Mitochondrial fragmentation drives selective removal of deleterious mtDNA in the germline

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Mitochondria contain their own genomes that, unlike nuclear genomes, are inherited only in the maternal line. Owing to a high mutation rate and low levels of recombination of mitochondrial DNA (mtDNA), special selection mechanisms exist in the female germline to prevent the accumulation of deleterious mutations1–3. However, the molecular mechanisms that underpin selection are poorly understood4. Here we visualize germline selection in Drosophila using an allele-specific fluorescent in situ-hybridization approach to distinguish wild-type from mutant mtDNA. Selection first manifests in the early stages of Drosophila oogenesis, triggered by reduction of the pro-fusion protein Mitofusin. This leads to the physical separation of mitochondrial genomes into different mitochondrial fragments, which prevents the mixing of genomes and their products and thereby reduces complementation. Once fragmented, mitochondria that contain mutant genomes are less able to produce ATP, which marks them for selection through a process that requires the mitophagy proteins Atg1 and BNIP3. A reduction in Atg1 or BNIP3 decreases the amount of wild-type mtDNA, which suggests a link between mitochondrial turnover and mtDNA replication. Fragmentation is not only necessary for selection in germline tissues, but is also sufficient to induce selection in somatic tissues in which selection is normally absent. We postulate that there is a generalizable mechanism for selection against deleterious mtDNA mutations, which may enable the development of strategies for the treatment of mtDNA disorders.

To visualize germline selection, we designed fluorescently labelled DNA probes that bind specifically to unique regions of the D-loops of mtDNA from either Drosophila melanogaster or a closely related species, Drosophila yakuba (Extended Data Fig. 1a–e). We then transplanted mitochondria from wild-type D. yakuba into a strain of D. melanogaster in which the mtDNA contained a temperature-sensitive point mutation in cytochrome c oxidase subunit 1 (Co18)3,5,7, thereby generating heteroplasmic animals that contained mixtures of wild-type and mutant mtDNA (Extended Data Figs. 1f, g, 4a, b). At the permissive temperature (18 °C) the mutation does not grossly affect cytochrome oxidase activity, and is consequently not selected against in the germline3,5,7. At the restrictive temperature (29 °C) cytochrome oxidase activity is greatly reduced, and the mutation is selected against when paired with wild-type mtDNA from either D. melanogaster3,5,7 or D. yakuba8. This heteroplasmic animal model and mtDNA-specific fluorescent in situ-hybridization (FISH) assay enable us to directly observe and analyse mtDNA selection in vivo.

Drosophila ovaries comprise two types of tissue: germline, which gives rise to eggs and the next generation; and somatic cells, which surround the germline (Fig. 1a). Because our heteroplasmic strain contained largely mutant D. melanogaster mtDNA (93%), at the permissive temperature the ovaries remained largely mutant in both the germline and the soma (Fig. 1b, Extended Data Fig. 1h–i”). At the restrictive temperature, the proportion of wild-type D. yakuba mtDNA relative to mutant D. melanogaster mtDNA increased markedly in the germline but not in the soma (Fig. 1c, Extended Data Fig. 1i–"), which demonstrates that mtDNA selection is germline-specific. Male mtDNA is not inherited, and mtDNA FISH and quantitative PCR (qPCR) analyses of heteroplasmic Drosophila testes indicate that mtDNA selection is largely absent in the male germline (Fig. 1d, e, Extended Data Fig. 2). mtDNA selection is therefore female-germline-specific.

mtDNA selection is thought to occur early during oocyte development1–3. In Drosophila, germline stem cells divide asymmetrically during this time to self-renew and to produce differentiating daughters that undergo four rounds of divisions with incomplete cytokinesis to form germline cysts (Fig. 1f). mtDNA FISH analysis showed no increase in wild-type D. yakuba mtDNA relative to mutant D. melanogaster mtDNA in germline stem cells. However, selection was observed when germ cells differentiated first into cysts and thereafter into egg chambers (Fig. 1g, i, Extended Data Figs. 3a–c” , b–b”, d–d”, e). Inhibition of cyst formation by reducing the expression of the key early differentiation factor, Bag of marbles (Bam), blocked selection (Fig. 1h, Extended Data Fig. 3c–c” , f). Our results show that mtDNA selection occurs after the stem cell stage, early in oogenesis, during germline cyst differentiation.

Germline selection could occur at the cellular level as a result of cell death. Cyst cells that inherit too many mutant mitochondrial genomes could die, and would therefore not be represented in subsequent progeny9. However, a previous study did not observe the death of cyst cells during selection in Drosophila10, and we found that inhibiting cell death by overexpressing the cell-death inhibitor p35 did not block selection (Fig. 2a). Alternatively, the unit of selection could be the mitochondrial genome. To investigate this, we tested whether expression of the Ciona intestinalis protein alternative oxidase (AOX)—which can partially complement loss of complex IV11,12—influenced selection (Extended Data Fig. 4a–c). In effect, we bypassed the function of complex IV while leaving the mutant gene in place. Expression of AOX largely blocked selection by rescuing the mutant mitochondria (Fig. 2b, Extended Data Fig. 4d), which indicates that the selection mechanism senses defects in the oxidative phosphorylation process. Consistent with previous reports11, our data show that the unit of selection is the mitochondrion itself.

We therefore asked whether morphological changes in mitochondria could be observed during selection in differentiating cysts. Using a mitochondrially targeted enhanced yellow fluorescent protein (eYFP) and live confocal microscopy, we observed that cyst mitochondria were rounder and more discrete than stem-cell mitochondria, which were more often clustered, tubular and branched (Fig. 2c, d, Supplementary Video 1). In accordance with previous findings12, these results indicate that germline cyst mitochondria become fragmented. We propose that fragmentation enables mutant mitochondrial genomes to be distinguished from wild-type genomes. During the 2- to 8-cell cyst stage, mtDNA does not replicate3; consequently, fragmentation causes a reduction in the number of genomes per mitochondrion, which decreases the probability that both mutant and wild-type genomes reside in the same mitochondrion and improves the efficacy of selection. To facilitate selection it is also necessary for fragmentation.

https://doi.org/10.1038/s41586-019-1213-4
to prevent mitochondria from sharing their contents. To assess this we targeted a photoactivatable GFP to the mitochondrial matrix and photoactivated a subset of mitochondria in stem cells and cysts. In stem cells, the photoactivatable GFP diffused rapidly throughout the mitochondrial network, which indicates that these mitochondria share contents (Fig. 2c, g). In cysts, the photoactivatable GFP rarely passed from one mitochondrion to another (Fig. 2f, g), indicating that, at this stage, mitochondria do not readily share contents. From these observations it can be suggested that germline cyst mitochondrial fragmentation generates functionally distinguishable units for selection.

To directly test whether the fragmentation that is observed in cysts is necessary for selection, we increased the interconnectedness of cyst mitochondria by overexpressing the pro-fission protein Mitofusin13 (Extended Data Fig. 5a, b). Using mtDNA FISH analysis, we found that the overexpression of Mitofusin largely abolished selection (Fig. 3a, b, Extended Data Fig. 5e–f). Consistent with our FISH data, qPCR analysis indicated that the overexpression of Mitofusin increased the amount of mutant mtDNA while not grossly affecting the amount of wild-type mtDNA (Fig. 3f, Extended Data Fig. 5h). To exclude the possibility that our results were influenced by the fact that both the nuclear and mutant mitochondrial genomes were from D. melanogaster whereas the wild-type mitochondrial genome was from D. yakuba, we repeated the experiment in a heteroplasmic strain in which both wild-type and mutant mitochondrial genomes were from D. yakuba. In this D.-melanogaster-only background, the overexpression of Mitofusin blocked selection in a similar manner (Extended Data Fig. 5i). Increasing the connectedness of cyst mitochondria by reducing expression of the pro-fission factor Drp114 (Extended Data Fig. 5c) also blocked the selective removal of mutant mtDNA (Extended Data Fig. 5f–g). These findings indicate that promoting mitochondrial fusion or inhibiting fission enables mutant mtDNA to hide and to escape selection during oogenesis. Therefore, a sustained fragmented phase is necessary for mtDNA selection.

To test whether mitochondrial interconnectedness could underlie the absence of mtDNA selection in germline stem cells, we promoted fragmentation in stem cells by reducing the expression of Mitofusin (Extended Data Fig. 6a, b). This induced selection in germline stem cells (Fig. 3c, Extended Data Fig. 6c–f). A marked reduction in mutant mtDNA was observed, which suggests that—once fragmented—an elimination pathway acts to degrade mutant mtDNA. Although the reduction in expression of Mitofusin also caused defects...
in germline development (Fig. 3c, Extended Data Fig. 6c–e), qPCR analysis of mtDNA from the few young embryos obtained (Fig. 3f) and of whole ovaries (Extended Data Fig. 6f, e) confirmed that it enhanced germline selection. The overexpression of Drp1 similarly enhanced selection (Extended Data Fig. 6d, e). Control experiments indicated that Mitofusin and Drp1 do not regulate selection through processes other than fusion and fission (Extended Data Fig. 6f–h, Supplementary Note 1). Together, these data show that mitochondrial fragmentation is not only necessary but is also sufficient for germline mtDNA selection.

There is no robust selection against mutant mtDNA in most somatic tissues\(^5,16\). Given that a prolonged fragmented phase is sufficient to induce selection in the germline, we asked whether it might also be sufficient in the soma. Notably, reducing Mitofusin expression induced strong selection against mutant mtDNA in somatic follicle cells (Fig. 3d, e), which demonstrates that sustained fragmentation is sufficient to induce mtDNA selection in somatic cells. Our data indicate that the key determinant, which permits selection in the germline but not the soma, is the marked decrease in fusion of germline mitochondria during early oogenesis that results in an extended phase of mitochondrial fragmentation.

To test whether this fragmented phase is caused by a decrease in Mitofusin expression, we measured the amounts of Mitofusin protein and RNA and found that both were selectively reduced in cyst mitochondria (Fig. 3g, h, Extended Data Fig. 7). Downregulation of Mitofusin was not affected by reducing the expression of known posttranslational regulators Pink1, Parkin, VCP\(^17\) or Mul1\(^18\) (Extended Data Fig. 8). Mitofusin expression is therefore downregulated in germline cysts, which drives mitochondrial fragmentation and—in turn—germline mtDNA selection.

It is not known how mutant mitochondria are recognized and selected against once they are fragmented. The mitochondrial genome encodes proteins that are required for the generation of a proton motive force (PMF) and the synthesis of ATP. Mutations in mtDNA would therefore be expected to directly affect the PMF, the amount of ATP or both. Indeed, both the PMF and the amount of ATP were reduced in germline cysts, in predominantly mutant heteroplasmic flies (Fig. 4a, b, d, e). Inhibition of mitochondrial fragmentation by overexpressing Mitofusin blocked this reduction, further highlighting the importance of sustained mitochondrial fragmentation in exposing mutant genomes (Extended Data Fig. 9a–d). To determine whether a reduction in PMF marks mutant mitochondria for selection, we tested the effect of restoring the PMF in mutant mitochondria\(^19,20\). Therefore, to restore the PMF, we reduced expression of IF1 (Fig. 4c). We observed...
Fig. 5 | The mitophagy proteins Atg1 and BNIP3 are necessary for germline mitochondrial DNA selection. a–d, mtDNA FISH analysis of control heteroplasmic germaria (a) and of heteroplasmic germaria in which the expression of Atg1 (b), Atg8a (c) or BNIP3 (d) was reduced. The dashed circles demarcate the germline, and arrows point to wild-type mtDNA (Extended Data Fig. 10). e, BNIP3 protein localization in a heteroplasmic germlarum; the right panel also shows mitochondria (blue) as visualized with anti-ATP5a antibody. f, Percentage of mutant mtDNA, as assayed by qPCR, of control heteroplasmic ovaries (Ctrl) and of ovaries in which the expression of Atg1 (Atg1 KD), Atg8a (Atg8a KD) or BNIP3 (CG5059 knockdown (BNIP3 KD1 and BNIP3 KD2)) was reduced. g, The amount of mutant and wild-type mtDNA, as assayed by qPCR, in ovaries in which the expression of Atg1 or BNIP3 was reduced, normalized to the amount of mutant and wild-type mtDNA in control ovaries.

no effect on selection (Fig. 4g) and no increase in the amounts of wild-type or mutant mtDNA (Fig. 4h), which indicates that a loss of PMF is not necessary for selection against mutant mitochondria. We then explored whether a reduction in the amount of mitochondrial ATP could provide a signal to select against mutant mitochondria. It is not possible to restore ATP levels in mutant heteroplasmic animals without also restoring the PMF; therefore, we tested whether reducing ATP was sufficient to make wild-type mitochondria appear mutant and promote their elimination. We generated a transgenic strain that conditionally expressed a dominant negative form of ATP synthase in both mutant and wild-type mitochondria (Fig. 4f. Extended Data Fig. 9e–g). Reduction in the amount of mitochondrial ATP reduced mutant mtDNA and, notably, wild-type mtDNA (Fig. 4g, h), which indicates that a reduction in mitochondrial ATP is sufficient to induce selection.

We next sought to determine how mutant mitochondria are selected against once they have been fragmented and their ATP has been depleted. Mitophagy would seem to be a good candidate for the mechanism, as it is the main pathway for the elimination of dysfunctional mitochondria from somatic tissues. However, Parkin-mediated mitophagy has little effect on the clearance of mutant mtDNA in somatic tissues or in the germline. Nevertheless, because mitochondrial fragmentation in stem cells causes a marked reduction in mutant mtDNA (Fig. 3c), we asked whether other mitophagy pathway components are required for germline mtDNA selection. Notably, we found that reduction in the expression of Atg1—the major regulator of autophagy—blocked selection, whereas reduced expression of Atg8—a key structural component of the autophagosome that interacts with selective autophagy receptors—did not (Fig. 5a–c, f, Extended Data Fig. 10a–c). Instances of Atg1-dependent, Atg8- and Parkin-independent mitophagy have previously been described, notably in the clearance of mitochondria during the maturation of red blood cell. Furthermore, which also requires the outer mitochondrial membrane protein BNIP3L (also known as NIX). Given these parallels, we assessed whether BNIP3 (also known as CG5059)—the Drosophila protein that is most homologous to BNIP3L—was required for selection in the germline. Reducing the expression of BNIP3 inhibited selection (Fig. 5d, f, Extended Data Fig. 10d–f). Consistent with these findings, BNIP3 is upregulated in differentiating cysts, in which it is associated with mitochondria (Fig. 5e).

Bypassing mutant complex IV (Extended Data Fig. 4d) or preventing mitochondrial fragmentation by overexpressing Mitofusin (Extended Data Fig. 5b, i) blocked selection, primarily by preventing the elimination of mutant mtDNA. However, we found that, instead of preventing the elimination of mutant mtDNA, reducing the expression of Atg1 or BNIP3 predominantly decreased the levels of wild-type mtDNA (Fig. 5g). This was also the case when Atg1 expression was reduced in a heteroplasmic strain in which both wild-type and mutant mtDNAs were from D. melanogaster (Extended Data Fig. 10c, f). It has previously been proposed that Pink1 inhibits the replication of mutant mtDNA, enabling wild-type mtDNA to outcompete their mutant counterparts. Our results indicate that the turnover of mitochondria is coupled to replication, such that the elimination of defective mitochondria may trigger the replication of active mitochondria and ultimately selection.

Our findings indicate that developmentally regulated fragmentation of cyst mitochondria is required to isolate their genomes and proteomes, so that mitochondria possessing mutant mtDNA can be selected against through a process requiring the mitophagy proteins Atg1 and BNIP3. Given the benefits of mitochondrial fragmentation on mtDNA selection, the question arises as to why mitochondria are not always fragmented. Enhanced fragmentation comes at a cost, as substantial reduction in the expression of Mitofusin causes mitochondrial and cellular dysfunction in both germline and somatic tissues. In addition, evidence suggests that frequent fusion and swapping of mitochondrial content is important to maintain the health of the network and to efficiently generate ATP. It has previously been shown that, during early oogenesis, the germline does not have a strong requirement for mitochondrialy generated ATP. It may have evolved an alternative energy metabolism to tolerate the possible negative energetic consequences of reduced mitochondrial function caused by sustained fragmentation. It will be interesting to explore whether inducing mitochondrial fragmentation in somatic tissues can be used as a treatment for those suffering from mtDNA disorders. Recent work indicates that this may be the case: inducing mitochondrial fragmentation in the soma temporarily during midlife improved health and prolonged lifespan in Drosophila and Caenorhabditis elegans, possibly by promoting the removal of deleterious mtDNA. In conclusion, we have uncovered a key driver of mtDNA-purifying selection in the female germline, and our findings suggest therapeutic approaches for the treatment of mtDNA disorders.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41586-019-1213-5.

Received: 4 October 2017; Accepted: 18 April 2019; Published online 15 May 2019.

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Acknowledgements We thank J. Chung, M. Guo, P. O’Farrell, H. Jacobs, H. Ma, the Drosophila Species Stock Center, the Bloomington Drosophila Stock Center and the Vienna Drosophila Stock Center for fly stocks; members of the Lehmann laboratory and K. Lau for discussions; Y. Abdu, L. Barton, A. Blum, S. Burden, S. Kidd, M. Murphy, A. McQuibban and D. Siekhaus for comments on the manuscript; and A. Steir for experimental suggestions to address the reviewers’ comments. This work was supported by Canadian Institutes of Health Research grant FRN 159510 to T.R.H. and by National Institutes of Health grant R37HD41900 to R.L. T.R.H. is part of the University of Toronto Medicine by Design initiative, which receives funding from the Canada First Research Excellence Fund. R.L. is a Howard Hughes Medical Institute investigator.

Reviewer information Nature thanks Rachel Cox, Yukiko Yamashita and the other anonymous reviewer(s) for their contribution to the peer review of this work.

Author contributions S.P.J. and J.M.P. contributed equally to this work, T.R.H., T.L. and R.L. designed the experiments; T.R.H., T.L., S.P.J. and J.M.P. performed the experiments; and T.R.H., T.L. and R.L. wrote the manuscript with input from all authors.

Competing interests The authors declare no competing interests.

Additional information Extended data is available for this paper at https://doi.org/10.1038/s41586-019-1213-4.

Supplementary information is available for this paper at https://doi.org/10.1038/s41586-019-1213-4.

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METHODS

Fly stocks. For a list of fly stocks used in this paper see Supplementary Table 1.

To generate UAS.CV-DN, the coding sequence of AT synthase subunit C (CG1746) was amplified using Phusion High Fidelity PCR system (NEB, M0530L), the proton-accepting glutamic acid 121 mutated to a glutamine (numbered according to the start of the preprotein; E121Q) and the mutated coding sequence cloned into pVAlUM22B using Gibson Assembly master mix (NEB, E2611S). Plasmid DNA was then injected into BestGene into 3 strain carrying Ubi promoter driving sites and integrated into the second chromosome using phiC31 integrate34.

Heteroplasmic flies. Heteroplasmic flies were generated by germ plasm transfer from either wild-type D. yakuba or wild-type D. melanogaster (w1118) embryos into mutant D. melanogaster (mtCoPm + mtDNAk6) embryos as described previously35–38. GAL4 drivers were crossed into the heteroplasmic fly lines. Heteroplasmy of the animal being measured. This percentage varies from fly to fly. Because we are interested in the percentage decrease in mutant mtDNA in the germline relative to the starting heteroplasmy, the percentage mutant mtDNA in each ovary was normalized to the percentage mutant mtDNA in its corresponding carcass. The percentage wild-type mtDNA in each ovary was then derived by subtracting that value from 100%. The soma (carcass) represents the starting carcass.

qPCR quantification of mitochondrial DNA. One- to three-day-old flies were dissected. For Figs. 3f, 5f, g and Extended Data Figs. 1g, 2e, 3f, 5h, mtDNA was extracted from pools of embryos, dissected ovaries and fly carcasses as previously described39. Samples were mechanically homogenized with a plastic pestle in 100 μl of homogenization buffer (100 mM Tris–HCl pH 8.8, 0.5 mM EDTA, 1% SDS) and incubated for 30 min at 65°C. Potassium acetate was added to 1 M and samples were incubated for 30 min on ice, before centrifugation at 20000×g for 15 min at 4°C. DNA was then precipitated from the supernatant by adding 0.5 volumes of isopropanol followed by centrifugation at 20000×g for 5 min at room temperature. The resultant pellet was washed with 70% ethanol and suspended in water. qPCR was carried out using 25 ng of nucleic acid and 300 nM of each primer pair with a Roche LightCycler 480 machine and LightCycler 480 SYBR Green 1 Master 2X (Roche, 04887352001). The PCR program was: 10 min at 95°C, 45 cycles of 90 s at 95°C, 30 s at 55°C and 30 s at 72°C. Dissociation curves generated through a thermal denaturation step were used to verify amplification specificity. For Figs. 2a, b, 4g, h and Extended Data Figs. 4d, 5i, 6d–f, 10e, f, individual ovaries and carcasses were homogenized in 10 μl of pH 8.0, 1 mM EDTA, 10 mM NaCl, 200 μg ml−1 Proteinase K, incubated at 25°C for 30 min and 95°C for 2 min42. qPCR was carried out as described above with 1/25 of an ovary and 1/50 of a carcass. For the primers used, see Supplementary Table 4.

Antibodies. Primary antisera used were rabbit anti-Vasa (from the laboratory of R.L.), mouse monoclonal anti-Hts (1B1, DSHB)40, mouse monoclonal anti-HA (Abcam, ab130275), rabbit anti-GFP (Aves Labs, GFP-1020), mouse monoclonal anti-ATP5A [15H4C4] (Abcam, ab14748), mouse anti-PDH E1α (Abcam, ab11034) and rabbit anti-phospho-PDH E1α (S293) (Millipore, AP1062). Secondary antibodies were DyLight 405 donkey anti-rabbit, DyLight 405 donkey anti-mouse, Cy3 donkey anti-mouse (Jackson ImmunoResearch, and Alexa Fluor 488 goat anti-chicken from Thermo Fisher Scientific.

qPCR quantification of mitochondrial DNA. One- to three-day-old flies were dissected. For Figs. 3f, 5f, g and Extended Data Figs. 1g, 2e, 3f, 5h, mtDNA was extracted from pools of embryos, dissected ovaries and fly carcasses as previously described39. Samples were mechanically homogenized with a plastic pestle in 100 μl of homogenization buffer (100 mM Tris–HCl pH 8.8, 0.5 mM EDTA, 1% SDS) and incubated for 30 min at 65°C. Potassium acetate was added to 1 M and samples were incubated for 30 min on ice, before centrifugation at 20000×g for 15 min at 4°C. DNA was then precipitated from the supernatant by adding 0.5 volumes of isopropanol followed by centrifugation at 20000×g for 5 min at room temperature. The resultant pellet was washed with 70% ethanol and suspended in water. qPCR was carried out using 25 ng of nucleic acid and 300 nM of each primer pair with a Roche LightCycler 480 machine and LightCycler 480 SYBR Green 1 Master 2X (Roche, 04887352001). The PCR program was: 10 min at 95°C, 45 cycles of 90 s at 95°C, 30 s at 55°C and 30 s at 72°C. Dissociation curves generated through a thermal denaturation step were used to verify amplification specificity. For Figs. 2a, b, 4g, h and Extended Data Figs. 4d, 5i, 6d–f, 10e, f, individual ovaries and carcasses were homogenized in 10 μl of pH 8.0, 1 mM EDTA, 10 mM NaCl, 200 μg ml−1 Proteinase K, incubated at 25°C for 30 min and 95°C for 2 min42. qPCR was carried out as described above with 1/25 of an ovary and 1/50 of a carcass. For the primers used, see Supplementary Table 4.
Live imaging, photoactivation and measurement of membrane potential. For live imaging of mito-eYFP tagged mitochondria, ovaries were removed from females and the ovarioles were teased apart using tungsten needles in Halocarbon 200 oil (Halocarbon Products, 9002-83-9) on a cover-slip. For photoactivation and measurement of the membrane potential, ovaries were removed from females and incubated in Schneider’s medium (Life Technology, 21700) containing 1 μM tetrathenylborate (Sigma, T25402) and 20 nM tetramethylrhodamine, methyl ester (TMRM) (Invitrogen, T668) for 30 min at room temperature in the dark. 10 μg ml⁻¹ CellMask Deep Red Plasma membrane stain (Invitrogen, C10046) was then added and the ovaries were incubated for an additional 10 min. The ovaries were washed once with Schneider’s Medium before being teased apart as above in Halocarbon 200 oil on a cover-slip. For all live imaging, the samples were then mounted on a slide with a gas-permeable membrane (YSI Membrane Kit Standard) before imaging with a Zeiss LSM780 confocal microscope with Plan-Apochromat 40×/1.4 Oil DIC and Plan-Apochromat 63×/1.4 Oil DIC objectives. For all live-imaging experiments and for measurement of membrane potentials, at least three biological replicates were imaged. For photoactivation, the background signal (before photoactivation) was subtracted from all images in the time series using Fiji. Images were corrected for chromatic shifting using 0.1 nm TetraSpeck microspheres (Thermo Fisher) and deconvolved using Huygens Essential X11. For the quantification of the diffusion of photoactivated mito-PAGFP in stem cells and cysts, the standard deviation of the PAGFP fluorescence intensity was calculated using Fiji as previously described4,14. An increase in diffusion of PAGFP leads to a decrease in the standard deviation and indicates an increase in the number of productive fusion events. Imaris software (Bitplane) was used to quantify mitochondrial motility in germainia. Individual mitochondria were tracked using the autoregressive motion algorithm, and for each mitochondrion the distance moved (μm) in one second was measured (displacement delta length). Mean displacement of all mitochondria over one minute of live imaging is reported.

Clear native gel electrophoresis. The oligomerization of ATP synthase was assessed by clear native PAGE (CN-PAGE)45. Ten pairs of ovaries were homogenized in 50 μl PBS and mixed with 50 μl 0.1% digitonin (Thermo Fisher, BN20061) in PBS. After incubation on ice for 15 min, samples were centrifuged (10,000 g) for 15 min at 4°C. The pellets (mitoplast fraction) were washed in 200 μl PBS and centrifuged (10,000 g) for 15 min at 4°C, then solubilized in 25 μl 1× NativePAGE sample buffer (Thermo Fisher, BN20032) supplemented with 10 μl 5% digitonin (Thermo Fisher, BN20061). After incubation on ice for 15 min, samples were centrifuged (20,000 g) for 30 min at 4°C. Samples (15 μl) were then resolved on 1.0-mm, 10-well NativePAGE 3–12% Bis–Tris Gels (Thermo Fisher, BN20118X10) with 1× NativePAGE running buffer (Thermo Fisher, BN2001) according to the manufacturer’s instructions, at 4°C. The cathode buffer was supplemented with 0.02% (w/v) n-dodecylβ-D-maltoside (Sigma, D4641) and 0.05% (w/v) sodium deoxycholate (Sigma, 30970). After CN-PAGE, proteins were transferred to 0.2 μm PVDF membranes (Bio-Rad, 162-0174) using an XCell SureLock Mini-Cell Electrophoresis System (Thermo Fisher, EI0001) in buffer comprising 48 mM Tris (Sigma, T1503), 39 mM glycine (Thermo Fisher, BP381-1), 0.05% (w/v) SDS (Sigma, L3771), 20% (v/v) methanol (Thermo Fisher, A412-4), pH 8.3. The membrane was blocked in PBS, 0.1% Tween 20 with 5% skimmed milk powder and incubated with primary antibody for 1 h at room temperature. Blots were incubated with the appropriate secondary antisera for 1 h at room temperature, treated with Pierce ECL Western Blotting Substrate (Thermo Fisher, 32106) according to the manufacturer’s instructions, and visualized on the ChemiDoc MP Imaging System (BioRad, 170-8280).

ATP/ADP determination. To measure ATP and ADP, embryos were dechorionated in bleach for 2 min, washed in PBS containing 0.1% Triton X-100, and homogenized (10 embryos per sample) with a pestle in 12 μl Assay Buffer (Sigma, MAK135A). Samples were then analysed using ADP/ATP Ratio Assay Kit (Sigma, MAK135) according to the manufacturer’s instructions. Luminescence was recorded using the Synergy H1 Microplate Reader (BioTek, BTH1M). Data were analysed using paired t-tests.

Data availability. Source Data for all graphs are provided with the paper. The Cp values associated with each primer pair and DNA, and confocal image data are available upon request.

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Extended Data Fig. 1 | FISH probes are specific for either D. yakuba or D. melanogaster mitochondrial DNA. a, Schematics of the mitochondrial genome and the D-loops of D. yakuba and D. melanogaster. In the schematic of the D-loop of D. melanogaster, the boxed regions denote two classes of repeated sequences. The open boxes are unique to D. melanogaster. The hatched boxes contain a 300-bp sequence, that is conserved in other Drosophilids and is depicted by solid bars above the repeats in D. melanogaster and by a single solid bar above the D. yakuba D-loop. The FISH probes are directed against unique regions of the D-loops; the D.-yakuba-specific probe is depicted as a green bar and the D.-melanogaster-specific probe is depicted as a magenta bar beneath the respective D-loops. b–e, Confocal images of D. yakuba (b, e) and D. melanogaster (c, d) stage 7 egg chambers hybridized with D.-yakuba-specific probes (green; b, c) and D.-melanogaster-specific probes (magenta; d, e). All egg chambers were also hybridized with probes recognizing mtDNA of both species (common; middle panels, blue). The merged images are in the right panels. The D. yakuba probe hybridizes to D. yakuba mtDNA (b) but not D. melanogaster mtDNA (c). The D. melanogaster probe hybridizes to D. melanogaster mtDNA (d) but not D. yakuba mtDNA (e). f, Schematic illustrating the generation of heteroplasmic flies by the transfer of germ plasm that contains wild-type mitochondria (green) from D. yakuba (D. yak) into D. melanogaster (D. mel) embryos that are homoplasmic for mt:CoI+mt:ND2del1 mutant mitochondria (magenta). g, Bar plots showing the percentage of mutant and wild-type mtDNA, as assayed by qPCR, in adult female carcasses without ovaries from the original mutant D. melanogaster strain and the heteroplasmic line generated by pole plasm transplantation. The data are an average of four biological replicates. h, h’, h”, i, i’, i”, Ovarioles of flies heteroplasmic (Het) for D. melanogaster mt:CoI+mt:ND2del1 (mut) and D. yakuba (wt) genomes that were shifted to 18 °C (permissive temperature) for 10 days or maintained at 29 °C (restrictive temperature), hybridized with fluorescent probes that detect either wild-type D. yakuba (green) or mutant D. melanogaster (magenta) genomes. Selection against the mutant genome is observed in the germline when flies were raised at 29 °C. For mtDNA FISH, at least eight control and experimental ovarioles, germaria or testes were imaged for each experimental condition. Imaging parameters are presented in Supplementary Table 3. Here and in all subsequent Extended Data Figs. the greyscale images are non-background-subtracted and unnormalized.
Extended Data Fig. 2 | Selection against mutant mitochondrial DNA does not occur in the male germline. a–d, Testes of heteroplasmic (Het) flies that were shifted to 18 °C for 7 days (a, c) or maintained at 29 °C (b, d) hybridized with fluorescent probes that detect either wild-type D. yakuba (green) or mutant D. melanogaster (magenta) genomes. The higher magnification images in c and d include the stem cells and spermatogonial cysts. Selection against mutant mtDNA is not observed in testes of flies raised at the restrictive temperature (29 °C). e, Scatter plots showing the percentage of mutant mtDNA, as assayed by qPCR, of adult ovaries (n = 5) and testes of heteroplasmic flies raised at 29 °C (n = 5), and of adult testes of heteroplasmic flies shifted to 18 °C for 7 days (n = 5). The mtDNA qPCR data throughout are presented as medians with interquartile range and compared by two-tailed unpaired t-tests. In Supplementary Table 2, we also present 95% confidence intervals of the difference between the control and experimental means for all datasets and the number of biologically independent samples used to derive the statistics. The dashed line denotes the percentage mutant mtDNA in whole adult-female carcasses lacking ovaries. All testes are oriented with the stem-cell niche towards the left.
Extended Data Fig. 3 | Selection manifests in germline cyst cells and does not occur when cyst formation is blocked. a, b, Germaria of heteroplasmic females (Het), raised at 29 °C, were hybridized with fluorescent probes that detect either wild-type *D. yakuba* or mutant *D. melanogaster* mtDNA, and reacted with anti-Vasa antisera to mark the germline (a–a‴) or anti-Hts (1B1) antisera to mark the fusome and somatic cells (b–b‴). The dashed outlines delineate the germline in the germarium (a), and egg chambers surrounded by somatic follicle cells (a, b). Wild-type mtDNA (arrows) can first be strongly detected in cysts. c–c‴. A germarium of a heteroplasmic fly (Het), raised at 29 °C, in which cyst formation was blocked by expression of an RNAi against *bag-of-marbles* (*Bam*; UAS-*bam*shRNA TRiP .HMJ22155) in the germline under the control of nos-GAL4. The germarium was hybridized with fluorescent probes directed against wild-type (c, c‴) and mutant mtDNAs (c′, c‴) and reacted with anti-Vasa antisera to mark the germline (c‴). No increase in wild-type mtDNA is observed. d–d‴. A germarium of a heteroplasmic fly, raised at 29 °C, hybridized with fluorescent probes that detect either wild-type *D. yakuba* mtDNA (d, d‴) or mutant *D. melanogaster* mtDNA (d′, d‴), and reacted with anti-Orb antisera (d‴, blue in d‴) to demarcate all cells of the developing cysts and the oocyte in later egg chambers. Arrows in d and d‴ point to wild-type mtDNA, and dashed outlines delineate the germline in the egg chambers. e, Scatter plots showing the relative amounts of wild-type *D. yakuba* and mutant *D. melanogaster* mtDNA, as assayed by FISH, in cysts (n = 7) and egg chambers (EC, n = 6) compared to the amount in stem cells (SC, n = 7). f, Scatter plot showing percentage of mutant mtDNA, as assayed by qPCR, of control (Ctrl; nos-GAL4 driving UAS-mCherry RNAi) heteroplasmic ovaries (n = 5) and of heteroplasmic ovaries in which cyst formation was blocked by the knockdown of *bam* (*Bam* KD; n = 5). Here and in all subsequent images, ovarioles are oriented with the stem-cell niche towards the left.
Extended Data Fig. 4 | Expression of the *C. intestinalis* alternative oxidase (AOX) rescues mutant mitochondria. **a**, In wild-type mitochondria the electron transport chain complexes (I–IV) that reside in the inner mitochondrial membrane couple the transfer of electrons to the transfer of protons across the membrane. The resulting proton motive force drives the synthesis of ATP by complex V. **b**, At the restrictive temperature the CoI" mutation blocks the transfer of electrons through complex IV (cytochrome oxidase, purple) resulting in the absence of both the generation of a proton motive force and ATP production. **c**, AOX (yellow) catalyses the transfer of electrons from ubiquinone to molecular oxygen, bypassing complexes III and IV. This restores the transfer of protons at complex I and the generation of ATP. **d**, Scatter plot of the amount of mutant *D. melanogaster* (purple) and wild-type *D. yakuba* (green) mtDNA, as assayed by qPCR, in ovaries expressing AOX under the control of nos-GAL4, normalized to the amount of mutant and wild-type mtDNA in control ovaries (Ctrl) expressing mCherry RNAi (Ctrl, \( n = 20 \); AOX, \( n = 23 \)). Expression of AOX rescues the mutant genomes.
Extended Data Fig. 5 | Mitochondrial fragmentation is necessary for germline mitochondrial DNA selection. a–c, Stills of live images illustrating the effect that overexpressing Mitofusin (b) or reducing the expression of Drp1 (c) in the germline compared to controls (a, nos-GAL4 driving UAS-mCherry RNAi; the stills in Fig. 2c, d are higher magnifications of this image). When Mitofusin is overexpressed (nos-GAL4 driving UAS-marf47), or when the expression of Drp1 is reduced (nos-GAL4 driving UAS-Drp1.miRNA.CDS), the mitochondria in the cysts are no longer discrete as they are in control cysts. The mitochondria (white) were labelled with a mitochondrially targeted eYFP and cell membranes (blue) were labelled with CellMask Deep Red Plasma membrane Stain. Stem cells and cysts are outlined in red. d, d’, d'', Germarium of a control heteroplasmic female (nos-GAL4 driving UAS-mCherry RNAi), raised at 29 °C, hybridized with fluorescent probes that detect either wild-type D. yakuba mtDNA (greyscale in d; green in d’’); or mutant D. melanogaster mtDNA (greyscale in d’; magenta in d’’). The dashed outlines delineate the germline. g, Scatter plot showing the percentage of mutant D. melanogaster mtDNA, as assayed by qPCR, in carcasses (carc.) and ovaries of heteroplasmic flies in which wild-type mtDNA was either from D. yakuba or D. melanogaster. mCherry RNAi was expressed in the ovaries under control of nos-GAL4. h, Scatter plot of the amount of mutant D. melanogaster (purple) and wild-type D. yakuba (green) mtDNA, as assayed by qPCR, of young embryos laid by heteroplasmic females in which Mitofusin was overexpressed in the germline (Mfn OE, n = 4) normalized to the amount of mutant and wild-type mtDNA in young embryos laid by control heteroplasmic females (Ctrl; nos-GAL4 driving UAS-mCherry RNAi, n = 4). i, Same as g, except the analysis was performed on ovaries in which both wild-type and mutant mtDNAs were from D. melanogaster (Ctrl, n = 24; Mfn OE, n = 21). Mitofusin overexpression increases the levels of mutant mtDNA.
Extended Data Fig. 6 | Mitochondrial fragmentation is sufficient for germline mitochondrial DNA selection. a, b, Stills of live images illustrating the effect that reducing the expression of Mitofusin in the germline (b) has on the morphology of mitochondria compared to controls (a). When mitofusin is knocked down (nos-GAL4 driving UAS-mfn shRNA2 TRiP.HMC03883) the mitochondria in the stem cells are fragmented. The mitochondria (white) were labelled with a mitochondrially targeted eYFP and cell membranes (blue) were labelled with CellMask Deep Red Plasma membrane Stain. Stem cells and cysts are outlined in red. c, c’, c”, The knockdown of mitofusin in the germline by expressing mitofusin RNAi (nos-GAL4 driving UAS-mfn shRNA2 TRiP.HMC03883) results in selection for wild-type mtDNA (green) occurring in stem cells. The germarium was also reacted with anti-Vasa antiserum (c” to mark the germline and delineate the stem cells and cysts. Wild-type D. yakuba mtDNA, greyscale in c, green in c”, mutant D. melanogaster mtDNA, greyscale in c’, magenta in c”. Mutant mtDNA is readily detected in the soma but not in the germline. d, Scatter plot comparing the percentage of mutant mtDNA, as assayed by qPCR, of ovaries in which mitofusin was weakly knocked down in the germline (Mfn KD; nos-GAL4 driving UAS-mfn long hairpin RNA1 TRiP.JF0165049; Ctrl, n = 23; Mfn KD, n = 24) and of ovaries in which Drp1 was overexpressed in the germline (Drp1 OE; nos-GAL4 driving UAS-Drp1.E; Ctrl, n = 11; Drp1 OE, n = 11). The percentage of mutant mtDNA in each case was normalized to the percentage of mutant mtDNA in control ovaries to illustrate that the overexpression of Drp1 enhances selection to a similar extent as does a weak reduction in the expression of Mitofusin. e, Scatter plot of the amount of mutant D. melanogaster (purple) and wild-type D. yakuba (green) mtDNA, as assayed by qPCR, in ovaries in which the expression of Mitofusin was weakly reduced (Ctrl, n = 20; Mfn OE, n = 21) or in which Drp1 was overexpressed in the germline (Ctrl, n = 10; Drp1 OE, n = 10), normalized to the amount of mutant and wild-type mtDNA in control ovaries (Ctrl) expressing mCherry RNAi in the germline. Reducing the expression of Mitofusin or overexpressing Drp1 results in a decrease in mutant mtDNA. f–h, The effect of germline overexpression of Mitofusin (Mfn) and Drp1 on copy number (f), ATP levels (g), and mitochondrial motility (h) in homoplasmic wild-type D. melanogaster ovaries (see Supplementary Note 1). f, Scatter plot of the amount of mtDNA, as assayed by qPCR, in homoplasmic ovaries in which Mfn (n = 24) or Drp1 (n = 24) was overexpressed in the germline, normalized to the amount of mtDNA in control ovaries (Ctrl; nos-GAL4 driving UAS-mCherry RNAi, n = 23). g, Scatter plot of the amount of ATP in homoplasmic ovaries overexpressing Mfn (n = 5) or Drp1 (n = 5) in the germline under control of Maternal α-Tubulin Gal4, normalized to the amount of ATP in control ovaries (Ctrl; Maternal α-Tubulin Gal4 driving UAS-mCherry RNAi, n = 5). h, Scatter plot of mitochondrial motility in homoplasmic ovaries overexpressing Mfn (n = 5) or Drp1 (n = 5) in the germline. Motility was assessed by measuring mean mitochondrial displacement using live confocal microscopy and Imaris analysis software.
Extended Data Fig. 7 | Mitofusin is downregulated in germline cysts. 

a, a′, a″, a‴. A germarium of a female fly expressing haemagglutinin-tagged Mitofusin (Mfn), under control of the Mitofusin promoter (Marf-gHA48), and mitochondrially targeted eYFP (mito-eYFP50), was reacted with anti-haemagglutinin antisera to detect Mitofusin (a), anti-GFP antisera to detect mitochondria (a′) and anti-Vasa antisera to delineate the germline (a″). In a‴, the ratio of the levels of Mitofusin to mito-eYFP is presented in pseudocolour. The colours correspond to the ratios indicated on the pseudocolour bar. The dashed red circles outline the cysts and the dashed white circles demarcate the germline in the egg chambers.

b. Scheme for quantifying the levels of mitofusin RNA at different time points during early oogenesis. Females mutant for the differentiation factor Bam—which is required for cyst formation—and carrying a rescuing transgene expressing Bam under the control of a heat-shock promoter were heat-shocked at 37 °C for 2 h, and then allowed to recover for the indicated times. This enables the isolation of ovaries that contain staged cysts, predominantly at the 2-, 4- or 8-cell cyst stage. The morphology of the spectrosome and fusome, as revealed by staining with anti-Hts (1B1) antisera, was used to confirm the staging. RNA for RT-qPCR was isolated from ovaries from flies before heat shock and at the indicated times following heat shock.
Extended Data Fig. 8 | The downregulation of Mitofusin in cysts is not mediated by known regulators of Mitofusin protein. a–h", Germaria of females expressing haemagglutinin-tagged Mitofusin, under control of the Mitofusin promoter (Marf-gHA48) reacted with anti-haemagglutinin antisera to detect Mitofusin (greyscale in a–h, magenta in a--h") and anti-Vasa antibody to delineate the germline (greyscale in a’–h’, blue in a’–h’). The indicated known regulators of Mitofusin protein levels were knocked down in the germline using RNAi under the control of nos-GAL4. The numbers in parentheses are Bloomington Drosophila Stock Center stock numbers. All ovarioles are oriented with the stem-cell niche towards the left.
Extended Data Fig. 9 | Inhibiting mitochondrial fragmentation blocks the decrease in proton motive force and ATP levels in cysts of heteroplasmic flies. a–d, Germaria of heteroplasmic control flies (a, c; w1118) and heteroplasmic flies in which Mitofusin was overexpressed in the germline (b, d; nos-GAL4 driving UAS-marf47), reacted with TMRM to visualize mitochondrial membrane potential (pseudocoloured in a, b) or with antibodies to phosphorylated pyruvate dehydrogenase (PDH P, purple) and pyruvate dehydrogenase (PDH, green) to measure ATP levels (c, d). e, Diagram showing the essential glutamate at position 121 in c-ring subunit that acts as the proton donor and acceptor in the proton translocation pathway. In the dominant negative c-ring (CV-DN) this glutamate was mutated to a glutamine, which can no longer bind the protons. f, Scatter plot illustrating the reduction in ATP/ADP ratio in embryos laid by mothers expressing CV-DN in the germline under the control of Maternal α-Tubulin Gal4. The ratios were measured using an ADP/ATP Ratio Assay Kit (Abcam ab65313). Data presented are median and interquartile range and were analysed using paired t-tests (ATP, n = 5; ADP, n = 4; ATP/ADP, n = 4). g, Blue native polyacrylamide gel illustrating that the expression of the dominant negative inhibitor of complex V (CV-DN) does not disrupt the Complex V dimer. For gel source data, see Supplementary Fig. 1.
Extended Data Fig. 10 | The mitophagy proteins Atg1 and BNIP3 are necessary for germline mitochondrial DNA selection. a–d″. Germaria of a control heteroplasmic female (a, a″; nos-GAL4 driving UAS-mCherry RNAi) and of heteroplasmic females in which the expression of Atg1 (b, b″), Atg8a (c, c″), or BNIP3 (d, d″) was reduced in the germline, raised at 29 °C, hybridized with fluorescent probes that detect either wild-type *D. yakuba* mtDNA (greyscale in a–d, green in a″–d″) or mutant *D. melanogaster* mtDNA (greyscale in a′–d′, magenta in a″–d″). The dashed circles demarcate the germline in the early egg chambers. The arrows point to wild-type mtDNA.

e, f, Scatter plots showing the percentage of mutant mtDNA and the amount of mutant (magenta) and wild-type (green) mtDNA, as assayed by qPCR, of control heteroplasmic ovaries (Ctrl, n = 24 in e, f) and of ovaries in which the expression of Atg1 was reduced in the germline (Atg1 KD, n = 24 in e; n = 23 in f). In the left panel in f, the amount of mutant and wild-type mtDNA of heteroplasmic ovaries overexpressing Mitofusin (Mfn OE) is plotted to illustrate that overexpressing Mitofusin primarily inhibits selection by increasing the amount of mutant mtDNA, whereas reduced expression of Atg1 primarily inhibits selection by decreasing the amount of wild-type mtDNA. The control and Mitofusin-overexpression data are the same as that presented in Extended Data Fig. 5h. All the dissections and analyses were carried out at the same time. The right panel of f is a magnified view to illustrate the effect of reducing the expression of Atg1 on the level of wild-type mtDNA. In e and f, both wild-type and mutant mtDNAs were from *D. melanogaster*. 
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| ☒   | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials

- Obtaining unique materials: in material and method section

Antibodies

- Antibodies used: in materials and method section
- Validation: Describe the validation of each primary antibody for the species and application, noting any validation statements on the manufacturer’s website, relevant citations, antibody profiles in online databases, or data provided in the manuscript.

Eukaryotic cell lines

Policy information about cell lines

- Cell line source(s): non used
- Authentication: Describe the authentication procedures for each cell line used OR declare that none of the cell lines used were authenticated.
- Mycoplasma contamination: Confirm that all cell lines tested negative for mycoplasma contamination OR describe the results of the testing for mycoplasma contamination OR declare that the cell lines were not tested for mycoplasma contamination.
Commonly misidentified lines
(See ICLAC register)
Name any commonly misidentified cell lines used in the study and provide a rationale for their use.

Palaeontology

Specimen provenance
Provide provenance information for specimens and describe permits that were obtained for the work (including the name of the issuing authority, the date of issue, and any identifying information).

Specimen deposition
Indicate where the specimens have been deposited to permit free access by other researchers.

Dating methods
If new dates are provided, describe how they were obtained (e.g. collection, storage, sample pretreatment and measurement), where they were obtained (i.e. lab name), the calibration program and the protocol for quality assurance OR state that no new dates are provided.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals
Provide details on animals observed in or captured in the field; report species, sex and age where possible. Describe how animals were caught and transported and what happened to captive animals after the study (if killed, explain why and describe method; if released, say where and when) OR state that the study did not involve wild animals.

Wild animals
For laboratory work with field-collected samples, describe all relevant parameters such as housing, maintenance, temperature, photoperiod and end-of-experiment protocol OR state that the study did not involve samples collected from the field.

Human research participants

Policy information about studies involving human research participants

Population characteristics
Describe the covariate-relevant population characteristics of the human research participants (e.g. age, gender, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."

Recruitment
Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.

ChIP-seq

Data deposition
☐ Confirm that both raw and final processed data have been deposited in a public database such as GEO.
☐ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links
For "Initial submission" or "Revised version" documents, provide reviewer access links. For your "Final submission" document, provide a link to the deposited data.

Files in database submission
Provide a list of all files available in the database submission.

Genome browser session
Provide a link to an anonymized genome browser session for "Initial submission" and "Revised version" documents only, to enable peer review. Write "no longer applicable" for "Final submission" documents.

Methodology

Replicates
Describe the experimental replicates, specifying number, type and replicate agreement.

Sequencing depth
Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.

Antibodies
Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.

Peak calling parameters
Specify the command line program and parameters used for read mapping and peak calling, including the ChiP, control and index files used.

Data quality
Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.
### Software

Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.

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### Flow Cytometry

#### Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

#### Methodology

| Sample preparation | Describe the sample preparation, detailing the biological source of the cells and any tissue processing steps used. |
|--------------------|-----------------------------------------------------------------------------------------------------------------|
| Instrument         | Identify the instrument used for data collection, specifying make and model number.                               |
| Software           | Describe the software used to collect and analyze the flow cytometry data. For custom code that has been deposited into a community repository, provide accession details. |
| Cell population abundance | Describe the abundance of the relevant cell populations within post-sort fractions, providing details on the purity of the samples and how it was determined. |
| Gating strategy    | Describe the gating strategy used for all relevant experiments, specifying the preliminary FSC/SSC gates of the starting cell population, indicating where boundaries between “positive” and “negative” staining cell populations are defined. |

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

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### Magnetic resonance imaging

#### Experimental design

| Design type | Indicate task or resting state; event-related or block design. |
|-------------|-----------------------------------------------------------------|
| Design specifications | Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials. |
| Behavioral performance measures | State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects). |

#### Acquisition

| Imaging type(s) | Specify: functional, structural, diffusion, perfusion. |
|-----------------|-------------------------------------------------------|
| Field strength  | Specify in Tesla                                       |
| Sequence & imaging parameters | Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle. |
| Area of acquisition | State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined. |

#### Preprocessing

| Preprocessing software | Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.). |
|------------------------|-------------------------------------------------------------------------------------------------|
| Normalization          | If data were normalized/standardized, describe the approach(es); specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization. |
| Normalization template | Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI152, ICBM152) OR indicate that the data were not normalized. |
| Noise and artifact removal | Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration). |
### Volume censoring
Define your software and/or method and criteria for volume censoring, and state the extent of such censoring.

### Statistical modeling & inference

| **Model type and settings** | Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation). |
|-----------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| **Effect(s) tested**        | Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used. |
| **Specify type of analysis:** | Whole brain | ROI-based | Both |
| **Statistic type for inference** | Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods. |
| **Correction** | Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo). |

### Models & analysis

| **Models & analysis** | Involved in the study |
|-----------------------|------------------------|
|                       | Functional and/or effective connectivity |
|                       | Graph analysis |
|                       | Multivariate modeling or predictive analysis |

| **Functional and/or effective connectivity** | Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information). |
|-----------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------|
| **Graph analysis**                           | Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.). |
| **Multivariate modeling and predictive analysis** | Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics. |