Mitochondrial RNA granules are fluid condensates positioned by membrane dynamics

Timo Rey, Sofia Zaganelli, Emilie Cuillery, Evangelia Vartholomaiou, Marie Croisier, Jean-Claude Martinou and Suliana Manley

Mitochondria contain the genetic information and expression machinery to produce essential respiratory chain proteins. Within the mitochondrial matrix, newly synthesized RNA, RNA processing proteins and mitoribosome assembly factors form punctate sub-compartments referred to as mitochondrial RNA granules (MRGs). Despite their proposed importance in regulating gene expression, the structural and dynamic properties of MRGs remain largely unknown. We investigated the internal architecture of MRGs using super-resolution localization microscopy and correlated electron microscopy, and found that the MRG ultrastructure consists of compacted RNA embedded within a protein cloud. Using live-cell super-resolution structured illumination microscopy and fluorescence recovery after photobleaching, we reveal that MRGs rapidly exchange components and undergo fusion, characteristically properties of fluid condensates. Furthermore, MRGs associate with the inner mitochondrial membrane and their fusion coincides with mitochondrial remodelling. Inhibition of mitochondrial fission or fusion leads to an aberrant accumulation of MRGs into concentrated pockets, where they remain as distinct individual units despite their close apposition. Together, our findings reveal that MRGs are nanoscale fluid compartments, which are dispersed along mitochondria via membrane dynamics.

RNA in eukaryotic and bacterial cells can be sequestered into ribonucleoprotein granules that exhibit a wide range of forms and functions, under both physiological and stress conditions. For example, in the nucleus, speckles and paraspeckles are involved in RNA splicing and transcriptional regulation, whereas the nucleolus creates a compartment for ribosomal assembly. RNA-protein granules often form by liquid–liquid phase separation (LLPS), and multivalent weak interactions between disordered RNA-binding protein (RBP) domains, and RNA itself, have been identified as hallmark factors for the formation of biomolecular condensates in many in vitro and in silico studies. However, phase behaviour is sensitive to a number of environmental parameters, such as concentration, ionic strength, pH or crowding, native conditions of which are challenging to reproduce in test tubes. Therefore, studies in living cells are fundamental to understanding the formation mechanisms, which may go beyond LLPS, and biological functions of such structures.

In mitochondria, mitochondrial RNA granules (MRGs) comprise newly synthesized mitochondrial RNAs (mtRNAs), transcribed from the 16-kb mitochondrial DNA (mtDNA) as long polycistronic precursors, as well as mitochondrial RBPs. It was previously demonstrated that mtRNA is essential for MRG formation. However, both the structural organization of and the dynamic interplay between MRG components remain unknown. Mitochondria undergo dramatic shape changes through fusion, fission and branching, of which fission directly impacts the distribution of mtDNA. How MRGs respond to this dynamicity and complex architecture is also unknown, due in part to their size (below the diffraction limit). Here, we investigate the molecular organization, distribution and positioning mechanism of MRGs within the mitochondrial network, using super-resolution and correlated fluorescence and electron microscopy. We show that MRGs are ~130-nm, sub-compartmentalized liquid condensates. They associate with the inner mitochondrial membrane (IMM) and mislocalize following perturbation of mitochondrial fission and fusion dynamics.

To assess the MRG dimensions and overall organization, we examined two MRG-associated RBPs—FASTKD2 and GRSF1 together with mtRNA in fixed COS-7 cells. We stained endogenous proteins using immunofluorescence and newly synthesized RNA by incubation with 5 mM bromouridine (BrU) for 1 h, and anti-BrU staining as previously described. We found that the MRG RNA within the MRGs occupied a region with a median diameter of 92 nm (±40 nm s.d.), n = 431; Fig. 1b and Supplementary Fig. 1). To assess the MRG shape we determined the ratio between the long and short axes for each granule and measured a median eccentricity of 1.7 (±0.6 s.d.) for mtRNA (Extended Data Fig. 1). We found that the mtRNA within the MRGs occupied a region with a median diameter of 88 nm (±32 nm s.d.), n = 310; Fig. 1b and Supplementary Fig. 2), when stained with antibodies against mtDNA. This is consistent with previous reports of ~100-nm nucleid diameters, as is our estimated eccentricity of 1.7 (±0.7 s.d.), reflecting a slightly ellipsoidal shape. Thus, mtDNA serves as an internal reference or positive control for our STORM-based size and shape measurements.

We then quantified the nanostructure of MRGs immunolabelled for GRSF1 or FASTKD2 (bona fide MRG markers), where mutations in the latter are associated with severe mitochondrial diseases. Our measurements showed that the MRG diameter marked by either protein is significantly larger than that of mtRNA: 139 nm...
for FASTKD2 and 123 nm (±31 nm (s.d., n = 338) for GRSF1 foci in COS-7 cells (Fig. 1b and Supplementary Figs. 3 and 4). Alternative size descriptors, including radius of gyration and convex hull area, showed the same trends (Extended Data Fig. 1). Furthermore, protein punctae are significantly rounder, with an eccentricity of 1.4 (±0.4 s.d.) and 1.5 (±0.5 s.d.) for FASTKD2 and GRSF1, respectively (Extended Data Fig. 1), underlining the differences between RNA and protein organization in MRGs.

To further investigate the MRG architecture and the relationship between mtRNA- and FASTKD2-foci dimensions, we next performed two-colour hSTORM (Fig. 1c and Extended Data Fig. 2). We found no correlation between the size (R = 0.26, n = 26) or eccentricity (R = 0.42) of the protein components and newly synthesized RNA for individual granules (Extended Data Fig. 2). Yet, in 80% of measured MRGs, the projected area of mtRNA was smaller and contained within (>80% overlap) the FASTKD2 area (Fig. 1d–f and Supplementary Table 1). These quantifications led us to schematize the ultrastructure of a typical MRG as compacted RNA surrounded by and commingled with RBPs (Fig. 1f).

Liquid phase properties, such as the ability to rapidly exchange components while maintaining a high local concentration of selected molecules, play a functional role in stress and other RNA granules by enabling mRNA sequestration, enzyme buffering and tuning of reaction kinetics. These roles may apply to MRGs and their function in gene expression, if they are indeed liquid-like. To test this hypothesis, we generated stable FASTKD2-eGFP expressing cell lines and assessed the common hallmarks of fluids: content exchange and droplet fusion. We examined the molecular exchange of MRG components by fluorescence recovery after photobleaching (FRAP). To monitor MRG fluorescence inside highly mobile mitochondria, we developed a software tool for FRAP analysis with motion tracking (Fig. 2a). We found FASTKD2-eGFP molecules within MRGs to recover on average to ~64% of their bleached intensity, with a median half-recovery time of 5.8 s (±3.3 s (s.d.)) and 4.6 s (±1.1 s (s.d.)) in U2OS and COS-7 cells, respectively (Fig. 2c, Extended Data Fig. 3 and Supplementary Videos 1 and 2). To test whether dynamic exchange is generalizable beyond FASTKD2, we created two additional stable cell lines expressing MRG markers—ERAL1 and DDX28—and both fused to eGFP. Both recovered at a timescale similar to FASTKD2 (half-recovery time of 1.9 s (±0.7 s (s.d.)) for ERAL1 and 3.1 s (±1.9 s (s.d.)) for DDX28) (Fig. 2c, Extended Data Fig. 3 and Supplementary Videos 3 and 4). For comparison, we overex-

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**Fig. 1** The nanoscopic architecture of MRGs consists of compacted RNA surrounded by RNA binding proteins. a, Wide-field immunofluorescence of FASTKD2 (left) shows punctate, diffusion-limited MRGs within mitochondria (white dashed lines). Scale bar, 10 μm. Zoomed view (centre) of a representative MRG: overlay of STORM (green) and wide-field images (grey-scale). Right: the MRG is segmented by its high density of STORM localizations (right) using DBSCAN. Scale bar, 200 nm. The images are representative of the dataset shown in b. b, MRG (mtRNA, FASTKD2 or GRSF1) and nucleoid (mtDNA) diameters determined as mean full-width at half-maximum (FWHM) from hSTORM images. The numbers (n) of clusters quantified for each condition are shown in the figure and are pooled from 24, 13, 7 and 14 fields of view (FOVs) and 4, 4, 3 and 2 samples for BrU, GRSF1, FASTKD2 and mtDNA, respectively. The median diameter and number of analysed clusters are noted. Box plots denote the first and third quartiles and the median; the whiskers comprise the rest of the distributions, except outliers. A two-sided Mann-Whitney U-test was used and **** denotes P values < 0.0001; NS, non-significant (P_{BrU-mtDNA} = 1, P_{BrU-FASTKD2} = 1.0 × 10^{-4}, P_{BrU-GRSF1} = 4.5 × 10^{-5}, P_{GRSF1-FASTKD2} = 3.1 × 10^{-3}). Representative images are shown on the right. Scale bar, 200 nm. c, Example of two-colour hSTORM of MRGs (anti-FASTKD2, green) and mtRNA (anti-BrU, blue) overlaid on wide-field images (grey). Scale bar, 500 nm. Images are representative of the dataset shown in f, d, Scatter plots of localizations from the clusters marked d and e in c. Areas were estimated using a convex hull (dashed line) and used to find the percent overlap between channels. Diameter and eccentricity were also quantified (Supplementary Information). Scale bars, 100 nm. f, Schema of MRG organization and median ± s.d. values for all analysed two-colour hSTORM clusters (n = 26 MRGs were examined over eight independent experiments). Statistical source data are provided in Source Data Fig. 1.
Fig. 2 | MRGs exchange content and undergo fusion in live CoS-7 cells. **a,b,** Examples of FRAP time-lapse series of MRGs (**a,** stable expression, FASTKD2-eGFP, green) or nucleoids (**b,** transient transfection, TWINKLE-eGFP, magenta), from the datasets represented in **c** and **d,** respectively. An individual MRG or nucleoid (arrowhead) was partially photobleached to allow tracking during recovery. Scale bars, 2 μm. **c,d,** FRAP intensity, where symbols represent mean data points, lines are single exponential fits, and shaded areas are standard deviations at each time point, for FASTKD2-eGFP (**n** = 44 MRGs examined over eight independent experiments) and ERAL1-eGFP (**n** = 17 MRGs examined over three independent experiments), DDX28-eGFP (**n** = 17 MRGs examined over three independent experiments) (**c**), and TWINKLE-eGFP (**n** = 50 nucleoids examined over five independent experiments) (**d**). **e,** Representative SIM time-lapse series of an MRG fusion event (white arrowheads) in cells stably expressing FASTKD2-eGFP. Cells were imaged at 1/3 Hz. Yellow and blue arrowheads highlight mitochondrial network dynamics (top). MRGs enriched in FASTKD2-eGFP stand out in yellow; pixel intensity values are shown with a linear Fire lookup table (LUT). Line profiles of MRGs along the mitochondrial axis (middle, dashed lines) show the intensity values at each time point (bottom). Scale bar, 2 μm. **f,** Temporal evolution of the integrated intensity of MRGs in COS-7 cells before and after fusion. Pre-fusion integrated intensities were summed for each granule pair (**n** = 9 biologically independent cells) and the sums were used for normalization (grey dashed line). Data are presented as mean and s.d. for each MRG type for four time points before fusion (light green, **t**−4-**t**−1) as well as after fusion (dark green, **t**+1-**t**+4). Statistical source data are provided in Source Data Fig. 2.
Fig. 3 | MRGs are associated with inner mitochondrial membranes and are randomly spaced along the mitochondrial network. a, Correlated fluorescence (axially projected confocal image, green) and transmission electron micrograph (TEM, greyscale), in a FASTKD2-tRFP expressing COS-7 cell. Electron densities corresponding to MRGs are visible (arrowheads; n = 7 MRGs from three mitochondria). Scale bars, 10 μm (top) and 1 μm (bottom). b, Western blot of fractionated mitochondria, probed with antibodies against MRG (FASTKD2, GRSF1, FASTKD5) and nucleoid transcription factor A, mitochondrial (TFAM), mtSSB) components. IMM-integrated proteins (complex IV, prohibitin (PHB)) and soluble matrix protein pyruvate dehydrogenase E1 α (PDHE1α) served as controls. Blots were processed simultaneously and compared quantitatively from the same gel. The experiment was performed twice with similar results.

c, FASTKD2-eGFP expressing HeLa cells treated with 100 μM antimycin A for 1 h, then fixed. IMM (anti-complex IV, cyan) and outer mitochondrial membrane (OMM, anti-TOMM20, magenta), reveal swollen mitochondria with few inner membranes. MRGs appear proximal to the IMM (arrowheads). The experiment was performed twice with similar results. Scale bars, 10 μm (top), 2 μm (bottom).

d, FASTKD2-eGFP expressing COS-7 cells treated with 100 μM antimycin A for 1 h, imaged live. Pixel intensity values are shown with a linear Fire LUT as in Fig. 2e. Time-lapse series are shown for two exemplary mitochondria (dashed boxes). Kymographs (from the yellow dashed line) highlight the co-mobility of individual granules. Scale bars, 10 μm (top) and 1 μm (bottom). The experiment was performed twice with similar results.

e, MRG movement over time, measured as the distance between the MRG and the closest membrane, for swollen mitochondria as exemplified in d (n = 20 MRGs examined from three cells). Data are presented as mean (blue line) ± s.d. (error bars).
f, Histogram of the distance between neighbouring pairs of MRGs for unbranched mitochondria (n = 206 mitochondria over three independent experiments) and simulated randomly distributed granules (grey). g, Correlation between MRG number and length of unbranched mitochondria (n = 206 mitochondria over three independent experiments). Each box plot denotes the median and first and third quartiles, and diamonds show outliers for each bin (for example, bin_1 = 0–1 μm and so on). The linear regression (green dashed line) was computed on non-binned data. Unprocessed blots and statistical source data are provided in Source Data Fig. 3.
pressed the mitochondrial helicase TWINKLE fused to eGFP as a nucleoid marker with high DNA-binding affinity. TWINKLE foci only slightly (~29%) recovered over the course of our FRAP assay (50 s), and served as negative control to highlight that fast protein exchange is particular to MRGs (Fig. 2b,d). Thus, MRG components exchange rapidly, on a fast timescale, even compared to stress granules (recovery half-time, 18.5–35 s).

Whereas liquid drops may fuse upon contact, solid granules will instead remain inert or aggregate while maintaining their shape. We followed FASTKD2 foci by live-cell super-resolved structured illumination microscopy (SIM), which provides the necessary resolution to discern the two cases. We observed MRG fusion in multiple instances in both U2OS and COS-7 cells, where two individual foci merged to form a single spot (Fig. 2e, Extended Data Fig. 4 and Supplementary Videos 5 and 6). We also noted MRG splitting on some occasions (Extended Data Fig. 4 and Supplementary Video 7). Imaging TWINKLE-eGFP by SIM, we observed nucleoid ‘kiss-and-runs’ and nucleoid splitting, as previously described, as well as one fusion event (Extended Data Fig. 4 and Supplementary Videos 8 and 9). We determined the photobleaching-corrected integrated intensity of FASTKD2-eGFP in the merged droplet to be approximately the sum of the initial droplets, as expected if no

Fig. 4 | Impaired mitochondrial fission leads to aberrant MRG positioning. a, HeLa cells imaged by confocal microscopy in control settings (top) or after 48 h of Drp1(K38A) overexpression (OE, bottom), stained for MRGs (anti-FASKD2, green), nucleoids (anti-DNA, magenta) and mitochondria (MitoTracker Deep Red, cyan). The second and fourth rows show zoomed views of the regions indicated by grey boxes in the first and third rows. The experiment was performed three times with similar results. Scale bars, 10 μm (first and third rows) and 1 μm (second and fourth rows). b, Confocal (left) and STED (right) images of mito-bulbs containing MRGs (anti-FASKD2, green) and nucleoids (anti-mtDNA, magenta) in fixed Drp1(K38A)-overexpressing COS-7 cells. The experiment was performed twice with similar results. Scale bars, 10 μm (left) and 2 μm (right, zoom). c, Example of FRAP time-lapse images from the datasets represented in d of mito-bulb-associated MRGs (arrowheads), in FASTKD2-tRFP (green) stably expressing COS-7 cells transiently transfected with Drp1(K38A) for 24 h. Scale bar, 2 μm. d, Comparison of FASTKD2-tRFP FRAP between control (n = 31 MRGs examined over four independent experiments) and Drp1(K38A) (n = 40 MRGs examined over three independent experiments) overexpressing cells. Symbols represent mean data points, lines are single exponential fits and shaded areas are s.d. values for each time point. e, Confocal fluorescence and TEM (CLEM) of MRGs (stable expression, FASTKD2-tRFP, green) in COS-7 cells, fixed 24 h after Drp1(K38A) transfection. Zoom of a single mitochondrion shows several MRGs (arrowheads), resembling a bunch of grapes (10 MRGs were analysed from three mitochondria). Scale bars, 10 μm (left), 1 μm (right). Grey dashed boxes indicate magnified regions. Statistical source data are provided in Source Data Fig. 4.
material was lost during fusion (Fig. 2f). Notably, 75% of fusion events coincided with visible mitochondrial rearrangements such as fusion, fission or bulging (Supplementary Table 2).

Infoldings of the IMM called cristae densely populate the mitochondrial interior. By correlative fluorescence and electron microscopy (CLEM), we observed that displaced cristae accommodate MRGs in open spaces (Fig. 3a), as was recently reported for nucleoids by live-cell stimulated emission depletion (STED) microscopy16. MRGs are clearly distinguishable as round electron-dense granules, with dimensions consistent with our hSTORM data (Figs. 1 and 3a and Extended Data Fig. 5) and an ultrastructure reminiscent of that of stress granules and P-bodies22.

Noticing their close proximity to the IMM and intrigued by the concurrence between MRG fusion and membrane dynamics, we assessed their association with the membrane using both biochemical and image-based approaches. First, we performed a biochemical fractionation to enrich for IMM or matrix-associated proteins. We found MRG components FASTKD2 and FASTKD5 primarily in the IMM fraction, while GRSF1 was present in both IMM and matrix fractions (Fig. 3b), similar to the nucleoid associated protein TFAM, in accordance with previous reports18. Next, we swelled mitochondria by antimycin A treatment of live COS-7 and HeLa cells to test whether MRGs would diffuse freely within the cristae-devoid enlarged lumen19 or remain associated with the IMM. We observed that most MRGs decorated the perimeter (Fig. 3c) and retained their relative position within mitochondria over time (Fig. 3d,e, Extended Data Fig. 6 and Supplementary Videos 10 and 11), consistent with IMM association. Thus, as nucleoids10, MRGs appear to be physically attached to the IMM.

We then assessed the distribution of MRGs along the mitochondrial network. A semiregular nucleoid spacing was found to be important in fusion yeast to circumvent the problem that random spacing would contribute to binomial errors in genome partitioning and thus a high probability of total mtDNA loss20. We used the same method to compare inter-MRG distances with those of simulated randomly placed granules (Fig. 3f). Our analysis shows that the positions of individual MRGs within their respective mitochondria are not distinguishable from a random distribution (Extended Data Fig. 6), while the distances between MRGs inside the same mitochondrion are at the borderline of being considered different from random (P = 0.04997; Fig. 3f). In the case of a random distribution along the entire mitochondrial network, we would expect an equal probability of finding an MRG anywhere along the network, so that larger mitochondria should have more granules. Indeed, we found on average one MRG for every 2.00 μm (±1.65 μm) of mitochondria (n = 131) and a positive correlation between MRG number and mitochondrial length (correlation coefficient = 0.62; Fig. 3g and Extended Data Fig. 6). Although nucleoids are accurately positioned14, our data suggest that MRGs are not.

Because MRGs appear relatively immobile within the matrix, yet are randomly distributed, we decided to investigate the interplay between MRG positioning and mitochondrial dynamics. We inhibited mitochondrial fission by overexpression of a dominant negative mutant of the fission factor dynamin-related protein 1 (Drp1), Drp1T317K. Under this condition, we observed highly elongated mitochondria with enlarged domains, as previously described and termed ‘mito-bulbs’25. We found that these domains contain not only nucleoids, but also MRGs (Fig. 4a). With super-resolved STED microscopy, we discovered that mito-bulbs are better described as resembling bunches of grapes, composed of many interspersed MRGs and nucleoids, rather than as a single enlarged and coalesced structure as previously proposed25 (Fig. 4b). Intrigued by the absence of MRG fusion (illustrated in Fig. 2) in such a confined space, we assessed whether MRGs may have solidified, as stress granules can26. We found that fluorescence recovery of stably expressed FASTKD2-tRFP was similar in control and Drp1T317K-overexpressing cells, suggesting that the liquid nature of MRGs within the mito-bulbs had not changed (Fig. 4c,d and Supplementary Videos 12 and 13). Using CLEM, we confirmed that the MRGs remained as distinct individual units, despite their close apposition, and did not observe the presence of stress granules that could have formed physical barriers to the fusion of these granules (Fig. 4e and Extended Data Fig. 5). Furthermore, this EM analysis showed that a number of these electron-dense granules were closely apposed to membranes, consistent with the results shown in Fig. 3a–c. Tightly stacked cristae could be seen adjacent to mito-bulbs in affected mitochondria (Fig. 4d). Knockdown of mitochondrial fusion factor Mitofusin 2 (Mfn2) evoked similar MRG clustering, further supporting a role for mitochondrial dynamics in maintaining an even distribution of MRGs along mitochondria (Extended Data Fig. 7).

In conclusion, our data show that MRGs share several properties with phase-separated condensates, albeit the exact mechanism of their formation is not yet fully elucidated. We propose that condensation of mtRNA and RBPs into MRGs may allow mammalian cells to regulate positioning of these components along the mitochondrial network via membrane association (Fig. 3). Mitochondrial dynamics via fission and fusion is critical for maintaining a random positioning of MRGs, and its perturbation leads to their accumulation in small domains, while their individual stability and capacity for molecular exchange is maintained (Fig. 4). Our findings show that changes in positioning can arise, decoupled from changes in the biophysical properties of RNA sub-compartments. This insight could be important for understanding mitochondrial disorders that are reported to feature aberrant mitochondrial RNA and DNA distribution into clusters. Adequate positioning of genetic material and transcripts may be crucial for proper synthesis of the respiratory chain and oxidative phosphorylation17.

Online content
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Methods

Plasmids and reagents. All cell culture reagents and chemicals were purchased from Sigma unless stated otherwise.

The following plasmids were cloned in the laboratory, using pWPT lentiviral vector (Addgene 12259) as a backbone: FASTK2D-eGFP, FASTK2D-1RFP, DDX28-eGFP, ERAL1-eGFP and TWINKLE-eGFP. CFP-Dnp1 (K38A) plasmid was a gift from A. van der Bliek. Plasmids for lentiviral production pMD2.G and psPAX2 were gifts from D. Trono (Addgene 12259 and 12260, respectively).

Cell culture and transfection. HEK293T cells were maintained in 5% CO₂ at 37 °C in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10−25 mM glucose, 4 mM GlutaMAX (Gibco) or 2 mM t-glutamine, 10% heat-inactivated fetal bovine serum (FBS), 100 μM penicillin and 100 mg/ml streptomycin. All cells were maintained in culture for a maximum of 20 passages, and routinely assessed for mycoplasma contamination.

Stable cell line generation. HELa Coxlta-SNAP cells were a gift from A. Van der Bliek. Plasmids for lentiviral production pMD2.G and psPAX2 were gifts from D. Trono (Addgene 12259 and 12260, respectively).

Cell culture and transfection. HEK293T cells was performed with Lipofectamine LTX (Invitrogen) or FuGENE 6 (Promega) according to the manufacturer’s instructions (typically 4–6 μl FUGENE reagent and 100–500 ng of plasmid DNA were used per well of a six-well plate). Cells were analysed 12–48 h after transfection. siRNA complete culture medium for 1 h before fixation, as previously described1,2. BrU assay was performed, cells were incubated with 5 mM 5-bromouridine (BrU) in Bromouridine tagging of RNA.

Live-cell treatments. Cytometry Core Facility at EPFL, to enrich cells expressing eGFP at the desired level. DDX28-eGFP cell lines, FACS sorting was performed with the help of the Flow Cytometry Core Facility at EPFL, to enrich cells expressing eGFP at the desired level.

For antimycin A treatment, cells were incubated with 25 or 250 nM antimycin A. Antimycin A was resuspended in EtOH at 1 mM and stored at −20 °C as 250 mM aliquots, and was heated and vortexed before dilution in culture medium when used.

Antimycin A treatment. Cells were incubated with 25 or 100 μM antimycin A (Abcam) in complete culture medium for 24 h or 1 h, respectively, before fixation or live imaging. Live imaging was performed in Leibovitz L-15 medium (Gibco) for SIM microscopy or Live Cell Imaging Solution (Thermo Fisher Scientific) for STED microscopy, supplemented with adequate amount of antimycin A. Antimycin A was resuspended in ETOH at 1 mM and stored at −20 °C.

Immunofluorescence. Cells were seeded on glass coverslips and grown to a confluence of 40–80%. Following live-cell treatments if indicated, fixation of cultured cells was performed in warm 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 15 min. Then, cells were rinsed with PBS, and cell permeabilization was executed by incubating the fixed cells in PBS containing 0.3% Triton X-100 and 10% pre-immune goat serum for 15 min. The same buffer was used to incubate cells with the specified primary antibody (see antibody list below). After incubation with primary antibodies overnight in a humid chamber and at 4 °C or 2 h at room temperature, the cells were washed with PBS and incubated with the appropriate secondary antibody conjugated with a fluorophore. Where indicated, the mitochondrial network was stained before fixing the cells using MitoTracker Deep Red FM (Thermo Fisher Scientific), according to the manufacturer’s instructions.

Antibodies used for immunofluorescence. The following primary antibodies were used for immunofluorescence: anti-FASTK2D (Proteintech, 17464-1-A: 1250 dilution), anti-bromodeoxyuridine (Roche, 11170376001; 1:250 to 1:500 dilution), anti-GRFS1 (Sigma, HPA036985; 1:250 dilution), anti-DNA (ProGen, 61014; 1:250 to 1:400 dilution), anti-TOMM20 (Abcam, ab186734; Santa Cruz Biotech, SC-17764; 1:200 dilution for both antibodies), anti-OxPhos Complex IV subunit IV (clontech; 1:300 dilution), anti-ERAL1 (Eurogentec, A21348; 1:200 dilution) and anti-mtHSP70 (Thermo Fisher Scientific, MA3-028; 1:250 dilution).

The secondary antibodies differed depending on the microscopy technique applied and are detailed in the following. All secondary antibodies for immunofluorescence were diluted 1:500–1:1,000.

hSTORM. hSTORM experiments were performed as previously described31,40, using the same hardware. Microscope acquisitions were controlled by Micromanager software (v. 2.0 beta), interfaced with the Thorlabs AFT software (v. 3.21.4) to control the piezo stage. Immunofluorescence was performed as described above. The following primary antibodies were combined for one- or two-colour imaging, as stated in the figures: anti-FASTK2D, anti-bromodeoxyuridine, anti-GRFS1 and anti-DNA. For one-colour hSTORM we used Alexa Fluor 647 coupled anti-rabbit or anti-mouse secondary antibodies (Invitrogen), the respective number of foci analysed are stated in Fig. 1. To verify mitochondrial localization of the analysed foci, we co-stained the mitochondrial proteins TOMM20 or mtHSP70 using the respective primary antibodies and Alexa Fluor 488 coupled secondary antibody (Invitrogen). For two-colour hSTORM, we used Alexa Fluor 647 for BrU coupled with DyLight 755 (Invitrogen) to label FASTK2D and we analysed 12 FOVs from four distinct imaging days. To allow lateral drift-correction, 100-nm gold nanoparticles coated with polyl-lysine were incubated on each sample for 5 min before the sample was mounted on the microscope with fresh imaging buffer. Before acquiring each raw STORM stack (10-nm exposure, 20,000–40,000 frames), we collected a 50-ms wide-field reference image at low laser power. Manually incrementing the 405 nm activation laser power allowed prolonged imaging. Imaging conditions (excitation illumination powers of 500–1,500 mW) were adjusted according to sample type.

We analysed and plotted the obtained localizations by adapting published MATLAB38,44 and new Python scripts (Supplementary Fig. 1). In brief, adaptations of different image registration and super-resolution localisation algorithms were applied very restrictively, verifying the presence of a bright wide-field signal in both channels. For two-colour experiments, additional care was paid to reduce effects due to chromatic aberrations. For this, the localizations of every selected ROI were visually inspected, and ROIs were rejected from further analysis unless visually discernible systematic shift was found following DBCR validation.

All descriptors were computed from localizations belonging to each particular cluster. We calculated the FWHM from the summed eigenvalues as the diameter for each granule using a Python script provided here. The eccentricity was determined as the ratio between the long axis and the short axis. Axis lengths are the eigenvalues of the covariance matrix of clustered localizations. For example, the ratio of the y and x dimension as described in ref. 46. We used the MATLAB function ‘convexHull’ to compute granule area and identify the hull-defining edge points. Three data points >0.25μm² were omitted for the creation of Supplementary Fig. 1, but kept for all statistical analysis. The radius of gyration was computed as the sum of variances in x and y, divided by the number of localizations. Sigma was computed as the sum of the eigenvalues in x and y.

SIM live-cell microscopy. SIM was performed on a three-dimensional (3D) NSIM Nikon microscope with a CFI Apochromat TIRF objective (x100, numerical aperture (NA) 1.49, Nikon). The microscope was equipped with 400-mW, 561-nm and 488-mW, 488-nm lasers (Coherent Sapphire) and a back-illuminated electron-multiplying charge-coupled device camera (Nikon, 3, Acton Technology).

Live-cell imaging was performed at 37 °C, using 488- and 561-nm lasers for eGFP and TRP excitation, respectively. Imaging settings were adapted to yield the best image quality with minimal photobleaching at a laser power between 2 and 10% and 3–10 s per frame. Per field of view, 15 raw images were acquired in 3D-SIM imaging mode to ensure the highest signal-to-noise ratio and signal-to-background ratio. Super-resolved SIM images were reconstructed using the commercial Nikon NS-Elements software (v. 3.2.2) and analysed in Fiji (Image 2.0.0 -rc-69/152p). Open-source Microbe software47, originally developed for analysis of bacteria, was used for supervised automatic segmentation of mitochondria and location of their associated foci (Supplementary Fig. 4).

FRAP and confocal microscopy. For FRAP assays, cells were seeded on coverslips and grown to 60–80% confluence. Coverslips were mounted on a Zeiss LSM 700 inverted confocal microscope with a Plan-Apochromat oil objective (×63, NA 1.40). Microscope acquisitions were controlled by the Zen (2009 v. 6.0.0) software from Zeiss. The microscope was equipped with 488-nm and 555-nm solid-state lasers and three photomultipliers for simultaneous transmission and epifluorescence recording. A sliding prism and green and red bandpass filters were used to ensure clean fluorescence emission. For live assays, an Okolab stage top incubator H301 was used to maintain the sample temperature at 37 °C. Cells were maintained in CO₂ independent Leibovitz L-15 medium (Gibco). For FASTK2D (n = 44, FAST = 31, n = 40), ERAL1 (n = 17) and DDX28 (n = 16) FRAP, the pixel size was reduced to 70 nm (zoom = 12) and line scans were recorded at a pixel dwell time of 2.55 μs (maximum speed), resulting in a scan time of 97.75 ms per 128 x 128-pixel FOV. The pinhole was opened for FRAP recording. The 10 x 10-pixel ROIs were manually drawn around single MRGs for FRAP and two or three bleach time points were acquired for point Dye. eGFP in COS-7 (n = 75) and U2OS (n = 38), and TWINKLE-eGFP (n = 50) FRAP were acquired with twice as many pixels (254 x 254 and 20 x 20 ROI) but for the same sample region (zoom = 12). A different FOV was chosen for every FRAP experiment and multiple different cells could be imaged per sample, but samples...
times. Unbroken cells and nuclei were discarded in the pellet after centrifugation. Cell passage through the needle was repeated 20

Cells were then fixed at room temperature for 1 h in fresh fixative (2% PFA, 1%

Mitochondrial sub-fractionation and western blotting. To further proceed with sub-mitochondrial fractionation and remove the OMM, mitoplasts were prepared by gently resuspending the mitochondria in cold

Mitochondrial RNA granules), or upon reasonable request. Jupyter Notebooks are available in the online repository GitHub (https://github.com/TimoHenry/FRAPanalysis) or in the online repository Zenodo (https://doi.org/10.5281/zenodo.3747143) or through personal communication. All imaging as well as numerical data relevant to this study are publicly available; please contact the corresponding authors. Source data are provided with this paper.

Data availability
All code including adapted STORM-analysis code, TrackFRAP, FRAPtA and

Primary antibodies used for western blots. The primary antibodies used for western blots were anti-FASKTD2 (ProteinTech, 17464-1-AP), anti-GSRF1 (Sigma, HPA03985), anti-FASKTD5 (Sigma, SAB2700438), anti-TFAM (ProteinTech, 22985-1-AP), anti-nSSB (ProteinTech, 12212-1-AP), anti-PHb (Thermo Fisher Scientific, MS-261-PO), anti-OxPhos Complex IV subunit IV (clone 20EC12, Thermo Fisher Scientific, A21348) and anti-PDHEx (GeneTex, GTX104015). All primary antibodies were diluted 1:2,000.

Stimulated emission depletion microscopy. MitoTracker Red CMXROS was employed to perform a Leica TCS SP8 inverted microscope with an HC-PL APO glycerol motC. STED W objective (x93, NA 1.30) for fixed samples or an HC

For live-cell STED microscopy, we followed the sample preparation and adapted the imaging protocol as recently published in ref. 41. In brief, HeLa CosA-SNAP cells were seeded onto coverslips the day before imaging. For live-samples, cells were incubated in a 15% 30 min with 1 μM SNAP-Cell SiR fluorescent substrate (New England Bios), followed by a 15–30 min washing step in dye-free medium. Cells were imaged at 37 °C in Life cell imaging solution (Thermo Fisher Scientific).

Statistics and reproducibility. All plotting and statistical analysis was performed using Python 3, with Jupyter Notebook 6.0 used to document analyses. Mann–Whitney U, Students t and Kolmogorov–Smirnov tests were used as indicated, and computed using the Python scipy library. seaborn- and matplotlib-libraries were used to plot the figures, and to add statistical significance indicators we used the stastanoTools-library. To avoid conflicts with non-Gaussian distributions we report the median values, unless stated otherwise. Correlation coefficients are calculated as Pearson’s R using nmaply-library. Analysis is reproducible through automated analysis scripts.

All plotting and statistical analysis was performed using Python 3, with Jupyter Notebook 6.0 used to document analyses. Mann–Whitney U, Students t and Kolmogorov–Smirnov tests were used as indicated, and computed using the Python scipy library. seaborn- and matplotlib-libraries were used to plot the figures, and to add statistical significance indicators we used the stastanoTools-library. To avoid conflicts with non-Gaussian distributions we report the median values, unless stated otherwise. Correlation coefficients are calculated as Pearson’s R using nmaply-library. Analysis is reproducible through automated analysis scripts.

Mitochondrial sub-fractionation and western blotting. To perform mitochondrial sub-fractionation experiments, HeLa cells were grown to ~60% confluency in four 150-mm Petri dishes. Cells were washed in PBS and collected by

For confocal microscopy of fixed cells, samples were prepared as described above. Alexa Fluor 488, 594 or 647 secondary conjugated antibodies (Invitrogen) were used to visualize the immunolabelled targets. Imaging was performed using a Leica TCS SP8 inverted microscope with a Plan-Apochromat oil objective (x63, NA 1.40), the Light Air mode (Leica) to get deconvolved images. Microscope acquisitions were controlled by LAS X (v. 3.5.2) software from Leica. The microscope was equipped with 405-, 488-, 552- and 638-nm lasers. Imaging for Extended Data Fig. 7 was performed using a Molecular Devices ImageXpress Micro XL automated wide-field microscope, equipped with an air objective (x60).

FRAP analysis and software. For FRAP recording of moving objects, a custom Fiji script was co-developed with O. Burri from the Bioimaging and Optics Platform (BIOP) at EPEL. In brief, this script, ‘TrackFRAP’, is based on the Fiji plugin TrackMate and automatically follows the bleaching ROI during recovery. It allows the user to choose other foci as FRAP references for overall bleaching correction and outputs both a list of intensity values and metadata to allow reproducible data analysis. All tracks were manually inspected to ensure the bleached granule was recorded correctly over the full course of acquisition. If no reference granule could be tracked over the full acquisition period, the dataset was excluded from analysis. We then developed a Python script to load and analyse TrackFRAP data, which we termed FRAPtrackAnalyser (FRAPtA) and which is based on the FRAPAnalyser tool. Single or double differential tracks were plotted and compared for each dataset, as well as used to extract recovery times.

Correlative confocal light and electron microscopy. Cells were seeded on a gridfed coverslip (MatTek, P35-1.5-14-CGRD-D), transfected with CFP-Drp1(K38A) plasmid if applicable, and grown to 50–60% confluence. Cells were then fixed at room temperature for 1 h in fresh fixative (2% PFA, 1%

The blots were further incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Dako, P0447 and 100,000 dilution), and visualized using an ECL system (GE Healthcare). Where required, images of western blots were treated for contrast enhancement and band intensities were quantified using ImageJ.

Data availability
All imaging as well as numerical data relevant to this study are publicly available in the online repository Zenodo (https://doi.org/10.5281/zenodo.3747143) or upon reasonable request. A README-file on Zenodo will guide the reader. All remaining other data supporting the findings of this study are available from the corresponding author on reasonable request. Plasmids and cell lines are available; please contact the corresponding authors. Source data are provided with this paper.

Code availability
All code including adapted STORM-analysis code, TrackFRAP, FRAPtA and

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Author contributions

S.Z., T.R., J.-C.M. and S.M. conceived and designed the study and wrote the manuscript. All authors reviewed and edited the manuscript. T.R. and S.Z. designed, executed, analysed and validated the experiments. E.C. executed and coded FRAP experiments and analysis. E.V. performed fractionation and western blotting. M.C. embedded, sectioned and acquired transmission electron microscopy samples. T.R. and S.Z. prepared the figures and plots. S.M. and J.-C.M. supervised the project.

Competing interests

The authors declare no competing interests.

Additional information

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Supplementary information is available for this paper at https://doi.org/10.1038/s41556-020-00584-8.

Correspondence and requests for materials should be addressed to T.R., J.-C.M. or S.M.

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Extended Data Fig. 1 | Workflow and quantification of nanoscopic architecture of MRGs. a, Workflow used for this study. Previously unpublished parts of the analysis are highlighted by asterisks, while other parts were previously published17,36. b–f Additional quantification of MRG and nucleoid (mtDNA) architecture from hSTORM data. Markers (mtRNA, FASTKD2 and GRSF1), number of granules n and median values are indicated for each condition; n.s. denotes p-values > 0.05, * denotes p-values ≤ 0.05, ** denote p-values ≤ 0.01, *** denote p-values ≤ 0.001, **** denote p-values ≤ 0.0001 of two-sided Mann-Whitney-U test. Individual data points are plotted grey, box plots denote first and third quartiles, and the median, whiskers comprise rest of distributions except outliers. Multiple acquisitions, samples and imaging days were pooled. b, Median eccentricity of both MRG-proteins differ slightly (p = 5.1e−3), with largely overlapping boxes and can be approximated by spheres. Nucleoids and nascent RNA components of MRGs are more elongated (pBrU-FASTKD2 = 1.4e−15, pBrU-GRSF1 = 1.7e−6). c, Comparison of areas described by convex hull. MRG-protein foci are significantly larger than nascent-RNA foci (pBrU-FASTKD2 = 2.7 × 10−61, pBrU-GRSF1 = 6.5 × 10−49), yet less different from one another (pGRSF1-FASTKD2 = 6.9 × 10−7). The distribution of mtRNA- and mtDNA-foci areas strongly overlap, though their medians are significantly different with pmtRNA-mtDNA = 1.2 × 10−6. Three outliers for FASTKD2 (> 2.5 µm²) were removed for better visualisation, but included in all quantitative analysis. d, and e, Comparison of alternative standard point-cloud descriptors Radius of gyration (Rg), and Sigma as the average of the eigenvalues in two dimensions, and multiplied by two to yield a diameter. f, Density of localisations was also compared, and both GRSF1 and FASTKD2-foci follow a narrow normal distribution, while mtDNA & BrU show a larger variance of density. Number (n) of clusters quantified for each condition is represented in the figure and is pooled from 24, 13, 7, and 14 FOVs, and 4, 4, 3, and 2 samples for BrU, GRSF1, FASTKD2, and mtDNA respectively. Statistical source data are provided in Source data Extended data Fig. 1.
Extended Data Fig. 2 | Comparison and correlation of two-colour htSTORM data. 

a, Nine additional examples of two-colour htSTORM of MRGs. Scatter plots of localisations (right) are shown next to corresponding clusters of FASTKD2 (green) and mtRNA (BrU, blue) overlaid on widefield images (left). Convex hull areas are represented with dashed lines. 
b and c, Scatter plots of all FASTKD2-mtRNA (BrU) pairs with regression-fit (black) and standard deviation (grey). Histograms of the distribution for FASTKD2 (y-axis, right, green), and mtRNA (x-axis, top, blue), including a kernel density estimate are shown. No correlation of Diameter (FWHM) ($R = 0.26$) or eccentricity (length/width, $R = 0.43$) was found between FASTKD2 and BrU foci from individual granules ($n=26$ MRGs over 4 independent experiments). 
d-f, Comparison of foci characteristics for one- versus two-colour htSTORM by Two-sided Mann-Whitney-U test from two-colour to one-colour data. Number of granules $n$ (pooled from 20 FOVs, and 8 samples) and median values are indicated for each condition; n.s. denotes $p$-values $>0.05$, * denotes $p$-values $\leq 0.05$, ** denote $p$-values $\leq 0.01$, *** denote $p$-values $\leq 0.001$, **** denote $p$-values $\leq 0.0001$. 

d, FWHM is not significant for FASTKD2 ($p = 0.22$) but two-colour BrU foci were significantly larger ($p = 8.8e^{-4}$), and two-colour FASTKD2 were also larger than two-colour-mtRNA ($p = 0.0014$). 
e, Eccentricity is not significantly different ($p_{mtRNA} = 0.36$, $p_{FASTKD2} = 1.0$).
f, Size determined by convex hull, differed between one-colour and two-colour BrU ($p = 2.4e^{-8}$) as well as FASTKD2 ($p = 0.017$). This may in parts be due to the heavy weight of two outliers as visible in the plot. Statistical source data are provided in Source data Extended data Fig. 2.
Extended Data Fig. 3 | FRAP of MRG-associated proteins. **a**, Representative time-lapse images of MRGs FRAP experiments in U2OS cells stably expressing FASTKD2-eGFP (green) (n= 39 MRGs examined over 3 independent experiments). White arrowheads indicate the photobleached structures. Scale bar: 5 μm. **b**, FRAP analysis of FASTKD2-eGFP in U2OS (n= 39 MRGs examined over 3 independent experiments) and COS-7 cells (n = 44 MRGs examined over 8 independent experiments). Symbols in the graph represent mean data points. Single exponential fits (lines) and standard deviations for each time point (shaded area) are shown. **c, d**, Representative time-lapse images of ERAL1- (n = 17 MRGs examined over 3 independent experiments) and DDX28-eGFP (n = 17 MRGs examined over 3 independent experiments) FRAP experiments in COS-7 cells. White arrowheads indicate the photobleached structures. These images correspond to the data plotted in Fig. 2c. Scale bar: 2 μm.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | MRG and nucleoid dynamics in live cells. **a**, Representative time-lapse images of an MRG fusion event in a live U2OS cell, monitored by SIM. MRGs are visualized by stable expression of FASTKD2-eGFP (n=7 cells). **b**, Representative time-lapse images of an MRG splitting event in a live COS-7 cell, monitored by SIM. MRGs are visualized by stable expression of FASTKD2-eGFP (n=6 cells). **c, d**, Representative time-lapse images of nucleoid “kiss-and-run” (n=1 cell) and splitting events (n=2 cells), respectively, in COS-7 cells, monitored by SIM. Nucleoids are visualized by transient expression of TWINKLE-eGFP and mitochondrial outlines are highlighted by TOMM20-eGFP expression. **a–d**, Cells were imaged at 1/5 Hz. White arrowheads indicate the dynamic events. Dashed lines indicate the segments used to measure the intensity (grey values) represented in the plots below. Linear Fire LUTs are used to represent pixel intensity values. Scale bars: 2 μm. Statistical source data are provided in Source data Extended data Fig. 4.
Extended Data Fig. 5 | CLEM of FASTKD2-tRFP and MRG electron densities. Correlative confocal fluorescence micrograph of FASTKD2-tRFP and transmission electron micrograph (TEM) in COS-7 control (a - c)(7 MRGs were examined from 3 mitochondria of a single cell) and Drp1K38A-CFP positive (d, e) (10 MRGs were examined from 2 mitochondria of a single cell) cells. TEM-highlights correspond to the data presented in Figs. 3a and 4e respectively, and show additional examples. Contiguous 50 nm TEM microtome sections show electron densities corresponding to the MRGs visualized by fluorescence microscopy (yellow arrowheads). Scale bars: Confocal: 10 µm; Confocal zoom: 2 µm; CLEM and TEM sections: 500 nm.
Extended Data Fig. 6 | Membrane association and distribution of MRGs within mitochondria. a, STED microscopy of HeLa cells stably expressing Cox8a-SNAP (grey) and FASTKD2-eGFP (green), treated with 100 µM antimycin A for 1 hour, prior to labelling with SIR-SNAP dye and live cell imaging (The experiment has been performed twice with similar results). A zoomed field of view on the right (dashed box). Scale bar: 2 µm. b–c, FASTKD2-eGFP expressing COS-7 cells untreated (b) or treated with 25 µM antimycin A for 24 hours (c), imaged live using SIM microscopy. The same linear Fire LUT is used for pixel intensity values as in Fig. 2e. Zoomed time-lapse series are shown for two mitochondria (dashed boxes) (The experiment has been performed twice with similar results). Kymographs below (plotted lines are represented on the analysed mitochondria as yellow dashed lines). Scale-bar: 2 µm. d, Example FOV of semi-automated mitochondria segmentation and MRG-association with their parent organelle with the ImageJ plugin, MicrobeJ. (The experiment has been performed three times with similar results). e, and f, Histograms of absolute or relative position of MRGs (n = 231 MRGs examined over 3 independent experiments) within their host mitochondria (green) and simulated, randomly positioned granules (grey). The observed distribution of MRG positions is not significantly different from the simulated random distribution (Kolmogorov-Smirnov test: $p_{\text{abs}} = 0.057$, $p_{\text{norm}} = 0.053$). Statistical source data are provided in Source data Extended data fig. 6.
Extended Data Fig. 7 | Effect of perturbations to mitochondrial dynamics on MRG distribution. Representative widefield-microscopy images of HeLa cells silenced using siRNAs against Drp1 (second row) or Mfn2 (third row). Cells were fixed after 72 hours of silencing. A negative control siRNA was used in parallel, and is shown in the first row. MRGs and nucleoids were immunolabeled using anti-FASTKD2 (green) and anti-DNA (magenta), respectively. Mitochondria were labelled using MitoTracker Deep Red staining (cyan) (The experiment has been performed twice with similar results). Scale-bar: 10 µm. Disrupted MRG and nucleoid positioning, and clumped appearance as described for mito-bulbs is apparent when either Drp1 and Mfn2 are silenced, but the negative control shows well-dispersed MRGs and nucleoids.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
- STORM: Micromanage 2.0 beta & APT Software (v. 3.21.4), Localization with MATLAB 2016b for Windows 10.
- FRAP: ZEN 2009.
- SIM: NIS-Elements (v. 3.2.2)
- Confocal: Zeiss ZEN (v 6.0.0)
- STED: LAS X (v. 3.5.2), With LIGHTNING deconvolution

Data analysis
- STORM: DIBSCAN MATLAB function; customized MATLAB script; MATLAB 2018a, Anaconda 3 & jupyter notebooks (Python3).
- FRAP: customized Fiji script; TrackFRAP, available on GitHub; ImageJ 2.0.0-rc-69/1.52p & Python3.
- SIM: Microbej (v. 5.131 [20] - beta; ImageJ plugin), Python3
- CLEM: Fiji (ImageJ 2.0.0-rc-69/1.52p)

All customized software will be made publicly available. Link: https://github.com/TimoHenry/MitochondrialRNAgrules

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- A list of figures that have associated raw data
- A description of any restrictions on data availability

All imaging as well as numerical data relevant to this study are publicly available on the online repository Zenodo (https://doi.org/10.5281/zenodo.3747143), or upon reasonable request. All code including adapted STORM-analysis code, TrackFRAP, FRAPIA and other Python scripts and Fiji macros for figure generation are
available in the online repository Github (https://github.com/TimolHenny/MitochondrialRNAgranules), or upon reasonable request. Plasmids and cell lines are available to share, do not hesitate to contact the corresponding authors should you have any further enquiries.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size: No hypothesis-based experiment was performed. Therefore, the sample size was not predetermined. Sample sizes were chosen based on the distribution of the data-points. For morphology data, we collected images and quantified the features within. We then plotted histograms of the feature properties, and ensured that the sample sizes were large enough that the distribution was well-sampled. For FRAP data, experiments were performed in triplicate. Sample size was based on experience in prior studies and sized to allow significance in biologically relevant effect sizes.

Data exclusions: STORM data acquisition was stopped on a case-by-case basis. E.g. when STORM buffer exceeded its optimal capacity and dye blinking diminished. SMMLM data were also filtered for quality using common metrics in the field (number of localizations, signal). Out of focus data was excluded. These are standard procedures in the field. For live imaging, data with visual deterioration of cellular health were excluded. Loss of focus also led to exclusion of FRAP FOVs. These are standard procedures in the field. Exclusion of data can be retraced through metadata of filters applied.

Replication: Multiple replicates were performed with each antibody and the staining results were in general reproducible. Stained samples that did not show the expected localization to mitochondria were excluded, and often could be attributed to using previously prepared, rather than fresh, solutions. Br-UdR is particularly sensitive to rigorous adherence to published protocols. In parallel, we used comparable results, confirming that the variability was due to sample preparation and not intrinsic variability. All experiments presented in the paper have been reproduced multiple times (a minimum of three replicates), except the correlative light and electron microscopy, as indicated in the manuscript. To ensure reproducibility, we carefully controlled the experimental conditions (passages of cells, stable cells to control expression levels, etc.). All attempts at replication were successful.

Randomization: This is not applicable to our study. Randomization is introduced to reduce bias. There is no risk of bias in our study because all analyses were quantitative, and not subjective.

Blinding: There was not a group allocation component to the study.

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|-----|-----------------------|
| ☐   | Antibodies            |
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| ☒   | Palaeontology         |
| ☐   | Animals and other organisms |
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Antibodies

Antibodies used are commercially available and referenced in the main manuscript. Please refer to materials and methods section for more information. anti-FASAKD2 (ProteinTech, 17464-1-AP), anti-bromodeoxyuridine (Roche, 11170376001), anti-GRSF1 (Sigma, HPA036985), anti-DNA (ProGen, S1014), anti-TOMM20 (Abcam, ab186734; Santa Cruz Biotech., SC-17764), anti-Complex IV (Thermo Fisher Scientific, A21348), anti-Hsp70 (Thermo Fisher Scientific, MA3-028), anti-GRSF1 (Sigma, HPA036985), anti-FASAKD2 (Sigma, SA92-00438), anti-TFAM (ProteinTech, 22386-1-AP), anti-mS88 (ProteinTech, 12212-1-AP), anti-PBH (Thermo Fisher Scientific, MS-261-PO), anti-OxPhos Complex IV subunit IV (clone 20E8C12, Thermo Fisher Scientific, A21348), anti-PDHE1α (GeneTex, GTX104015)
Validation

• Proteintech: We use affinity chromatography, which makes use of the binding strength of a protein for its ligand. Another popular method is protein A/G purification, which uses the recombinant fusion protein A/G from E. coli. Protein A/G purification will yield all IgGs in the serum, not just the desired antibodies. Affinity chromatography selects for just the ones with the highest affinity toward the target. We also validate our antibodies through knockout and knockdown (KO/KD) studies. Proteintech was the first company to implement siRNA knockdown validation experiments, the gold standard for testing antibody specificity. The validation process involves using small interfering RNA to knock down gene expression in an antibody product – assessing whether the signal subsides with the expression of the target gene.

  1. anti-FASTKD2 [Proteintech, 17464-1-AP]; for WB, IP, IHC, IF, ELISA (used in our study for WB, IF)
     species specificity: human, mouse, rat, zebrafish
  2. anti-TFAM [Proteintech, 22586-1-AP]; for WB, IP, IHC, ELISA (used in our study for WB)
     species specificity: human, mouse, chicken
  3. anti-mtSSB [Proteintech, 12212-1-AP]; for WB, CHIP, IF, ELISA (used in our study for WB)
     species specificity: human, mouse, rat

• Sigma: Prestige Antibodies® are highly characterized and extensively validated antibodies with the added benefit of all available characterization data for each target being accessible via the Human Protein Atlas portal linked just below the product name at the top of this page. The uniqueness and low cross-reactivity of the Prestige Antibodies® to other proteins are due to a thorough selection of antigen regions, affinity purification, and stringent selection. Prestige antigen controls are available for every corresponding Prestige Antibody and can be found in the linkage section. Every Prestige Antibody is tested in the following ways:
   - IHC tissue array of 44 normal human tissues and 20 of the most common cancer type tissues.
   - Protein array of 364 human recombinant protein fragments.
   - Validated for multiple commonly used applications such as IHC (Immunohistochemistry), IF (Immunofluorescence), and WB (Western Blot)

  1. anti-GRSF1 (Sigma, HPA036985); also validated by orthogonal RNaseq; validated for: WB, IF, IHC (used in our study for WB)
     species specificity: human
  2. anti-FASTKD5 (Sigma, SA82700438);
     for: WB, IHC (used in our study for WB)
     species specificity: human
  3. anti-bromodeoxyuridine (Sigma/Roche, 111703760D1/BMC9318)
     The antibody specifically binds to bromodeoxyuridine and crossreacts with iodouridine (10%). Anti-bromo-deoxyuridine does not crossreact with fluorescein-uridine, nor with any endogenous cellular components such as thymidine or uridine.
     For: FC, IHC/IF, cryosections, paraffin sections (used in our study for IF)
     Produced in mice, general species specificity

• ProGen:
   Purified by size exclusion chromatography and validated in publications by WB, IHC since 1996.
   anti-DNA (ProGen, 61014)
   for: ICC, IF, IHC (used in our study for IF)
   species specificity: all

• Abcam:
   Produced recombinantly [animal-free] for high batch-to-batch consistency and long-term security of supply.
   anti-TOMM20 (Abcam, ab186734)
   for: FC, ICC, IF, IHC, WB (used in our study for IF)
   species specificity: mouse, rat, human

• Santa Cruz Biotech:
   Validated by knockdown + WB, and by WB, IF, etc in >300 publications.
   anti-TOMM20 (Santa Cruz Biotech, SC-17764)
   for: IP, ELISA, IF, IHC, WB (used in study for IF)
   species specificity: mouse, rat, human

• Thermo Fisher Scientific:
   anti-Complex IV (Thermo Fisher Scientific, A21348)
   Validated by knockdown + WB
   for: ELISA, ICC, IF, IHC, WB (used in our study for WB)
   species specificity: mouse, rat, human

anti-Hsp70 (Thermo Fisher Scientific, MA3-028)
MA3-028 has been successfully used in Western blot, immunocytochemical, immunofluorescence, immunohistochemical (paraffin), and immunoprecipitation procedures. It has been thoroughly tested and validated for cellular immunofluorescence (IF) applications.
   for: ELISA, ICC, IF, IHC, WB (used in our study for WB)
   species specificity: mouse, rat, human

anti-PI3K (Thermo Fisher Scientific, MS-261-PQ)
   Validated by knockdown + WB
   for: ELISA, ICC, IF, IHC, WB (used in our study for WB)
   species specificity: mouse, rat, human, pig, chicken
Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s) | COS7 - ECACC - 87021302  
|                     | HEK293T - ATCC - CRL-11268
|                     | HeLa - ATCC - CCL-2
|                     | U2OS - ATCC - HTB-96 |

Authentication

ATCC, the provider of U2OS and HeLa cell lines, uses STR profiling for authentication.

ECACC, the provider of the HEK293T cell line, uses STR profiling for authentication.

HPA culture collections, the provider of the COS7 cell line, operates in tandem with ECACC and uses STR profiling for authentication.

Mycoplasma contamination

FACSed cell lines were tested for mycoplasma contamination with negative results. Mycoplasma contamination was checked for routinely in all cell lines.

Commonly misidentified lines

(See ICPLC register)

Not applicable as no commonly misidentified cell lines were used in the study.