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

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Abstract

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 (MZT). During this time many maternal RNAs are degraded and transcription of zygotic RNAs ensues¹. A longstanding question has been, what factors regulate these events? The recent findings that microRNAs^{2,3} and Smaugs⁴ 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 sites⁵ brought up 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 plays a key role in the activation of the early zygotic genome, and suggests that Zld may also regulate maternal RNA degradation during the MZT.

In *Drosophila*, an initial wave of zygotic gene transcription occurs between 1-2 hours of development during mitotic cleavage cycles 8-13. This is followed by a major burst of activity between 2-3 hours (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 patterning⁵⁻⁷. ten Bosch *et. al.*⁵ demonstrated that TAGteam sites are required for the early

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expression of the dorsoventral (DV) gene *zen*, and the sex determination genes *sisB* and *Sxl*. To isolate the TAGteam binding factor, we performed a yeast one-hybrid screen with a 91 bp fragment (Fig. 1a, sequences in uppercase) from the *zen* cis-regulatory region^{8,9}, which contains four TAGteam sites⁵ (Fig. 1a in red, the first two are the reverse complement). *zld* (*CG12701* on the X-chromosome) was selected as the only candidate of the 11 recovered that had the potential to bind specific DNA sequences since 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 C-terminal region of Zld fused to GST (GST-ZldC; Fig. 1b, stippled region). They all formed complexes with GST-ZldC, though with different affinities (Fig. 1c lanes 1-9), while mutations (Fig. 1a, in purple) in the heptanucleotide sequence abolished binding (Fig. 1c, lanes 10-12). Interestingly, the site with the strongest affinity, CAGGTAG, is the site most over represented in regulatory elements of pre-blastoderm genes versus postblastoderm genes⁵. A plasmid expressing full-length Zld protein promoted transcriptional activation of a *zen91-lacZ* reporter but not a mutated *zen91m-lacZ* reporter after co-transfection in *Drosophila* S2 cells (Fig. 1d). Taken together, these data strongly suggest that Zld activates transcription of *zen*, and likely 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 shown¹⁰. To analyze *zld* function, we generated deletion alleles of *zld* by imprecise excision (schematized in Fig. 1b). Hemizygous embryos showed abnormal CNS 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). *M⁻Z⁻zld* embryos never expressed *zld* (Fig. 2h) unlike *M⁻Z⁺zld* embryos, which began to express *zld* ubiquitously in cycle 14 (Fig. 2i). However, regardless of their zygotic genotypes, all *M⁻zld* embryos showed a severe abnormal morphology after cycle 14 (Fig. 2h,j), and did not survive to make cuticle.

Before cycle 14, *M⁻zld* embryos are similar to wild-type, except for sporadic nuclear fallout (Fig. 3c'). However, at early cycle 14 the hexagonal-actin network becomes disorganized (Fig. 3c) and begins to degenerate (Fig. 3d) resulting in a multinucleated phenotype (Fig. 3d'', arrow) resembling *nullo*¹² and *serendipity-α*¹³ (*sry-α*) mutants. Cellularization does not proceed as furrow canals never move inward (Fig. 3d, arrow) like in wild-type (Fig. 3b, arrow), and Neurotactin (Nrt) accumulates abnormally in the apical cytoplasm (Fig. 3h, arrow), reminiscent of the *slam* mutant phenotype^{14,15}. Staining with α-Slam antibody

confirmed that Slam protein is mostly absent by mid-cycle 14 (Fig. 3f) while in wild-type, Slam has moved basally (Fig. 3e'). In addition, nuclei do not elongate but instead become rounded, enlarged, and clump together (Fig. 3f and h' arrow). Regions of higher nuclear density were observed (data not shown), a phenotype similar to that obtained by injection of dsRNA against CG1270110, which we noticed resembles a *frühstart (frs)* phenotype¹⁶. Despite their aberrant morphology, *M' zld* embryos attempt to form a ventral furrow (Fig. S1b,c,e) but soon become highly disorganized with only pole cells recognizable (Fig. S1f). We rescued the *M' zld* cellularization defects by driving a wild-type copy of *zld* in the germline using the *ovarian tumor (otu)* promoter¹⁷. The cytoskeleton becomes well structured (Fig. 3i) and furrow canal ingression are normal (Fig. 3i') as Slam protein is restored (data not shown).

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*, *Sxl*, *zen* and *dpp*. None of these genes were activated in *M' zld* embryos (data shown for *sry-α*, *sisB*, and *zen* in Fig. 4b, d, f, respectively), except at the poles in some cases. However, *sna* and *sog*, which are activated by Dorsal¹⁸, were not absent but delayed in expression by at least 2 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 where Dorsal protein levels are low, a combinatorial mechanism involving both Dorsal and Zld establishes the broad *sog* domain. Interestingly, the two TAGteam sites in the 393bp *sog* enhancer¹⁹ lie close to four Dorsal binding sites.

Our results hinted 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. 120 genes were down-regulated at least 2-fold, and surprisingly 176 genes were up-regulated, in the absence of Zld ($p < 0.05$; Fig. 4k). The down-regulated set (Table S1) was strongly enriched in genes that are zygotically expressed (Fig. 4l) and involved in early developmental processes (Fig. S2), including most of the genes we assayed by *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 genes²⁰), 6 sex determination genes (*sisA*, *sisB*, *sisC*, *run*, *Sxl*, *dpp*), and 8 DV genes (*dpp*, *tld*, *tok*, *tsg*, *tsg-like*, *scw*, *zen*, *zen-2*) are in our down-regulated dataset. Overall, 75% of the early genes previously described as pre-cellular^{5,6,21} are included. This number may be an underestimate since there may be many genes like *sna* and *sog* that did not make the 2-fold cut-off (Fig. 4k), but are indeed regulated by Zld.

About 80% of the down-regulated genes have TAGteam sites within 2 Kb upstream of the transcription start site (Table S2), 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 down-regulated genes are direct Zld targets. In addition, the TAGteam sites upstream of the down-regulated genes tend to be located very close to the transcription start site, within 200 bp (Table S2), consistent with the previous finding that early zygotic genes have a statistical overrepresentation of TAGteam sites close to the start site^{5,6}.

In contrast to the down-regulated genes, the up-regulated 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. Since the miR-309 enhancer²² 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 miRs become abundant during cycle 14, and play a role in maternal transcript turnover in 2-4 hr embryos³. Not surprisingly, our 1-2 hr (cycles 8-13) dataset had no overlap with the 44 published miR-309 targets³, however 2-4 hr profiling experiments should reveal whether they are up-regulated in the absence of *zld*. We also compared our up-regulated genes to those affected by *smaug4*, another gene required for removal of maternally supplied RNAs. We found there was little overlap with the published Smaug targets⁴, suggesting that Zld is involved in a parallel pathway of maternal RNA degradation.

In summary, we have demonstrated that Zld functions as a key transcriptional activator during the 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 preblastoderm embryo is to set the stage for key processes such as cellular blastoderm formation, counting of X chromosomes for dosage compensation and sex determination, and pattern formation, by ensuring the coordinated accumulation of batteries of gene products during the MZT. This early preparedness should allow sufficient time for the formation of molecular machines²³ 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}24 element UM-8171-3 (Flybase, Szeged stock center). The *ovoD FRT*^{19A} stock was generated by transposition of *P{mini w+, ovo*^{D1-26}}25 onto *y w sn FRT*^{19A}, *hsFLP122*. Germ-line clones were induced in *zld*²⁹⁴ *FRT 19A* / *ovo*^{D1} *FLP122 FRT19A* by the FLP-FRT technique²⁶. Virgin females were collected and mated to *yw*, *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 following the Matchmaker One-Hybrid System (Clontech) protocol with the 91 bp *zen*-promoter and a 0-6 hours *Drosophila* embryonic cDNA library fused to the Gal4 activation domain²⁷ (gift from L. Pick).

Molecular Biology

DNA binding assays⁹ and *Drosophila* S2 cell transient transfection assays²⁸ 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 CuSO₄ 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 online 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 hr *yw* and *M⁻ zld* embryos by TRIzol (Invitrogen). cDNA was prepared using the GeneChip® HT One-Cycle cDNA Synthesis Kit (Invitrogen), labeled with the BioArray™ HighYield™ RNA Transcript Labeling Kit (Enzo), and hybridized to Affymetrix *Drosophila* Genome 2 arrays and processed by a GeneChip Fluidics Station 400.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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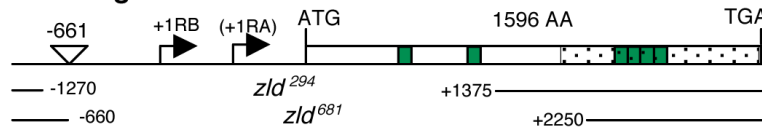
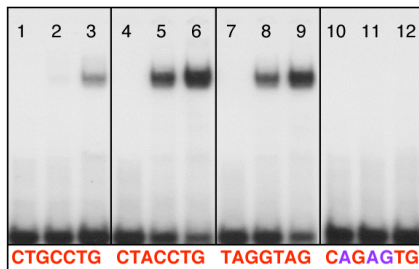
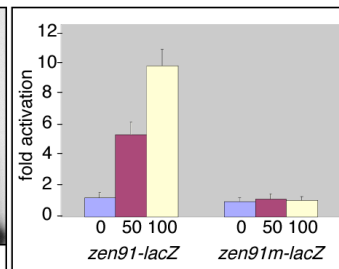
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a zen enhancer sequence

ttgcaccagCTGCCTGTGTTTATTCACCTACCTGAAACCCATACTCTTATCGCCT
 A AG G T
 GATCCTCGCGGGTGCACACTATTAGGTAGACACTGTACAGGCAGcactagcg
 A CA A CA

b zelda gene structure**c Zelda DNA binding****d Zelda transactivation****Figure 1. TAGteam sites bind Zld and mediate transcriptional activation**

(a) DNA sequence of the 91 bp *zen* enhancer (uppercase) plus surrounding sequences (lowercase). Base substitutions are in purple. (b) Schematic organization of the *zlda* locus (*CG12701*; Flybase) with the two predicted transcription start sites, RB and RA. The P{RS3}UM8171-3 insertion site is between -661 and -660. The nucleotides deleted in *zld*²⁹⁴ and *zld*⁶⁸¹ are indicated as 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 10ng GST-ZldC, the third 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 *zlda* under control of the inducible *metallothionein* promoter, the *zen91-lacZ* or *zen91m-lacZ* reporter plasmids, and the *luciferase* control. Error bars, s.e.m.; n=3.

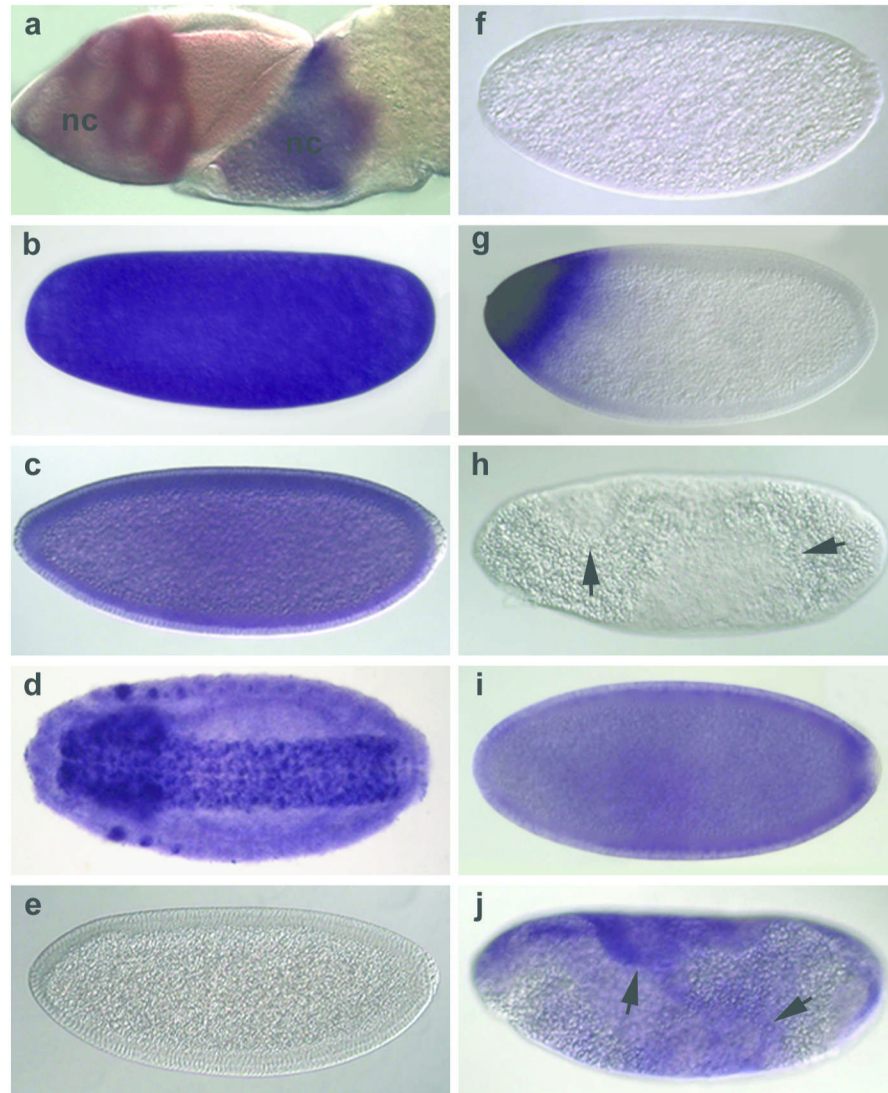


Figure 2. Maternal *zld* transcripts are lost as zygotic *zld* is activated in cycle 14

Wild-type (wt; a-d) and *zld*²⁹⁴ (e-j) ovaries (a) and embryos (b-j) were hybridized with *zld* (all but g) or *bcd* (g) RNA probes. (a) mid (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) Unfertilized egg. (c) cycle 14 embryo undergoing cellularization. (d) late-stage embryo. (e) *M*⁺*Z*⁻ *zld* cycle 14 embryo. Maternal *zld* transcripts have disappeared. (f) *M*⁻ *zld* cycle 10-11 embryo. (g) *M*⁻ *zld* cycle 14 embryo has a normal *bcd* pattern. (h) *M*⁻*Z*⁻ *zld* late cycle 14 embryo showing anomalous distribution of cytoplasm (arrows). (i) *M*⁻*Z*⁺ *zld* early cycle 14 embryo showing onset of zygotic *zld* expression. (j) *M*⁻*Z*⁺ *zld* late cycle 14 embryo showing abnormalities (arrows).

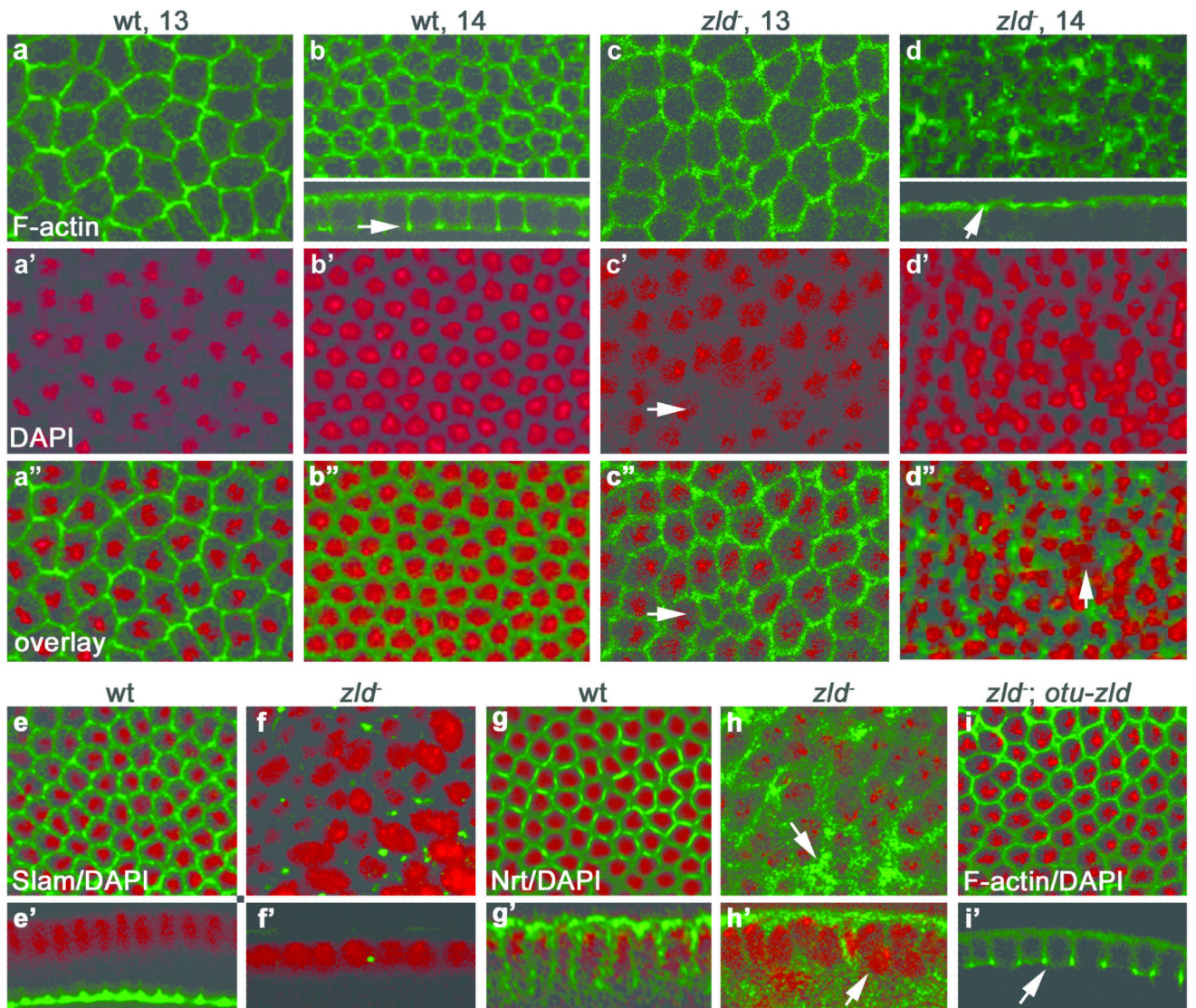


Figure 3. Maternal *zld* is required for cellularization

Confocal images of wt, *zld* (M^zld^{294}) and rescued ($M^zld^{294}; otu-zld$) embryos (as indicated) stained with phalloidin to detect F-actin, α -slam, or α -Nrt antibodies (green), and DAPI to detect DNA (red). In M^zld embryos the cytoskeletal network is disorganized and quickly degenerates in early cycle 14 (d) accompanied by nuclear fallout (d', arrow). Slam protein disappears in cycle 14 (f, f'), while Nrt accumulates apically (h, arrow). In $M^zld^{294}; otu-zld$ embryos the cytoskeleton is organized (i) and cellularization proceeds (i', arrow).

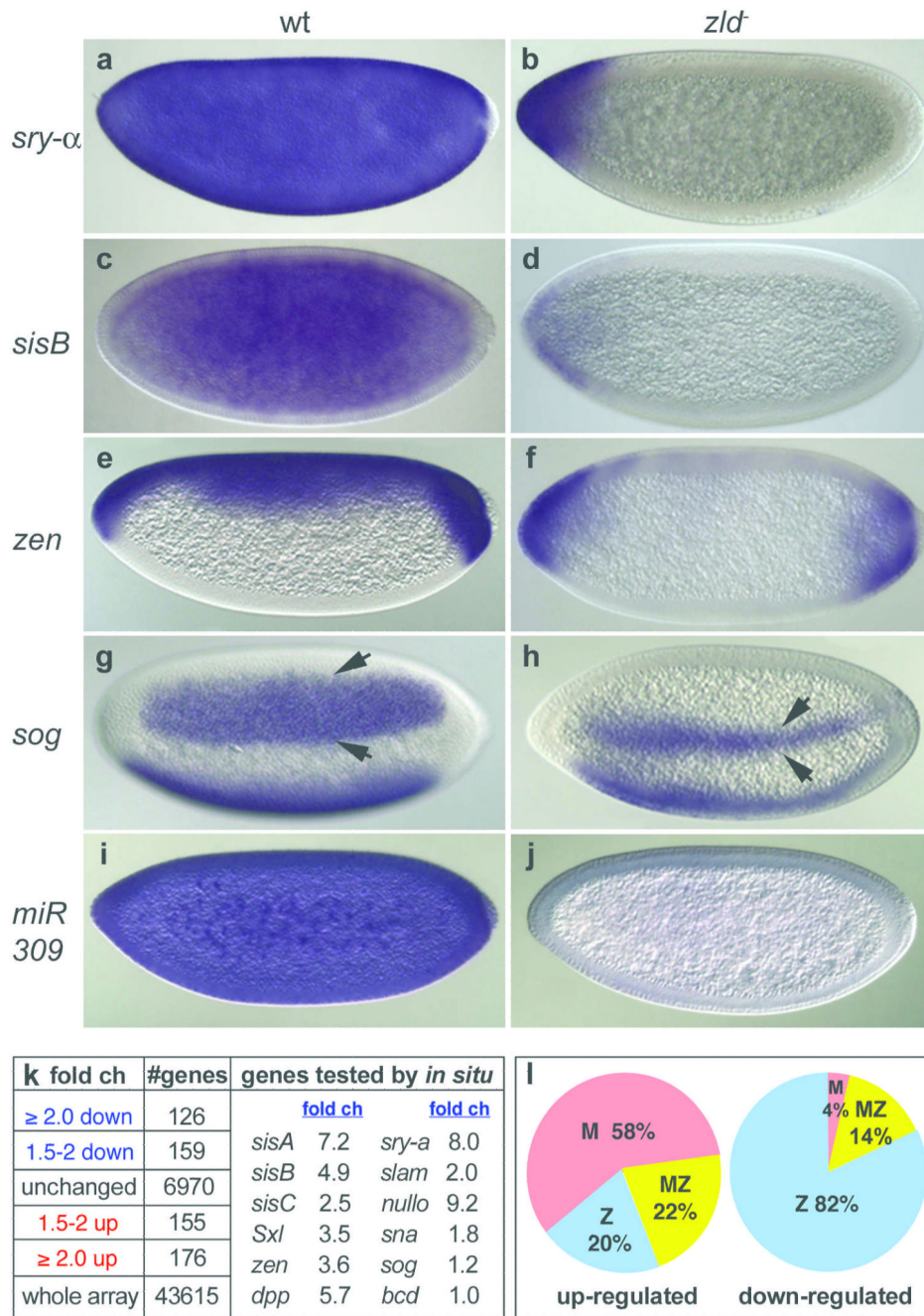


Figure 4. Zld plays a role in zygotic gene activation and maternal RNA degradation during the MZT

wt (left) and *M-Z zld²⁹⁴* (right) mitotic cycle 12-14 embryos were hybridized as indicated (309 = pri-miR-309). (k) Summary of expression profiles of 1-2 hr wt and *M-zld²⁹⁴* embryos. fold ch = fold change with respect to wt (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 down- (≥ 2 fold) and up-regulated (1.5 fold) gene sets.