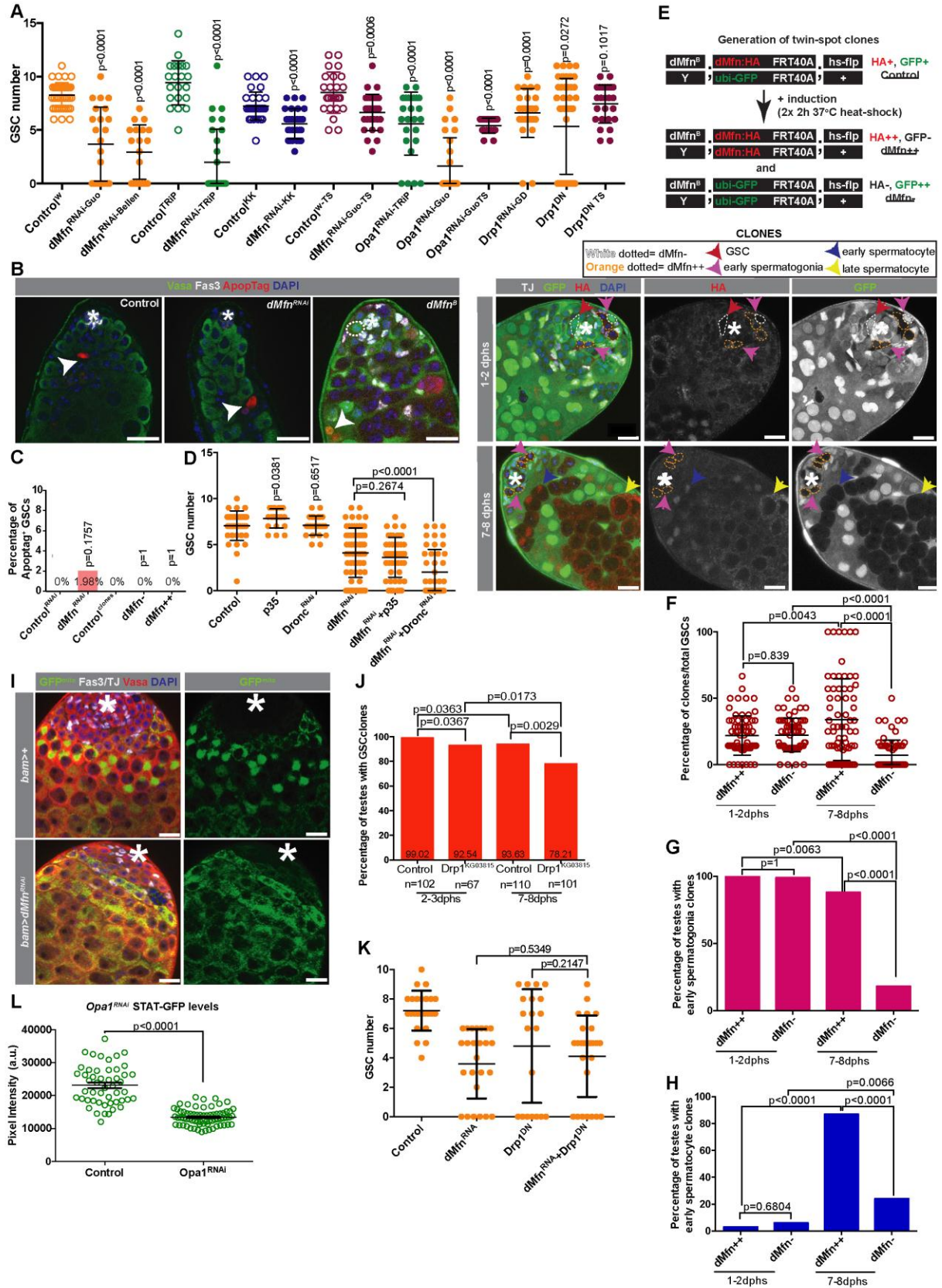


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Mitochondrial fusion regulates lipid homeostasis and stem cell maintenance in the *Drosophila* testis

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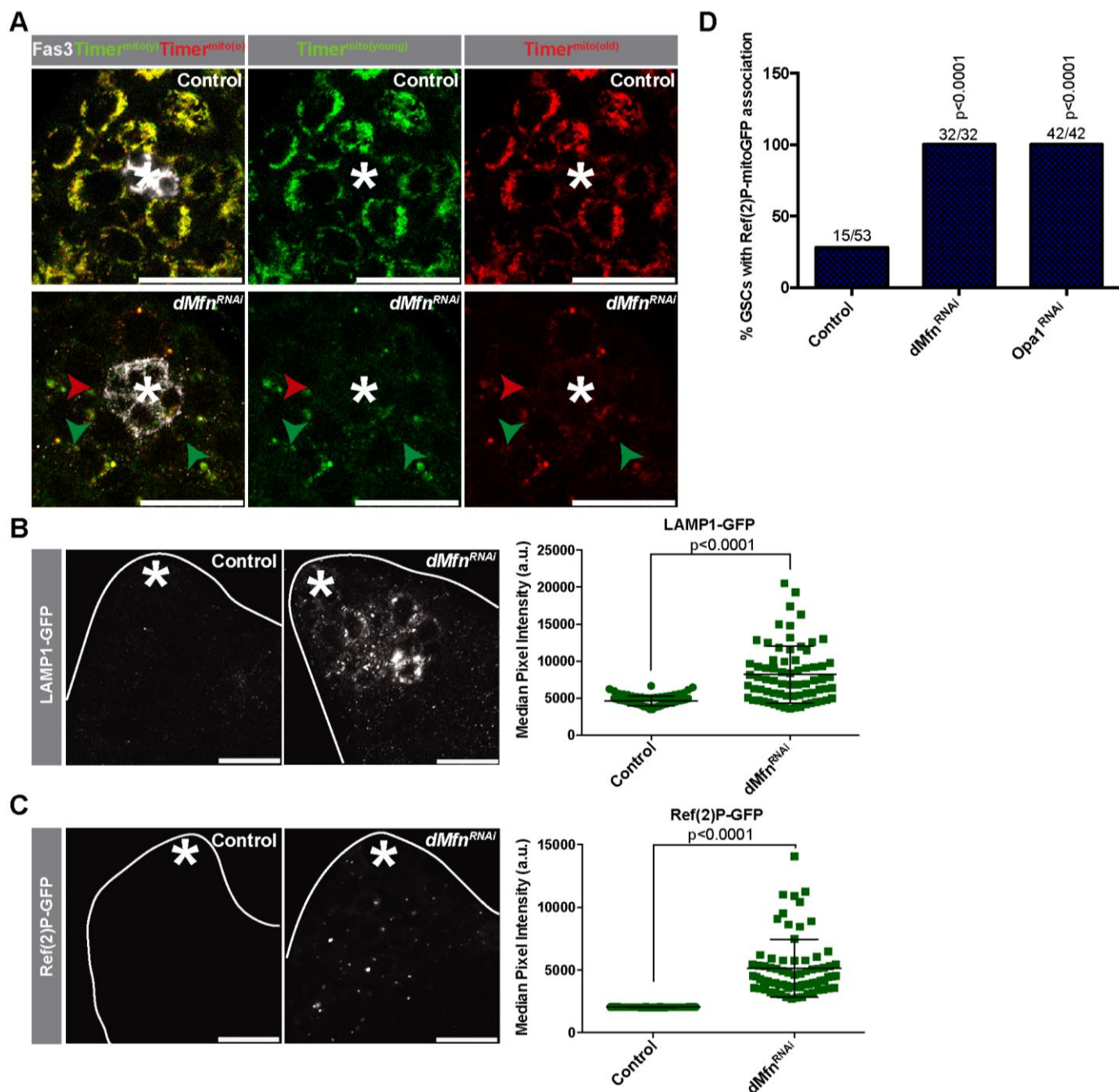
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Supplementary Figure 1

Mitochondrial fusion is required for GSC maintenance

A) Quantification of GSCs per testes in 10 days old (do) *nos-GAL4:VP16* animals expressing transgenes targeting the down regulation of *dMfn*, *Opa1* and *Drp1*. Symbol colors refer to appropriate control. Dark-red symbols represent similar experiments done in 10do *nos-Gal4; Gal80^{TS}* animals, in which the transgene expression was only induced after eclosion (see Methods). N=45 testes for Control^W, n=23 testes for *dMfn*^{RNAi-Guo}, n=19 testes for *dMfn*^{RNAi-Bellen}, n=21 testes for Control^{TRIP}, n=28 testes for *dMfn*^{RNAi-TRIP}, n=32 testes for Control^{KK}, n=27 testes for *dMfn*^{RNAi-KK}, n=24 testes for Control^{W-TS}, n=29 testes for *dMfn*^{RNAi-GuoTS}, n=25 testes for *Opa1*^{RNAi-TRIP}, n=28 testes for *Opa1*^{RNAi-Guo}, n=17 testes for *Opa1*^{RNAi-GuoTS}, n=27 testes for *Drp1*^{RNAi-GD}, n=46 testes for *Drp1*^{DN}, n=25 testes for *Drp1*^{DN TS}. Two-tailed t-test used. **B)** Representative IFs of testes stained with ApopTag. Arrowheads denote cells positive for ApopTag. Dotted circle in right panel denotes a *dMfn*^{RNAi} clone. 3 biological replicates. **C)** Quantification of ApopTag⁺ GSCs. Two-sided fisher's exact test was used. N=142 GSCs for control^{clones}, n=103 GSCs for *dMfn*^{RNAi}, n=91 GSCs for control^{clones}, n=40 GSCs for *dMfn*^{RNAi}, n=58 GSCs for *dMfn*^{RNAi} (+GFP^{mito}) data reproduced from Figure 6C for comparison. N=20 testes for *p35*, n=25 testes for *Dronc*^{RNAi}, n=48 testes for *dMfn*^{RNAi}+*p35*, n=43 testes for *dMfn*^{RNAi}+*Dronc*^{RNAi}. Two-tailed t-test used. **E)** Schematics and IF examples of the twin-spot clonal generation of *dMfn* loss-of-function clones. In representative IFs of testes with *dMfn*^{RNAi} and *dMfn*^{RNAi} clones, arrowhead and trace colors are keyed at the bottom panel. 3 biological replicates. **F-H)** Clonal quantification. Quantification of the percentage of clonal GSCs per total GSCs in **F** (two-tailed t-test used), testes with early spermatogonial clones in **G**, and testes with early spermatocyte clones in **H**. Two-sided fisher's exact test was used in G and H. n=68 testes for all conditions. **I)** Representative IF of testes from 5do animals expressing a mitochondrially targeted GFP in early spermatogonia with the driver *bam-GAL4:VP16*. Note that when *dMfn* is depleted, the germline mitochondria of late spermatogonia/early spermatocytes do not fuse and fail to aggregate in one side of the cell. Representative of 30 testes per genotype, analyzed in 3 independent experiments. **J)** Quantification of the percentage of testes containing control GSC clones or GSCs homozygous for a null allele of *Drp1*. N=102 testes for control 1-2dphs and n=110 testes for control 7-8dphs; n=67 testes for *Drp1*^{KG03815} 1-2dphs and n=101 for *Drp1*^{KG03815} 7-8dphs. Two-sided fisher's exact used. **K)** GSC quantification in animals expressing *nos-GAL4:VP16>dMfn*^{RNAi} (+GFP^{mito}), *Drp1*^{DN} (+GFP^{mito}), *nos-GAL4:VP16>dMfn*^{RNAi}+*Drp1*^{DN} and control. Two-tailed t-test was used. N=24 testes for control, n=25 testes for *dMfn*^{RNAi}, n=20 testes for *Drp1*^{DN}, n=27 testes for *dMfn*^{RNAi}+*Drp1*^{DN}. **L)** Quantification of STAT-GFP pixel intensity in nuclei of control or *Opa1*-depleted GSCs from 2do animals. N=50 GSCs for control and n=68 GSCs for *Opa1*^{RNAi}. Two-tailed t-test used. In all figures, asterisk (*) denotes the hub; scale bar, 20μm. Raw data and statistical detail for data plots in A, C, D, F-H, J-L available in Supplemental Table 2. Plots in A, D, F, K and L display mean with error bars displaying standard deviation. C, G, H and J display graphical representations of proportion.

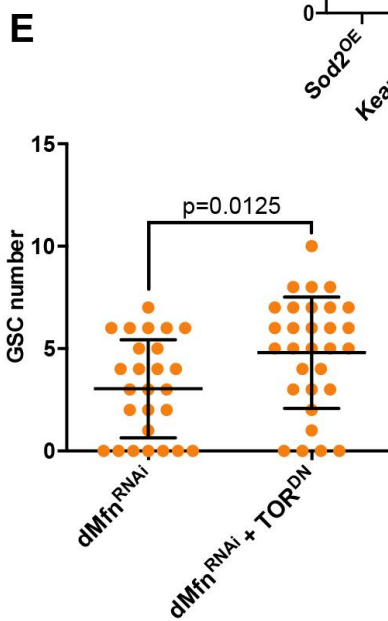
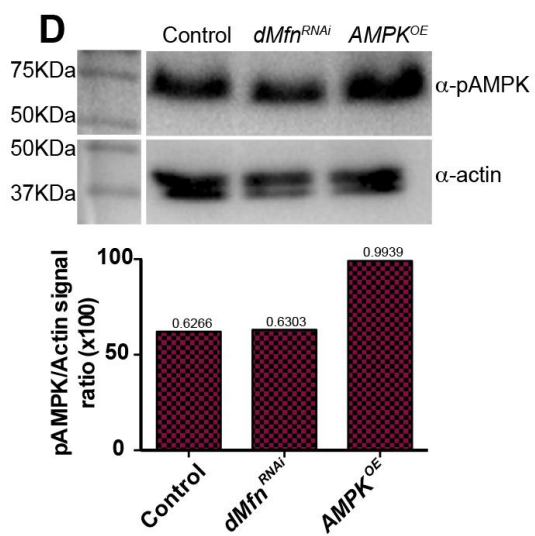
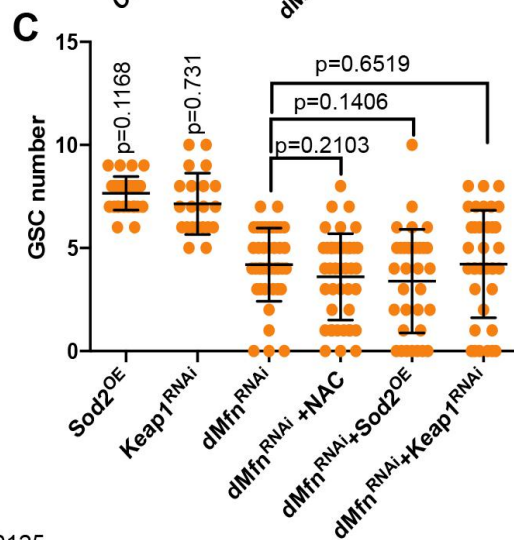
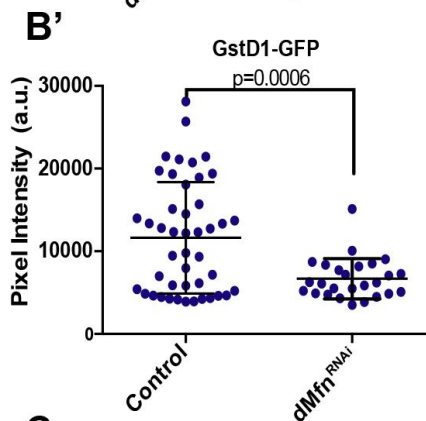
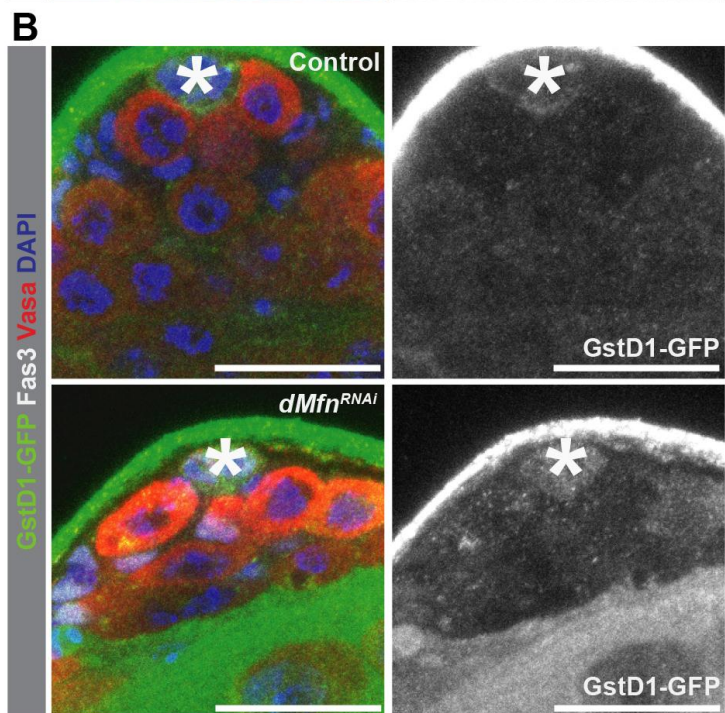
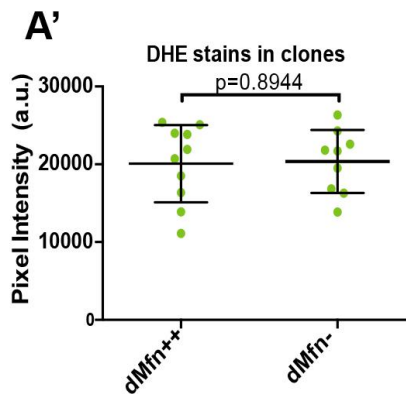
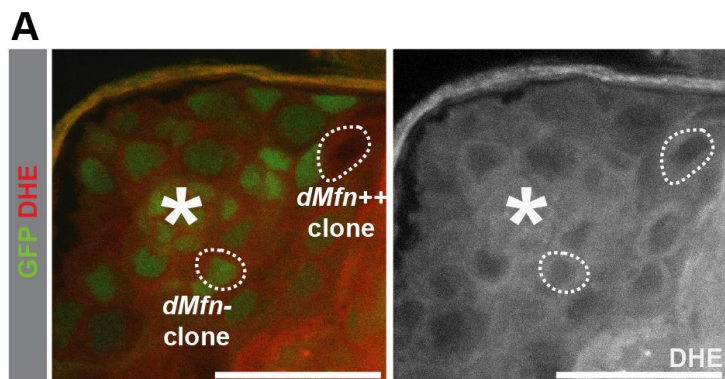


Supplementary Figure 2

Block in mitochondrial fusion leads to loss of mitochondrial function and induction of mitophagy

A) Representative images of GSCs expressing Timer^{mito} in either control or *dMfn*^{RNAi} animals. Recently folded (“young”) Timer^{mito} fluoresces green (Timer^{mito(y)}), but with maturation (“old”), Timer^{mito} shifts fluorescence to red (Timer^{mito(o)}). Note the accumulation of mitochondria with primarily red (red arrowhead) or green (green arrowhead) Timer^{mito} signal (i.e., no mixing) upon *dMfn* depletion. N=30 testes per genotype, analyzed in 3 individual experiments. **B-C)** Images of testes from 5d animals of the noted genotypes also expressing UAS-LAMP1:GFP (B) or UAS-Ref(2)P:GFP (C) transgenes. Fluorescence intensity was calibrated using the maximal signal emitted from the *dMfn*^{RNAi} samples for comparison. Pixel quantifications of LAMP1 (B) or Ref(2)P (C) signal in GSCs displayed to the right of each IF panel (two-tailed t-test used). For LAMP1, n=81 GSCs in control, n=71 GSCs in *dMfn*^{RNAi}; for Ref(2)P, n=75 GSCs for control, n=74 GSCs for *dMfn*^{RNAi}. **D)** Quantification of the percentage of GSCs with at least one mitochondrial unit associated with

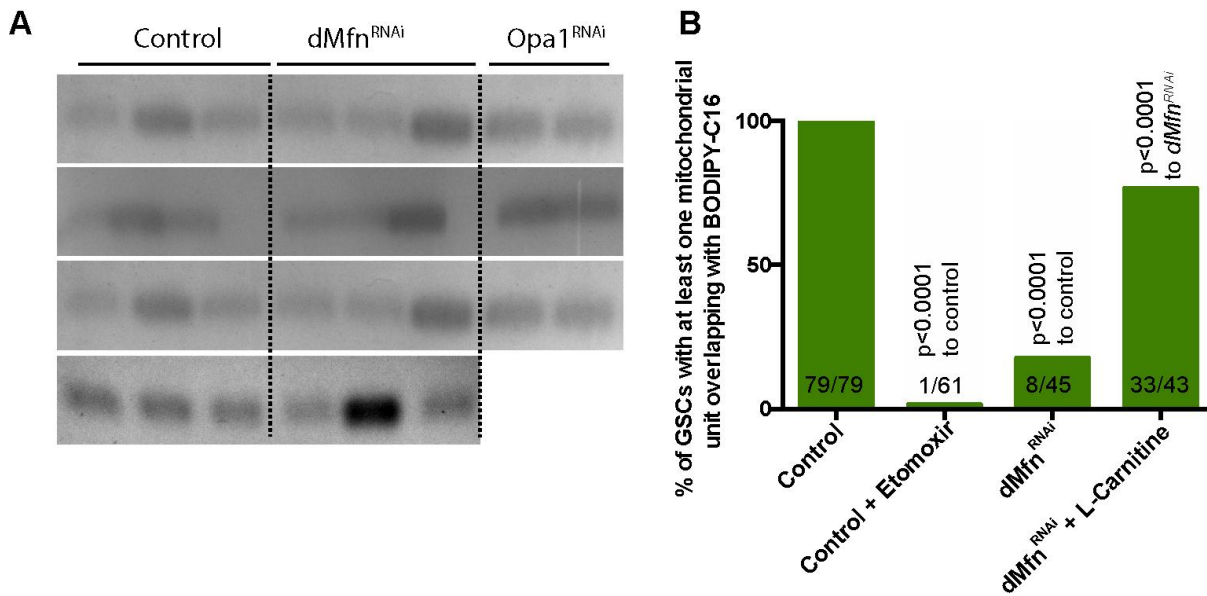
Ref(2)P aggregates (see Methods). In testes from both *dMfn*^{RNAi} and *Opa1*^{RNAi}, there was a significant increase in co-localization of the two signals in GSCs ($p < 0.0001$ compared to controls). Two-sided fisher's exact was used. N=53 GSCs in control, n=32 GSCs in *dMfn*^{RNAi}, n=42 GSCs in *Opa1*^{RNAi}. In all figures, asterisk (*) denotes the hub; scale bar, 20 μ m. Raw data and statistical detail for data plots in B-D available in Supplemental Table 2. Plots in B and C display mean with error bars displaying standard deviation. D displays graphical representation of proportion.



Supplementary Figure 3

No changes in ROS nor AMPK activation were observed upon *dMfn* depletion

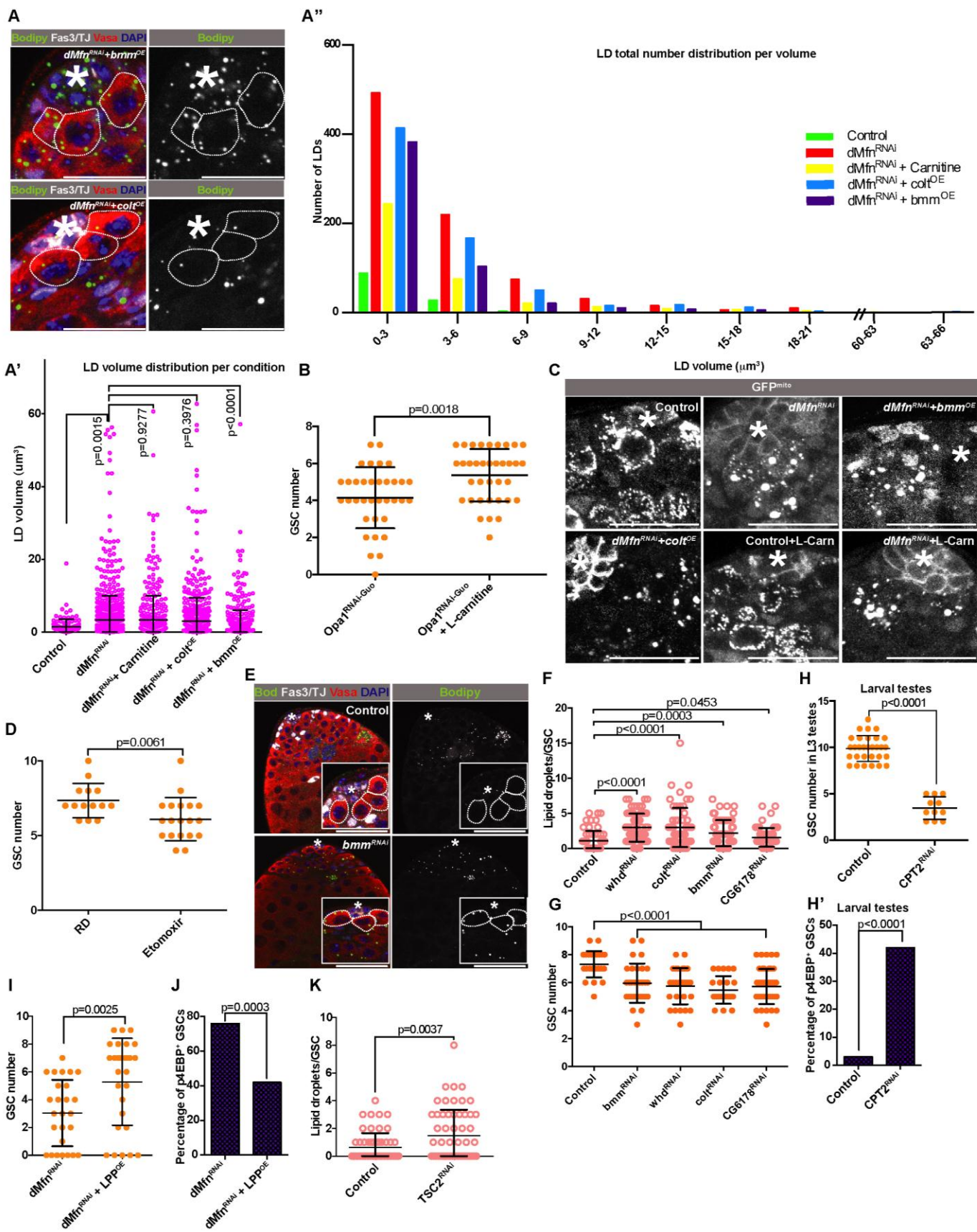
A) DHE stains showed no difference in ROS levels between GSCs of control vs *dMfn* loss of function clones. Quantification shown in **A'** (two-tailed t-test used). N=10 clones for *dMfn*⁺⁺, n=9 clones for *dMfn*⁻. **B)** The reporter for antioxidant gene-regulation *GstD1-GFP* showed no difference between control and *dMfn*-depleted GSCs. Pixel intensity quantification is shown in **B'**. N=45 GSCs for control, n=26 GSCs for *dMfn*^{RNAi}. Two-tailed t-test used. **C)** Quantification of GSCs in testes that expressed *nos-GAL4:VP16>dMfn*^{RNAi} (+*GFP*^{mito}) and were fed either a regular diet (n=41 testes) or food containing 100mM of the antioxidant NAC (n=43 testes) for 5 days; or that co-expressed *dMfn*^{RNAi} with either the overexpression of the ROS-scavenger *Sod2* (n=33 testes) or the knockdown of the CnC/NRF2 inhibitor *Keap1* (n=36 testes) in the germline. No statistically significant difference was observed among any groups with *dMfn*^{RNAi}. Two-tailed t-test was used. For comparison, *nos-GAL4:VP16>Sod2*^{OE} (n=29 testes) and *nos-GAL4:VP16>Keap1*^{RNAi} (n=21 testes) are also displayed (no statistically significant difference to control represented in Figure 4C). **D)** Western blot analysis shows no change in pAMPK levels when *dMfn* was depleted in 2do animals. Experiment repeated 3x with similar trends. **E)** Quantification of GSCs per testes in 5do animals show that the co-expression of a dominant-negative TOR construct with *dMfn*^{RNAi} rescued the GSC loss (two-tailed t-test used). N=26 testes for *dMfn*^{RNAi} and n=30 testes for *dMfn*^{RNAi}+*TOR*^{DN}. In all figures, asterisk (*) denotes the hub; scale bar, 20μm. Raw data and statistical detail for blots in D and data plots in A', B', C and E available in Supplemental Figure 7 and Supplemental Table 2, respectively. Plots in A', B', C and E display mean with error bars displaying standard deviation. D displays graphical representation of pixel intensity ratio.



Supplementary Figure 4

FA utilization by mitochondria is disrupted when mitochondrial fusion is blocked

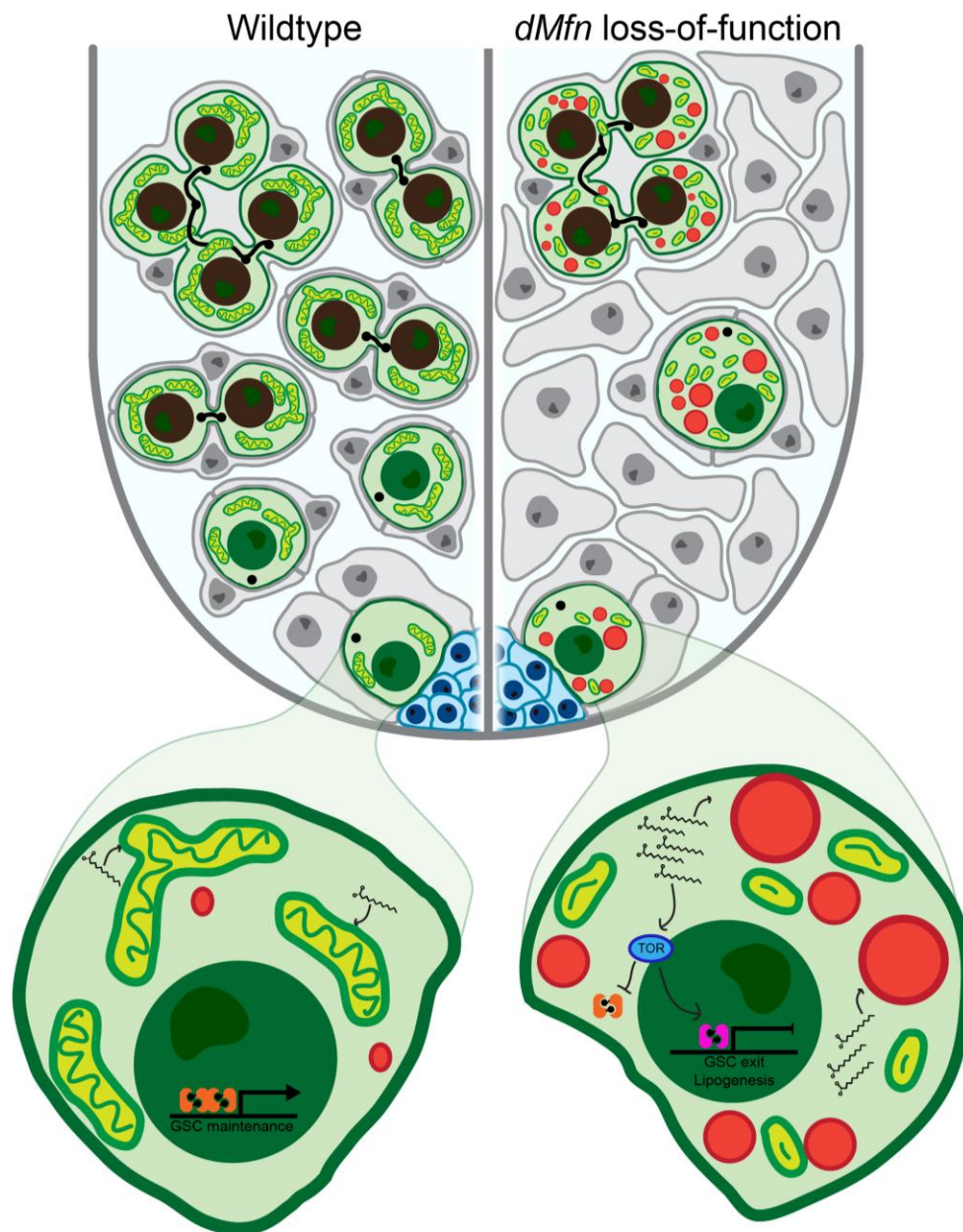
A) Thin-layer chromatography blots for triglyceride level measurements displayed in Figure 5B. Raw blots available in Supplemental Figure 7. **B)** Quantification of the percentage of GSCs with at least one mitochondrial unit overlapping with BODIPY-C16 for Figure 5C. For etomoxir feeding or dMfn^{RNAi}, p<0.0001 compared to control; dMfn^{RNAi}+L-Carnitine p<0.0001 compared to dMfn^{RNAi}. Two-sided fisher's exact was used. Plot display graphical representations of proportion. N=79 GSCs for control, n=61 GSCs for control+etomoxir, n=45 GSCs for dMfn^{RNAi}, n=43 GSCs for dMfn^{RNAi}+L-carnitine. Raw data and statistical detail available in Supplemental Table 2.



Supplementary Figure 5

FA utilization by mitochondria is required for GSC maintenance

A) IFs of testes from 5do animals stained with the lipophilic dye BODIPY 493/503 of the noted genotypes. Compare to Figure 6A. Representative of 30 testes per condition, analyzed in 3 individual experiments. **A')** Plot representation of volume of individual LDs in tips of testes of the noted genotypes. n=10 testes per condition. Experiment was repeated 3x with similar trends. Two-tailed t-test used. **A'')** Distribution of the total number of LDs of a given volume in the noted genotypes (see Methods for detail on quantification). N=122 LDs for control, n=881 LDs for *dMfn^{RNAi}*, n=391 LDs for *dMfn^{RNAi}* + L-carnitine, n=697 LDs for *dMfn^{RNAi}*+*colt^{OE}*, n=540 LDs for *dMfn^{RNAi}*+*bmm^{OE}*. **B)** Quantification of GSCs from 5do animals expressing *nos-GAL4:VP16>Opa1^{RNAi}* fed either a regular diet (n=35 testes) or a diet supplemented with L-carnitine (n=36 testes). Two-tailed t-test used. **C)** Representative images of GSCs expressing *nos-GAL4:VP16>GFP^{mito}* and the described genotypes. Note that the mitochondrial network in rescue experiments remains similar to the one observed in *dMfn^{RNAi}* alone. 30 testes per condition. Experiment was repeated 3x with similar trends. **D)** Quantification of GSCs from 10do *w¹¹¹⁸* (control) animals fed either a regular diet (n=14 testes) or a diet supplemented with 100μM etomoxir (n=19 testes) (two-tailed t-test used). N=14 testes (control), n=19 testes (etomoxir); 3 biological replicates. **E-G)** Depletion of lipid catabolism genes in GSCs. Representative examples of testes from either control or animals expressing *bmm^{RNAi}* for 10 days in early germ cells (*nos-GAL4:VP16*) shows accumulation of LDs in the germline (E). Image representative of 30 testes, analyzed in 3 individual experiments. Quantification of LDs per GSCs shown in **F**, and GSCs per testes shown in **G** (two-tailed t-test used). For **F**, n=63 GSCs for control, n=81 GSCs for *whd^{RNAi}*, n=62 GSCs for *colt^{RNAi}*, n=65 GSCs for *bmm^{RNAi}*, n=76 GSCs for *CG6178^{RNAi}*; for **G**, n=25 testes for control, n=28 testes for *whd^{RNAi}*, n=23 testes for *colt^{RNAi}*, n=30 testes for *bmm^{RNAi}*, n=43 testes for *CG6178^{RNAi}*. **H-H')** Depletion of *CPT2* in early germ cells with *nos-GAL4:VP16* leads to a significant decrease in GSC number in third instar larvae (H; two-tailed t-test used) and an increase in GSCs positive for p4E-BP (H'; two-sided fisher's exact used). For **H**, n=30 testes for control and n=11 testes for *CPT2^{RNAi}*, for **H'**, n=60 GSCs for control and n=38 GSCs for *CPT2^{RNAi}*. **I)** Quantification of GSCs in testes from 5do animals expressing *dMfn^{RNAi}*(+*GFP^{mito}*) (reproduced from Figure S3E) (n=26 testes) or *dMfn^{RNAi}*+*LPP^{OE}* (n=28 testes) with *nos-GAL4:VP16* (two-tailed t-test used). **J)** Quantification of p4E-BP⁺ GSCs per total GSCs (two-sided fisher's exact used). N=49 GSCs for *dMfn^{RNAi}*, n=69 GSCs for *dMfn^{RNAi}*+*LPP^{OE}*. **K)** Quantification of LDs per GSCs in controls (n=56 GSCs) or flies expressing *nos-GAL4:VP16>TSC2^{RNAi}* (n=58 GSCs) for 10 days (two-tailed t-test used). In all figures, asterisk (*) denotes the hub; scale bar, 20μm. Raw data and statistical detail for data plots in A-B, D, F-K available in Supplemental Table 2. Plots in A', B, D, F-H, I, K display mean with error bars displaying standard deviation. H', J display graphical representations of proportion. A'' displays absolute number of LDs across binning volumes of 3 μm³.



LEGEND

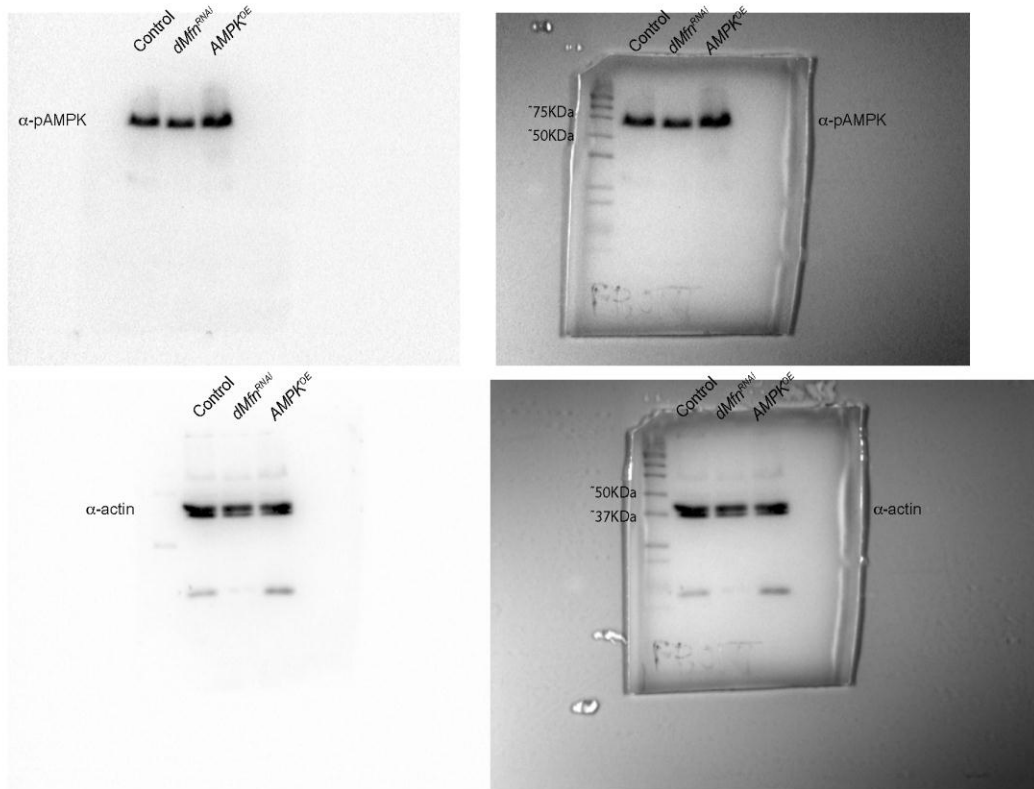
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	Hub cell		Free fatty acid
	Target of Rapamycin		STAT92E
			SREBP

Supplementary Figure 6

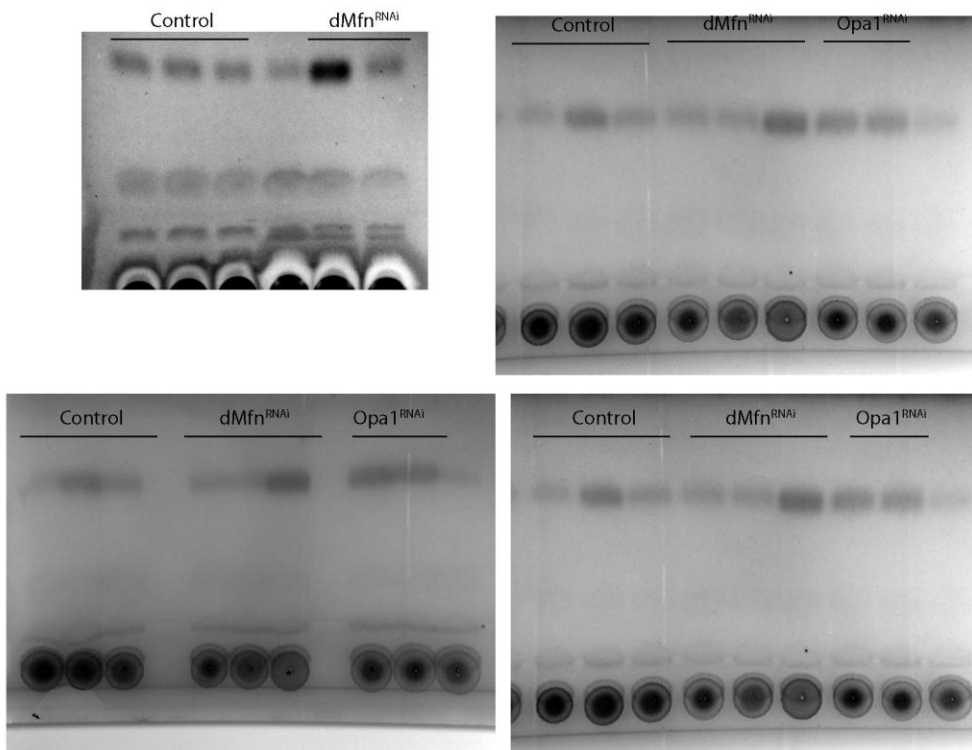
Mitochondrial fusion influences lipid homeostasis to regulate GSC maintenance

Schematic showing that disruption of mitochondrial fusion leads to loss of GSCs in *Drosophila* males.

A - raw files from Figure S3D



B - raw files from Figure 5B and S4A



Supplementary Figure 7

Raw blot data related to Figures 5, S3 and S4

A) Unprocessed western blots related to Figure S3D. **B)** Unprocessed TLC blots related to Figures 5B and S4A.

Excel file

Supplementary Table 1

Screen results for regulators of germline stem cell (GSC) metabolism

Results of the RNAi-based genetic screen targeting regulators of mitochondrial dynamics (categories described in column 1) using the *nanos-GAL4:VP16* driver with *UAS-GFP^{mito}* to downregulate the noted genes (columns 2 and 3) using UAS-based lines (column 4) for 10 days post-eclosion. GSC numbers and standard deviations described in column 5; statistical p-value (derived using two-tailed t-test against appropriate control) noted in column 6, and number of testes analyzed noted on column 7.

Excel file

Supplementary Table 2

Statistics source data

Raw data and statistical analysis information for all data presented throughout the manuscript.