supernatant into a new 1.5-mL tube and save as the “cytosolic extract” (Fraction B-1); keep on ice until cell fractionation is completed. Transfer 50 μL of B-1 to a separate 1.5-mL tube for western blotting (Fraction B-2) and keep on ice until DNA extraction is completed.

15. Wash the pellet from step 13 three times with 1 mL of ice cold PBS, spinning at 950 x g for 3 minutes at 4˚C between washes.

16. Resuspend the pellet in 500 μL of NP-40 Lysis Buffer, incubate on ice for 10 min, and then spin at max speed (~21,000 x g) for 10 minutes at 4˚C.

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

17. Without disturbing the pellet, gently transfer the supernatant into a new 1.5-mL tube and save as the “mitochondrial extract” (Fraction B-3); keep on ice until cell fractionation is completed. Transfer 50 μL of to a separate 1.5-mL tube for western blotting (Fraction B-4) and keep on ice until DNA extraction is completed.

18. Wash the nuclear pellet from step 17 three times with 1 mL of ice cold PBS, spinning at max speed for 3 minutes at 4˚C between washes.

19. Resuspend the pellet in 500 μL of SDS Lysis Buffer and boil at 95˚C for 15 minutes to lyse the nucleus and extract DNA.

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

20. Sonicate the nuclear extract in a water bath sonicator at room temperature at high intensity for 5 minutes (alternating 30 seconds ON/ 30 seconds OFF).

21. Spin the SDS lysate at max speed (~21,000 x g) for 5 minutes at room temperature and save the supernatant in a new 1.5-mL tube as the “nuclear extract” (Fraction B-5). Transfer 50 μL of B-5 to a separate 1.5-mL tube for western blotting (Fraction B-6) and keep at room temperature until DNA extraction is complete.

DNA extraction

22. Beginning with 400 μL of each fraction (whole cell (A-1), cytosolic (B-1), mitochondrial (B-3), and nuclear extract (B-5)), treat each with 4 μL of 5 mg/mL RNase A stock for 1.5 hours at 37˚C.

23. Add 4 μL of 20 mg/mL proteinase K stock into each sample. Incubate at 55˚C for 1 hour.

24. Add 400 μL of phenol/chloroform/isoamyl alcohol. Vortex vigorously for 1 min and spin at max speed (~21,000 x g) for 5 minutes at RT.

25. Remove 320 μL of the top aqueous solution and place into a new tube. Avoid picking up any of the lower phenol/chloroform/isoamyl alcohol phase. The lower organic phase may be discarded.

26. Add an equal volume (320 μL) of chloroform/isoamyl alcohol to the aqueous solution. Vortex vigorously for 1 min and spin at max speed for 5 minutes at RT.

27. Remove as much of the top aqueous solution as possible and place into a new tube. Avoid aspirating any of the lower chloroform/isoamyl alcohol phase. The lower organic phase may be discarded.

28. Add an appropriate volume of 7.5 M NH_4OAc for a final concentration of 0.75 M. Calculate the volume of 7.5 M stock required by determining the volume transferred from Step 27.

29. 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.

30. Add 2.5X volume of 100% ethanol and mix well.

31. Incubate at −80 °C for 1 hour (or −20°C overnight) to precipitate DNA.

32. Spin for 20 minutes in a 4°C centrifuge at max speed to pellet DNA precipitate.

33. Decant supernatant carefully without disturbing the pellet.

34. Wash the pellet with 300 μL of 95% EtOH and vortex 3 times.

35. Spin tubes for 15 minutes at 4°C max speed.

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36. Decant supernatant carefully without disturbing the pellet.

37. Repeat steps 34–36 for a second 95% EtOH wash.

38. After decanting, quick spin to draw residual EtOH to the bottom.

39. Remove residual EtOH with a P20 pipette. Be careful not to disturb the pellet.

40. Air dry for 2–5 mins.

41. Resuspend in 20–100 μL of nuclease-free water or Tris-EDTA buffer.

    Buffer may vary depending on the downstream analysis.

42. Measure DNA concentration with Biotek Epoch plate reader and Take3 plate or appropriate spectrophotometer. Store at −80°C until fractions are verified to be pure (Steps 43–45).

    From 3×10^6 MEFs, the users should get 1–2 μg of cytosolic DNA, 13–15 μg of mitochondrial DNA and 30–35 μg of nuclear DNA. Absorbance of 260/280 should be between 1.8–1.9.

Immunoblotting to determine purity of cellular fractions

43. Quantify protein concentration from whole cell (A-2), cytosolic (B-2), mitochondrial (B-4), and nuclear extracts (B-6) using the Micro BCA Protein Assay kit per the manufacturer's instructions.

44. Run approximately 10–30 μg of denatured and reduced protein extract on SDS polyacrylamide mini gels and perform western blotting using preferred method. Antibodies against GAPDH (cytosolic extract), TFAM (mitochondrial extract), and Lamin (nuclear extract) can be used for immunoblotting to assess purity (Table 2).

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

qPCR to detect DNA in cellular fractions

45. If fractions are free of cross-contamination as determined by immunoblotting, dilute purified DNA from the whole cell (A-1), cytosolic (B-1), mitochondrial (B-3), and nuclear extracts (B-5) from Step 42 to 2 ng/μL in nuclease-free water.

46. 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 of SYBR mix plus 0.125 μL each of 10 μM forward and reverse primers, for a total volume of 2.75 μL.

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Volume of master mix needed = 2.75 x # samples x # replicates. Prepare accordingly and pipette into a 96-well plate. It is useful to make 5–10% extra to account for pipetting errors.

47. Using the E1-ClipTip Electronic Adjustable Tip Spacing Multichannel Equalizer pipette, add 2.25 μL of diluted DNA from step 45 to each well of a 384-well plate.

When adding DNA to a well, gently touch the pipette tip to one side of the well before adding DNA. 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 of the well.

48. Spin down the 384-well plate for 1 min at 2,000 x g at room temperature.

49. Add 2.75 μL of the SYBR green and primer master mix to the other side of each well in the 384-well plate.

50. Spin down the plate for 1 min at 2,000 x g at room temperature.

51. Seal the plate with optical film, making sure that all the edges are tightly sealed.

52. Tap the plate gently to mix the DNA, SYBR Green, and primers. Spin down the plate for 1 min at 2,000 x g at room temperature.

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

Example program: Step 1– 95°C for 30 sec, Step 2– 95°C for 5 sec then 60°C for 30 sec, repeated 40 times, Step 3– 65°C for 5 sec, Step 4– 60°C to 95°C increasing at increments of 0.5°C for 5 sec.

54. For SYBR Green-based qPCR analysis, calculate mtDNA abundance relative to nuclear DNA using the delta delta Cq (ΔΔCq) method.

For sample results, see Figure 5, containing 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 extracts (B-3) 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 depletion of mtDNA and inhibition of release from mitochondria

This protocol describes two pharmacological methods to inhibit mtDNA replication and release into the cytosol. 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

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(ddC), a chain terminating nucleoside analog that inhibits mtDNA replication, to deplete mtDNA from mitochondria. Although prolonged culture in ddC will dramatically reduce cellular mtDNA content, most primary cells and cell lines can tolerate the ddC concentration described below for up to 5 days without dramatic loss of respiratory chain function and/or increased cell death. 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. 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.

Materials:

Primary mouse embryonic fibroblasts (MEFs) from day 13.5–15.5 embryos, isolated as previously described (Xu, 2005) or Human foreskin fibroblasts (ATCC, HFF-1, SCRC-1041)

Complete media (referred to as “DMEM-FBS”): Dulbecco’s Modified Eagle’s Medium high glucose (Sigma-Aldrich, D5796), with 10% Fetal bovine serum (Avantor Seradigm, 97068-085, or another extremely low endotoxin, high-quality fetal bovine serum)

Dulbecco’s Phosphate Buffered Saline (PBS, Sigma-Aldrich, D8537)

Trypsin/EDTA (Sigma-Aldrich, T3924)

Biosafety cabinet (NuAire, BSC Class II, Type A2 or equivalent)

Benchtop microcentrifuge (Eppendorf 5424 or equivalent)

Swinging bucket rotor centrifuge (Eppendorf 5702 or equivalent)

Tissue culture microscope (Nikon Eclipse Ts2 or equivalent)

Hausser Scientific Bright-Line hemocytometer or equivalent

Single channel pipettes (1000, 200, 20, and 10 μL)

Sterile filter tips (1000, 200, 20, and 10 μL)

Sterile serological pipettes (10 and 5 mL)

Motorized pipet controller (Corning Stripettor or equivalent)

10-cm cell culture dishes (USA Scientific, 5666-4160)

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12-well or 24-well cell culture plates (USA Scientific, 5666-5180 and 5666-2160)

15-mL conical tubes (USA Scientific, 5618-8271)

1.5-mL microcentrifuge tubes (USA Scientific, 1615-5500)

Corning LSE vortex mixer or equivalent

Dimethyl sulfoxide (DMSO) (Corning, 25-950-CQC)

10-mL syringe (BD Biosciences, 302995)

0.2-μm PES syringe filter (Corning, 431229)

2',3′-Dideoxycytidine (ddC, Sigma-Aldrich, D5782) (10 mg/mL (47 mM) stock in PBS, 0.2 μm filter-sterilized)

VBIT-4 (Selleckchem, S3544) (10 mM stock in DMSO, 0.2 μm filter-sterilized)

Protocol Steps

ddC or VBIT-4 treatment to inhibit mtDNA replication or release into cytosol

1. Seed approximately 1×10^6 primary MEFs or HFFs onto a 10-cm dish in 10 mL of DMEM-FBS. Incubate at 37 °C, 5% CO_2 until confluent.
2. Aspirate media and wash plate with 5 mL of PBS.
3. Aspirate PBS and add 1 mL of trypsin/EDTA. Incubate at 37 °C for 5 minutes or until cells have detached.
4. Quench trypsin/EDTA by adding 3 mL of DMEM-FBS, and then transfer the cells to a 15-mL tube, centrifuge at 200 x g for 5 minutes at room temperature, and decant media.
5. Resuspend the cells in 5 mL of DMEM-FBS and count using hemocytometer or preferred method.
6. Dilute cells to 6×10^4 cells/mL in DMEM-FBS.
7. Plate cells in 12-well dishes, adding 1 mL each to the required number of wells (6×10^4 cells/well). Incubate at 37 °C, 5% CO_2 for 6 hours to allow the cells to attach.

   You will need at least two sets of paired wells (control and treated) per condition: one pair will be used for RNA extraction and the other, for protein extraction, following Basic Protocol 1. In other words, for each chemical (ddC or VBIT-4), you will need four wells.

   If immunofluorescence staining is desired, additionally sterilize two 18-mm coverslips and plate cells at 6×10^4 cells/coverslip, as described in Basic Protocol 1.

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8. Add ddC to the appropriate wells at a final concentration of 150 μM. Add an equal volume of PBS to the control group (e.g., add 3.2 μL of a 47 mM ddC stock to 1 mL of treatment cells and 3.2 μL of PBS to 1 mL of control cells).

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

9. Add VBIT-4 to the appropriate wells at a final concentration of 10 μM. Add an equal volume of DMSO to the control group (e.g., add 1 μL of a 10 mM VBIT-4 stock to 1 mL of treatment cells and 1 μL of DMSO to 1 mL of control cells).

10. Place plate in the cell culture incubator for 2–4 days at 37 °C, 5% CO_2.

11. If incubating for longer than 48 hours, aspirate the media from the ddC and matched control wells and add fresh DMEM-FBS containing 150 μM ddC every two days to maintain the active concentration of ddC. For VBIT-4 treatment, no further manipulation is needed until collection.

    For ddC treatment, users will need to empirically determine the most appropriate ddC concentration and incubation time. Users may examine total mtDNA content by microscopy (Basic Protocol 1 *) or determine mtDNA abundance in whole cell extracts using qPCR (* Basic Protocol 2 *) to identify the optimal conditions for each cell line used.*

    For VBIT-4 exposure, it may not be necessary to treat cells for the full four days to see an effect. One to two days is often enough time to observe a decrease in cytosolic mtDNA and any coordinate downregulation in ISG transcript expression. Please see published studies for additional information and protocols on VBIT-4 (Kim et al., 2019; Sprenger et al., 2021; Torres-Odio et al., 2021).

12. At the end of incubation, lyse cells for downstream RNA and protein analyses as described in Steps 18–32 and Steps 33–39 of Basic Protocol 1, respectively, or fix cells for analysis by immunofluorescence, as described in Basic Protocol 1, Steps 40–66.

    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.*

    If users also desire to examine mtDNA abundance in cellular fractions following 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 in steps 8–11 above.*

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REAGENTS AND SOLUTIONS:

SDS Lysis Buffer

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

To prepare, mix:

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

Adjust pH to 8.0 with concentrated HCl and bring final volume to 100 mL with nuclease-free water. Filter through 0.2 μm PES bottle top filter (ThermoFisher, 569-0020).

SDS lysis buffer may be stored at room temperature for 6 months. Before use, add protease inhibitors (Sigma-Aldrich, 11836170001).

Digitonin Lysis Buffer

Composition: 50 mM HEPES pH 7.4, 150 mM NaCl, 18 μg/mL digitonin, protease inhibitors

Digitonin lysis buffer should be prepared fresh each time.

To prepare, mix:

- 8 mL of nuclease-free water (Sigma-Aldrich, W4502)
- 500 μL of 1M HEPES, pH 7.4 (Sigma-Aldrich, H0887)
- 87.7 mg of NaCl (Dot scientific, DSS23020-5000)
- 3.6 μL of 50 mg/mL digitonin stock (Prepare digitonin stock by adding 50 mg digitonin (EMD Chemicals, 300410) to 1 mL of nuclease-free water and heating to 95°C for 15 minutes or until dissolved. Digitonin stock should be prepared fresh each time)
- Bring final volume to 10 mL with nuclease-free water
- Add to 10-mL syringe (BD Biosciences, 302995) fitted with 0.2 μm PES syringe filter (Corning, 431229) and filter sterilize
- Before use, add protease inhibitors (Sigma-Aldrich, 11836170001)

NP-40 Lysis Buffer

Composition: 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40 (v/v), and 10% Glycerol (v/v), protease inhibitors

To prepare, mix:

- 350 mL of nuclease-free water (Sigma-Aldrich, W4502)

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- 3.03 g of Tris base (Dot Scientific, DST60040-5000)
- 4.38 g of NaCl (Dot Scientific, DSS23020-5000)
- 1 mL of 500 mM Ethylenediaminetetraacetic acid disodium salt solution (EDTA) (Sigma-Aldrich, E7889)
- 5 mL of Nonidet P 40 Substitute (NP-40) (Sigma-Aldrich, 74385)
- 50 mL of Glycerol (Dot Scientific, DSG22020-4000)

Adjust pH to 7.5 with concentrated HCl and bring final volume to 500 mL with nuclease-free water. Filter through 0.2 μm PES bottle top filter (ThermoFisher, 569-0020).

NP-40 lysis buffer may be stored at 4°C for up to 6 months. Before use, add protease inhibitors (Sigma-Aldrich, 11836170001).

COMMENTARY:

Background Information

Over the last decade, interest in the intersection of mitochondria and innate immunity has grown considerably. Mitochondrial DNA, in particular, has garnered significant interest for its role as a DAMP in numerous inflammatory processes and human diseases (West, 2017; Riley and Tait, 2020; Bai and Liu, 2019; Hopfner and Hornung, 2020). Despite growing roles for the mtDNA-innate immune axis in human disease, assays to quantify mtDNA release into 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 they are highly adaptable to a wide range of mammalian cell types. Our desire here is to provide researchers working in varied 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 et al. found that mtDNA released into the circulation during systemic inflammatory response syndrome could engage TLR9 on neutrophils, activating IL-8 secretion and chemotaxis (Zhang et al., 2010a, 2010b). 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; West et al., 2015; Torres-Odio et al., 2021). 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

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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 being 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 transcription factor A mitochondrial (TFAM). Experimental approaches to modestly deplete TFAM have the advantage of inducing mtDNA release into the cytosol, while minimizing off-target effects to other mitochondrial functions. Depletion of TFAM by 50–70% only has modest effects on mitochondrial RNA expression and does not result in significant impairments in mitochondrial respiration in most cultured cells (West et al., 2015). In addition to serving as a mitochondrial transcription factor, TFAM tightly binds mtDNA and compacts it into a higher order protein/DNA structure known as the nucleoid (Kasashima et al., 2011). Each nucleoid typically consists of a single copy of the mitochondrial genome, but nucleoids may contain 2–10 mtDNA genomes depending on cell type and condition (Kukat et al., 2011). TFAM expression level and mtDNA abundance are directly correlated (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, nucleoid morphological changes, and mtDNA instability (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 also 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, deletions, and fragmentation of the mitochondrial genome (Nissanka et al., 2018; Kujoth et al., 2005; Trifunovic et al., 2004), which is also coupled to the activation of cGAS and downstream IFN-I signaling (Lei et al., 2021; Martinez et al., 2018). Alternatively, deletion of caseinolytic mitochondrial matrix peptidase proteolytic subunit (CLPP), a component of the mitochondrial unfolded protein response (Shpilka and 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 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. ABT-737 and Q-VD-OPH act synergistically to permeabilize mitochondria, allowing release of mtDNA while simultaneously inhibiting 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). MOMP, in turn,

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allows the release of mtDNA and cytochrome c into the cytosol. Unchecked, cytochrome c will initiate a caspase cascade leading to apoptosis-mediated cell death (Jiang and 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 will lead to cell death, this compound also 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 (Riley and Tait, 2020; McArthur et al., 2018; Ader et al., 2019).

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 2',3′-dideoxycytidine (ddC). This nucleoside analogue was first developed as an antiviral therapy to treat HIV, but resulted in high levels of toxicity to patients (Lewis and Dalakas, 1995). It was determined that the mitochondrial DNA polymerase POLG efficiently incorporates ddC into the mitochondrial genome, resulting in depletion of mtDNA (Dalakas et al., 2001; Johnson et al., 2001; Lee et al., 2003). Despite this being an undesirable clinical outcome for a therapeutic, 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 analogues, 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 (King, 1996; Seidel-Rogol and Shadel, 2002; Chandel and Schumacker, 1999), but ethidium may exhibit toxicity to nuclear DNA at higher concentrations and, therefore, has a greater propensity for non-mitochondrial, off-target effects (Turner and Denny, 1996).

The inhibition of mtDNA release into the cytosol is somewhat complicated since there have been multiple mechanisms proposed. The first mechanism by which mtDNA may exit mitochondria is through Bax/Bak MOMP (McArthur et al., 2018; Tait and Green, 2010; Riley et al., 2018), which we have exploited in Alternate Protocol 1. Inhibiting the release of mtDNA through this pathway may be problematic, however. Several inhibitors of Bax/Bak pore formation have been described in the literature (Jensen et al., 2019; Niu et al., 2017; Parone et al., 2006; Spitz et al., 2021), but these have not proven universally effective at limiting mtDNA release. Another potential route for mtDNA release is through the mitochondrial permeability transition pore (mPTP), a non-specific pore which typically only allows passage of components smaller than 1.5 KDa (Bernardi, 1999; Halestrap et al., 2002). The mPTP has been shown to form in response to changes in cellular calcium homeostasis or irradiation, and allow the release of fragmented mtDNA (Patrushev et al., 2006, 2004). 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 and Baines, 2012), but VDACs also mediate mtDNA release and cGAS-STING mediated IFN-I responses independently of apoptosis, as shown in mice lacking mitochondrial Endonuclease G (*EndoG*$^{-/-}$)(Kim et al., 2019).

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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). VBIT-4, therefore, appears to be an effective way to pharmacologically inhibit mtDNA release, but 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 cell 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 basally and after stimulation can also be influenced by the type of serum that is used. Fetal bovine serum contains a large array of different components, including growth factors, cytokines, and endotoxins that can vary widely depending on the source, manufacturer, and even lot of serum that is being used. Prior to purchasing any FBS, 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 in Basic Protocols 1 and 3 and Alternate Protocol 1, it is important for users 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, suggested cell numbers and antibody dilutions described in Basic Protocol 1 may need to be adjusted during optimization of the immunofluorescence microscopy methods.

Regarding Basic Protocol 2, robust cellular subfractionation, in which cytosolic, mitochondrial, and nuclear extracts are separated without cross-contamination, requires precise control of timing and buffer/detergent conditions. The timing of membrane solubilization steps and detergent concentrations described in Basic Protocol 2 have been used successfully for MEFs and HFFs, but other cell types may require additional optimization. A proper concentration of digitonin to 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 for digitonin ranges between 10–30 μg/mL digitonin for plasma membrane permeabilization. Moreover, it is critical that the Digitonin Lysis Buffer be prepared fresh before use. Digitonin has limited solubility in aqueous solutions and will begin to precipitate within several hours of buffer preparation (Kun et al., 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, adjustment of solubilization times and

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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 and 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 the gene *Rpl37*, 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 et al., 2004).

One final consideration is that not all cell types express a functional cGAS-STING signaling pathway. Many transformed cell lines, including HeLa, HEK293T, or 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

Please see Table 6 for a list of common problems with the protocols, their 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 both the RNA and protein levels, in both MEFs (Figure 2A) and HFFs (Figure 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 (Figure 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 (Figure 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 their size and distribution (Figure 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 (Figure 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 (Figure 6).

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Time Considerations:

The minimum time to complete all the procedures outlined 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 approximately 1 hour to complete. Transfection of cells with siRNA on day 2 will take about 30 minutes. After the 3-day incubation following siRNA transfection, collection of RNA/protein and cell fixation/staining can be completed on the same day. Fixation and staining of cells usually take between 5–6 hours. RNA and protein isolation can easily be completed during the 1-hour primary or secondary antibody incubation steps. At this 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 coverslips with fingernail polish takes less than 10 minutes. Mounted coverslips can be stored in the dark at 4°C for a month before imaging without a significant loss in quality.

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

Cellular fractionation and isolation of DNA from cellular compartments in Basic Protocol 2 can be completed in a single day once cells have reached confluency. Cellular fractionation will take 2 hours, and DNA extraction will take 5 hours.

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 hour. Addition of ddC or VBIT-4 in the afternoon of day 1 will take 10 minutes. For ddC treatment, aspiration of spent media and addition of fresh media with ddC will take 10 minutes. Collection of cells on day 5 for RNA or protein analysis will take about an hour.

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

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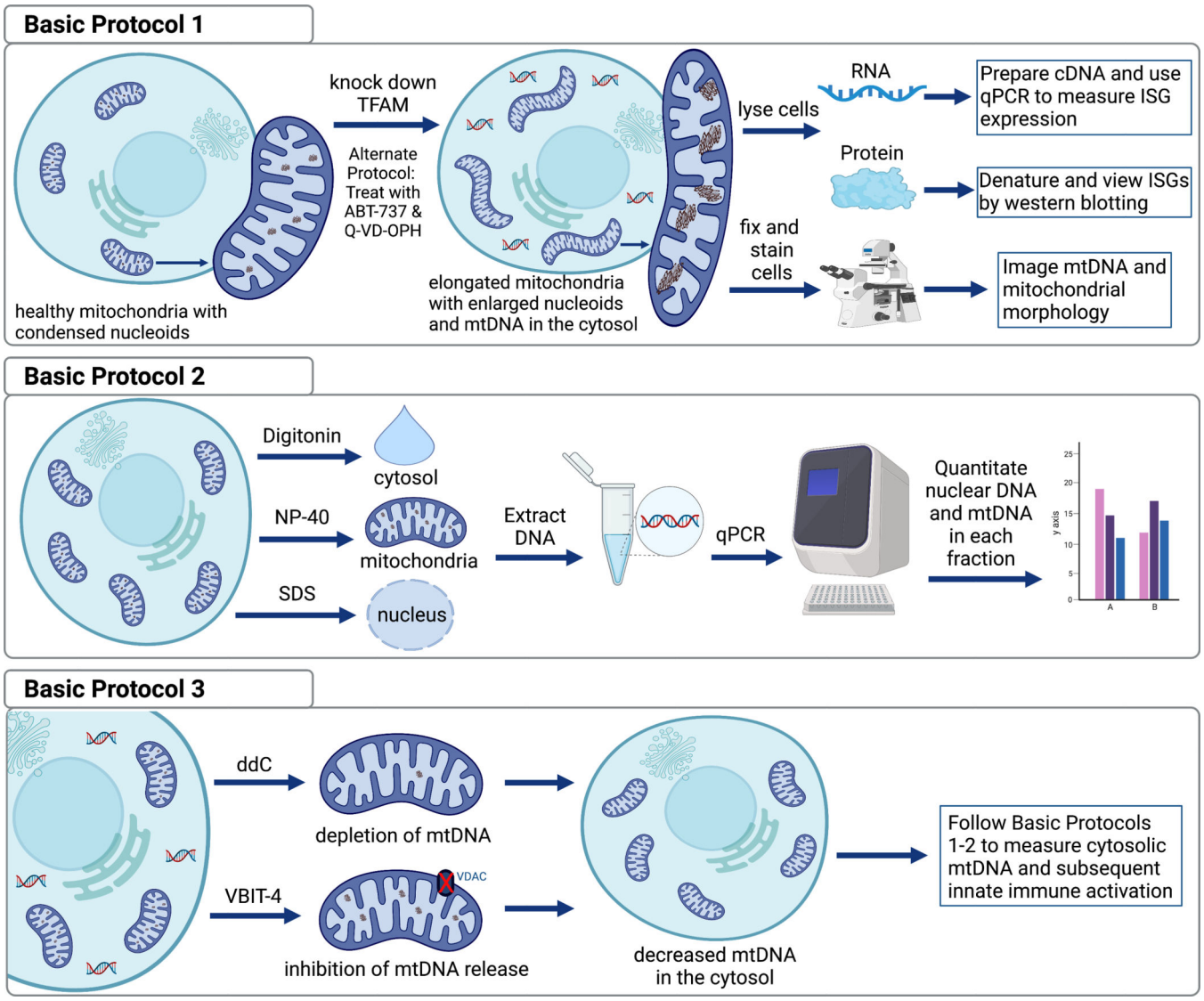

Figure 1: Graphical overview of the protocols and experimental workflows described in this article.

Mitochondrial DNA (mtDNA); cyclic GMP-AMP synthase (cGAS); stimulator or interferon genes (STING); interferon-stimulated gene (ISG); ABT-737 (Bcl-2 inhibitor); Q-VD-OPH (pan-caspase inhibitor); Nonidet P-40 (NP-40); sodium dodecyl sulfate (SDS); 2',3′-Dideoxycytidine (ddC); VBIT-4 (Voltage-dependent anion channel inhibitor).

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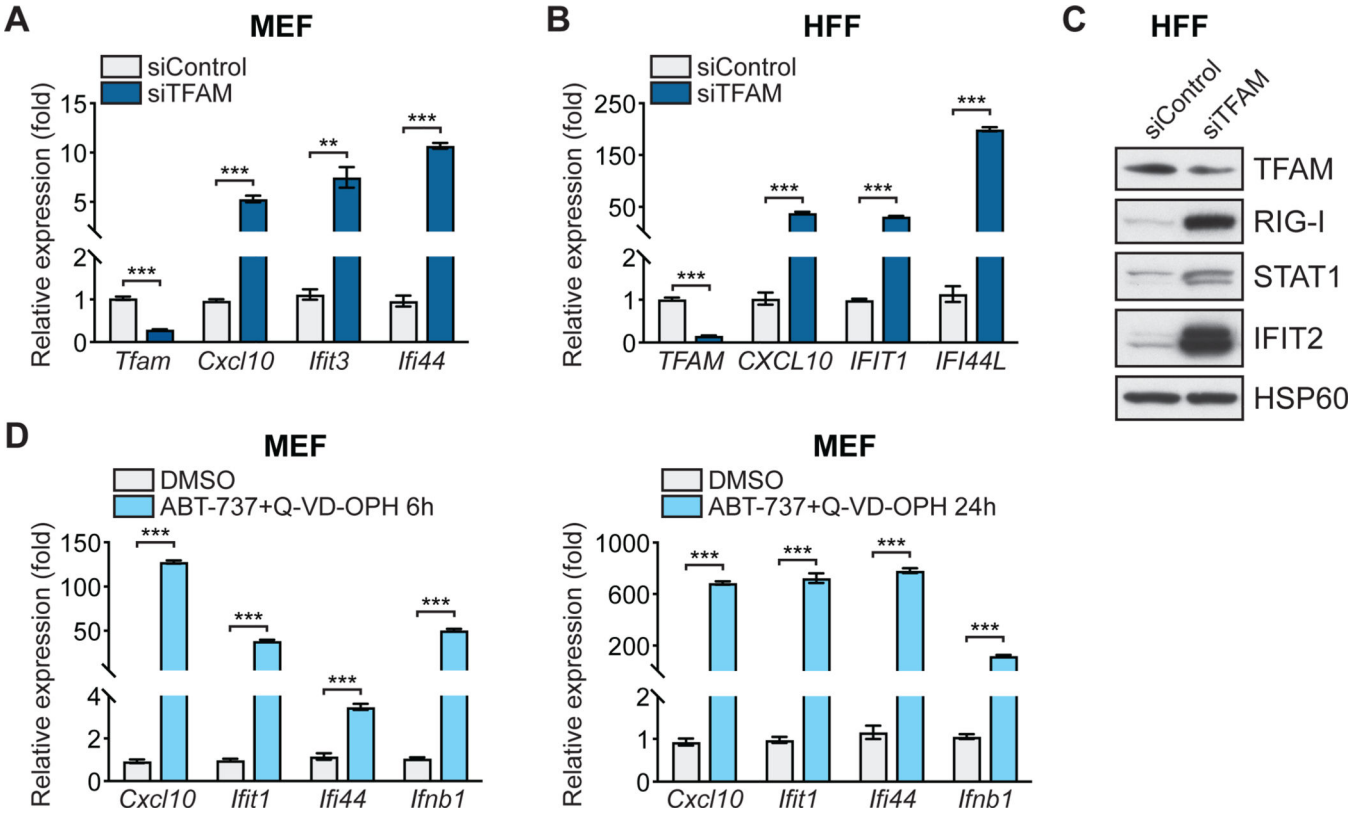

Figure 2: Triggering mtDNA release into the cytosol induces type I interferons (IFN-I) and interferon-stimulated gene (ISG) expression in mouse and human fibroblasts.

(A-B) Mouse embryonic fibroblasts (MEFs) (A) or human foreskin fibroblasts (HFFs) (B) were transfected with Control (siControl) or Transcription Factor A, mitochondrial (TFAM) (siTFAM) siRNAs for 72 hrs. RNA was extracted and reverse transcribed into cDNA, and cDNA was then subjected to SYBR Green-based qPCR analysis to profile *TFAM* and ISG (e.g. *Cxcl10, Ifit1, Ifit3, Ifi44*) expression. (C) HFF cells were transfected with siRNAs for 72 hrs. Proteins were extracted 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) hours. RNA was extracted and reverse transcribed into cDNA, and cDNA was then subjected to SYBR Green-based qPCR analysis to profile ISG (e.g., *Cxcl10, Ifit1, Ifi44, Ifnb1*) expression.

In A, B and D, plots show mean fold change and error bars represent standard error of the mean. N=3. Statistical significance was determined using unpaired Student's *t* test after Shapiro-Wilk normality test. **P < 0.01 and ***P < 0.001.

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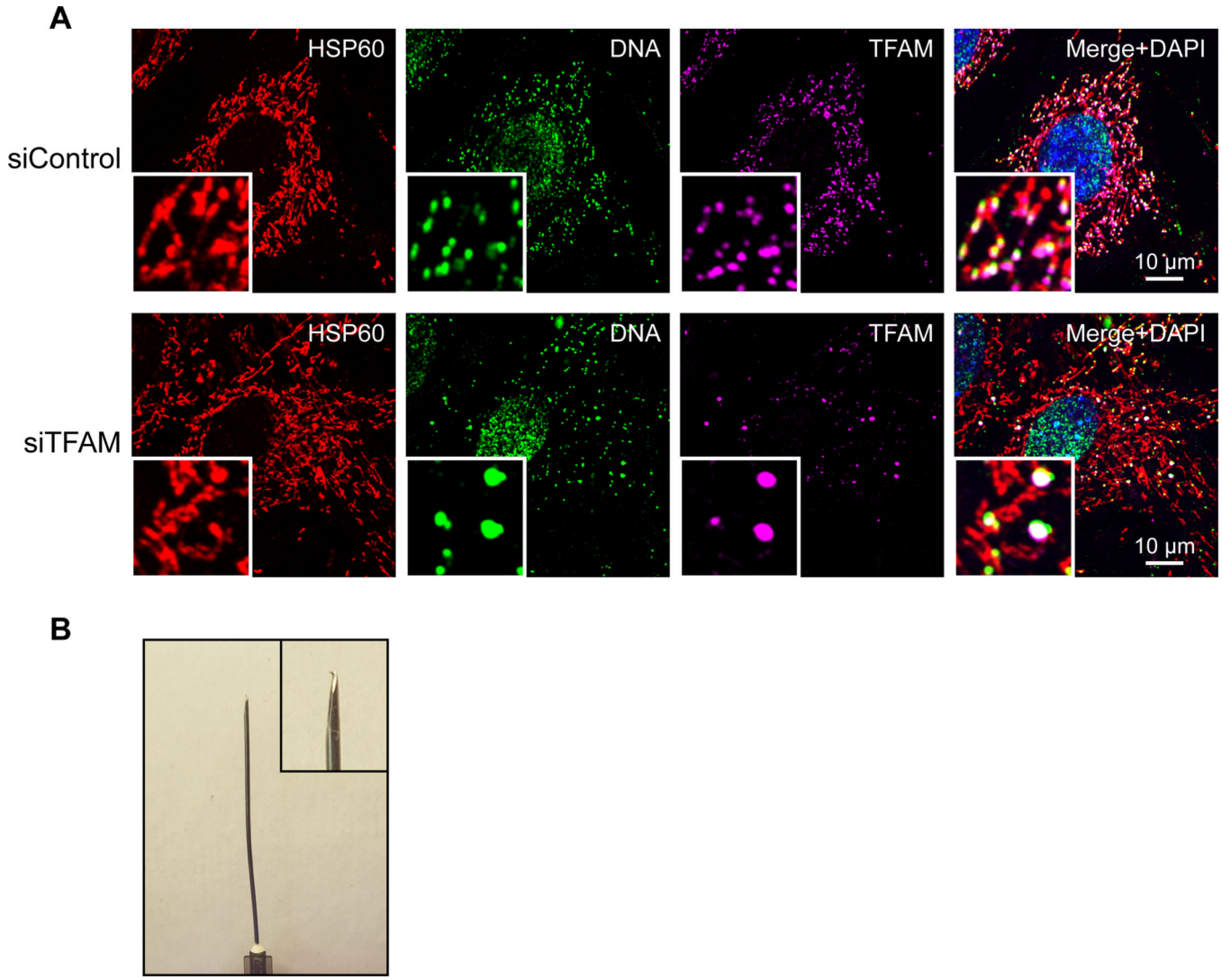

Figure 3: Knockdown of transcription factor A, mitochondrial (TFAM) results in mitochondrial DNA (mtDNA) nucleoid enlargement and mitochondrial network elongation in mouse fibroblasts.

(A) Mouse embryonic fibroblasts (MEFs) were mounted on coverslips and transfected with Control (siControl) or TFAM (siTFAM) siRNAs for 72 hrs. 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 60X oil-immersion objective. Inset panels represent 3X digital zoom. (B) Example image of how to bend the tip of a 21G needle to aid in picking up coverslips.

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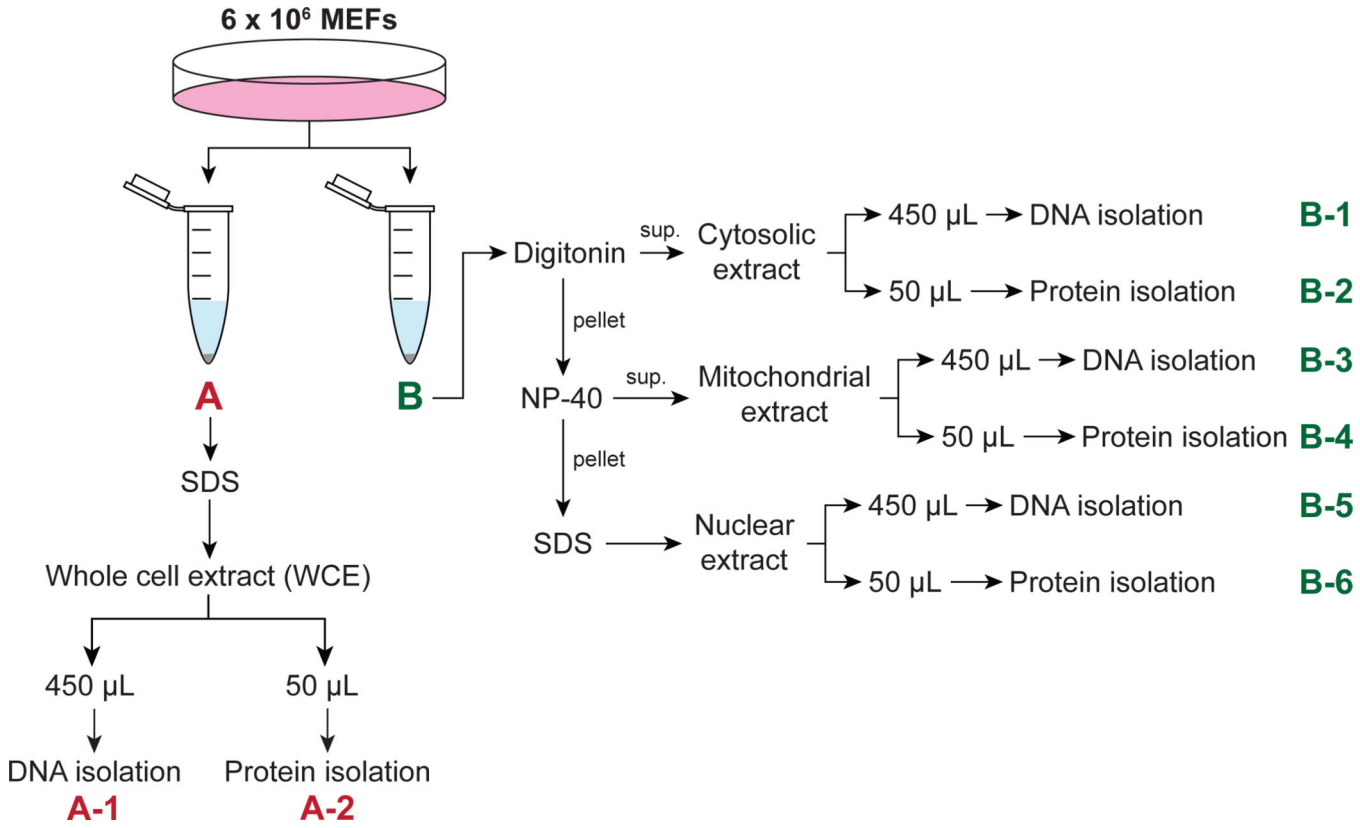

Figure 4: Graphical overview of the experimental workflow described in Basic Protocol 2.
Western blot (WB); Nonidet P-40 (NP-40); sodium dodecyl sulfate (SDS); supernatant (sup.); mouse embryonic fibroblasts (MEFs).

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A

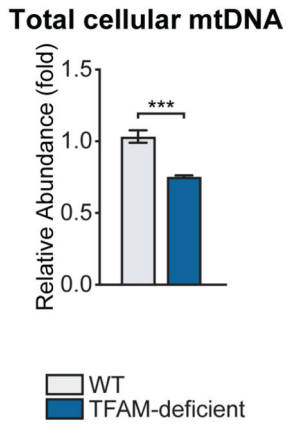
	Whole Cell Extract Cq values		Cytosolic Extract Cq values
	TERT	D-Loop	D-Loop
WT	21.67	14.10	25.65
	21.93	14.20	25.30
	21.84	14.30	24.96
TFAM-deficient	21.82	14.69	23.69
	21.84	14.69	24.03
	21.76	14.56	23.81

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)

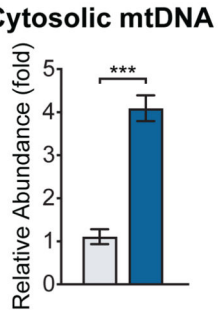


Figure 5: Transcription factor A, mitochondrial (TFAM) deficient cells exhibit elevated levels of mitochondrial DNA (mtDNA) in the cytosol.

(A-C) TFAM knockdown mouse embryonic fibroblasts (MEFs) were subjected to subcellular fractionation as described in Basic Protocol 2. DNA was isolated from whole cell (WCE) and cytosolic extracts, and subjected to SYBR Green-based qPCR analysis to quantitate nuclear (TERT) and mitochondrial (D-loop) DNA using specific primers. Raw Cq values from WCE and cytosolic extracts are shown (A). Raw values and formulas for calculating total cellular mtDNA abundance in WCE are shown, and plotted on the right, as mean values (B). Raw values and formulas for calculating mtDNA abundance in cytosolic

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extracts are shown, and plotted on the right, as mean values (C). N=3. Statistical significance was determined using unpaired Student's *t* test after Shapiro-Wilk normality test. **P < 0.01 and ***P < 0.001. Error bars represent standard error of the mean.

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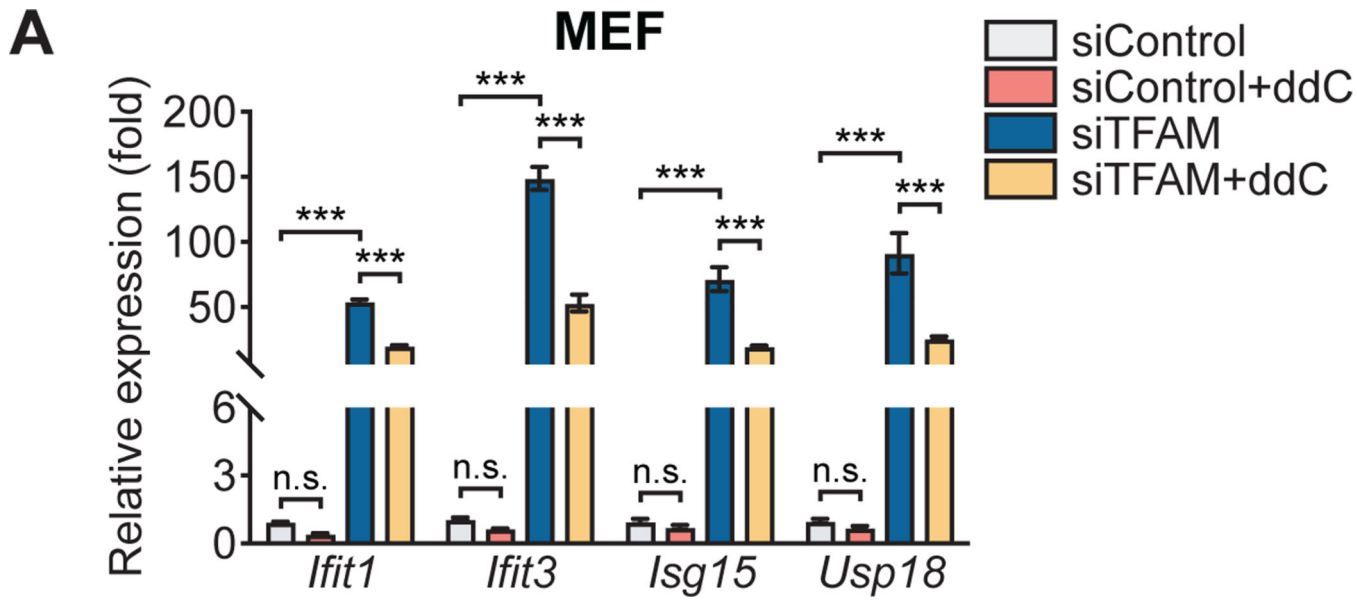

Figure 6: Depletion of mitochondrial DNA reduces interferon-stimulated gene (ISG) expression induced by cytosolic mtDNA.

Mouse embryonic fibroblasts (MEFs) (A) or human foreskin fibroblasts (HFFs) (B) were transfected with Control (siControl) or Transcription Factor A, mitochondrial (TFAM) (siTFAM) siRNAs for 72 hrs in the presence or absence of 150 μM 2',3′-Dideoxcytidine (ddC). RNA was extracted and reverse transcribed into cDNA, and cDNA was then subjected to SYBR Green-based qPCR analysis to profile ISG (*Ifit1, Ifit3, Isg15, Usp18*) expression. Plots show mean fold change and error bars represent standard error of the mean. N=3. Statistical significance was determined using unpaired Student's *t* test after Shapiro-Wilk normality test. **P < 0.01 and ***P < 0.001.

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Table 1 –

Optimized primer sequences to assess ISG expression in human and mouse cells by RT-qPCR.

Name	Species	RefSeq ID	Use	Forward sequence 5′–3′	Reverse Sequence 5′–3′
Cmpk2	Mouse	NM_020557.4	ISG	AAAGAATCAACCAACTTT	GGCCTCCACTCACCTCAGTA
Ifi44	Mouse	NM_133871.3	ISG	CTGATTACAAAAGAAGACATGACAGAC	AGGCAAAACCAAAGACTCCA
Ifit1	Mouse	NM_008331.3	ISG	CAAGGCAGGTTTCTGAGGAG	GACCTGGTCACCATCAGCAT
Ifit3	Mouse	NM_010501.2	ISG	TTCCCAGCAGCACAGAAAC	AAATTCCAGGTGAAATGGCA
Rsad2	Mouse	NM_021384.4	ISG	ATAGTGAGCAATGGCAGGCCT	AACCTGCTGATGCAAGCTGT
Zbp1	Mouse	NM_021394.2	ISG	TCAAAGGGTGAAGTCATGGA	GTGGAGTGGCTTCAGAGCTT
Gapdh	Mouse	NM_008084.3	Control	GACTTCAACAGCAACTCCCAC	TCCACCACCCTGTTGCTGTA
Rpl37	Mouse	NM_026069.3	Control	CATCCTTTGGTAAGCGTCGCA	TGGCACTCCAGTTATACTTCCT
CMPK2	Human	NM_207315.4	ISG	ACCCAGTCAGTGGCAGATTC	TGAGCAGCAGGATAAGGTCA
IFIT44l	Human	NM_006820.4	ISG	CAATTTAAGCCTGATCTAACCCC	CAGTTGCGCAGATGATTTTC
IFIT1	Human	NM_001548.5	ISG	GCAGCCAAGTTTTACCGAAG	GCCCTATCTGGTGATGCAGT
IFIT3	Human	NM_001549.6	ISG	TGGGAACAGCAGAGACACAG	AAGTTCCAGGTGAAATGGCA
RSAD2	Human	NM_080657.5	ISG	GCCAAAACATCCTTTGTGCT	TGGCTCTCCACCTGAAAAGT
ZBP1	Human	NM_030776.3	ISG	TGGACACGGGAACATCATTA	GAATCACCTGGTGCCATTG
GAPDH	Human	NM_002046.7	Control	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
RPL37	Human	NM_000997.5	Control	AGTGCCTTCTCTTCCGGTCT	TTCCAAACGATGACGTTCC

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Table 2 –

Suggested antibodies to assess cGAS-STING pathway activation, ISG expression, and cellular fractions by western blot.

Name	Use	Company	Catalog number	WB Dilution	Species Reactivity
cGAS	ISG	Cell Signaling	31659	1:1,000	M only
cGAS	ISG	Cell Signaling	15102	1:1,000	H only
GAPDH	Cytosolic loading control	Proteintech	60004-1-Ig	1:1000	H, M
IFIT2	ISG	Proteintech	12604-1	1:1,000	H, M
IFITM1	ISG	Proteintech	60074-1-Ig	1:5,000	H
IRGM1	ISG	Cell Signaling	14979	1:1,000	M
ISG15	ISG	Cell Signaling	2743	1:1,000	H, M
LAMIN B1	Nuclear loading control	Proteintech	66095-1-Ig	1:1000	H, M
RIG-I	ISG	Cell Signaling	4200	1:1,000	H, M
STAT1	ISG	Cell Signaling	9172	1:1,000	H, M
STAT2	ISG	Cell Signaling	72604	1:1,000	H, M
TFAM	Mitochondrial loading control	Proteintech	22586-1-AP	1:2,000	H
TFAM	Mitochondrial loading control	Millipore	ABE483	1:1,000	M
ZBP1	ISG	Adipogen	AG-20B-0010	1:1,000	M
β-Actin	Cytosolic loading control	Proteintech	66009-1-Ig	1:5,000	H, M
Calnexin	ER loading control	Proteintech	10427-2-AP	1:1,000	H, M
HSP60	Mitochondrial loading control	Santa Cruz	sc-1052	1:5,000	H, M
VDAC1	Mitochondrial loading control	Proteintech	55259-1-AP	1:2000	H, M

Species Reactivity Key H: Human **M**: Mouse

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Table 3 –

Primary antibody dilution for immunofluorescence staining of mtDNA.

Primary Antibody	Host Species	Suggested Dilution	Supplier	Species Reactivity
Anti-DNA	Mouse	1:300	Millipore, cat. no. CBL186	H, M, R
Anti-HSP60	Goat	1:300	Santa Cruz, cat. no. sc-1052	H, M, R
Anti-TFAM	Rabbit	1:800	Millipore, cat. no. ABE483	M
Anti-TFAM	Rabbit	1:800	Proteintech, cat. no. 22586-1-AP	H

Species Reactivity Key H: Human **M**: Mouse **R**: Rat

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Table 4 –

Secondary antibody dilution for immunofluorescence staining of mtDNA.

Suggested Secondary Antibody	Conjugate	Suggested Dilution	Supplier
Anti-Mouse IgM, μ chain specific	Rhodamine Red™ X	1:600	Jackson ImmunoResearch Labs, cat. no. 715-295-020
Anti-Goat IgG (heavy + light chains)	Alexa® 647	1:600	Jackson ImmunoResearch Labs, cat. no. 805-165-180
Anti-Rabbit IgG (heavy + light chains)	Alexa® 488	1:800	Jackson ImmunoResearch Labs, cat. no. 711-545-152

IgM: Immunoglobulin M **IgG:** Immunoglobulin G

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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	CTAGCTCATGTGTCAAGACCCTCTT	GCCAGCACGTTTCTCTCGTT
MT-D-Loop	Mouse	mtDNA control region, non-coding	mtDNA	AATCTACCATCCTCCGTGAAACC	TCAGTTTAGCTACCCCCAAGTTTAA
MT-CYTB	Mouse	17711	mtDNA	GCTTTCCACTTCATCTTACCATTTA	TGTTGGGTTGTTTGATCCTG
MT-RNR2	Mouse	17725	mtDNA	CTAGAAACCCCGAAACCAAA	CCAGCTATCACCAAGCTCGT
KCNJ10	Human	3766	Nuclear DNA control	GCGCAAAAGCCTCCTCATT	CCTTCCTTGGTTTGGTGGG
MT-ND1	Human	4535	mtDNA	GAACTAGTCTCAGGCTTCAACATCG	CTAGGAAGATTGTAGTGGTGAGGGTG
MT-D-Loop	Human	mtDNA control region, non-coding	mtDNA	CATAAAGCCTAAATAGCCCACACG	CCGTGAGTGGTTAATAGGGTGATA

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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 transected
Poor TFAM knockdown	Inefficient siRNA transfection	Increase ratio of RNAiMAX: siTFAM
		Increase amount of siTFAM transected
Poor ISG induction	Insufficient TFAM knockdown	See above
	Cells do not express cGAS and/or STING	Use primary cells (e.g., MEFs, macrophages or 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 digitonin lysis buffer the day of with a freshly made stock of digitonin.
		Increase incubation time with digitonin lysis buffer or increase digitonin concentration (Basic Protocol 2, step 10–11)
	Incomplete washing	Complete all washes before moving on to next step in procedure. During each wash, lightly agitate tubes by inversion to increase stringency of wash
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 (Basic Protocol 2, step 10–11)
	Centrifugation speed too high, leading to ruptured mitochondria	Reduce centrifugation speed (Basic Protocol 2, steps 12 and 13)
	Incomplete clearing of mitochondria from cytosolic fraction	Repeat centrifugation step, making sure not to transfer any of the pellet to the new tube (Basic Protocol 2, step 13)
Mitochondrial contamination in nuclear fraction	Incomplete washing	Complete all washes before moving on to next step in procedure. During each wash, lightly mix by inversion to increase stringency of wash
Nuclear contamination in mitochondrial fraction	Centrifugation speed too high, leading to ruptured nuclei	Reduce centrifugation speed (Basic Protocol 2, step 16)
ddC treatment does not reduce ISG expression	Incomplete depletion of mtDNA	Increase ddC concentration
		Change media with fresh ddC each 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