Regulation of mitochondrial morphology and function by stearoylation of TFRI

Deniz Senyilmaz1, Sam Virtue2, Xiaojun Xu†, Chong Yew Tan2, Julian L. Griffin3, Aubry K. Miller1, Antonio Vidal-Puig2,4 & Aurelio A. Teleman1

Mitochondria are involved in a variety of cellular functions, including ATP production, amino acid and lipid biogenesis and breakdown, signalling and apoptosis5,6. Mitochondrial dysfunction has been linked to neurodegenerative diseases, cancer and ageing4. Although transcriptional mechanisms that regulate mitochondrial abundance are known7, comparatively little is known about how mitochondrial function is regulated. Here we identify the metabolite C18:0 (Fig. 1a) and human transferrin receptor 1 (TFR1; also known as TFRC) as mitochondrial regulators. We elucidate a signalling pathway whereby C18:0 stearoylates TFRI, thereby inhibiting its activation of JNK signalling. This leads to reduced ubiquitination of mitofusin via HUWE1, thereby promoting mitochondrial fusion and function. We find that animal cells are poised to respond to both increases and decreases in C18:0 levels, with increased C18:0 dietary intake boosting mitochondrial fusion in vivo. Intriguingly, dietary C18:0 supplementation can counteract the mitochondrial dysfunction caused by genetic defects such as loss of the Parkinson's disease genes Pink or Parkin in Drosophila. This work identifies the metabolite C18:0 as a signalling molecule regulating mitochondrial function in response to diet.

To study the function of very long chain fatty acids, we analysed Drosophila lacking Elovl6 (refs 6, 7), the enzyme elongating C16 fatty acids to C18. Sequence analysis identified nod as fly Elovl6 (herein referred to as Elovl6). On standard laboratory food, Elovl6 loss-of-function animals (l(3)02281/+/; Elovl6- ) die as early larvae (Fig. 1a). We confirmed that Elovl6- mutants have impaired C16:0–C18:0 elongase activity and reduced C18:0 levels (Extended Data Fig. 1a, b), and that their lethality is rescued by human ELOVL6 (Extended Data Fig. 1c, d). Survival to pupation was rescued by supplementing fly food (containing little lipid), with C18:0 (Fig. 1a), but not C18:1 or C20:0 (Extended Data Fig. 1e), confirming that the larval lethality is due to C18:0 deficit.

We serendipitously discovered that removing antifungal agents from fly food improved survival of Elovl6- mutants (Fig. 1a). Since these agents are mitotoxins, this suggested that Elovl6- mutants might be hypersensitive to mitochondrial inhibition. Indeed, sub-lethal concentrations of rotenone, a mitochondrial respiratory chain complex I inhibitor, killed Elovl6- mutants when added to antifungal-free food (Fig. 1b), but other drugs did not (Extended Data Fig. 1f). Thus, mitochondrial function is limiting in Elovl6- mutants (Extended Data Fig. 1g), suggesting that Elovl6- mutants suffer from a complex III defect.

If the main cause of Elovl6- lethality is reduced mitochondrial function, then viability should be rescued by restoring mitochondrial functional capacity. Indeed, Elovl6- viability was rescued by expressing AOX or Spargel (Drosophila PGClA), driving mitochondrial

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1German Cancer Research Center (DKFZ), 69120 Heidelberg, Germany. 2University of Cambridge Metabolic Research Laboratories, Wellcome Trust-MRC Institute of Metabolic Science, Cambridge CB2 0QQ, UK. 3The Department of Biochemistry, Tennis Court Road, Cambridge CB2 1GA, UK. 4Wellcome Trust Sanger Institute, Hinxton, Cambridge, Cambridgeshire CB10 1SA, UK. †Present address: State Key laboratory of Natural Medicines, China Pharmaceutical University, 210009 Nanjing, China.

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**Figure 1 | Drosophila lacking C18:0 have impaired mitochondrial function.** a, Elovl6- mutant larval lethality rescued by dietary C18:0 (10% in food) or by removal of mitoxic antifungal reagents (n = 4 × 60 animals per vial). b, Elovl6- mutants are sensitive to sub-lethal concentrations (100 μM) of rotenone (n = 4 × 30 animals per vial). c, d, Elovl6- mutants have impaired respiration (c, left), rescued by supplementing with C18:0 (10%) (c, right), or by expressing Ciona intestinalis alternative oxidase (AOX) (d), allowing bypass of complexes III and IV. e, f, Survival to pupation of Elovl6- mutants is rescued by ubiquitous expression of Spargel (e) or AOX (f), χ² tests, P = 0.05, n = 195 (e) or 81 (f). g, Amino- or carboxy-terminus-tagged Drosophila Elovl6 localizes to mitochondria, visualized with mitoGFP in S2 cells. DAPI, 4',6-diamidino-2-phenylindole; HA, haemagglutinin. Scale bar, 10 μm (n = 4). For details, see Supplementary Methods. Error bars show standard deviation (s.d.). a–d, **P < 0.01, *P < 0.05, not significant (NS) P > 0.05, two-tailed t-test.
biogenesis (Fig. 1c, f and Extended Data Fig. 1h, i). Thus, the organismal function of C18:0 is less pleiotropic than expected. Interestingly, Drosophila Elovl6 localizes to mitochondrial outer membranes (Fig. 1g and Extended Data Fig. 1j).

Lipidomic analysis of purified larval mitochondria (Extended Data Fig. 5b) revealed that their membranes have little C18:0 (Extended Data Fig. 1j), indicating that the effects of C18:0 are independent of lipoic acid. Thus, C18:0 regulates mitochondrial morphology in fly and human cells.

Mitochondrial fragmentation is due to either hyperactive fission or impaired fusion. Blocking fission with mdivi-1, a DRP1 inhibitor, induced mitochondrial fusion in control cells, but not in HeLa cells cultured without C18:0 (Fig. 2d), indicating impaired mitochondrial fusion in this condition. Mitochondria labelled with photoactivatable mitochondrionally targeted green fluorescent protein (mitoGFP) rapidly fused with the rest of the network (stained with MitoTracker, red), indicating that mitochondrial fusion, not fission. Pharmacological inhibition of mitochondrial fission with mdivi-1 does not cause mitochondrial network fusion in cells growing without C18:0. Bottom, quantification. n = 15. d, C18:0 affects mitochondrial fusion, not fission. Pharmacological inhibition of mitochondrial fission with mdivi-1 does not cause mitochondrial network fusion in cells growing without C18:0. Bottom, quantification. n = 15. e, Direct observation of mitochondrial fusion, monitored as dispersion of locally photoactivated mitoGFP (green) into the rest of the mitochondrial network (MitoTracker, red). Bottom, quantification (representative of ten experiments). For details, see Supplementary Methods. Error bars show s.d. a, c, d, bottom, **P < 0.01, not significant (NS), * P < 0.05 two tailed t-test. Scale bars: 20 μm (a); 10 μm (b–e).

Figure 2 | C18:0 is required for mitochondrial fusion. a, Elovl6 mutants have fragmented mitochondria (top), rescued by dietary C18:0 (10% in food). Bottom, fragmentation quantified (8 fields from 4 animals). b, Elovl6 knockdown in Drosophila cells causes mitochondrial fragmentation, reversed by supplementing medium with 100 μM C18:0 for 120 min. Bottom, quantification. RNAi, RNA interference. n = 50. ***P < 0.001, Mann–Whitney test. c, C18:0 removal by delipidating serum (Delipid. serum) causes mitochondrial fragmentation in human cells, rescued by resupplementing with 100 μM C18:0 for 2 h. Bottom, quantification. n = 15. ** ** C18:0 affects mitochondrial fusion, not fission. Pharmacological inhibition of mitochondrial fission with mdivi-1 does not cause mitochondrial network fusion in cells growing without C18:0. Bottom, quantification. n = 15. e, Direct observation of mitochondrial fusion, monitored as dispersion of locally photoactivated mitoGFP (green) into the rest of the mitochondrial network (MitoTracker, red). Bottom, quantification (representative of ten experiments). For details, see Supplementary Methods. Error bars show s.d. a, c, d, bottom, **P < 0.01, not significant (NS), * P < 0.05 two tailed t-test. Scale bars: 20 μm (a); 10 μm (b–e).
die as early stage larvae that do not grow (Extended Data Fig. 3a–d). Dietary supplementation with C18:0 had no effect on the growth or viability of dMfn knockouts (Fig. 3d).

We asked whether C18:0 affects Mfn via post-translational modifications (PTMs). Mfn from Elovl6 mutant larvae, or from HeLa cells growing with delipidated serum, migrated differently in SDS–polyacrylamide gel electrophoresis (SDS–PAGE) compared with control conditions (Extended Data Fig. 3e, f). Immunoprecipitating MNF2 from HeLa cells treated with or without C18:0 and probing without C18:0 is hyper-ubiquitinated (Fig. 3e, lanes 1, 3, 5 (endogenous proteins) and Extended Data Fig. 3g (tagged proteins)). Several ubiquitin ligases target MNF2 (refs 16–18). Only knockdown of HUWE1 rescued the mitochondrial fragmentation (Fig. 3f and Extended Data Fig. 4a–c) and MNF2 hyper-ubiquitination (Fig. 3e, lane 3 versus 4) caused by C18:0 removal, as well as lethality of Elovl6 mutant flies (CG8184 in Drosophila; Extended Data Fig. 4d), identifying HUWE1 as the C18:0-responsive ubiquitin ligase. As expected, increased MNF2 ubiquitination caused MNF2 protein destabilization (Extended Data Fig. 3h). C18:0 removal did not dramatically drop MNF2 steady-state levels, partly due to compensatory increases in MNF2 expression (Extended Data Fig. 3i), suggesting that ubiquitination additionally blocks MNF2 function in a degradation-independent manner, as for other HUWE1 targets30. HUWE1 only ubiquitinates MNF2 phosphorylated on Ser 27 by JNK19. Inhibition of JNK prevented mitochondrial fragmentation upon C18:0 removal (Fig. 3g). In sum, C18:0 regulates MNF2 ubiquitination via HUWE1, and thereby mitochondrial morphology and function. Elovl6 mutant flies display other dMfn loss-of-function phenotypes, such as reduced endoplasmic-reticulum–mitochondrial connections and abnormal cristae11,22 (Extended Data Fig. 5).

We asked how C18:0 affects JNK or HUWE1 activity. Endoplasmic reticulum stress can activate JNK. However, C18:0 removal did not lead to an unfolded protein response (UPR) (Extended Data Fig. 6a, b) and neither knockdown of UPR effectors nor treatment with tauroursoxycholic acid (TUDCA), an endoplasmic reticulum chaperone that inhibits endoplasmic reticulum stress13, blunted mitochondrial fragmentation upon C18:0 removal (Extended Data Fig. 6c, d). Instead, we hypothesized that C18:0 might regulate proteins via covalent binding (‘stearoylation’), analogous to protein palmitoylation. We synthesized C18:0 derivatives with azide or alkyne functionalities, which inhibit endoplasmic reticulum stress23, blunted mitochondrial fragmentation upon C18:0 removal (Extended Data Fig. 6c, d). Inhibition of JNK prevented mitochondrial fragmentation upon C18:0 removal (Fig. 3g). In sum, C18:0 regulates MNF2 ubiquitination via HUWE1, and thereby mitochondrial morphology and function. Elovl6 mutant flies display other dMfn loss-of-function phenotypes, such as reduced endoplasmic-reticulum–mitochondrial connections and abnormal cristae11,22 (Extended Data Fig. 5).

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function, activating JNK in response to the ligand gambogic acid.

transferrin with mitochondria (Extended Data Fig. 8h), suggesting that transferrin uptake (Extended Data Fig. 8g), or reduced association of containing iron–sulfur clusters (Extended Data Fig. 8c–f), impaired (Extended Data Fig. 8b), a drop in protein or activity levels of enzymes grow for days in medium lacking C18:0 but die in medium lacking iron could affect mitochondria via iron uptake or delivery. However, cells for cells to sense the absence of C18:0.

Since TFR1 is important for cellular iron uptake, TFR1 stearoylation could affect mitochondria via iron uptake or delivery. However, cells grow for days in medium lacking C18:0 but die in medium lacking iron (Extended Data Fig. 8a), suggesting that iron uptake is not markedly impaired in the absence of C18:0. Indeed, cells in medium lacking C18:0 do not show an iron deficiency transcriptional response (Extended Data Fig. 8b), a drop in protein or activity levels of enzymes containing iron–sulfur clusters (Extended Data Fig. 8c–f), impaired transferrin uptake (Extended Data Fig. 8g), or reduced association of transferrin with mitochondria (Extended Data Fig. 8h), suggesting that the effects of C18:0 are independent of iron. TFR1 also has a signalling function, activating JNK in response to the ligand gambogic acid. Low concentrations of gambogic acid that do not induce apoptosis (Extended Data Fig. 9a) induced rapid mitochondrial fragmentation in HeLa cells (2 h; Fig. 3i). This was suppressed by adding C18:0 (Fig. 3i and Extended Data Fig. 9b), indicating that C18:0 blocks this signalling function of TFR1. Indeed, treatment of HeLa cells with C18:0 reduced JNK activation, using JNK phosphorylation and phospho-JNK nuclear translocation as readouts (Extended Data Fig. 9c, d). JNK inhibition blocked the ability of gambogic acid to induce mitochondrial fragmentation (Fig. 3j and Extended Data Fig. 9e). In sum, these data suggest that TFR1 induces mitochondrial fragmentation via JNK, and that this is inhibited by TFR1 stearoylation (Fig. 3k).

Palmitoyl-transferases covalently bind C16:0 before transferring it to substrates. We found one member of this family, ZDHHC6, in our C17:0–azide pulldowns, suggesting that it is a C18:0 transferase. Indeed, knockdown of ZDHHC6 blunted TFR1 stearoylation (Extended Data Fig. 7c, lane 5). Further work is required to study this in detail.

We noticed that elevating C18:0 levels in control cells increases mitochondrial fusion (Fig. 2b). Supplementing the diet of wild-type flies with C18:0 also increased mitochondrial fusion, whereas starvation of larvae led to mitochondrial fragmentation (Fig. 4). Thus, fly cells respond to both increases and decreases in levels of C18:0.

We asked whether dietary C18:0 supplementation could improve mitochondrial function in pathological conditions. Flies mutant for Parkin are established Parkinson’s disease models. They have impaired mitochondrial function, and recapitulate Parkinson’s disease phenotypes (reduced lifespan, neurodegeneration and impaired motor control). Dietary supplementation with C18:0 rescued the longevity, ATP levels and climbing defects of Parkin flies and the longevity of Parkin flies (Fig. 4 and Extended Data Fig. 10; other Parkin phenotypes not tested).

We identify C18:0 as a regulator of mitochondrial function. Upon loss of C18:0, TFR1 de-stearoylation activates JNK, leading to HuwE1-dependent MFN2 ubiquitination, impaired MFN2 activity, and mitochondrial fragmentation. Loss of C18:0 in flies specifically impacts mitochondrial function, since Elovl6 lethality can be rescued by Sargel, Aox or Dmfn expression or HuwE1 knockdown. To our knowledge, this is the first time stearoylation of a human protein has been found to regulate its function. The link between TFR1 and mitochondria perhaps makes sense, because iron enters cells via TFR1 and then mainly travels to mitochondria for iron–sulfur clusters. Flies are sensitive to dietary C18:0; increased dietary C18:0 leads to increased mitochondrial fusion in vivo. Thus, the metabolite C18:0 acts as a signalling molecule linking diet to mitochondrial function. Intriguingly, dietary C18:0 can also improve mitochondrial function in some pathological conditions in the fly, since dietary supplementation with C18:0 improved the Parkinson’s disease-related
phenotypes observed in Pink and Parkin mutant flies (see Supplementary Discussion).

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Extended Data Figure 1 | noa/Elovl6 is the functional homologue of human ELOVL6. a, C16:0 to C18:0 elongase activity is significantly blunted in Elovl6 mutants, whereas elongase activities on other fatty acids measured are not affected. Microsomal preparations from control or Elovl6 mutant animals were incubated with radioactive malonyl-CoA and the indicated fatty-acyl-CoA. Elongation was quantified by incorporation of the aqueous metabolite malonyl-CoA into lipid-soluble fatty acids, as described previously30. Values represent biological triplicates. b, Gas chromatography flame ionization detector (GC-FID) analysis reveals that Elovl6 mutant larvae have reduced levels of C18:0, the product of Elovl6 elongase activity. Values are the averages of technical duplicates on biological duplicates. Error bars represent standard error of the mean (s.e.m.).
c, Lethality of Elovl6 mutants is fully rescued to expected Mendelian ratios by ubiquitous expression (with actin-GAL4) of human ELOVL6 from a UAS transgene (χ² test, 1.149 < 3.841 = χ², where P = 0.05, n = 141; ****P < 0.0001). d, Human ELOVL6 and Drosophila Elovl6 are functionally equivalent, since the lethality of Elovl6 mutant flies is fully rescued to expected Mendelian ratios by ubiquitous expression (with actin-GAL4) of Elovl6 from a UAS transgene (χ² test, 2.38 < 3.841 = χ², where P = 0.05, n = 76; **P < 0.01). e, The lethality of Elovl6 mutant flies is most strongly rescued by C18:0, the product of Elovl6. Synchronized 1st instar larvae of indicated genotypes were grown on standard food supplemented with indicated fatty acids (5%). The percentage of total animals surviving to pupation was calculated. Values represent average of biological triplicates. f, Elovl6 mutants are not hypersensitive to drugs such as G418 (protein biosynthesis inhibitor) or etoposide (topoisomerase inhibitor). Thirty synchronized L1 larvae were grown in vials with food supplemented with either G418 (50 μg ml⁻¹) or etoposide (25 μM). Percentage of animals that reach pupation was quantified. Values represent average of four biological replicates. g, Complex IV activity of Elovl6− larvae is not impaired. Complex IV activity of female pre-wandering larvae was measured with Oroboros high-resolution respirometry. Oxygen consumption was measured in the presence of only N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) as substrate, which can be directly oxidized by complex IV. The values were corrected for non-mitochondrial oxygen consumption (oxygen consumption in the presence of complex IV inhibitor potassium cyanide (KCN)) and normalized to tissue weight. n = 3. h, i, Overexpression of Spargel in Elovl6 mutant female pre-wandering larvae leads to increased mitochondrial abundance, assessed by porin levels (h; representative of six biological replicates) and citrate synthase activity (i; n = 4) in pre-wandering larvae. See Supplementary Fig. 12 for image of the uncropped full western blot. j, Drosophila Elovl6 (either N- or C-terminally tagged) localizes to the mitochondrial outer membrane. S2 cell lysates (‘total’) were successively fractionated to yield crude mitochondria (which include mitochondrial-associated membranes (MAMs)), pure mitochondria (lacking MAMs), and mitochondrial outer membranes (OM), inner membranes (IM) and inter-membrane space (IMS). Endogenous porin and ATPsyn-a were used as positive controls for outer membranes and inner membranes, respectively. 7.5 μg of protein from each fraction was loaded per lane. See Supplementary Fig. 13 for image of the uncropped full western blot. Representative of two biological replicates. k, Lipidomic analysis of standard fly food reveals low levels of C18:0 in the food. a, c, d, e, g, i, Error bars represent s.d. a, b, e–g, i, *P < 0.05, **P < 0.01, not significant (NS) P ≥ 0.05, two-tailed t-test.
**Figure 1.**

**a** Comparison of fatty acid composition in control and dElov6* mitochondria. The graph shows the percentage of different fatty acids (C10:0, C12:0, C14:0, C16:0, C18:1, C18:2 cis, C18:3) in control and dElov6* mitochondria.

**b** Western blot analysis of porin and tubulin in whole larva samples. The blot shows the expression levels of these proteins in control and dElov6* samples.

**c** Bar graph comparing citrate synthase activity per mg protein in control and dElov6* samples. The graph indicates a nonsignificant difference (n.s.).

**d** Confocal microscopy images of the fat body in control and dElov6* samples with and without C18 fatty acid supplementation. The images show control samples with no C18 fatty acid and dElov6* samples with and without C18 fatty acid supplementation.

**e** Bar graph showing mitochondrial fragmentation index in control, Delipidated serum, +C18:0, +C12:0, +C18:1, and +C20 samples. The graph indicates a significant difference (**) in +C18:0 samples compared to other conditions (n.s.).

**f** Table showing the effects of control siRNA and LIAS siRNA on mitochondrial fragmentation index in medium control and Delipidated serum conditions. The table includes conditions such as -C18:0, +C18:0, -C18:0, and +C18:0.

**g** Western blot analysis of lipoyc acid, tubulin, lipoic acid, and actin in C18:0 control samples. The same extracts were used for ED 2h and ED 8c.

**h** Western blot analysis of DLAT, KGDH, NDUFA1, IBA57, and Actin in C18:0 control samples. The same extracts were used for Delipidated serum (24h) conditions.
Extended Data Figure 2 | C18:0 regulates mitochondrial morphology.

a, Lipidomic (GC-FID) profiles of purified mitochondria from *Elovl6* mutant 3rd instar larvae do not show major differences compared with control animals. Mitochondrial membranes from both control and mutant animals have very low levels of C18:0. Controls for purity of mitochondrial prep are shown in Extended Data Fig. 6b. b, *Elovl6* mutant larvae do not have reduced amounts of mitochondria, quantified via levels of porin (b, representative of three biological replicates) or citrate synthase activity (c, n = 3). See Supplementary Fig. 14 for image of the uncropped full western blot. d, *Elovl6* mutant larvae have fragmented mitochondria, which is rescued by dietary C18:0 supplementation. Mitochondrial morphology from fat bodies of control or *Elovl6* mutant female larvae, fed control or C18:0 (10%) supplemented food, visualized with mitoGFP. Images are representative of eight areas of four larvae from each genotype and food conditions. Equivalent pictures for body wall are shown in Fig. 2a. e, Only C18:0, and not shorter, longer or desaturated fatty acids, restores mitochondrial fragmentation to control levels in HeLa cells grown in medium containing delipidated serum. Mitochondria were visualized with MitoTracker (red) (top) and mitochondrial fragmentation was quantified by normalizing the number of mitochondrial particles to total mitochondrial area (bottom) (n = 15). f, Reduced lipoic acid (LA) levels do not lead to mitochondrial fragmentation. Lipoic acid synthase (*LIAS*) was knocked down by RNAi in HeLa cells, leading to significantly reduced lipoic acid levels, assayed by immunoblotting of total cell lysates with antibody detecting lipoic acid (bottom left). Unlike removal of C18:0, this does not lead to mitochondrial fragmentation. Representative images (top left) are quantified (top right) (n = 6). See Supplementary Fig. 14 for image of the uncropped full western blot. g, h, HeLa cells growing in medium containing delipidated serum for either 24 h (the same time point used for all other experiments in which mitochondrial fragmentation was assessed) (g, h), or for an extended period of time: 4 days (h). Lipoic acid levels were assayed by immunoblotting total cell lysates with an anti-lipoic acid antibody (g), and levels of lipoylated proteins were assessed with specific antibodies (h). See Supplementary Fig. 15 for image of the uncropped full western blot. c, e, f, *P < 0.05, **P < 0.01, not significant (NS) P > 0.05, two-tailed t-test. Error bars represent s.d. Scale bars, 10 μm.
Extended Data Figure 3 | Mitofusin loss of function phenocopies Elov16 mutation or removal of C18:0. a, dMfn knockout larvae (1st instar) have fragmented mitochondria, visualized with mitoGFP. Representative of ten images. b, Endogenous dMfn runs as a main band plus a laddering of apparently increasing molecular weights on an SDS–PAGE gel. Specificity is controlled by blotting lysates from control and Mfn-knockout female larvae with anti-Mfn antibody. c, Homozygous mutation of dMfn is lethal. Mfn-knockout larvae survive for several days as small L1/L2 larvae and eventually die. Synchronized 1st instar larvae were grown on standard fly food and examined every 24 h for developmental stage and per cent survival (n = 30). d, dMfn-knockout animals have impaired oxygen consumption. Oxygen consumption of inverted, digitonin permeabilized, female larval tissues was measured with an Oroboros oxygraph chamber and normalized to tissue weight. Oxygen consumption was measured in the presence of the following substrates: GMN (glutamate and malate), GMD (glutamate, malate and ADP), GMcD (glutamate, malate, cytochrome c and ADP), GMScD (glutamate, malate, succinate, cytochrome c and ADP), ETS (glutamate, malate, cytochrome c, ADP and rotenone), and Sc(Rot)u (glutamate, malate, cytochrome c, ADP and rotenone). n = 5. e, Endogenous dMfn is post-translationally modified in a C18:0-dependent manner in Drosophila. dMfn from Elovl6 female mutants migrates in an SDS–PAGE gel differently, compared with Mfn2 from control animals. This is reversed by supplementing the diet with C18:0. All indicated bands are dMfn, since they disappear in lysates from dMfn-knockout animals (see Extended Data Fig. 3b). Flies were grown on antifungal-free food. f, Endogenous MFN2 is post-translationally modified in a C18:0-dependent manner in human HeLa cells. MFN2 immunoprecipitated from HeLa cells treated for 24 h with medium containing standard or delipidated serum, and then for 2 h in the absence or presence of C18:0 (100 μM), lysed in 8M urea (see Methods). g, C18:0 affects ubiquitination of MFN2. MFN2 is more heavily ubiquitinated in cells treated with delipidated serum than in control cells and this is reversed by supplementing the medium with C18. HeLa cells were cotransfected with tagged versions of MFN2 (myc) and ubiquitin (HA). Tagged MFN2 was immunoprecipitated and blots were probed with HA antibody to detect ubiquitination. Quantification of ubiquitination, normalized to Myc–MFN2 in the immunoprecipitate (IP) is shown below the lane. h, C18:0 removal destabilizes MFN2 protein. A cyclohexamide (CHX) chase experiment was performed to block de novo synthesis of MFN2, thereby looking at turnover of existing MFN2 protein in vivo. HeLa cells treated with medium containing delipidated serum plus or minus C18:0 were treated with 100 μM CHX and then lysed at the indicated time points to compare MFN2 protein levels. Bottom, densitometric quantification of the blots normalized to loading control. i, dMfn expression is upregulated in Elovl6−/− flies compared with controls. dMfn transcript levels in 24 h female pre-wandering larvae were determined by quantitative polymerase chain reaction with reverse transcription (RT–PCR), normalized to rp49 (in triplicates). Scale bar, 10 μm. d, i, *P < 0.05, **P < 0.01, ***P < 0.001, not significant (NS) $P ≥ 0.05$, two-tailed t-test. Error bars represent s.d. See Supplementary Fig. 16 for images of the uncropped full western blots.
Extended Data Figure 4 | HUWE1 is required for hyperubiquitination of MFN2 in response to C18:0 withdrawal.  

**a**, Short interfering RNA (siRNA) depletion of other ubiquitin ligases targeting MFN (besides HUWE1, shown in Fig. 3) does not rescue the mitochondrial fragmentation induced by removal of C18:0 (top). Bottom, quantification.  

**b**, siRNA depletion of PARK2 in HEK293 cells, as in HeLa cells (**a**), does not rescue the mitochondrial fragmentation induced by removal of C18:0 (top). Bottom, quantification.  

**c**, HUWE1 knockdown efficiency controlled by detecting HUWE1 protein levels. See Supplementary Fig. 17 for image of the uncropped full western blot.  

**d**, Survival to pupation of Elovl6 mutants is fully rescued by ubiquitous expression (daughterless-GAL4) of RNAi targeting Huwe1 (CG8184). Elovl6 mutants expressing HUWE1 RNAi survive to pupation at expected Mendelian frequencies ($\chi^2$ test, 0.86 < 3.841 = $\chi^2$, where $P = 0.05$). Flies were grown on antifungal-free food. Values represent average of four biological replicates. Scale bars, 10 μm.  

**a**, **b**, **d**, *P < 0.05, **P < 0.01, ***P < 0.001, not significant (NS) P ≥ 0.05, two-tailed $t$-test. Error bars represent s.d.
**Extended Data Figure 5** | *Elovl6* mutants have other Mfn loss-of-function phenotypes such as reduced mitochondrial-associated membranes and abnormal cristae. **a**. The mitochondrial associated membranes (MAM) band is strongly reduced or absent in Percoll gradients of crude mitochondrial fractions from *Elovl6*-mutant animals, compared with controls. **b**, Purity control of mitochondrial preparations show that pure mitochondrial fractions are lacking markers of other subcellular organelles such as calnexin (endoplasmic reticulum) and lamin (nuclei). See Supplementary Fig. 17 for image of the uncropped full western blot. Right, quantification shows that levels of the endoplasmic reticulum marker calnexin are reduced in crude mitochondrial fractions from *Elovl6* mutants, compared with controls, in agreement with reduced MAMs in *Elovl6* mutants. Values show densitometry ratios of calnexin levels in crude mitochondrial fractions, normalized to total lysate calnexin. **c**, Electron microscopy of *Drosophila* S2 cell mitochondria (left) reveals cristae abnormalities in *Elovl6*-depleted cells. Middle, quantification (*n* = 200). Significance of the difference was calculated with a Mann–Whitney test (*P* < 0.05). Right, average circularity of mitochondria was calculated with ImageJ software. Scale bar, 1 μm. *n* = 200, ****P < 0.0001, two-tailed t-test. Error bars show s.d.
**Figure a:** Relative expression (log scale) of PERK, BiP, CHOP, and ATF-4 under control, delipidated serum, and tunicamycin conditions.

**Figure b:** Western blot of p-eIF2α and loading control under control, delipidated serum, and tunicamycin conditions.

**Figure c:** Mitochondrial fragmentation index under control and delipidated serum conditions with siRNA knockdown of IRE1, PERK, and ATF6.

**Figure d:** Mitochondrial fragmentation index under control and delipidated serum conditions with TUDCA treatment.
Extended Data Figure 6 | C18:0 removal does not lead to endoplasmic reticulum stress, and inhibiting UPR does not inhibit mitochondrial fragmentation upon C18:0 removal. a, C18:0 removal for 24 h does not induce expression of UPR target genes, quantified by quantitative RT–PCR, normalized to RPL19. BiP (also known as HSPA5) is a readout for IRE1 (also known as ERN1) activation, CHOP (also known as DDIT3) is a readout for ATF6 activation, and PERK (also known as EIF2AK3) is a readout of its own activation due to a positive transcriptional feedback loop. Tunicamycin serves as a positive control. y axis is displayed in a logarithmic scale to fit all data points on one graph. The experiment was done in triplicates. b, p-eIF2α, a UPR marker, does not increase upon removal of C18:0 whereas it is induced by tunicamycin, a positive control. See Supplementary Fig. 18 for image of the uncropped full western blot. c, Knocking down mediators of the UPR response does not inhibit mitochondrial fragmentation upon C18:0 removal. HeLa cells were transfected with either control siRNAs or siRNAs targeting UPR mediators as indicated. Left, the mitochondrial fragmentation index; right, representative images. n = 15. d, Inhibiting endoplasmic reticulum stress by means of a chemical chaperone, TUDCA, does not rescue mitochondrial fragmentation upon C18:0 removal. HeLa cells were pre-treated with 500 μg ml⁻¹ TUDCA 30 min before delipidated serum treatment. Left, mitochondrial fragmentation index (n = 15); right, representative images. a, c, d, *P < 0.05, **P < 0.01, two-tailed t-test. Error bars show s.d.
Extended Data Figure 7 | TFR1 is the mediator of C18:0 signalling to mitochondrial morphology. **a**, C17:0–azide is a functional analogue of C18:0 in that it induces mitochondrial fusion in HeLa cells, whereas other C18:0 derivatives are not. Cn:0–azide = HO2C(CH2)n–1N3; Cn:0–alkyne = HO2C(CH2)n–1CCH.

**b**, TFR1 is the most enriched protein in a C17:0–azide pulldown, and it regulates mitochondrial morphology. HeLa cells were treated with C17:0–azide for 2 h, and covalently bound proteins were precipitated by lysing cells under denaturing conditions (8 M urea), and linking the C17:0–azide to an alkyne-labelled resin via click chemistry (left). Precipitated proteins were identified by mass spectrometry, and peptide counts were normalized to peptide counts in a negative control pulldown from cells not treated with C17:0–azide (n = 3) (right; column 2). Indicated proteins were also tested by siRNA-mediated knockdown for effects on mitochondrial morphology (column 3).

**c**, TFR1 is covalently bound to the C18:0 derivative C17:0–azide in HeLa cells in a ZDHHC6-dependent manner. HeLa cells were treated with C17:0–azide for 2 h, and subsequently lysed in denaturing conditions (8 M urea). Similar to b, the C17:0–azide was ‘clicked’ onto a biotinylated alkyne, and the labelled proteins were pulled-down with streptavidin beads. After washing, immunoprecipitated proteins were eluted off beads in Laemmli buffer containing biotin, and analysed by immunoblotting. The palmitic acid analogue C15:0–azide was used as a positive control since TFR1 is known to also be palmitoylated. C17:0–azide pulls down more TFR1 than equal amounts of C15:0–azide, indicating that TFR1 palmitoylation cannot account for the C17:0 signal. The C17:0–azide–TFR1 interaction is completely blunted upon ZDHHC6 knockdown. See Supplementary Fig. 18 for image of the uncropped full western blot.

**d**, TFR1 is required for C18:0 removal to induce mitochondrial fragmentation. HeLa cells were transfected with either control or TFR1 targeting siRNAs before treatment with medium containing delipidated serum plus or minus C18:0. Representative images are shown here and quantification of mitochondrial fragmentation is shown in Fig. 3h. n = 15.
Extended Data Figure 8 | C18:0 removal does not affect iron uptake or delivery. a, HeLa cells cannot grow in the presence of deferoxamine (DFO), an iron chelator (top) whereas they grow in delipidated serum lacking C18:0 at a comparable rate to cells in control medium (bottom). n = 3. b, Treatment of HeLa cells with medium containing delipidated serum (lacking C18:0) for 24 h does not lead to transcriptional activation of iron deficiency response genes (bottom), which are activated by DFO-mediated iron chelation (24 h) as a positive control (top). n = 3. c, Treatment of HeLa cells with medium containing delipidated serum for 24 h or 4 days does not lead to a drop in levels of succinate dehydrogenase b (SDHB), which contains an Fe–S cluster. See Supplementary Fig. 19 for image of the uncropped full western blot. d–f, Treatment of HeLa cells with medium containing delipidated serum for 24 h or 4 days does not lead to a drop in activities of enzymes containing lipoylated subunits (PDH and OGDH) (d, e) or Fe–S-cluster-containing subunits (SDH) (f). DFO treatment to chelate iron from the medium, or siRNA-mediated depletion of the enzymes were used as positive controls (d–f, bottom). n = 4. g, Treatment of HeLa cells with medium containing delipidated serum (24 h) does not cause a reduction in transferrin uptake. Cells were treated with 25 μg ml⁻¹ Alexa-488-coupled transferrin for 30 min. Representative images (left) and quantification of the amount of transferrin per cell (right) (n = 5). h, Treatment of HeLa cells with medium containing delipidated serum (24 h) does not reduce association of transferrin-containing vesicles with mitochondria. Crude mitochondria were fractionated from cells growing in medium containing or lacking C18:0, and the amount of transferrin that copurifies with mitochondria was analysed and quantified by immunoblotting. See Supplementary Fig. 19 for image of the uncropped full western blot. a, b, d–g, *P < 0.05, ***P < 0.001, ****P < 0.0001, not significant (NS) P ≥ 0.05, two-tailed t-test. Error bars show s.d.
a

| Gambogenic Acid (uM) | Control | Delipid. Serum |
|---------------------|---------|----------------|
| 10                  | 10      | 0              |
| 0.5                 | 0.5     | 0              |
| 1                   | 1       | 0              |
| 2                   | 2       | 0              |
| 0                   | 0       | 0              |

Cleaved Caspase-3

non-specific loading control

Tubulin

b

control

1μM Gambogenic Acid (GA)

1μM GA + C18:0

mitotracker/DAPI

C18:0

loading control

relative pJNK quantification 1 0.51

c

d

pJNK

DAPI

phalloidin

merge

Control

-C18:0

+ C18:0

5 μM GA

nuclear/cytoplasmic pJNK ratio

C18:0

- +

***

e

control

5μM GA

10μM SP600125

5 μM GA + 10μM SP600125

mitotracker/DAPI
Extended Data Figure 9 | JNK signalling is required for mitochondrial fragmentation induced by C18:0 removal. 

| a | Treatment of HeLa cells with 1 μM gambogic acid does not induce apoptosis. 10 μM gambogic acid was used as a positive control for apoptosis induction, assessed by cleaved caspase-3 levels. 1 μM gambogic acid neither induces caspase cleavage (shown here) nor causes cells to die (data not shown). Cells were treated with 10 μM gambogic acid for 1 h, or for all other concentrations for 3 h. See Supplementary Fig. 20 for image of the uncropped full western blot.

| b | Activation of TFR1 by treating cells with 1 μM gambogic acid leads to mitochondrial fragmentation that is reversed by 1 h C18:0 pre-treatment. Representative images are shown here and quantification of mitochondrial fragmentation is shown in Fig. 3i (n = 15).

| c | Treatment of HeLa cells with C18:0 to inhibit TFR1 causes reduced JNK signalling activity, assayed by p-JNK levels on an immunoblot. See Supplementary Fig. 20 for image of the uncropped full western blot.

| d | Removal of C18:0, as well as treatment with gambogic acid, induces shuttling of phosphorylated JNK into the nucleus. Cells were stained with phospho-JNK antibody (left) and relative levels of nuclear to cytosolic phospho-JNK signal was quantified (right) (n = 37 cells). ***P < 0.001, two-tailed t-test. Error bars show s.d.

| e | JNK signalling is required for TFR1 activation to induce mitochondrial fragmentation. HeLa cells were treated with the JNK inhibitor SP600125 30 min before gambogic acid treatment to activate TFR1. Representative images are shown here and quantification of mitochondrial fragmentation is shown in Fig. 3j (n = 15).
Extended Data Figure 10 | Dietary C18:0 improves Parkinson’s disease phenotypes of Pink and Parkin mutant flies. a, b, Dietary C18:0 supplementation (10%) significantly increases lifespan of male Parkin25 (a) and Pink1B9 (b) mutant flies. n = 8–10 animals. c, Dietary C18:0 supplementation rescues ATP levels of 1-week-old male Pink1B9 mutant adult flies. n = 3 × 3 animals. d, Dietary C18:0 supplementation significantly improves locomotor defects of 2-week-old male Pink1B9 mutant flies. Locomotion quantified as animals climbing up past a threshold in a given amount of time (technical duplicates, biological quadruplicates, ten animals per assay). e, Parkin loss of function in flies leads to mitochondrial fragmentation, which is rescued by dietary supplementation with C18:0. Guts from 14-day-old female control or park25 mutant adult flies expressing mitoGFP and grown on food supplemented with or without C18:0 (10%) were dissected and mitochondria were imaged. Quantification of mitochondrial fragmentation is shown (3 animals per condition, 6 optical areas per animal). b–d, Control flies are the revertant line Pink1RV. Error bars show s.d. Not significant (NS) P ≥ 0.05, *P < 0.05.