Supplementary Methods

Fly strains. The *zld*^{294} and *zld*^{681} alleles were generated by imprecise excision of the P{RS3}[24] element UM-8171-3 located in 18F2 (Flybase, Szeged stock center) using a Δ2,3 source on the *CyO* chromosome (gift from J. Treisman). These were balanced over *FM7c-ftz-lacZ* (gift from S. Roth). Germline clones were induced in *zld*^{294} *FRT 19A* / *y w sn P{mini w+, ovo*$^{D1\text{-}26}$*}*[25] *FRT*^{19A}*, hsFLP122* (see below) second or third instar larvae by the FLP-FRT technique[26]. Virgin females were collected and mated to either *yw*, *FM7/Y*, or *FM7c-ftz-lacZ/Y* (to identify the zygotic genotype with respect to *zld*). Escapers (*zld*$^{+}$ germ-line clone embryos) varied between experiments (1 to10%). To rescue the *zld* phenotype, one copy of the *otu-zld* transgene on chromosome II (see below) was present in germ-line clones.

Confirmation of *zld* null alleles. The exact insertion site of UM-8171-3 was confirmed by sequencing the PCR product of genomic DNA using primers covering the 3' LTR of the P-element and genomic sequences from -1186 to -1162 upstream of the CG12701 RB start site (Flybase). The P-element inserted between -660 and -661. Deletion breakpoints of *zld*^{294} and *zld*^{681} were determined by genomic PCR analysis and sequencing of the PCR product using primers outside of the deleted region. *zld*^{294} deletes sequences -1270 to +1376 and *zld*^{681} deletes sequences from -660 to +2250 and leaves part of the P-element. Nucleotide numbering is according to the *Drosophila melanogaster* X chromosome sequence (release v5.1).

Construction of the *ovoD FRT*^{19A} chromosome. We made the *ovoD FRT*^{19A} stock by transposition of *P{mini w+, ovo*^{D1}*}*[25] onto *y w sn FRT*^{19A}*, hsFLP122*. Briefly, males of genotype *y w sn FRT*^{19A}*, hsFLP122/Y ; +/ CyO, Δ2-3 ; FRT*^{82B} *ovo*$^{D1\text{-}18}$*/Sb*^{1} were crossed to *C(1)DX, y*^{1} *w*^{1} *f*^{1}*/Y* females. Individual male offspring that were *w*$^{+}$ *Cy*$^{+}$ *Sb* (genotype *y w sn FRT*^{19A}*, hsFLP122 *; */+ ; Sb*^{1} **/+*, where * represents potential *P{mini w+, ovo*^{D1}*}* insertions) were crossed to *C(1)DX, y*^{1} *w*^{1} *f*^{1}*/Y* females. Insertions onto the X chromosome were identified as lines in which the *w*$^{+}$ was transmitted to all the sons and to no daughters. These chromosomes were maintained as *y w sn P{mini w+, ovo*^{D1}*} FRT*^{19A}*, hsFLP122/Y* and *C(1)DX, y*^{1} *w*^{1} *f*^{1}*/Y* females. To test for penetrance of the *ovoD* insertion, and to confirm that the *FRT*^{19A} or *hsFLP122* elements were not inadvertently mobilized, we crossed the *y w sn P{mini w+, ovo*^{D1}*} FRT*^{19A}*, hsFLP122* males to *y w* and *y w FRT*^{19A} females at 25°C. We subjected a brood from each of these crosses (24-48 hr old larvae) to a heat shock at 37°C

for 1 Hr, while other broods were not heat shocked. Female offspring were then assayed for fertility and egg laying. We selected a line (4.1) in which *y w sn P{mini w+, ovo^{D1}} FRT^{19A}, hsFLP122/ y w*, either plus or minus heat shock, showed complete sterility and laid almost no eggs, while the *y w sn P{mini w+, ovo^{D1}} FRT^{19A}, hsFLP122/ y w FRT^{19A}* showed almost complete sterility in the absence of heat shock, but showed good fertility upon heat shock. The male offspring from these fertile females were all *w sn^{+}*, indicating they contained the *y w FRT^{19A}* and not the *y w sn P{mini w+, ovo^{D1}} FRT^{19A}, hsFLP122* chromosome, as would be expected if the *P{ovoD}* were dominantly blocking female germline development.

***zld* rescue construct.** The *zld* rescue construct was made by subcloning the full-length *zld* coding region +642 to +6345 relative to the RB +1 start site into the *Eco*RI site of the **pCOG** plasmid, which lies between the *ovarian tumor* promoter and the *K10* 3'UTR[17] (gift from C. Navarro). Full length *zld* was prepared by PCR from genomic DNA (Clonetech), cloned into the **pCR2.1-TOPO vector** (Invitrogen), and verified by sequencing.

Site-directed mutagenesis. The following *zen* promoter fragments were subcloned into the *Eco*RI site of the **pCaSpeR*hsp43-lacZ*** transformation vector (gift from M. Frasch): two tandem copies of the 91 bp fragment (Fig. 1a in upper case) prepared by PCR of subcloned *zen* genomic DNA, two copies of a mutated version of the 91 bp fragment whereby the base substitutions shown in Fig. 1a (in purple) were introduced by PCR site-directed mutagenesis.

***in situ* hybridization.** Embryos were hybridized with digoxygenin-UTP (Roche Biochemicals) RNA probes synthesized from cloned cDNA sequences, or in the case of miR-309, primary transcript sequences spanning the cluster[22]. *lacZ* staining indicated embryos that harbored the *FM7-ftz-lacZ* chromosome and were thus *M'Z^{+} zld*. Stained embryos were mounted in Aquamount (Polysciences), or embedded in araldite (Polysciences). After hybridization, embryos were stained with DAPI (Sigma) to determine mitotic-cycle. Embryos were visualized by fluorescence microscopy using a Nikon FX-A microscope and by Nomarski optics using a Zeiss Axiophot microscope.

Antibody staining. Dilution of antibodies was as follows: mouse α-Nrt (Developmental Studies Hybridoma Bank) 1/50; rabbit α-Slam (gift from R. Lehmann) 1/200; rat α-Dorsal 1/50; rabbit α-Vasa 1/500. F-actin and DNA were visualized by TRITC- labeled phalloidin (Sigma) and Hoechst 33258 (Sigma), respectively. Embryos were viewed by fluorescence microscopy using a Nikon FX-A microscope for whole embryo views, or an Improvision Yokogawa CSU-10 spinning disk confocal system for grazing and sectional views. Images

were prepared using Velocity, ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http:rsb.info.nih.gov/ij/, 1997-2008), and Adobe Photoshop software.

Yeast one-hybrid assay. The yeast one-hybrid screen was done using the Matchmaker One-Hybrid System (Clontech). The *zen* 91-bp fragment with four TAGteam sites was amplified by PCR and cloned into the *Eco*RI site of the target-reporter vectors (**pLaci** and **pHisi-1**). The TAGteam reporter vectors were integrated into the yeast YM4271 strain to generate the yeast reporter strain YM4271[*TAG-lacZ*, *TAG-his*]. The YM4271[*TAG-lacZ*, *TAG-his*] reporter strain was used to screen a 0-6 hours embryonic cDNA library fused to the Gal4 activation domain[27]. All 121 His3 positive clones from the first screen were subjected to β-Galactosidase activity assays for the second screening, and the final 34 positive clones were sequenced to identify the candidates.

Transient transfection assays. The wild-type and mutated 91 bp *zen* promoter fragments described above were subcloned into the *Eco*RI site of the **pCaSpeR*hsp43-lacZ*** reporter vector[28] (gift from A. Laughon). The full-length *zld* coding region was cloned between the *Kpn*I and *Xho*I sites of **pMT/V5-His B** expression vector (Invitrogen). *Drosophila* S2 cells were grown at 28° C in Schneider's medium (Invitrogen) supplemented with 10% FCS. $3X10^6$ cells were transfected using Effectene Transfection reagent (Qiagen) with a 250 ng plasmid DNA mix containing 100 ng reporter plasmid, 50 ng plasmid constitutively expressing firefly *luciferase*, expression plasmid as indicated in Fig. 1E and the plasmid **pcDNA3** to bring the total amount of DNA to 250 ng. The expression of Zld protein was induced 24 hours post-transfection by adding 0.5 mM $CuSO_4$ directly to the medium. The cells were lysed after 24 hours and the β-Galactosidase and Luciferase activities were assayed (Promega) following the manufacturer's protocols. The fold activation was calculated as a ratio of the normalized (for transfection efficiency) *lacZ* activity in cells treated with 0.5 mM CuSO4 and untreated cells.

DNA binding assays. Electrophoretic mobility shift assays were performed as previously described[9] using affinity purified C-terminal part of the Zld protein containing four zinc fingers (amino acids 1240-1470) fused to GST (GST-Zld-C) and 26 bp oligonucleotide probes overlapping TAGteam sites from the *zen* promoter, except that EDTA was omitted from the binding buffer and $ZnSO_4$ was added to 10 μM. The incubation reactions contained 0.1 ng of ^{32}P labeled oligonucleotides and varying amounts of recombinant GST-Zld-C protein (see

Fig. 1G for nucleotide sequences).

Microarray analysis. Total RNA was extracted from three independent collections of 1-2 hr *yw* and *M- zld* embryos by TRIzol (Invitrogen). A portion of the collected embryos was fixed and stained with DAPI; 90% were in nuclear cycles 8 to13. cDNA was prepared using the GeneChip® HT One-Cycle cDNA Synthesis Kit (Manufactured by Invitrogen for Affymetrix) and labeled with the BioArray™ HighYield™ RNA Transcript Labeling Kit (Enzo). Labeled probes were hybridized to Affymetrix *Drosophila* Genome 2 arrays and processed by a GeneChip Fluidics Station 400. Data were acquired by a GeneChip® Scanner 3000 and processed/normalized by Affymetrix GeneChip Operating Software (GCOS). Genes were identified as present when at least two of the three replicates had present (P) assignment ($p<0.05$). Two-tailed t-test analysis was performed on the data from the three biological replicates.