SUPPLEMENTAL FIGURES

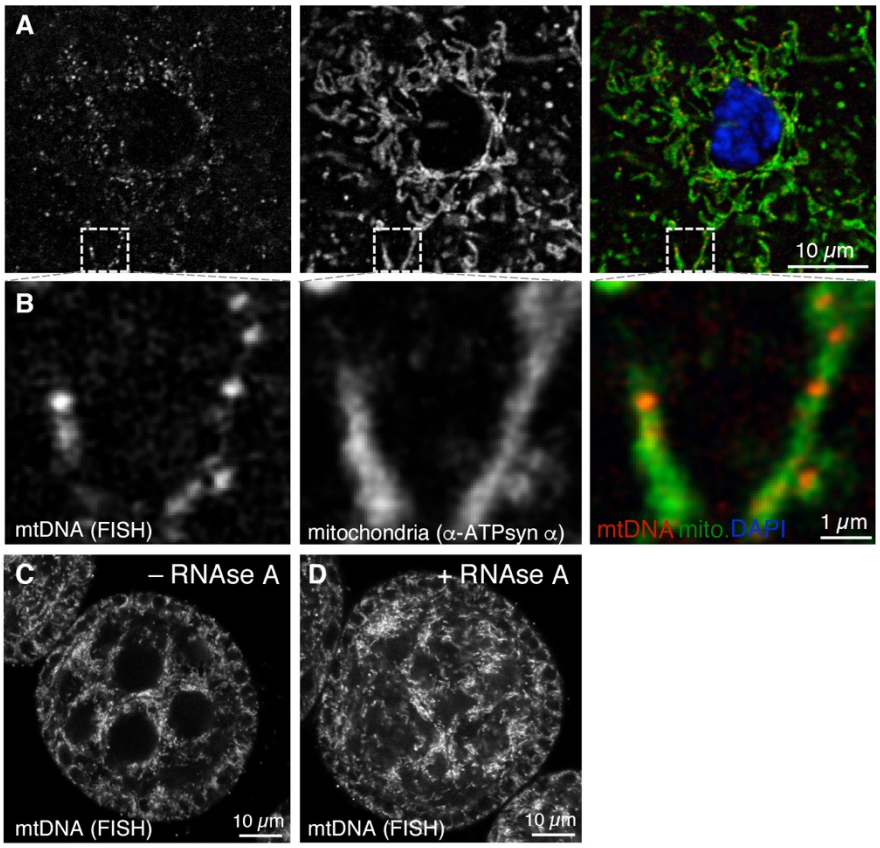

Figure S1 related to figure 1. mtDNA FISH foci co-localize with mitochondria and are insensitive to RNAase A treatment.

(**A** and **B**) mtDNA, mitochondria and nuclear DNA were visualized in the somatic follicle cells of stage 9 egg chambers using mtDNA FISH, α-ATP synthase α and DAPI, respectively. The white boxes indicate the region displayed in B.

(**C** and **D**) mtDNA was visualized in egg chambers treated without (**C**) or with (**D**) RNAseA.

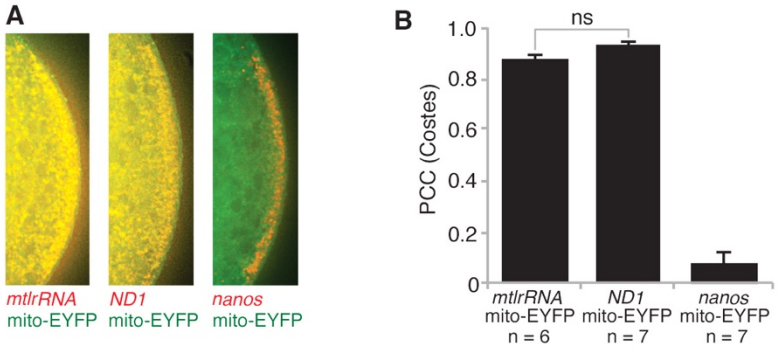

Figure S2 related to figure 2. *mtlrRNA* is present inside mitochondria at the embryo posterior.

The sub-cellular localization of the *mtlrRNA* at the posterior of 0 to 1 hour old control embryos was measured by single molecule RNA FISH with probes against: the *mtlrRNA*; *ND1* mRNA, a mtDNA-encoded RNA known to be present inside mitochondria; and *nanos* mRNA, a cytoplasmic germ plasm mRNA.

(**A**) *mtlrRNA*, *ND1* and *nanos* were imaged at the posterior of wildtype embryos using single molecule FISH and mitochondria were visualized with mito-EYFP.

(**B**) Co-localization of *mtlrRNA*, *ND1* and *nanos* with mito-EYFP as measured using Costes PCC. 1 represents perfect colocalization and 0 represents random colocalization. Near perfect overlap between the *mtlrRNA* and mito-EYFP and *ND1* mRNA and mito-EYFP was observed. If the *mtlrRNA* were present mostly outside of mitochondria the PCC would be near 0 as is the case for *nanos* RNA. Data are expressed as the mean +/– SD. Significance was assessed using an independent two-tailed Student's t-test.

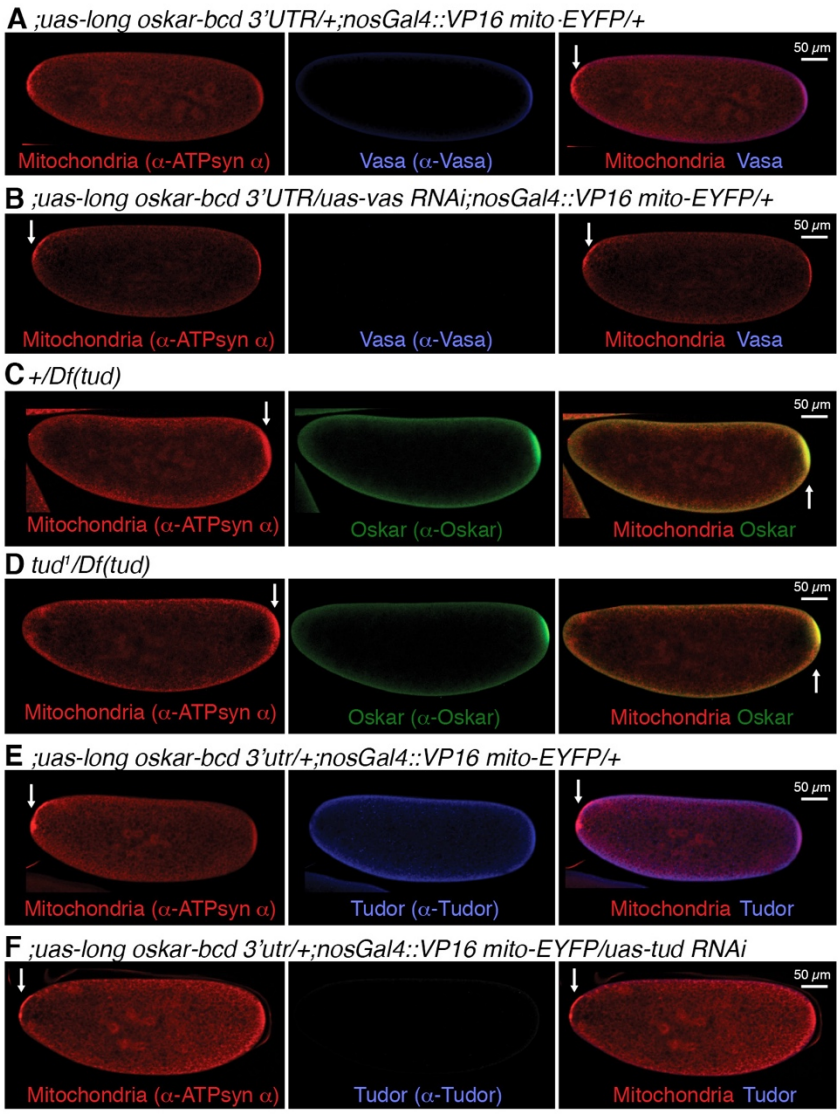

Figure S3 related to figure 4. Vasa and Tudor are not required for Long Oskar-mediated asymmetrical mitochondrial positioning.

Images of 0 to 1 hour old embryos of the indicated genotypes immunostained with α-ATPsyn α (mitochondria), α-Vasa, α-Oskar and α-Tudor. Arrows indicate mitochondrial accumulation.

(**A** and **B**) Knockdown of Vasa neither perturbs mitochondrial accumulation at the posterior nor the anterior when Long Oskar is expressed anteriorly. Long Oskar does not recruit Vasa to the anterior.

(**C** and **D**) Mitochondria accumulate normally at the posterior of *tudor* mutant embryos.

(**E** and **F**) Knockdown of Tudor neither perturbs mitochondrial accumulation at the posterior nor the anterior when Long Oskar is expressed anteriorly. Long Oskar does not recruit Tudor to the anterior.

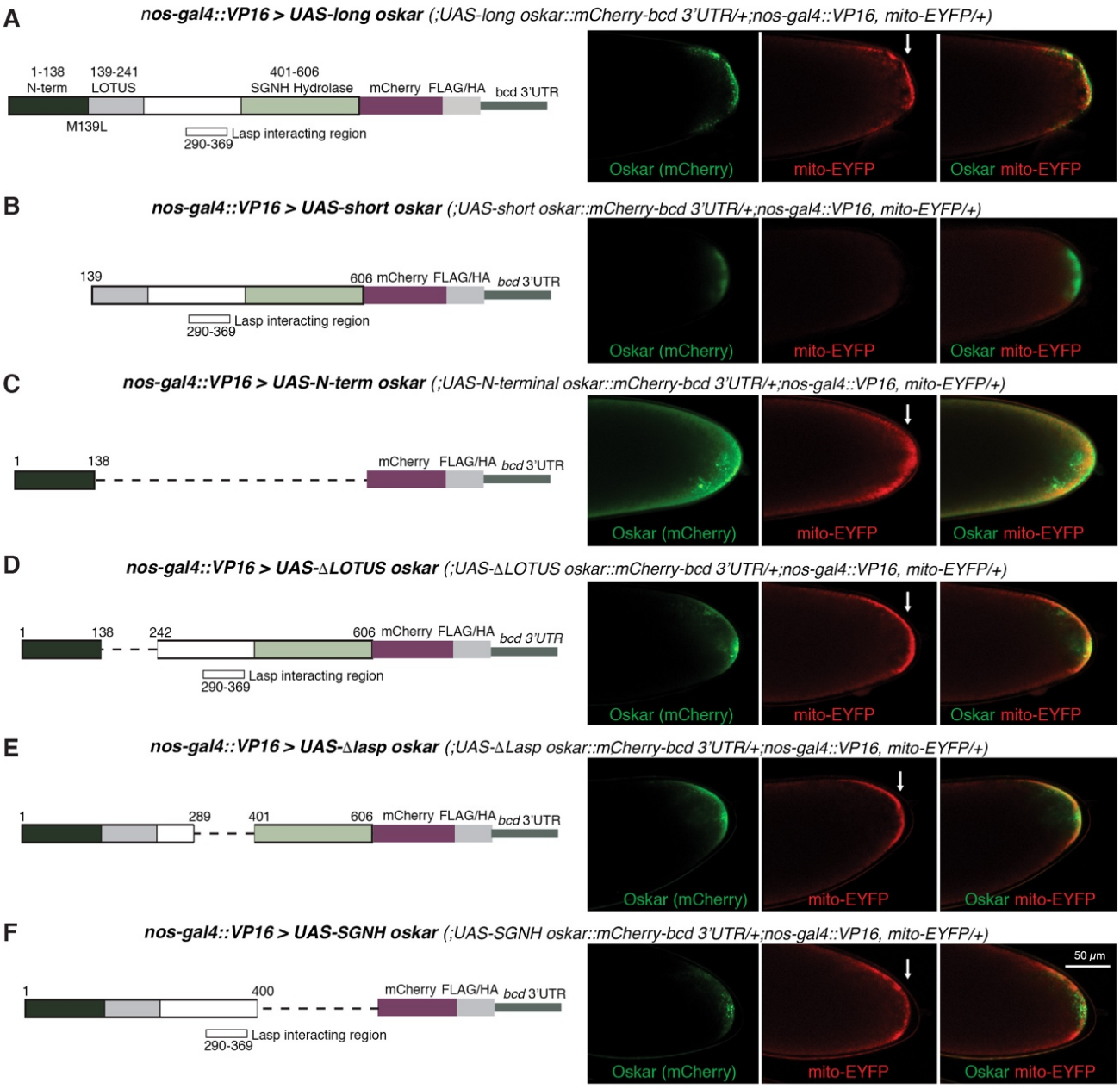

Figure S4 related to figure 4. N-terminal domain of Oskar is sufficient to trap mitochondria at the anterior.

Unlike all other figures, anterior is to the right in this figure. Images are of embryos expressing mito-EYFP to mark mitochondria and mCherry to mark the UAS expressed transgenic Oskar. Embryos were imaged live by confocal microscopy. Arrows indicate mitochondrial accumulation.

(**A**) Long Oskar traps mitochondria at the anterior when expressed there.

(**B**) Short Oskar does not trap mitochondria at the anterior when expressed.

(**C**) N-terminal domain of Long Oskar traps mitochondria at the anterior when expressed there.

(**D**) Oskar lacking its LOTUS domain traps mitochondria at the anterior when expressed there.

(**E**) Oskar lacking its Lasp interacting domain traps mitochondria at the anterior when expressed there.

(**F**) Oskar lacking its SGNH domain traps mitochondria at the anterior when expressed there.

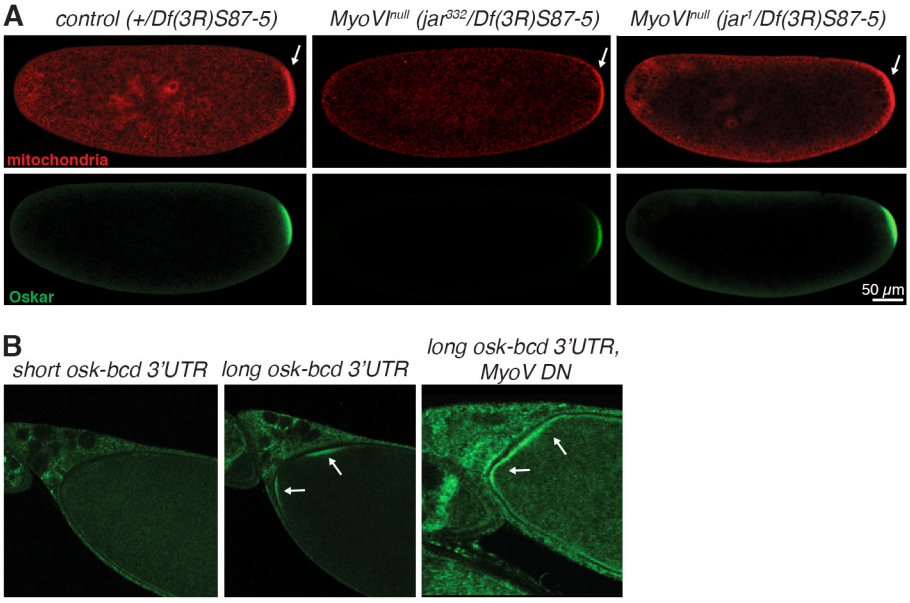

Figure S5 related to figure 6. Myosin VI and V are not required for mitochondrial accumulation.

(A) Embryos from MyoVI null mothers accumulate mitochondria at the posterior normally. Images are of 0 to 1 old embryos immunostained with α-ATPsyn α (mitochondria) and α-Oskar.

(B) Mitochondria accumulate at the anterior of late stage egg chambers expressing short oskar (short osk-bcd 3'UTR), long oskar (long osk-bcd 3'UTR) or long oskar (long osk-bcd 3'UTR) and a dominant negative form of MyoV (the N-terminal peptide of MyoV). Mitochondria were detected using a mitochondrially-targeted EYFP.

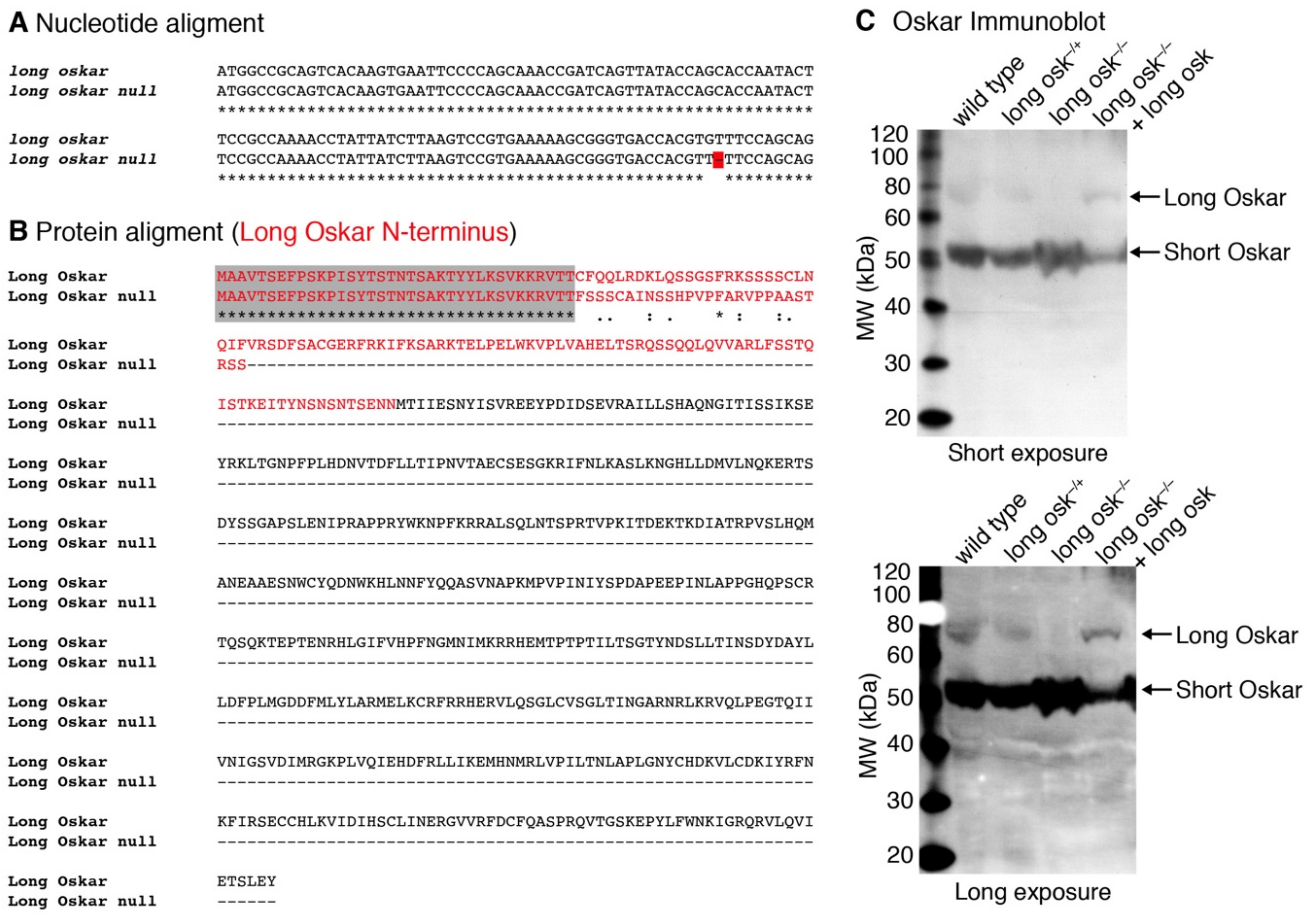

Figure S6 related to figure 7. Characterization of *long oskar* null mutant.

(A) Nucleotide sequence alignment of wildtype and long oskar null mutant. Red box indicate the mutated nucleotide.

(B) Protein sequence alignment of Oskar proteins predicted to be produced in wildtype and *long oskar* null mutants. Red, indicates the N-terminal sequence unique to Long Oskar. Grey box indicates the amino acids common to both wildtype Long Oskar and the truncated product generated by the oskar null mutation.

(C) Immunoblots of 0 to 2 hour embryo extracts from wild type (w^{1118}), *long osk* null heterzygotes (*long osk*$^{-}$*/TM3*), *long oskar* null mutants (*long osk*$^{-}$*/long osk*$^{-}$) and *long oskar* null mutants expressing transgenic *long oskar* (;long osk/long osk;*long osk*$^{-}$*/long osk*$^{-}$) probed with an antibody that detects both Long and Short Oskar.

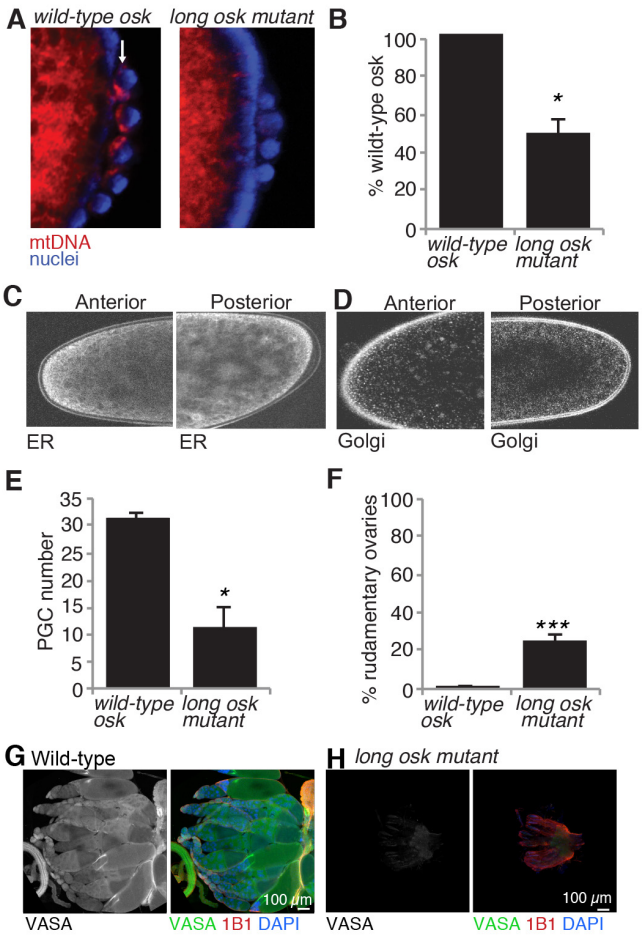

Figure S7 related to figure 7. Most mitochondria require Long Oskar to enter PGCs.

(**A**) mtDNA FISH of *oskar null* mutant embryos transgenically expressing either the wild type or the Short isoform of Oskar just after PGC formation.

(**B**) The number of mtDNA copies relative to nuclear genome in *oskar null* mutant PGCs transgenically expressing either wild type or the Short isoform of Oskar. Data are the mean of four independent replicates +/– SEM. *p < 0.05 (two tailed Student's *t*-test for pairwise comparsion).

(**C** and **D**) Images of live 0 to 1 hour old embryos expressing EYFP targetted to the ER (C) or the Golgi (D).

(**E**) The number of PGCs in 130 to 180 min old embryos from *oskar null* mutants transgenically expressing either wild type (n=12) or the Short isoform (n=19) of Oskar. Data are the mean +/– SEM. *p < 0.05 (two tailed Student's *t*-test for pairwise comparsion).

(**F**) Percentage of rudamentary ovaries in *oskar null* mutants transgenically expressing either wild type or the Short isoform of Oskar. Ovaries with no visible egg chambers were considered rudimentary. Data are the mean of 11 independent replicates +/– SEM. In total, 752 and 604 ovaries were analyzed for wild-type and *long oskar* mutants, respectivaly.***p < 0.001 (two tailed Student's *t*-test for pairwise comparsion).

(**G** and **H**) Representative images of wild-type and rudimentary ovaries stained with αVasa, 1B1 and DAPI to visualize germ cells ,somatic cells and DNA, respectively. One in four oskar null mutants transgenically expressing the Short isoform of Oskar (long osk mutant) had rudimentary ovaries which often resemble H.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Drosophila strains

Figure	Name	Genotype	Sources
1A,D-G; 2A-C; 3B-F; 6A-D; S2	*mito-EYFP*	*w*;; P{sqh-EYFP-Mito}3, nos-gal4::VP16*	b7194 R. Lehmann
1B, G	*nos-EGFP::moe*	*nos-EGFP::moe*	R. Lehmann
4A-C, S6C	*wild-type*	w^{1118}	R. Lehmann
4A, S6C, S7E	$osk^{-/-}$	osk^{A87}, ru^1 st^1 e^1 ca^1/*Df(3R)p-XT103*, ru^1 st^1 e^1 ca^1	A. Ephrussi R. Lehmann
4A	$tudor^{-/-}$	$tudor^{tux46}$/*Df(tudor)*	P. Lasko
4A	*vasa RNAi*	*nosgal4::VP16, mito-EYFP/uas-vasa RNAi*	b32434
4B	*short* $osk^{-/-}$	;osk^{M139L} *(go M2-12)/+;* osk^{A87}/*Df(3R)p-XT103*	A. Ephrussi
4B; S7A, B, E, F, H	*long* $osk^{-/-}$/*long osk mutant*	;osk^{M1L}*(go M1-7)/+;*osk^{A87}/*Df(3R)p-XT103*	A. Ephrussi
4C; 5A-C; S4C	*N-term osk-bcd 3'UTR*/N-term IP	*;uas-osk N-term::mCherry::3xFLAGHA/+; nosgal4::VP16, mito-EYFP/+*	This study
5A-C; S4A	long osk IP	*;uas-long oskar::mCherry::3xFLAGHA/+; nosgal4::VP16, mito-EYFP/+*	This study
5A-C; S4B	short osk IP	*;uas-short oskar::mCherry::3xFLAGHA/+; nosgal4::VP16, mito-EYFP/+*	This study
6E,F	*osk-bcd 3'UTR,* $TmII^{-/+}$	*P{ry+, osk-bcd}*^{42}*/+;*$TmII^{gs1}$/+	A. Ephrussi
6E,F	*osk-bcd 3'UTR,* $TmII^{-/-}$	*P{ry+, osk-bcd}*^{42}*/+;*$TmII^{gs1}$	A. Ephrussi
7B	*mito-PAGFP*	*;pSqh-mito-PAGFP*	This study
1B; 7C,E,F	*long* $osk^{-/+}$	*long* osk^{D6-1}/*TM6b*	This study
7D,E,F; S6C	*long* $osk^{-/-}$	*long* osk^{D6-1}	This study
S3A,E		*;uas-long oskar-bcd 3'UTR/+;nos-gal4::VP16/+*	A. Nakamura
S3B		*;uas-long oskar-bcd 3'UTR/uas-vas RNAi;nos-gal4::VP16/+*	A. Nakamura b38924
S3F		*;uas-long oskar-bcd 3'UTR/+;nos-gal4::VP16/ UAS-tud RNAi*	A. Nakamura b42800
S3C	*+/Df(tud)*	*+/Df(tud)*	R. Lehmann
S3D	tud^1*/Df(tud)*	tud^1*/Df(tud)*	R. Lehmann
S4D	*Δlotus osk*	*;uas-Δlotus osk::mCherry::3xFLAGHA/+; nosgal4::VP16, mito-EYFP/+*	This study
S4E	*ΔLasp osk*	*;uas-ΔLasp osk::mCherry::3xFLAGHA/+; nosgal4::VP16, mito-EYFP/+*	This study
S4F	*ΔSGNH osk*	*;uas-ΔSGNH osk::mCherry::3xFLAGHA/+; nosgal4::VP16, mito-EYFP/+*	This study
S5A	control	*;;+/Df(3R)S87-5*	b2363
S5A	$MyoVI^{null}$	*;;*jar^{322}*/Df(3R)S87-5*	b8776
S5A	$MyoVI^{null}$	*;;*jar^1*/Df(3R)S87-5*	b11793
S5B	*Long osk-bcd 3'UTR MyoV DN*	*;uas-long oskar-bcd 3'UTR/+;nos-gal4::VP16/ UAS-MyoV-Motor (line M14)*	A. Ephrussi A. Nakamura
S6C	*long* $osk^{-/+}$	*long* osk^{D6-1}/*TM3 Sb*	This study
S6C	*long* $osk^{-/-}$ *+ long osk*	;osk^{M139L} *(go M2-12);long* osk^{D6-1}	This study A. Ephrussi
S7A,B,E-G	*wild-type osk*	*;osk (go-12p);*osk^{A87}*/Df(3R)p-XT103*	A. Ephrussi
S7C	*ER*	w*; P{sqh-EYFP-ER}3	b7195
S7D	*golgi*	w*; P{sqh-EYFP-Golgi}3	b7193

b = Bloomington Stock Center

Generation of transgenic strains

Strains expressing various *UAS-oskar::mCherry3xFLAGHA bcd* 3'UTR constructs were generated as follows. Oskar, mCherry, 3xFLAGHA, and *bcd* 3'UTR were PCR amplified using Phusion High Fidelity PCR system (NEB, M0530L) and ligated together into the pVALIUM22 vector (Ni et al., 2011) using Gibson Assembly® master mix (NEB, E2611S). Mito-PAGFP was generated by PCR amplifying the *sqh* promoter and mitochondrial targeting sequence from genomic DNA from the *mito-EYFP* strain using Phusion High Fidelity PCR system (NEB, M0530L) and ligating with PAGFP into pVALIUM22 vector using Gibson Assembly® master mix. Plasmid DNA was then injected by BestGene Inc. into a strain carrying attP40 landing sites and integrated into the second chromosome using phiC31 integrase (Markstein et al., 2008).

Generation of long oskar CRISPR mutant

A CRISPR gRNA (GCGCAACTGCTGGAAACACG) was designed using flyCRISPR Target Finder (http://tools.flycrispr.molbio.wisc.edu/targetFinder/). Sense and antisense 5' phosphorylated oligonucleotides with overhangs sequences complementary to the overhangs generated by BbsI were annealed and ligated into a BbsI cleaved pU6-BbsI-chiRNA plasmid (Gratz et al., 2013). Plasmid DNA was injected by BestGene Inc. into a *vasa-cas9* strain (b55821). A mutation (*long oskar*$^{D6\text{-}1}$) that causes a frame-shift 36 amino acids after the starting methionine of *long oskar* was identified by PCR screening (Figure S6).

Embryo and ovary immunofluorescence

Embryos and ovaries were immunostained as follows. Embryos were dechorionated with a solution of 50% (v/v) Clorox® for 2 minutes. After thorough washing with H_2O, embryos were fixed in 5% formaldehyde (Fisher Scientific, F79-500)/50% heptane for 50 minutes. Embryos were gradually stepped into 100% methanol and incubated for 30 min or stored indefinitely at –20°C before being gradually rehydrated in PBST (0.1% Triton X-100 [Sigma, T8787], PBS) with 0.1% (w/v) bovine serum albumin (BSA, Affymetrix, 10857 500 GM). Samples were incubated in PBST with 0.1% BSA overnight at 4°C containing: mouse anti-ATP5A (1:5000, Abcam ab14748); chicken anti-GFP (1:1000, Aves GFP-1020); mouse anti-1B1 (1:20, Developmental Studies Hybridoma Bank); rabbit anti-Oskar (1:1000, A. Ephrussi); rabbit anti-Tudor (1:1000, P. Lasko); or rabbit anti-Vasa (1:5000, R. Lehmann). The next day, samples were incubated with appropriate secondary antibodies diluted in PBST with 0.1% BSA for 2 hours at room temperature. Embryos were mounted in Vectashield® media with or without DAPI (Vector Laboratories). Adult and larval ovaries were immunostained as above with the following exceptions: ovaries were dissected in PBS and immediately fixed in 5% formaldehyde in PBS for 25 min; ovaries were not subjected to methanol treatment; after fixation ovaries were permeabilized with 1% Triton X-100 in PBS for 1 hour; ovaries were incubated in PBST with 1% (w/v) BSA. Fluorescent images were acquired on a Zeiss 510META and LSM780 confocal microscopes with Plan-Apo40X/1.4 Oil DIC and EC Plan-Neofluar 10X/0.30 objectives.

S2R+ phalloidin staining

S2R+ cell fixation and staining was carried out according to standard procedures. S2R+ cells were seeded in eight-well chamber slides (Lab-Tek II, 154534) pre-coated with 0.1% poly-L-lysine (Sigma, P4707). Cells were then fixed with 4% paraformaldehyde (Electron Microscopy Sciences, 15713) in PBS for 10 min, permeabilized in 0.1% (v/v) Triton X-100 in PBS containing Alexa Fluor® 488 phalloidin (1:40; ThermoFisher Scientific,A12379). Cells were mounted in VECTASHIELD media containing DAPI, and fluorescent images were acquired with a Plan-Apochromat ×40/numerical aperture 0.8 objective on a Zeiss LSM 780 confocal microscope.

FISH

mtlrRNA FISH

mtlrRNA FISH was conduced on 0 to 1 hour embryo as previously described (Lécuyer et al., 2008) using the following primers:

Forward primer sequence (5′ to 3′)	Reverse primer sequence (5′ to 3′)	Size (bp)
CTTTAGGGATAACAGCGTAATTTTT	GATAATACGACTCACTATAGGGTTTTGATATTTGGTCCTTTCGT	223

Single molecule RNA FISH (smFISH) (Figure S2)

The single molecule RNA FISH protocol used in this study is as previously described (Trcek et al., 2015). Custom Stellaris® RNA smFISH Probes were designed against mitochondrial *mtlr* and *ND1* mRNAs and a germ plasm mRNA *nanos* (*nos*) by utilizing the Stellaris® RNA FISH Probe Designer (Biosearch Technologies, Inc., Petaluma, CA) available online at www.biosearchtech.com. The following smFISH RNA probes labeled with Quasar670 (Q670) (Biosearch Technologies, Inc.) were used:

mtlrRNA (5' to 3')	*ND1* (5' to 3')	*nos (5' to 3')*
GGCGAATATTATTTTTGCCG	AGCTACACTTACTAATACACAAA	GGTAAAGCTACGCGCCAACT
CCATTTAAAATTTTCAGTGG	ATATCCTAAAACTTTTCGTTCTA	ACGGCAATTCCAGGAATTTT
TTGCACAGTCAAAATACTGC	ATTAGGACCTTTACGAATTTGAA	ACTTGAAACAACTGCGAAGC
AGACTAATGATTTAGCTAAC	AGGAATTCCTATTAAACCAACTT	AAAATCGTATGTCCCTTAGA
GGGTCTTCTCGTCTTTTAAA	TTTAATCGCATCACAAAAAGGTT	AAATCGTGACGCAGAGGCAA
ACGCTGTTATCCCTAAAGTA	AAGGATAAGTTTGTTCTTTTGTA	AACTAAACTCGCTTTTGGGT
ATCGAGGTCGCAATCTTTTT	ACCACCCAAATTAAAAGAATATAA	TTCGCGATACTTCTTTATCT
GAACGGCTACACCCAAAATT	CCCCAATCTAGTACAACATAAAAA	GCACAGTTTATTCAACTGAA
AAAGTCGAACAGACTTAAAA	CAACCAGCTACTATAACAGTATAA	CAAATCCTCACCCAAAAACC
CCGGTTTGAACTCAGATCAT	TCGCAAACCTCCTAATAAAGCAT	TTATCGCGCACTCTACTTTC
AAGATAGAAACCAACCTGGC	ATAAGAAATAGTCTGAGCCACAG	AAATCCGGGTCGAAAGTTAC
GGTCCTTTCGTACTAAAATA	GATACTCAAACTAAAGCTATAGG	TAAACGCTGCAAAAGCTGCC
	CCGATTAGTTTCAGCTAATGAAA	GCTTTGATCGGAATGCGTAT
	TCTCCTTCAGCAAAATCAAAAGG	CCAGGCGCTATTTAAACGTT
	CTATATTCTACATTAAATCCTGA	GCACGGGATAACGCTCTAAA
	TAAAAATTAAAGCCAAACCCCCT	AGTGATCGTTCGTTGTCTAT
	ATAAAATTCTCGCATATTCAGCT	GTTTCCCTTTCACAGAAACA
	AACGAAATCGAGGTAAAGTTCCT	GCCACGACGATTGAACAAGT
		TCCATTCATCAACTTTCGGA
		AAATGAAGGCGACCAGTTGC
		CGAAATTTTTCGGCCGCAAG
		TTCAAAGTGTTCCTTTTTCA
		TGATACGATTGACAGTTCGA
		CTTGCTATTTCCTTTAGCAA
		ACAATGAATGCGTAGCCGAC
		TACTCTTCGCTTATCTATCA
		GCGGTTGTTTCATGTGTGAA
		CGAGCCATTGAATTTTTCAT
		AACCATTTCTTTATTTGGCA
		TCCAAGTTGCTGCGGAACAT
		TCCTCTGGCGTGAAAAGCAG
		TGCAGGCCCAGAATGTTGAG
		CCACTGGTATCCAAATACAT
		GTAATGGGCGGACTCAAAGT
		TCGGCCAGAAAAGGGAAGTG
		CATAAGGAGCGAATTGGCGG
		CAAGTGGTAGTGGTACTGTC
		TTGCTGGTGACTCGCACTAG
		AAGGATCGCGCAATCTCGTC
		CGTCACCTGCGCAAAGATTT
		CATAGCCATTGGTCGCGAAC
		TAGGACATGCGACCGAGATC
		CATTAAGTTGCCGCCATTGG
		AGTGGGTGGCGAGTGGAATG
		CACACGTTGTTCAGATGCTC
		GGCTGGTATATACGACATGT
		CTGCAAACCCATTGTATTGG
		CGAGATTGGTGGACACAGTG

Mixtures of 3′ labeled probes hybridizing along the transcript strongly amplified the signal-to-noise ratio and therefore enhanced our detection sensitivity. 0 to 1 hour old embryos were dechorionated and fixed

for 20 minutes at room temperature (RT) in a solution of 5 ml of 4% paraformaldehyde diluted in PBS and 5 ml heptane. Paraformaldehyde was removed and an equal volume of 100% methanol added. Vials were shaken vigorously for 15 s, and the embryos were collected and washed three times with 100% methanol and stored overnight in 100% methanol at 4 °C. The next day ~50 µl of embryos were rehydrated in a 1:1 mixture of methanol:PBST for 5 minutes and then twice in PBST. Embryos were then post-fixed for 20 minutes in 4% paraformaldehyde and PBST, followed by three 2 minutes washes in PBST. Afterwards, embryos were treated with 3 $\mu g \cdot ml^{-1}$ Proteinase K (Sigma-Aldrich, 03 115879 001) diluted in PBST at room temperature for 13 minutes and then for 1 hour on ice. During incubations, embryos were mixed gently several times by inverting the tube. Proteinase K was removed and embryos washed twice with 2 $mg \cdot ml^{-1}$ glycine. Embryos were post-fixed again for 20 minutes in 4% paraformaldehyde and 1X PBS and washed five times for 2 minutes each in PBST. During a pre-hybridization step, embryos were incubated in 10% deionized formamide (Life Technologies, 4311320) and 2 × SSC (300 mM NaCl, 30 mM sodium citrate, pH 7) for 10 minutes. Pre-hybridization solution was then removed, and a hybridization mix containing FISH probes was added to embryos, which were incubated overnight in the dark at 37 °C. Per ~50 embryos, hybridization mix was composed of 10% deionized formamide, 1 µl of competitor (5 $mg \cdot ml^{-1}$ *E. coli* tRNA (Roche, 10109541001) and 5 $mg \cdot ml^{-1}$ salmon sperm ssDNA (Applied Biosystems, MC01417)), 80 ng FISH probe mix, 10% of dextran sulfate (Pharmacia), 2 $mg \cdot ml^{-1}$ BSA (Sigma Aldrich, AM9680), 2X SSC, 10 mM vanadyl ribonucleoside complex (NEB, S1402S) and dH_2O to 60 µl. The hybridization mix was removed and embryos washed twice with 10% deionized formamide in 2X SSC pre-warmed to 37 °C for 15 minutes followed by two 1 hour washes in 1X PBS. Embryos were mounted in ProLong Gold Antifade Reagent (Molecular Probes, P36934) containing a 10-fold dilution of 100 nm TetraSpeck microspheres (Invitrogen, T-7279). Images were acquired in 3D using a Widefield Epifluorescence microscope, API DeltaVision personalDV system equipped with Photometrics CoolSNAP HQ2 CCD camera and Olympus PlanApo N 60x/1.42 oil and deconvolved and pixel shift-corrected in 3D using Huygens.

The degree of co-localization between mito-EYFP and the *mtlrRNA Q670, ND1 Q670*, and *nanos* Q670 RNAs was determined using the PCC (Costes) approach, which examines the spatial relationship between the fluorescent intensities of two overlapping objects labeled with spectrally distinct fluorophores rather than the frequency or duration of their co-occurrence (Costes et al., 2004; Trcek et al., 2015). PCC(Costes) is insensitive to object shape or the object number variability between two color images (Costes et al., 2004; Trcek et al., 2015). PCC (Costes) was determined using the JACoP Plugin in ImageJ (Bolte and Cordelières, 2006) on a 3D region of interest located in the center of the posterior pole was analyzed as previously described (Trcek et al., 2015).

Quantitative mtDNA FISH (Figures 1, 7 and S1)

The quantitative mtDNA FISH protocol used in this study is adapted from previously described methods (McKim et al., 2009; Trcek et al., 2015). A mixtures of 60 5′ labeled probes hybridizing along mtDNA strongly amplified the signal-to-noise ratio and therefore enhancing detection sensitivity. Briefly, the following 5' labeled CAL Fluor 590® DNA oligonucleotides were synthesized by Stellaris®:

Forward primer sequence (5′ to 3′)	Reverse primer sequence (5′ to 3′)	Size bp
GGGTAAATCATGCAGTTTTCTG	TTCAAGCTCCTAACCAAGAATTAGA	299
GAGCTATTGGAGGATTAAATCAAA	TGGTAAAAATCCTAAAAATGGAGGT	293
CGAATTTGTTATTCCGCTTTT	AGTCAATAATGATATCAAACTGCAA	266
CGCGACAATGATTATTTTCTACA	ATATTATTTATTCGTGGGAATGCT	292
GCTGGGACAGGATGAACTGT	CTCCCGCTGGGTCAAAAA	310
TTTTTGGTCATCCTGAAGTTTATATT	TCCTAAAGCTCATAAAATAGCTGGA	320
CCCAATTCAACTAAATTCATCA	ATTAAATATCCCACCAATACTGTAA	289
GGATTAACAGGAGTTGTTTTAGC	TTCCTAATAATGAAATAGTTGATCC	340
TTGAACTATTTTACCAGCAATTATTTT	TTCAAGAATGAATAACATCAGCA	299
TTTTTATTAATCGACCGGGTTT	GGTGCTATTTGTGGAATTAAAAA	291
TTCTTATATACCAAATTCACCTAAATC	TGACCTGATGGGCCTAATAAA	266

TCCATATATTTTTACAAGAACAAGACA	AGCAATTTGAGCTATTAATAAAAATGT	322
TTCAATCTTATGTGTTTGCTGT	CATCGTAAACCAATAGTTACTGCAT	300
TCACCCGCTATTGAATTAGGA	CGATTAATACATGAATTCCGTGA	313
CATCATTTTGGTTTTGAAGCA	CCACATTCAAATGGGGATCT	304
TTGATTGCAATTAGTTTCGACCT	TTTGACATTGAAGATGTTATGGAGA	312
TTTTTGATATAAATGTTGACCTC	TTGTAGGGGGTTTATTTGGA	227
AAGGAAAAGGAAAAATCAATCAA	CCTTTAACTTCAGCTTGTTTTAACGTA	331
TTATGAATAATAGCCCCAGCAC	TTCTTCTACATTAGTTACAGCTGGTG	300
GCTGCAGGTAACCAAGAAGAA	GGGTGAGATGGTTTAGGACTTG	281
AAATTTAAAGGCAATCAATGTAACAA	AACGTCTTGGAAGTCGAAGAATA	334
CCAGAAGAACATAAACCATGAGC	TTCTGGGTCTATAATTTTAGCAGGT	300
AATCCATAAGATAATATATCACAACCT	TGAAGGGGCCTTAGGTTTATC	266
CAAAATTATTTAAAGGACCTATTCG	CAACCATAATTAACGTCTCGACAA	274
TTACATGCTAACGGTGCATCA	GCAAGAACAATAAAAGGTAAAATAAAA	329
AAAATTCCTTTTCATCCTTATTTT	TGAATCCCTCGGAATTTTCTT	299
AACGAAATCGAGGTAAAGTTCC	TTTTATTTCCTATAGCTTTAGTTTGAA	294
GAATTAGACGACCAACCAGCTA	CAACCTTTTTGTGATGCGATT	236
TTGAATATATCCTAAAACTTTTCGTTC	GCCGTTCAAATTTTAAGTCTGTT	349
CATCGAGGTCGCAATCTTTT	TGAATGGTTGGACGAAATATTAAC	332

Genomic DNA, isolated from 50 *mito-EYFP* females using DNeasy Blood & Tissue Kit (Qiagen, 69506), acted as a template to amplify DNA with the primers listed above using Q5® high-fidelity DNA polymerase (NEB, M0491L). PCR products were then run on an agarose gel to verify purity, before QIAquick PCR purification (Qiagen, 28106). PCR products were pooled with equal molarity and stored at –80°C.

Samples (embryos, larval gonads and ovaries) were fixed in cacodylate fixative buffer (100 mM sodium cacodylate, pH 7.3, 100 mM sucrose, 40 mM potassium acetate, 10 mM sodium acetate, 10 mM EGTA, 5% formaldehyde) for 4 min. Samples were washed in 2 x SSC with 0.1% Tween-20 (2xSSCT), 2xSSCT/20% formamide, 2xSSCT/40% formamide and 2xSSCT/50% formamide for 10 min each. Hybridization solution (2xSSC, 50% formamide, 10% dextran sulfate, 2 mg ml^{-1} BSA, 10 mM vanadyl ribonucleoside complex) with 5 ng/μl CAL Fluor590® labeled probes was added to the samples and denatured by heating to 91°C for 2 min and then hybridized overnight at 37°C. The next day, the samples were washed at 37°C with pre-warmed 2xSSCT/50% formamide and then at room temperature with 2xSSCT/40% formamide, 2xSSCT/20% formamide and 2xSSCT. Samples were either mounted and imaged or immunostained as described above. Samples were mounted in ProLong Gold Antifade Reagent or Vectashield® Antifade mounting medium (H-1000). Images were acquired using a Zeiss LSM780, AxioOberver microscope equipped with and argon laser, HeNe 633 laser, a DPSS 561-10 laser, a Plan-Apo40X/1.4 Oil DIC and EC Plan-Neofluar 10X/0.30 objectives.

mtDNA foci were counted in the 3D image stack using a spot detection algorithm (Airlocalize) as previously described (Lionnet et al., 2011; Trcek et al., 2015). Briefly, to determine mtDNA concentrations in germ cells, after mtDNA FISH samples were immunostained with α-Vasa as described above. To exclude everything but germ cells, a Gaussian blur was applied to Vasa images before segmentation using the Minimum threshold method in ImageJ. Thresholded images were then divided by 255 and multiplying to their corresponding mtDNA images to exclude foci outside of germ cells. For whole embryos, to circumvent issues associated with imaging and analyzing very large data sets, representative 3D regions of interest of a known volume were acquired and proxies for the concentration of mtDNA across the entire embryo using volumes determined previously (Trcek et al., 2015).

Traditional mtDNA FISH (Figure S7)

Traditional mtDNA FISH was conducted as previously described (http://www.igh.cnrs.fr/equip/cavalli/Lab%20Protocols/p5.pdf) using the following mtDNA probes were amplified from w^{1118} genomic DNA with the Roche High Fidelity PCR system (Roche, 11732641001) and the following primers:

Forward primer sequence (5′ to 3′)	Reverse primer sequence (5′ to 3′)	Size bp
TGCAGTTTTCTGCATTCATTG	AAAGGAGAAGCTCTATCTTGTAAACC	3085
TTGCTCTTCCTTCTTTACGTTT	TTTGACATTGAAGATGTTATGGAGA	3071
TTTGATATAAATGTTGACCRCCAAAA	ATGTGAAGGGGCCRRAGGTT	3119
TTTCCGATTAGAAACAAAACAAAA	ATTTTGGGTGTAGCCGTTCA	3155

Live imaging

Ovaries were removed from females and the ovarioles teased apart using tungsten needles in Schneider's Medium (Life technology, 21720). Egg chambers were staged according to King (King, 1970). Embryos were collected and dechorionated as described above and transferred to a heptane glue coated coverslip before covering with Halocarbon Oil. Live imaging was performed on a Prairie Ultima (Prairie Technologies; www.prairie-technologies.com/) equipped with a pulsed 4W Ti:sapphire Chamelon laser (Coherent; www.coherent.com) and a Zeiss LSM510 confocal microscope.

Co-immunoprecipitation and mass spectrometry

All steps were conducted at 4°C with shaking unless otherwise specified. 0 to 3 hour old embryos were collected, dechorionated as described above, and resuspended in 500 µl NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-Cl, pH 8.0) with protease inhibitor (Roche, 04693159001). Homogenates were centrifuged at 20,000 *g* for 10 min and the supernatant filtered through a column (CRC, Affymetrix 13928) with a 35 µm pore (Affymetrix, 13912). The samples were incubated for 3 hours with 20 µl of Anti-FLAG® M2 affinity gel (Sigma, A2220) pre-equilibrated with NP40 buffer, and then washed four times with NP40 buffer. Proteins were eluted from the column with 100 µl 500 ng/µl 3xFLAG peptide for 15 minutes at room temperature. Proteins were electrophoresed through a NuPAGE® 4-12% Bis-Tris 1.0 mm gel (ThermoFisher Scientific, NP0321) using the NuPAGE® system and stained with SilverQuest Silver Staining Kit (ThermoFisher Scientific, LC6070).

Mass spectrometry analysis was conducted as follows. Protein samples were reduced with DTT at 57 ˚C for 1 hour and alkylated with iodoacetamide at room temperature in the dark for 45 minutes. One fourth of each sample was loaded onto NuPAGE® 4-12% Bis-Tris Gel 1.0 mm (ThermoFisher Scientific, NP0321) and electrophoresed for approximately 2 minutes at 200 V. This was repeated three times. The gel was stained using GelCode Blue Stain Reagent (Thermo Scientific) and bands were excised and de-stained in 1:1 (v/v) methanol/100 mM ammonium bicarbonate solution. The gel pieces were partially dehydrated with an acetonitrile rinse and further dried in a SpeedVac concentrator for 20 minutes. 300ng of sequencing grade-modified trypsin (Promega) was added to each gel sample. After the trypsin was absorbed, 200 µl of 100 mM ammonium bicarbonate was added to cover the gel pieces. Digestion proceeded overnight at room temperature on a shaker. A slurry of R2 20 µm Poros beads (Life Technologies Corporation) in 5% formic acid and 0.2% trifluoroacetic acid (TFA) was added to each sample at a volume equal to that of the ammonium bicarbonate added for digestion, and the samples were incubated for 3 hours at 4 ˚C with shaking. The beads were loaded onto equilibrated C18 ziptips (Millipore) and microcentrifuged for 30 seconds at 6,000 rpm. Gel pieces were rinsed three times with 0.1% TFA and each rinse was added to its corresponding ziptip followed by microcentrifugation. The extracted Poros beads were further washed with 0.5% acetic acid. Peptides were eluted by the addition of 40% acetonitrile in 0.5% acetic acid followed by the addition of 80% acetonitrile in 0.5% acetic acid. The organic solvent was removed using a SpeedVac concentrator and the sample reconstituted in 0.5% acetic acid.

One tenth of each sample was analyzed individually by liquid chromatography (LC) Orbitrap MS/MS. LC separation was inline with MS using the autosampler of an EASY-nLC 1000 (Thermo Scientific). Peptides were gradient eluted from the column directly to Orbitrap Elite mass spectrometer using a 1 hour gradient (Thermo Scientific) with Solvent A (2% acetonitrile, 0.5% acetic acid) and Solvent B (90% acetonitrile, 0.5% acetic acid). High resolution full MS spectra were acquired with a resolution of 60,000,

an AGC target of 1e6, with a maximum ion time of 120 ms, and scan range of 300 to 1500 m/z. Following each full MS twenty data-dependent low resolution CID MS/MS spectra were acquired. All MS/MS spectra were collected using the following instrument parameters: resolution of 15,000, AGC target of 1e4, maximum ion time of 100 ms, one microscan, 2 m/z isolation window, fixed first mass of 150 m/z, and NCE of 30. MS/MS spectra were searched using a Uniprot *Drosophilia* database with Oskar Full Length, C term, and N term sequences inserted, using Sequest (http://fields.scripps.edu/sequest/) within Proteome Discoverer (Thermo Scientific).

Protein gel electrophoresis, silver staining and immunoblotting

Co-immunoprecipitants were diluted in an equal volume of NuPAGE® LDS sample buffer (ThermoFisher Scientific, NP0007) containing 10% 2-mercapoethanol (Sigma, M-6250) and electrophoresed through a NuPAGE® 4-12% Bis-Tris Gel 1.0 mm (ThermoFisher Scientific, NP0321) according to the manufacturer's directions. Gels were stained using SilverQuest^{TM} staining kit (Invitrogen, LC6070). For immunoblotting, proteins were transferred to 0.2 µm polyvinylidene difluoride membranes (Bio-Rad, 162-0174) using a mini Bio-Rad Trans-Blot system in buffer comprising 48 mM Tris (Sigma, T1503), 39 mM glycine (Fisher Scientific, BP381-1), 0.05% (w/v) SDS (Sigma, L3771), and 20% (v/v) methanol (Fisher Scientific, A412-4) at pH 8.3. The membrane was then blocked in PBS, 0.1% Tween 20 with 2% skimmed milk powder and incubated with rabbit anti-Oskar (1:000, A.Ephrussi) overnight at 4°C. Blots were incubated with Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000) for 1 h at room temperature, treated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, 34080) according to the manufacturer's instructions, and visualized on HyBlot CL autoradiography film (Denville Scientific, E3012).

qPCR mtDNA copy number measurements

mtDNA copy number was determined in PGCs by qPCR as described previously (Hunter et al., 2010). Embryos were dechorionated as described above. PGCs were isolated by FACS (Figure 1) or manually (Figure S7B).

To isolate PGCs by FACS, dechorionated *nos-EGFP::moe* embryos were suspended in dissociation buffer (3.2 g/l NaCl, 3.0 g/l KCl, 0.69 g/l $CaCl_2.2H_2O$, 3.7 g/l $MgSO_4.7\ H_2O$, 1.79 Tricine buffer (pH 7), 3.6 g/l glucose, 17.1 g/l sucrose, 1 g/l BSA, sterilize by filtration and stored frozen) and homogenized using a glass Dounce homogenizer. The cell suspension was passed through a 100μm and then a 22μm filter. PGCs were FACS sorted (MoFlo cell sorter) into a minimal volume of TE buffer (approximately 10 μl) in DNA LoBind tubes (Eppendorf, 022431005). To confirm sample homogeneity and integrity, test samples were immunostained with α-Vasa and visualized using confocal microscopy. PGCs were diluted in 4X extraction buffer (80 mM Tris, pH 8, 20 mM EDTA, 0.4% Triton X-100, 4 mM DTT and 400 μg/ml proteinase K) and incubated for 25 min at 55°C and then for 4 min at 99°C. The number of cells determined by qPCR correlated well with the number of cells assayed as determined by FACS-sorting suggesting efficient DNA extraction.

For manual isolation (Figure S7B), PGCs were gently separated from the embryo just after formation with a tungsten needle and transferred using a mouth pipette assembly and pulled capillary to a DNA LoBind tube. Samples were then boiled for 5 minutes before the addition of 200 µg/ml proteinase K, 25 mM NaCl, 1 mM EDTA and 10 mM Tris-Cl, pH 8.0. Samples were then incubated for 20 minutes and boiled for 5 more minutes.

qPCR was performed in a Roche LightCycler® 480 using LightCycler® 480 SYBR Green I master (Roche, 04707516001) and the following primers:

Amplicon	Forward primer (5' to 3')	Reverse primer (5' to 3')	Size (bp)
mtDNA	gctcctgatatagcattcccacga	catgagcaattccagcggataaa	151

nuclear	cgagggatacctgtgagcagctt	gtcacttcttgtgctgccatcgt	152

The mtDNA and nuclear amplicons were cloned into pVALIUM22 vectors (Ni et al., 2011) using Gibson Assembly® master mix (NEB, E2611S). Standard curves were generated with known quantities of mtDNA and nuclear amplicons as determined using a Qubit® 2.0 fluorometer (Invitrogen).

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