Mitochondrial RNA stimulates beige adipocyte development in young mice
### Supplementary Table 1. Mouse qPCR primer sequences used in the study (Merck)

| Gene   | Forward Primer (fw) | Reverse Primer (rev) |
|--------|---------------------|----------------------|
| Bactin | GCACCAGGGTGATGATG   | CCAAGTTCTCATGCTGTTA  |
| Ppia   | ATTTCTTTGACTTGCGG   | AGACTTGAAGGGGAATG    |
| Gapdh  | TGACGTGCCCGCTGAGAA  | AGTGTAGCCAAGATGGCCT  |
| Aim2   | AGTTCAAAGTCAGGTG    | TCTGAGGGTACGCTGAGAC  |
| Ddx41  | ACAGGAGAACGGTGCTTTC | GACGCCAGTAATATCTCAG   |
| Ifi204 | CAGGGAAATAGGAGGTTG  | CAGAGGGTTCTCCAGGATG   |
| Zbp1   | AACCCTCAATCAAGTCCT  | TCTTCCAGCTGCTGCCATAG |
| Mb21d  | AGGAAAGCCTGCTGAAACT | AGCCAGGCTTGAAAGGTTAG |
| Tmem173| GGGGCCGTCACTTTGTC   | CCAGGTAGCTATGGTACTCC |
| Irf3   | GGCTTTGATGAGGTTCAAG | CATGTCTCTCCACAAAGTCT |
| Irf7   | CGACTTCAAGCATTCTCCG | AGGTGAGAAGGTTAGGTTAG |
| Il6    | GCTACCAAATGGATATAAT | GAATGAGTCTAGGGTTGT   |
| Tnfa   | TGCCATGTCTCAGCTCTTC | GAGGCCATTTGGGAACTT   |
| Ifnb   | CCAGCTCAAAGAAGGAGCA | CGCCCTGTAGTGAGGTTGAT |
| Ifna   | TGAAGGCAAGGAAAGGACTT | GAATGAGTCTAGGAGGTTG  |
| Rn16s  | ACACCCAGATGGCCTAAAG | ATACCGCGGCCCTAAACTT  |
| Nd1    | GCCGCTAGGCCTAGGCAA  | GGTCAGCAGCTGAGGAAGTA |
| Co3    | CCAAGGCCACCAACTCACT | GTGACGAGCTCCTGAGATCA |
| Ucp1   | CCTGGCTCTCTCGGAAACA | CTGTTACGGACGGTGATCC  |
| Ppargc1| GACCTAGTGCCACCAAGGC | TGAAACGAGAGCCATCCTT  |
| Cox7a1 | ATGAGGCGCCTACGGGTC | CATTGTCGGCCTGGAAGAG |
| Cidea  | TACTACCGGTCATCCATT  | ATCACAACCTGCGTGTTACG |
| Dio2   | GTCCGGAAAATGACCCCTT | CCCACCACCTCTGACTTTC |
Supplementary Information

| Target   | Primers                                             |
|----------|-----------------------------------------------------|
| Ifi205   | fw CAAGCAGGCCACTTCTGTGGA<br>rev TCAAAACGQTCTGGTTCAGT |
| Ddx58    | fw CAAACGGGCAACAGGAAAGT<br>rev ATCTCCGCTGCTCCTGAAATG |
| Zic1     | fw TTTCCCTGGCTCGGCCAAGGT<br>rev ACGTGCATGTCTTTGCGCTG |
| Fasn     | fw GGAGGTTGCTGATAGGCCTGAT<br>rev TGGTAATCCCATAGACCCGA |
| Prdm16   | fw ATCCACAGCGACGTAAGCCTG<br>rev ACATCTGCCACAGTCTTTGCA |
| Mda5     | fw GTCTTCTGCTGTGGGT<br>rev CAAACTTTGTTGCTTCTGTG |
| Cyp27b1  | fw AGCTCCTGCGACAAGGAAGT<br>rev ATCTTCACCACAGTCTTTGCA |
| Vdr      | fw ACTTGGACGGGTATGTCCT<br>rev CATGCTCCGCTGAAGAAC |

Supplementary Table 1. (cont.) Primers for measuring mouse mtDNA copy number

| Target | Primers                                             |
|--------|-----------------------------------------------------|
| Nd1    | fw GCTTTACGAGCCGTAGCCA<br>rev GGTCAGGCTGGCAGAAT |
| 16S    | fw ACACCGGAATGCCTAAAGGA<br>rev ATACCGCGGCCGTTAACTT |
| HK2    | fw GCCAGGCTCCTTCTGTATTTAGTG<br>rev GGGAACACAAAGACCT |

Supplementary Table 2. Antibodies used in the study (h, human; m, mouse)

| Target        | Cat. No.   | IgG type, source                                      |
|---------------|------------|-------------------------------------------------------|
| h/m STING     | NBP2-24683 | Rabbit polyclonal, 1:250 Novus Biologicals, Denver, CO |
|               | 13657      | Rabbit monoclonal, 1:250 Cell Signaling Technology, Danvers, MA |
| h/m AIM2      | 201708-T10 | Rabbit polyclonal, 1:125 Sino Biological, Eschborn, Germany |
| h/m DDX41     | 102459-T32 | Rabbit polyclonal, 1:50 Sino Biological, Eschborn, Germany |
| h/m p204 (IFI16) | NBP2-27153 | Rabbit Polyclonal, 1:100 Novus Biologicals, Denver, CO |
| h/m ZBP1      | 207744-T08 | Rabbit polyclonal, 1:150 Sino Biological, Eschborn, Germany |
| h/m LC3       | L8918      | Rabbit polyclonal, 1:500, Merck Sigma-Aldrich St. Louis, MO |
| h/m UCP1      | PA1-24894  | Rabbit polyclonal, 1:200 ThermoFisher Scientific, Rockford, IL |
| h/m DDX41     | 102459-T32 | Rabbit polyclonal, 1:150 Invitrogen, Carlsbad, CA |
| J2 (dsRNA)    | Anti-dsRNA [J2] | Mouse monoclonal, 1:100, clone J2 Absolute Antibody, Wilton, UK |
| m IRF7        | 12-5829-82 | PE-conjugated monoclonal IgG, clone MNGPKL, and matching isotype IgG, 1:500, ThermoFisher, Waltham, MA |
| h/m IRF7      | PA5-20280  | PE-conjugated monoclonal IgG, clone MNGPKL, and matching isotype IgG, 1:500, ThermoFisher, Waltham, MA |
Supplementary Information

Supplementary Methods

Activation and inhibition of cytosolic DNA/RNA sensors

To stimulate RIG-I/MDA5, we transfected adipocytes at 80% confluency with high molecular weight polynucleosine-polycytidylic acid (p(I:C)) or poly(deoxyadenylic-deoxycytidylic) acid (p(dA:dT)) using the LyoVec cationic lipid-based transfection reagent (InvivoGene, Toulouse, France). Control cells were treated with LyoVec transfection reagent only. We used 0.5-5 µg/ml p(dA:dT) or p(I:C), and cells were analyzed 2–24 h after transfection. IFI16/p204/IFI205 was activated with 1 µg/ml VACV-70 conjugated to LyoVec transfection reagent (InvivoGene; 18 h) \(^1\). To activate STING, we treated adipocytes with cGAMP (InvivoGene, Toulouse, France) for 18 h. Treatments are summarized in the table below. *Irf3* expression was suppressed by siRNA silencing, using oligos from Thermo Fisher (Silencer™ Pre-Designed siRNA, Cat. No.: AM16708, siRNA ID: 184585).

### Activation of cytosolic nucleic acid sensors with various ligands

| Receptor | Ligand | EC\(_50\) | Applied concentration |
|----------|--------|----------|----------------------|
| **STING** | 2’3 cGAMP | 20 nM | 10 µg/ml |
| cGAS | poly(dA:dT) | 40-200 ng/ml | 0.5-5 µg/ml |
| | human/mouse mtDNA | - | 2 µg/ml |
| | pCMV6 circular DNA | - | 1 µg/well |
| **RIG-I** | 3p-hpRNA | 5 ng/ml | 0.5 µg/ml |
| | 5’ppp-dsRNA | 1.2 nM | 1 µg/ml |
| | poly(I:C) HMW | 70±10 ng/ml | 0.5 µg/ml |
| **RIG-I and MDA5** | poly(dA:dT) 18 h low molecular weight poly(I:C) | 82±8 ng/ml | 2.5-5 µg/ml |
| | human/mouse mtRNA | - | 2 µg/well |
| **AIM2** | poly(dA:dT) | 40-200 ng/ml | 2.5-5 µg/ml |
| **DDX41** | poly(dA:dT) | 40-200 ng/ml | 2.5-5 µg/ml |
| | dsDNA (VACV-70) | - | 1 µg/ml |
| **IFI16 (p204 or IFI205)** | poly(dA:dT) | 40-200 ng/ml | 2.5-5 µg/ml |
| | dsDNA (VACV-70) | - | 1 µg/ml |
| **ZBP1** | poly(dA:dT) | 40-200 ng/ml | 2.5-5 µg/ml |
TLR3 was stimulated with naked p(I:C) (Sigma-Aldrich, 10 ng/ml, 18 h). As negative control we used ssRNA (InvivoGene). NFkB was inhibited with 5 µM BAY 11-7082 and JAK2/STAT3 with 280 nM ruxolitinib (Cayman Chemical Company, Ann Arbor, MI).

Vit-D3 and calcitriol were purchased from Sigma-Aldrich; IFNβ, IL-4, IL-6 and from ImmunoTools (Friesoythe, Germany). Isoproterenol and fumonisin B1 were purchased from Sigma-Aldrich and from Cayman Chemical Company, respectively. To test the inhibitory effect of Vit-D3 on IRF7 signaling, adipocytes were treated with 1 µM Vit-D3 for 48 h, and were transfected with mtRNA for 18 h. VDR was inhibited with PS121912, as described 2. Cellular uptake of cGAMP is dependent on the transporter Slc19a1 3, the level of which was similar in P6 and P56 adipocytes (GEO submission #GSE154925).

**ELISA assays**
Tissue samples were weighed and homogenized in RIPA buffer using a Roche bead mill homogenizer at 6,500 rpm for 1 min. Cell culture supernatants and plasma samples were centrifuged at 0.8 g for 10 min to remove cell debris, and supernatants were used for analysis. We used commercial ELISA kits to measure the levels of IL-6, TNFα (Fisher Scientific), IRF7, Vit-D3, calcitriol and VDR (MBS1605109, MBS268259, MBS288322, MBS2701844, MyBioSource). All samples were stored at -80°C until analysis.

**mtRNA isolation and in vitro transfection**
Adipocyte mitochondria were isolated with a commercial mitochondrial isolation kit (Thermo Fisher Scientific, Waltham, MA), using mouse adipocytes or human THP1 cells. Mitochondrial RNA (mtRNA) was isolated by lysing the mitochondrial pellet with TRI Reagent (Sigma-Aldrich), as described 4. Adipocytes were transfected with 2 µg of mtRNA in 6- or 24-well plates with cells at 80–90% confluency. As a transfection reagent we used Lipofectamine 3000 (Invitrogen) at a 1:3 ratio. Control cells received transfection reagent only. Cells were analyzed 18 h after transfection.

**mtDNA isolation and transfection**
Mitochondrial DNA (mtDNA) was isolated from mitochondria pellets using TRI Reagent (Merck Sigma-Aldrich) and reconstituted in TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0). Agarose gel electrophoresis was used to examine mtDNA integrity. Adipocytes were transfected for 18 h with 1-2 µg/ml mtDNA using the TurboFect Transfection Reagent. Control cells received transfection reagent only.

**Cytosolic mtRNA and mtDNA isolation**
Cytosol fractions of adipocytes were collected by subcellular fractionation of the cytoplasm and the cell organelles using digitonin, as described 5,6. Digitonin buffer contained 150 mM NaCl, 50 mM HEPES (pH 7.4) and 25 µg/ml digitonin (D141, Merck Sigma-Aldrich). Treated cells were processed until the step in which cytosol was obtained as described 4. Cytosolic fractions (250 µl) were added to 750 µl TRI Reagent (T3934, Merck Sigma-Aldrich) and total DNA and RNA extraction was performed as described 7. Isolated RNA was reverse transcribed into cDNA, *Bactin* and *Ppia* were used as reference genes. DNA was reconstituted in TE buffer and adjusted to 10 ng/µl. We performed qPCR using HK2 as a
reference nuclear genome-encoded gene, and measured the DNA copy number of mtDNA-encoded genes. We calculated the copy number according to the formula:

\[ \Delta C_t = C_{\text{Target gene}} - C_{\text{Reference gene}} \]
\[ \text{mtDNA copy number} = 2 \times 2^{\frac{\Delta C_t}{2}} \]

**Histology, immunofluorescence and image analysis**

Tissues were fixed with 4% paraformaldehyde and embedded in paraffin, as described (1). Sections were stained with hematoxylin and eosin (Carl Roth, Karlsruhe, Germany). Antibodies are listed in Supplementary Table 2. UCP1, IFI16, AIM2 immunohistochemistry was performed on paraffin-embedded tissue sections. For histomorphometry of fat cells we used Image J, with an image-processing algorithm that incorporated the Euclidean distance-based Watershed transformation to segment the images. Briefly, binarized images were generated using Otsu’s method for thresholding; enhanced images were generated using contrast limited adaptive histogram equalization (CLAHE), and finally segmented images were generated using the Watershed transformation (Extended Data Figure 8). For fluorescent microscopy of STING, cGAS, AIM2, DDX41, IFI205 and ZBP1 murine or human preadipocytes were grown on optical transparent glass-bottom plates (Greiner Bio-One GmbH, Frickenhausen, Germany) or glass coverslips, and labeled with antibodies listed in Supplementary Table 2. Negative control specimens of our fluorescent imaging and immunostaining are shown in Extended Data Figure 8. Histology images were adjusted to equal white balance after acquisition. Mitochondrial content and morphology was analyzed with ImageJ, as described (4). Beige adipose area was measured with our custom-developed image analysis software (BeAR™ v1.0, 4).

**Quantification of UCP1 staining**

*In vitro* UCP1 immunostaining was performed in 6-well culture plates, and samples were imaged and the optical density was measured using digital image analysis. Original images are available upon request through Figshare. Mitochondria were also labeled using an SDH-A histochemistry assay (BioOptica).

**Adipocyte differentiation**

Mouse or human preadipocytes of the stromal vascular fraction (SVF) were isolated and maintained as described (7-9). To ensure the depletion of adipose tissue macrophages (ATMs) from the harvested preadipocytes, we used magnetic bead cell purification of the SVF with an antibody against the F4/80 antigen (Miltenyi Biotec, Bergisch Gladbach, Germany) (10). Human subcutaneous adipose tissue preadipocytes were harvested as described (7,8). Preadipocytes were maintained in cell culture medium supplemented with 20 μg/ml insulin. To induce white differentiation of preadipocytes, we treated the cells with 50 μM IBMX, 1 μM dexamethasone, 1 μM rosiglitazone and 20 μg/ml insulin (all from Merck Sigma-Aldrich), as described (4). Lipid content was labeled with Oil red O.

**Flow cytometry analysis of DNA sensors, mitochondrial biogenesis, mitochondrial content and mitochondrial uncoupling**

Mitochondrial content was analyzed with MitoTracker dyes (Thermo Fisher Scientific). Mitochondrial biogenesis was detected with the MitoBiogenesis™ Flow Cytometry Kit.
(Abcam, Cambridge, UK). MitoThermo Yellow (MTY), a temperature-sensitive fluorescent probe, was used to assess mitochondrial thermogenesis and uncoupling, as described. Temperature difference between the control and the test groups was expressed as Mito-ΔT, and shown in the respective figures. MTY was developed and provided by Dr. Y-T. Chang (Center for Self-Assembly and Complexity, Institute for Basic Science & Department of Chemistry, Pohang University of Science and Technology, Pohang 37673, Republic of Korea). We used MTY for FACS analysis at 0.1 ng/ml to label 10⁶/ml cells. Cells were maintained at 37°C throughout the assay. DNA sensors (STING, p204, AIM2, DDX41) were detected with unconjugated antibodies (listed in Supplementary Table 2) and labeled with an FITC-conjugated secondary antibody for FACS analysis. Nucleic acids were labeled with Sytox Green (Thermo Fisher). Flow Repository identifiers of raw FACS data are as follows: #FR-FCM-Z236, #FR-FCM-Z2R6, #FR-FCM-ZYPU, #FR-FCM-ZYUU, #FR-FCM-Z5QA.

**Imaging of mitochondrial content**
For fluorescent microscopy of mitochondrial content and morphology, preadipocytes were grown on optical transparent glass-bottom plates (Greiner Bio-One GmbH, Frickenhausen, Germany) or glass coverslips. Functional mitochondria were labeled with MitoTracker Red. Mitochondria were also labeled with GFP using the BacMam 2.0 transfection system (Fisher Scientific). Mitochondrial respiration was evaluated with the WST-81 assay (Carl Roth), as described. SDH-A and COX-I level was measured with flow cytometry and spectrophotometry (Abcam MitoBiogenesis Kits). Inflammasome activity was measured with the Caspase-Glo 1 Inflammasome Assay (Promega Co., Madison, WI).

**High-fat diet feeding and indirect calorimetry**
Respiratory exchange rate (RER), oxygen consumption (VO₂) and energy expenditure (EE) were measured in each individual mouse for 24 h using a small animal indirect calorimetry system (CaloBox, Phenosys, Germany). Mean RER, VO₂ and EE values were determined over 7 h in the middle of both the day and the night phases. Basal glucose levels and glucose tolerance were measured as described. For HFD-feeding of mice (dams with litters P6 to P9, or mice at P28 for 12 weeks) we used a rodent HFD from SSNIFF Spezialdiäten (Soest, Germany, E15725-347). Vit-D3 was supplemented in diet, mtRNA was transfected in the iAT with magnetofection for 14 days. As a normal chow diet we used rodent diet from SSNIFF Spezialdiäten (Soest, Germany, E15051-047).

**Magnetofection of mtRNA**
In vivo delivery of mtRNA into the cytosol of adipocytes was achieved with magnetofection, using mtRNA–magnetic nanoparticle complexes (DogtorMag, OzBiosciences, San Diego, CA). Briefly, 15 μg mtRNA-nanoparticle complexes were injected into the iAT of mice, and enrichment of the magnetic nanoparticles was ensured by magnetic exposure of the fat depot, as described.

**Cell viability assay**
We used the Presto Blue Cell Viability Assay (Thermo Fisher Scientific) and the Rotitester Vital (Carl Roth) assays.
Institutional Review Board Statement
Research involving animals was approved by the regional governmental ethics and animal welfare committee in Tübingen, Germany (#1511; #1557; #1492; #1546; #0.232-1,2,4,5).

Software
We used the following software for data collection and analysis: QuantStudio™ Design & Analysis Software, Thermofisher Scientific v1.5.0 (for qPCR); Leica Application Suite X Leica 3.7.2.22383 (for microscopy of immunofluorescence); Olympus CellSense Entry Olympus 2.3 (for histology imaging and cell culture imaging); Leica Application Suite Z Leica 3.4.0 (for histology imaging); AUTOsoft Autobio Co. LTD 2.6.9 (for ELISA); Fusion FX6 Edge Vilber 18.02 and NanoDrop 2000/2000c Thermofisher Scientific 1.6.198 (for RNA quantity); RNAfold WebServer (University of Vienna, 2.4.18, for RNA structure prediction); ImageJ, Open Source, 1.52v (for histomorphometry), Motic Images Plus 3.0 V137 (for histology imaging), Adobe Photoshop and Adobe Illustrator (for figure assembly).

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