LETTERS

The zinc-finger protein Zelda is a key activator of the early zygotic genome in *Drosophila*

Hsiao-Lan Liang[1]*, Chung-Yi Nien[1]*, Hsiao-Yun Liu[1], Mark M. Metzstein[2], Nikolai Kirov[1] & Christine Rushlow[1]

In all animals, the initial events of embryogenesis are controlled by maternal gene products that are deposited into the developing oocyte. At some point after fertilization, control of embryogenesis is transferred to the zygotic genome in a process called the maternal-to-zygotic transition. During this time, many maternal RNAs are degraded and transcription of zygotic RNAs ensues[1]. There is a long-standing question as to which factors regulate these events. The recent findings that microRNAs[2,3] and Smaug[4] mediate maternal transcript degradation have shed new light on this aspect of the problem. However, the transcription factor(s) that activate the zygotic genome remain elusive. The discovery that many of the early transcribed genes in *Drosophila* share a *cis*-regulatory heptamer motif, CAGGTAG and related sequences[5,6], collectively referred to as TAGteam sites[5] raised the possibility that a dedicated transcription factor could interact with these sites to activate transcription. Here we report that the zinc-finger protein Zelda (Zld; Zinc-finger early *Drosophila* activator) binds specifically to these sites and is capable of activating transcription in transient transfection assays. Mutant embryos lacking *zld* are defective in cellular blastoderm formation, and fail to activate many genes essential for cellularization, sex determination and pattern formation. Global expression profiling confirmed that Zld has an important role in the activation of the early zygotic genome and suggests that Zld may also regulate maternal RNA degradation during the maternal-to-zygotic transition.

In *Drosophila*, an initial wave of zygotic gene transcription occurs between 1 and 2 h of development during the mitotic cleavage cycles 8–13. This is followed by a major burst of activity between 2 to 3 h of development (cycle 14) when the embryo is undergoing cellular blastoderm formation. Many pre-cellular genes contain TAGteam sites in their upstream regulatory regions including several direct targets of Bicoid, Dorsal and other key regulators of patterning[5–7]. It has been previously demonstrated[5] that TAGteam sites are required for the early expression of the dorsoventral gene *zen*, and the sex determination genes *sisB* (also known as *sc*) and *Sxl*. To isolate the TAGteam binding factor, we performed a yeast one-hybrid screen with a 91 base-pair (bp) fragment (Fig. 1a, sequences in upper-case) from the *zen cis*-regulatory region[8,9] (*zen(91)*), which contains four TAGteam sites[5] (Fig. 1a in red, the first two are the reverse complement). *zld*, encoded by the X chromosomal gene *CG12701* (also known as *vfl*), was selected as the only candidate of the 11 recovered that had the potential to bind specific DNA sequences because it encoded a protein with six C2H2 zinc fingers (represented as green boxes in Fig. 1b). Oligonucleotides (Fig. 1a, underlined sequences) with different TAGteam sites were tested in gel shift assays with the 357 amino acid carboxy-terminal region of Zld fused to glutathione *S*-transferase (GST–ZldC; Fig. 1b, stippled region). All oligonucleotides tested formed complexes with GST–ZldC, although with different affinities (Fig. 1c, lanes 1–9), whereas mutations (Fig. 1a, in purple) in the heptanucleotide sequence abolished binding (Fig. 1c, lanes 10–12). Notably, the site with the strongest affinity, CAGGTAG, is the site most over-represented in regulatory elements of pre-blastoderm genes versus post-blastoderm genes[5]. A plasmid expressing full-length Zld protein promoted transcriptional activation of a *zen(91)-lacZ* reporter but not a mutated *zen(91m)-lacZ* reporter after co-transfection in *Drosophila* S2 cells (Fig. 1d). Taken together, these data strongly suggest that Zld activates transcription of *zen* and probably other TAGteam-containing genes.

zld transcripts were detected in the germline cells of the ovary (Fig. 2a), in unfertilized eggs (Fig. 2b), and throughout early development (Fig. 2c). Later, *zld* becomes restricted to the nervous system and specific head regions (Fig. 2d), as previously shown[10]. To analyse

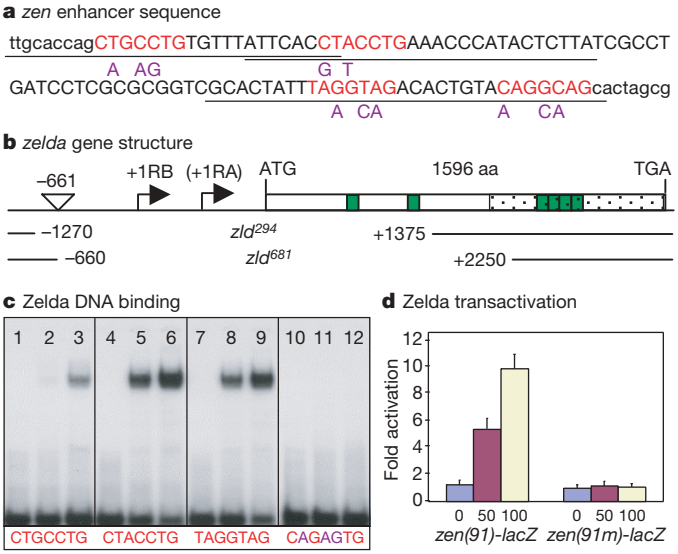


Figure 1 | TAGteam sites bind Zld and mediate transcriptional activation. **a**, DNA sequence of the 91-bp *zen* enhancer (upper-case) plus surrounding sequences (lower-case). Base substitutions are in purple. **b**, Schematic organization of the *zld* locus (*CG12701*; Flybase) with the transcription start sites for the RNA isoforms RB and RA. The *P{RS3}UM8171-3* insertion site is between −661 and −660 bp. The nucleotides deleted in zld^{294} and zld^{681} are indicated as a blank space between solid lines. **c**, Zld binding to oligonucleotides containing different TAGteam sites (denoted beneath each section of the gel). The first lane in each section contains free probe, the second lane contains probe plus 10 ng GST–ZldC, and the third lane contains probe plus 30 ng GST–ZldC. **d**, S2 cells were transfected with 0 ng (blue bar), 50 ng (red bar) or 100 ng (yellow bar) of plasmid expressing *zld* under control of the inducible metallothionein promoter, the *zen(91)-lacZ* or *zen(91m)-lacZ* reporter plasmids, and the luciferase control. Error bars, s.e.m.; $n = 3$.

[1]Department of Biology, New York University, 100 Washington Square East, New York, New York 10003, USA. [2]Institute of Human Genetics, University of Utah, Salt Lake City, Utah 84112, USA.
*These authors contributed equally to this work.

zld function, we generated deletion alleles of *zld* by imprecise excision (schematized in Fig. 1b). Hemizygous embryos showed abnormal central nervous system and head development (data not shown), consistent with previous reports of *CG12701* lethal P-insertion phenotypes[10,11]. *zld* transcripts were not observed in these embryos after cycle 14 (Fig. 2e). However, younger embryos had high levels of maternal *zld* transcripts (data not shown), indicating that maternally loaded *zld* transcripts are degraded during cellularization and replaced with zygotic *zld*.

To eliminate maternal *zld* from embryos, we induced clones of zld^{294} mutant germ cells in the adult female. All resulting embryos were null for maternal *zld* (M^- *zld*), and the male embryos were also null for zygotic *zld* (M^-Z^- *zld*). All early M^- *zld* embryos lacked *zld* transcripts (Fig. 2f) but had normal patterns of other maternally deposited factors such as *bicoid* RNAs (Fig. 2g) and the Dorsal protein gradient (data not shown). Unlike M^-Z^+ *zld* embryos, which began to express *zld* ubiquitously in cycle 14 (Fig. 2i), M^-Z^- *zld* embryos never expressed *zld* (Fig. 2h). However, regardless of their zygotic genotypes, all M^- *zld* embryos showed a severely abnormal morphology after cycle 14 (Fig. 2h and j) and did not survive to make cuticle.

Before cycle 14, M^- *zld* embryos are similar to wild type (compare Fig. 3a to 3c), except for sporadic nuclear fallout (arrow in Fig. 3c, middle). However, at early cycle 14 the hexagonal-actin network becomes disorganized and begins to degenerate (Fig. 3d, top) resulting in a multinucleated phenotype (arrow in Fig. 3d, bottom) resembling *nullo*[12] and *Serendipity α*[13] (*Sry-α*) mutants. Cellularization does not proceed as furrow canals never move inward (arrow in Fig. 3d, top) like in the wild type (arrow in Fig. 3b, top), and Neurotactin (Nrt) accumulates abnormally in the apical cytoplasm (arrow in Fig. 3h, top)—reminiscent of the *slam* mutant phenotype[14,15]. Staining with anti-Slam antibody confirmed that Slam protein is mostly absent by mid-cycle 14 (Fig. 3f, top), whereas Slam has moved basally in wild type (Fig. 3e, bottom). In addition, nuclei do not elongate but instead become rounded, enlarged and clump together (see Fig. 3f, and arrow in 3h, bottom). Regions of higher nuclear density were observed (data not shown), a phenotype similar to that obtained by injection of *CG12701* double-stranded RNA[10], which we noticed resembles a *frühstart* (*frs*, also known as *Z600*) phenotype[16]. Despite their aberrant morphology, M^- *zld* embryos attempt to form a ventral furrow (Supplementary Fig. 1b, c, e) but soon become highly disorganized with only pole cells recognizable (Supplementary Fig. 1f). We rescued the M^- *zld* cellularization defects by driving a wild-type copy of *zld* into the germ line using the *ovarian tumour* (*otu*) promoter[17]. The cytoskeleton becomes well structured (Fig. 3i, top) and furrow canal ingression is normal (Fig. 3i, bottom) as Slam protein is restored (data not shown).

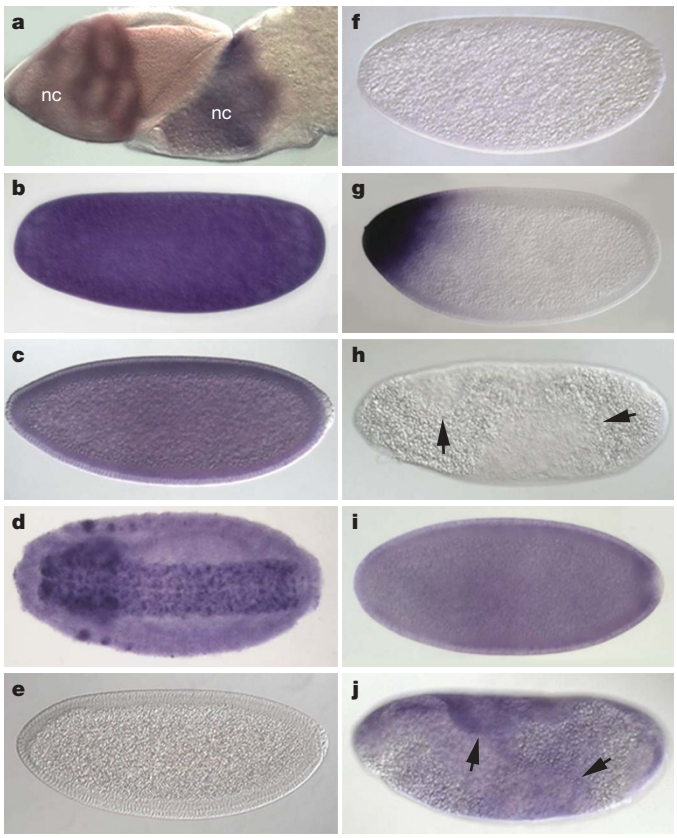


Figure 2 | Maternal *zld* transcripts are lost as zygotic *zld* is activated in cycle 14. **a–j**, Wild-type (**a–d**) and zld^{294} (**e–j**) ovaries (**a**) and embryos (**b–j**) were hybridized with *zld* (**a–f** and **h–j**) or *bcd* (**g**) RNA probes. **a**, Mid-stage (left) and late-stage (right) egg chambers with *zld* transcripts in the nurse cells (nc) but not the columnar follicle cells that overlay the oocyte. **b**, An unfertilized egg is shown. **c**, A cycle 14 embryo undergoing cellularization. **d**, A late-stage embryo is shown. **e**, An M^+Z^- *zld* cycle 14 embryo showing that maternal *zld* transcripts have disappeared. **f**, An M^- *zld* cycle 10–11 embryo is shown. **g**, An M^- *zld* cycle 14 embryo has a normal *bcd* pattern. **h**, An M^-Z^- *zld* late cycle 14 embryo showing anomalous distribution of cytoplasm (arrows). **i**, An M^-Z^+ *zld* early cycle 14 embryo showing onset of zygotic *zld* expression. **j**, An M^-Z^+ *zld* late cycle 14 embryo showing abnormalities (arrows).

The broad range of phenotypes strongly indicated that M^- *zld* embryos do not express genes essential for cellular blastoderm formation. We assayed the expression of *Sry-α*, *slam* and *nullo*, as well as *sisA*, *sisB*, *sisC* (also known as *os*), *Sxl*, *zen* and *dpp*. None of these genes was activated in M^- *zld* embryos (data shown for *Sry-α*, *sisB* and *zen* in Fig. 4b, d and f, respectively), except at the poles in some cases. However, *sna* and *sog*—which are activated by Dorsal[18]—were not absent but were delayed in expression by at least two cycles (data not shown), suggesting that Zld facilitates the onset of early gene transcription. Furthermore, the lateral stripes of *sog* were greatly reduced in width (Fig. 4h), indicating that in regions in which Dorsal protein amounts are low, a combinatorial mechanism involving both Dorsal and Zld establishes the broad *sog* domain. Notably, there are two TAGteam sites in the 393 bp *sog* enhancer[19] that lie close to Dorsal binding sites.

Our results indicated that Zld is a global activator of early genes. To test this directly we compared the expression profiles of wild-type and M^- *zld* embryos in mitotic cycles 8–13, a time point presumably enriched in genes that are direct Zld targets. One-hundred-and-twenty genes were downregulated and surprisingly 176 genes were upregulated at least twofold ($P \leq 0.05$), in the absence of Zld (Fig. 4k). The downregulated set (Supplementary Table 1) was strongly enriched in genes that are zygotically expressed (Fig. 4l) and involved in early developmental processes (Supplementary Fig. 2), including most of the genes we assayed *in situ* (Fig. 4k). For example, 12 genes involved in cellular blastoderm formation (*nullo*, *slam*, *Sry-α*, *bnk*, *frs*, *btsz*, *halo* and 5 *halo*-like genes[20]), 6 sex-determination genes (*sisA*, *sisB*, *sisC*, *run*, *Sxl* and *dpn*), and 8 dorsoventral genes (*dpp*, *tld*, *tok*, *tsg*, *tsg*-like, *scw*, *zen* and *zen2*) are in our downregulated data set. Overall, 75% of the early genes previously described as pre-cellular[5,6,21] are included. This number may be an underestimate because there could be many genes such as *sna* and *sog* that did not make the twofold cutoff (Fig. 4k) but are indeed regulated by Zld.

About 80% of the downregulated genes have TAGteam sites within 2 kilobases (kb) upstream of the transcription start site (Supplementary Table 2), and another 10% have TAGteam sites in introns, such as *slam* with two sites in its first intron, supporting the idea that most of our downregulated genes are direct Zld targets. In addition, the TAGteam sites upstream of the downregulated genes tend to be located very close to the transcription start site within 200 bp (Supplementary Table 2), consistent with the previous finding that early zygotic genes have a statistical over-representation of TAGteam sites close to the start site[5,6].

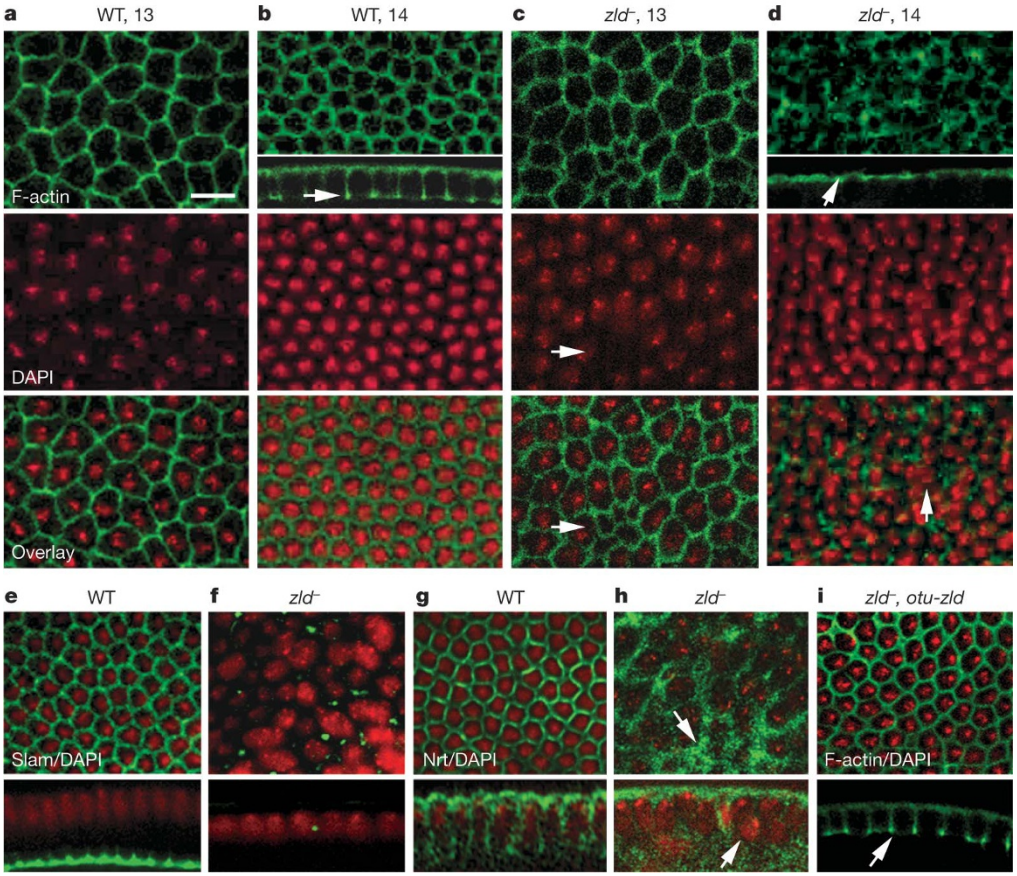
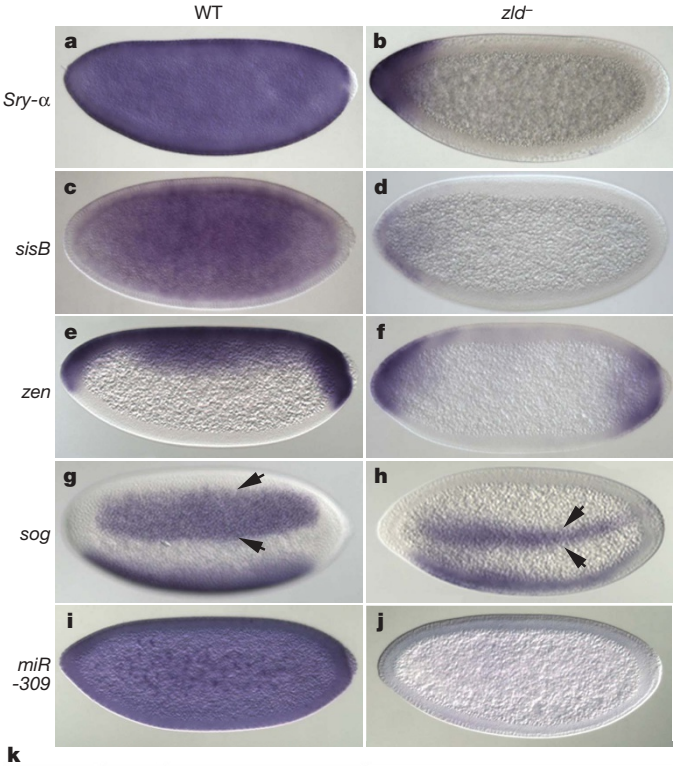


Figure 3 | Maternal *zld* is required for cellularization. **a**–**i**, Confocal images of wild-type (WT; **a**, **b**, **e**, **g**), M^{-} zld^{294} (zld^{-}; **c**, **d**, **f**, **h**) and rescued (M^{-} zld^{294}; *otu-zld*, **i**) embryos (as indicated) stained with phalloidin to detect F-actin, with anti-slam or anti-Nrt antibodies (green), and with DAPI (4,6-diamidino-2-phenylindole) to detect DNA (red). In M^{-} *zld* embryos the cytoskeletal network is disorganized and quickly degenerates in early cycle 14 (**d**, top) accompanied by nuclear fallout (**d**, middle). Slam protein disappears in cycle 14 (**f**, top and middle), whereas Nrt accumulates apically (**h**, top, arrow). In M^{-} zld^{294}; *otu-zld* embryos the cytoskeleton is organized (**i**, top) and cellularization proceeds (**i**, bottom, arrow). Scale bar, 10 μm.



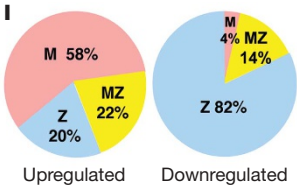
k

Fold ch	Genes
≥2.0 down	120
1.5–2 down	159
Unchanged	6,970
1.5–2 up	155
≥2.0 up	176
Whole array	13,615

Genes tested by *in situ*

Fold ch		Fold ch	
sisA	7.2	*Sry-α*	8.0
sisB	4.9	*slam*	2.0
sisC	2.5	*nullo*	9.2
Sxl	3.5	*sna*	1.8
zen	3.6	*sog*	1.2
dpp	5.7	*bcd*	1.0

l



In contrast to the downregulated genes, the upregulated set is strongly enriched in genes that are maternally expressed (Fig. 4l). We considered the possibility that Zld activates components of the RNA degradation machinery that in turn destabilize maternal RNAs. Because the microRNA (miRNA) *miR-309* enhancer[22] contains two TAGteam sites, we assayed for *miR-309* primary transcripts in M^{-} *zld* embryos (Fig. 4j), and indeed they were absent. It was recently shown that mature *miR-309* miRNAs become abundant during cycle 14, and are involved in maternal transcript turnover in 2–4 h embryos[3]. Not surprisingly, our 1–2 h (cycles 8–13) data set had no overlap with the 44 published *miR-309* targets[3], however 2–4 h profiling experiments should demonstrate whether they are upregulated in the absence of *zld*. We also compared our upregulated genes to those affected by *smaug*[4], another gene required for the removal of maternally supplied RNAs. We found there was little overlap with the published Smaug targets[4], suggesting that Zld is involved in a parallel pathway of maternal RNA degradation.

We have demonstrated that Zld functions as a key transcriptional activator during the maternal-to-zygotic transition (MZT) in *Drosophila*. This is the first demonstration of such an activator in any organism. We propose that the biological role of Zld in the pre-blastoderm embryo is to set the stage for vital processes such as cellular blastoderm formation, counting of X chromosomes for dosage compensation and sex determination, and pattern formation, by ensuring

Figure 4 | Zld plays a role in zygotic gene activation and maternal RNA degradation during the MZT. **a**–**j**, Wild-type (WT; left) and $M^{-}Z^{-}$ zld^{294} (zld^{-}; right) mitotic cycle 12–14 embryos were hybridized as indicated. **k**, Summary of expression profiles of 1–2 h wild-type and M^{-} zld^{294} embryos. Fold ch, fold change with respect to wild type (genes absent in the array data are not included). **l**, Percentage of genes for which there is expression data[3,5,19] described as maternal (M), zygotic (Z) or both (MZ) in the downregulated (≥2-fold; $n = 105$) and upregulated (≥1.5-fold; $n = 263$) gene sets.

the coordinated accumulation of batteries of gene products during the MZT. This early preparedness should allow sufficient time for the formation of molecular machines[23] involved in these processes, and so are ready to spring into action during the prolonged interphase of cycle 14.

METHODS SUMMARY

Fly strains. The *zld*^{294} and *zld*^{681} alleles were generated by imprecise excision of the *P{RS3}* (ref. 24) element *UM-8171-3* (FlyBase, Szeged stock centre). The *ovo*^{D} *FRT19A* stock was generated by transposition of *P{mini w*$^{+}$*, ovo*^{D1}*}* (ref. 25) onto *y w sn FRT19A*, *hsFLP122*. Germline clones were induced in *zld*^{294} *FRT19A*/*ovo*^{D1} *FLP122 FRT19A* by the FLP-FRT technique[26]. Virgin females were collected and mated to *y w*, *FM7* or *FM7c-ftz-lacZ* males. The *otu-zld* construct was microinjected into *w*^{1118} embryos.

Yeast one-hybrid assay. The yeast one-hybrid screen was performed using the Matchmaker One-Hybrid System (Clontech) protocol with the 91-bp *zen* promoter (*zen(91)*) and a 0–6 h *Drosophila* embryonic complementary DNA library fused to the Gal4 activation domain[27] (gift from L. Pick).

Molecular biology. DNA binding assays[9] and *Drosophila* S2 cell transient transfection assays[28] were performed as previously described. The fold activation was calculated as a ratio of the normalized (for transfection efficiency) LacZ activity in cells treated with 0.5 mM $CuSO_4$ and untreated cells.

Analysis of phenotypes. Various RNA probes, antibodies and molecular probes were used to detect gene expression or to visualize the cytoskeleton and nuclei (further described in the Methods). 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, and by Nomarski optics using a Zeiss Axiophot microscope.

Microarray analysis. Total RNA was extracted from three independent collections of 1–2 h *y w* and *M*$^{-}$ *zld* embryos by Trizol (Invitrogen). cDNA was prepared using the GeneChip HT One-Cycle cDNA Synthesis Kit (Invitrogen), labelled with the BioArray HighYield RNA Transcript Labelling Kit (Enzo), and hybridized to Affymetrix *Drosophila* Genome 2 arrays and processed by a GeneChip Fluidics Station 400.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 20 June; accepted 29 August 2008.
Published online 19 October 2008.

1. Newport, J. & Kirschner, M. A major developmental transition in early *Xenopus* embryos: II. Control of the onset of transcription. *Cell* **30,** 687–696 (1982).
2. Giraldez, A. J. *et al.* Zebrafish miR-430 promotes deadenylation and clearance of maternal mRNAs. *Science* **312,** 75–79 (2006).
3. Bushati, N., Stark, A., Brennecke, J. & Cohen, S. Temporal reciprocity of miRNAs and their targets during the maternal-to-zygotic transition in *Drosophila. Curr. Biol.* **18,** 501–506 (2008).
4. Tadros, W. *et al.* SMAUG is a major regulator of maternal mRNA destabilization in *Drosophila* and its translation is activated by the PAN GU kinase. *Dev. Cell* **12,** 143–155 (2007).
5. ten Bosch, J. R., Benavides, J. A. & Cline, T. W. The TAGteam DNA motif controls the timing of *Drosophila* pre-blastoderm transcription. *Development* **133,** 1967–1977 (2006).
6. De Renzis, S. D., Elemento, O., Tavazoie, S. & Wieschaus, E. F. Unmasking activation of the zygotic genome using chromosomal deletions in the *Drosophila* embryo. *PLoS Biol.* **5,** 1036–1051 (2007).
7. Li, X. *et al.* Transcription factors bind thousands of active and inactive regions in the *Drosophila* blastoderm. *PLoS Biol.* **6,** 365–388 (2008).
8. Jiang, J., Rushlow, C. A., Zhou, Q., Small, S. & Levine, M. Individual Dorsal morphogen binding sites mediate activation and repression in the *Drosophila* embryo. *EMBO J.* **11,** 3147–3154 (1992).
9. Kirov, N., Zhelnin, L., Shah, J. & Rushlow, C. Conversion of a silencer into an enhancer: evidence for a co-repressor in dorsal-mediated repression in *Drosophila. EMBO J.* **12,** 3193–3199 (1993).
10. Staudt, N., Fellert, S., Chung, H., Jäckle, H. & Vorbrüggen, G. Mutations of the *Drosophila* zinc finger-encoding gene *vielfältig* impair mitotic cell divisions and cause improper chromosome segregation. *Mol. Biol. Cell* **17,** 2356–2365 (2006).
11. Bourbon, H. M. *et al.* A P-insertion screen identifying novel X-linked essential genes in *Drosophila. Mech. Dev.* **110,** 71–83 (2002).
12. Simpson, L. & Wieschaus, E. F. Zygotic activity of the *nullo* locus is required to stabilize the actin-myosin network during cellularizatiron in *Drosophila. Development* **110,** 851–863 (1990).
13. Schweisguth, F., Lepesant, J. A. & Vincent, A. The *serendipity alpha* gene encodes a membrane-associated protein required for the cellularization of the *Drosophila* embryo. *Genes Dev.* **4,** 922–931 (1990).
14. Lecuit, T., Samanta, R. & Wieschaus, E. *slam* encodes a developmental regulator of polarized membrane growth during cleavage of the *Drosophila* embryo. *Dev. Cell* **2,** 425–436 (2002).
15. Stein, J. A., Broihier, H. T., Moor, L. A. & Lehmann, R. Slow as molasses is required for polarized membrane growth and germ cell migration in *Drosophila. Development* **129,** 3925–3934 (2002).
16. Grosshans, J., Müller, H. & Wieschaus, E. Control of cleavage cycles in *Drosophila* embryos by *frühstart. Dev. Cell* **5,** 285–294 (2003).
17. Robinson, D. N. & Cooley, L. Examination of the function of two kelch proteins generated by stop codon suppression. *Development* **124,** 1405–1417 (1997).
18. Stathopoulos, A. & Levine, M. Genomic regulatory networks and animal development. *Dev. Cell* **9,** 449–462 (2005).
19. Markstein, M., Markstein, P., Markstein, V. & Levine, M. S. Genome-wide analysis of clustered Dorsal binding sites identifies putative target genes in the *Drosophila* embryo. *Proc. Natl Acad. Sci. USA* **99,** 763–768 (2002).
20. Gross, S. P., Guo, Y., Martinez, J. E. & Welte, M. A. A determinant for directionality of organelle transport in *Drosophila* embryos. *Curr. Biol.* **13,** 1660–1668 (2003).
21. Pilot, F., Philippe, J. M., Lemmers, C., Chauvin, J. P. & Lecuit, T. Developmental control of nuclear morphogenesis and anchoring by charleston, identified in a functional genomic screen of *Drosophila* cellularization. *Development* **133,** 711–723 (2006).
22. Biemar, F. *et al.* Spatial regulation of microRNA gene expression in the *Drosophila* embryo. *Proc. Natl Acad. Sci. USA* **102,** 15907–15911 (2005).
23. Gunsalus, K. C. *et al.* Predictive models of molecular machines involved in *Caenorhabditis elegans* early embryogenesis. *Nature* **436,** 861–865 (2005).
24. Ryder, E. *et al.* The DrosDel collection: a set of P-element insertions for generating custom chromosomal aberrations in *Drosophila melanogaster. Genetics* **167,** 797–813 (2004).
25. Chou, T. B., Noll, E. & Perrimon, N. Autosomal *P[ovo*^{D1}*]* dominant female-sterile insertions in *Drosophila* and their use in generating germ-line chimeras. *Development* **119,** 1359–1369 (1993).
26. Chou, T. B. & Perrimon, N. The autosomal FLP-DFS technique for generating germline mosaics in *Drosophila melanogaster. Genetics* **144,** 1673–1679 (1996).
27. Yu, Y. *et al.* The nuclear hormone receptor Ftz-F1 is a cofactor for the *Drosophila* homeodomain protein. *Nature* **385,** 552–555 (1997).
28. Kirkpatrick, H., Johnson, K. & Laughon, A. Repression of *dpp* targets by binding of *brinker* to *mad* sites. *J. Biol. Chem.* **276,** 18216–18222 (2001).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank the following people for gifts of RNA probes, plasmids, antibodies and fly stocks: J. Erickson, R. Lehmann, R. Cinalli, R. Martinho, C. Navarro, J. Treisman and L. Pick. We thank J. Rhee and A. Chung for help with isolating and characterizing *zld* null mutants, and S. Fu for help locating TAGteam sites in Zelda target genes. We are grateful to M. Siegel and K. Birnbaum for help with the microarray analysis, and C. Desplan, P. Struffi, S. Small and R. Lehmann for critical reading of the manuscript. This work was supported by a grant from the National Institutes of Health (GM63024).

Author Information The microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession number GSE11231. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to C.R. (chris.rushlow@nyu.edu).

METHODS

Fly strains. The *zld*^{294} and *zld*^{681} alleles were generated by imprecise excision of the *P{RS3}* (ref. 24) element *UM-8171-3* located in 18F2 (Flybase, Szeged stock centre) 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} *FRT19A*/*y w sn P{mini w+, ovo*^{D1}*}* (ref. 25) *FRT19A, hsFLP122* (see later) second or third instar larvae by the FLP-FRT technique[26]. Virgin females were collected and mated to *y w, FM7/Y* or *FM7c-ftz-lacZ/Y* (to identify the zygotic genotype with respect to *zld*). Escapers (*zld*$^{+}$ germline clone embryos) varied between experiments (1% to 10%). To rescue the *zld* phenotype, one copy of the *otu-zld* transgene on chromosome II (see later) was present in germline 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′ long terminal repeat (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 *ovo*^{D} *FRT19A* chromosome. We made the *ovo*^{D} *FRT19A* stock by transposition of *P{mini w*$^{+}$*, ovo*^{D1}*}* (ref. 25) onto *y w sn FRT19A, hsFLP122*. In brief, males of genotype *y w sn FRT19A, hsFLP122/Y ; +/ CyO, Δ2-3 ; FRT82B 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 FRT19A, hsFLP122 *; */+ ; Sb*^{1} **/+*, in which * 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 of the sons and to no daughters. These chromosomes were maintained as *y w sn P{mini w*$^{+}$*, ovo*^{D1}*} FRT19A, 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 *FRT19A* or *hsFLP122* elements were not inadvertently mobilized, we crossed the *y w sn P{mini w*$^{+}$*, ovo*^{D1}*} FRT19A, hsFLP122* males to *y w* and *y w FRT19A* females at 25 °C. We subjected a brood from each of these crosses (24–48-h-old larvae) to a heat shock at 37 °C for 1 h, whereas 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}*} FRT19A, hsFLP122/ y w*, either with or without heat shock, showed complete sterility and laid almost no eggs, whereas the *y w sn P{mini w*$^{+}$*, ovo*^{D1}*} FRT19A, hsFLP122/y w FRT19A* showed almost complete sterility in the absence of heat shock, but showed good fertility after heat shock. The male offspring from these fertile females were all *w sn*$^{+}$, indicating that they contained the *y w FRT19A* and not the *y w sn P{mini w*$^{+}$*, ovo*^{D1}*} FRT19A, hsFLP122* chromosome, as would be expected if the *P{ovoD}* was 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 EcoRI site of the pCOG plasmid, which lies between the *otu* 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 EcoRI site of the pCaSpeRhsp43-lacZ transformation vector (gift from M. Frasch): two tandem copies of the *zen(91)* fragment (Fig. 1a in uppercase) prepared by PCR of subcloned *zen* genomic DNA, two copies of a mutated version of the *zen(91)* fragment (*zen(91m)*) in which 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 harboured 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 the 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 anti-Nrt (Developmental Studies Hybridoma Bank) 1:50; rabbit anti-Slam (gift from R. Lehmann) 1:200; rat anti-Dorsal 1:50; rabbit anti-Vasa 1:500. F-actin and DNA were visualized by TRITC-labelled phalloidin (Sigma) and by DAPI (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 (W. S. Rasband, http://rsb.info.nih.gov/ij/), and Adobe Photoshop software.

Yeast one-hybrid assay. The yeast one-hybrid screen was carried out using the Matchmaker One-Hybrid System (Clontech). The *zen* 91-bp fragment (*zen(91)*) with four TAGteam sites was amplified by PCR and cloned into the EcoRI 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 h 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 *zen* promoter fragments described above were subcloned into the EcoRI site of the pCaSpeRhsp43-lacZ reporter vector[28] (gift from A. Laughon). The full-length *zld* coding region was cloned between the KpnI and the XhoI 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. Three million 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. 1d and the plasmid pcDNA3 to bring the total amount of DNA to 250 ng. The expression of Zld protein was induced 24 h after transfection by adding 0.5 mM $CuSO_4$ directly to the medium. The cells were lysed after 24 h and the β-galactosidase and luciferase activities were assayed (Promega) according to 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 $CuSO_4$ and untreated cells.

DNA binding assays. Electrophoretic mobility shift assays were performed as previously described[9] using the affinity purified C-terminal part of the Zld protein containing four zinc fingers (amino acids 1240–1470) fused to GST (GST–ZldC) 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-labelled oligonucleotides (see Fig. 1a for nucleotide sequences) and varying amounts of recombinant GST–ZldC protein.

Microarray analysis. Total RNA was extracted from three independent collections of 1–2 h *y w* 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 to 13. cDNA was prepared using the GeneChip HT one-cycle cDNA synthesis kit (Invitrogen for Affymetrix) and labelled with the BioArray HighYield RNA transcript labelling kit (Enzo). Labelled 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. Genes were identified as present when at least two of the three replicates had present (P) assignment ($P \leq 0.05$). A Student's two-tailed *t*-test analysis was performed on the data from the three biological replicates.