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Zebrafish *cdc25a* is expressed during early development and limiting for post-blastoderm cell cycle progression

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Abstract

Cdc25 phosphatases are required for eukaryotic cell cycle progression. To investigate mechanisms governing spatiotemporal dynamics of cell cycle progression during vertebrate development, we isolated two *cdc25* genes from the zebrafish, *Danio rerio*, *cdc25a* and *cdc25d*. We propose that Zebrafish *cdc25a* is the zebrafish orthologue of the tetrapod *Cdc25A* genes, while *cdc25d* is of indeterminate origin. We show that both genes have proliferation promoting activity, but that only *cdc25d* can complement a *Schizosaccharomyces pombe* loss of function *cdc25* mutation. We present expression data demonstrating that *cdc25d* expression is very limited during early development, while *cdc25a* is widely expressed and consistent with the mitotic activity in previously identified mitotic domains of the post-blastoderm zebrafish embryo. Finally, we show that *cdc25a* can accelerate the entry of post-blastoderm cells into mitosis, suggesting that levels of *cdc25a* are rate limiting for cell cycle progression during gastrulation.

Keywords

zebrafish; cell cycle; Cdc25; mitotic domain; mitosis

Introduction

Over the last quarter-century, our understanding of the mechanisms that govern the cell division cycle has advanced rapidly. Studies in yeast and cultured cells have identified critical regulators of cell cycle progression and have provided detailed mechanisms underlying cell cycle control. However, the manner in which these factors interface with the genetic program of vertebrate development, although critical to a complete understanding of developmental processes is less clear, and requires analysis in intact embryos.

Early work in the yeast *Schizosaccharomyces pombe* identified many of the factors involved in eukaryotic cell cycle regulation (Nurse et al., 1976; Nurse and Thuriaux, 1980; Nasmyth and Nurse, 1981). In *S. pombe*, cell cycle progression is regulated in G2 by the Cdk1 kinase, the prototypic member of the cyclin-dependent kinase class of proteins (Simanis and Nurse, 1986). Cdks are regulated by the periodic availability of their obligate cyclin cofactors and by reversible inhibitory phosphorylation at adjacent threonine and tyrosine residues near the N-terminus (Gould and Nurse, 1989). This inhibitory phosphorylation is mediated by the Wee and Myt kinases (Gould and Nurse, 1989) and reversed by the dual-specificity phosphatase Cdc25 (Russell and Nurse, 1986; Gautier et al., 1991).

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Studies in Drosophila have established the importance of Cdc25 in coordinating cell proliferation with developmental events. Early syncitial cell cycles are driven by maternally provided mRNA and protein from the Drosophila *cdc25* homolog, *string* (Edgar and Datar, 1996). After the maternal-zygotic transition (MZT, cycle 10), maternal *string* mRNA is degraded (Edgar and Datar, 1996) and spatio-temporally patterned zygotic *string* expression begins (Edgar and O'Farrell, 1990). Expression of zygotic *string* mRNA anticipates mitoses in the early post-blastoderm cell cycles, and the highly stereotyped mitotic domains of the early Drosophila embryo correlate tightly with domains of *string* expression (Edgar and O'Farrell, 1990). Strong hypomorphic mutations in the Drosophila *string* gene cause an arrest in G2 of cycle 14 (Edgar and O'Farrell, 1990), suggesting a requirement for this protein in continued mitotic divisions during development. Further studies have shown that expression of *string* mRNA is directly controlled by patterning genes and that correct embryonic development requires precise control and integration of *string* expression and cell proliferation with patterning events (Edgar, 1994). Expression of a second Drosophila *cdc25* homologue, *twine*, is restricted to the germline, and loss-of-function mutations in this gene cause both male and female sterility (Edgar and Datar, 1996).

Tetrapod vertebrates posses three *Cdc25* genes, designated *Cdc25A*, *Cdc25B* and *Cdc25C*. The developmental role of these genes, however, remains unclear. *Cdc25B* knockout mice are largely phenotypically normal, although females are sterile, suggesting that this gene may play a role in female germ cell formation (Lincoln et al., 2002). Both *Cdc25C* (Chen et al., 2001) knockout and *Cdc25B/C* double knockout (Ferguson et al., 2005) mice develop normally, suggesting that either these phosphatases are dispensable for embryonic development or that *Cdc25A* can functionally compensate for loss of *Cdc25B* and C. The inaccessibility of the early mouse embryo and the difficulty of experimental manipulation in this model has made elucidation of the developmental roles of *Cdc25* genes problematic.

The role of Cdk phosphoregulation in vertebrate development has been most extensively investigated in the amphibian *Xenopus laevis*. As in Drosophila, maternal Cdc25A appears to be important in early cleavage phases and is degraded rapidly at the midblastula transition (MBT) (Kim et al., 1999; Shimuta et al., 2002). Later spatio-temporal regulation of expression of the inhibitory kinase Wee1 is critical for development (Leise and Mueller, 2002); disruption of Wee 1-mediated inhibition of cell proliferation by morpholino knockdown results in gastrulation defects (Murakami et al., 2004; Leise and Mueller, 2004). However, the extent to which the multiple Xenopus Cdc25 isoforms contribute to cell cycle progression and their potential role in coupling of cell proliferation and patterning during Xenopus development remains unclear.

In recent years, the zebrafish has emerged as a model for studies of vertebrate development. Despite the range of genetic tools available in this organism, including reverse and forward genetic techniques and a wide array of mutations in developmentally important genes, very little is known about the presence, expression and function of cell cycle regulatory genes and the placement of these cell cycle controls in the genetic hierarchy of development. Here we identify two zebrafish *cdc25* genes, *cdc25a* and *cdc25d*, and show that they are differentially expressed during early development, but neither transcript shows a dramatic redistribution at or shortly after the MBT within the embryonic cells. We conclude that *cdc25a* is a paralogue of the tetrapod *Cdc25a* gene, while the relationship between *cdc25d* and vertebrate *Cdc25* genes is unclear. Furthermore, we demonstrate that ectopic *cdc25a* mRNA expression can induce deep cells of mid-gastrula zebrafish embryos to rapidly enter mitosis, suggesting that Cdc25 activity is rate-limiting for cell cycle progression in these cells.

Materials and Methods

Fish husbandry

Adult zebrafish (*Danio rerio*) were maintained at 28°C as described in Westerfield (2000). Embryos were collected from natural mating and staged according to (Kimmel et al., 1995). All experiments were carried out in the DZ strain.

cDNA library screening and phylogenetic reconstruction

A mid-gastrula cDNA library (Sagerstrom et al., 2001) was plated to a density of 5000 colonies/ 200mm plate. These colonies were transferred to nitrocellulose filters, lysed using NaOH and crosslinked to the filter by UV irradiation. ^{32}P radiolabelled probes for *cdc25a* and *cdc25d* were synthesized by PCR followed by nick-end labelling (Takara Biosciences). Filters were incubated overnight with appropriate probes, washed and then exposed to film. Positive colonies were selected and plasmid DMA was isolated and sequenced using standard techniques. RACE was performed using the SMARTRACE kit (Clontech) following the manufacturer's directions. Protein sequence alignments were performed using ClustalX (Higgins and Sharp, 1988) and phylogenetic analysis of full-length protein sequences was performed in MEGA version 3.1 (Kumar etal., 2004).

In Situ Hybridization

Whole-mount in situ hybridization of wild-type embryos was carried out as described previously (Sagerstrom et al., 1996). Templates for zebrafish *cdc25a* and *cdc25d* in situ probe transcription were prepared by cloning the complete open reading frames into pBluescript KS + followed by linearization with either Xho1 (antisense) or Xba1 (sense) (New England Biolabs). Transcription was carried out using either T3 (Promega) or T7 (New England Biolabs) RNA polymerase in the presence of Digoxigenin labelling mix (Boehringer-Mannheim).

RT-PCR

Total RNA was extracted from collections of five embryos each at 256 cells, 512 cells, 1K cells, high and sphere stages using Trizol reagent (Ambion). cDNA synthesis was performed with the superscript III RT (Invitrogen) using random hexamer primers. Standard PCR reactions were performed for each stage, including a RT-minus control cDNA reaction using NovaTaq (Novagen) and an Eppendorf Mastercycler PCR machine. Aliquots were removed at 18, 21, 24, 27 and 30 cycles and analyzed by agarose gel electrophoresis. The following primers were used to amplify *cdc25a:* AACACGCCTGTCCGAGTGAAGAGG and CTCTAACGTAGCGACACATCCGTGG, amplifying a fragment of approximately 500bp, as well as previously described primers specific for Ornithine Decarboxylase (ODC) (Draper et al., 2001).

Yeast complementation assay

For expression in *S. pombe*, wild type zebrafish *cdc25a* and *cdc25d* genes were amplified by PCR with primers ATGGATATTGATATGGTTCCAGGCG and TCAGAGTTTTTTGAGACGGCTGTAC (*cdc25a*) and ATGGCCGGTGATGCGCTAGAGG and TTATCGTTTTGTTCTGTGATGTC (for *cdc25d*) and subcloned into the *S. pombe* pREP3x expression vector (Forsburg, 1993) in which transcription is regulated by the thiamine repressible *nmt1* gene promoter (Maundrell, 1990). Phosphatase-dead mutant versions *cdc25a*^{C470S} and *cdc25d*^{C295S} were constructed using the Quickchange site directed PCR mutagenesis kit (Promega). The *S. pombe cdc25* construct was previously described (Wolfe and Gould, 2004). The *S. pombe* cdc25-22 leu1-32 h temperature

sensitive strain (Nurse et al., 1976; Russell and Nurse, 1976) was grown to mid-log phase and transformed using Merlin Core Services™ EZ-Yeast Transformation kit.

Cells were grown on Edinburgh Minimal Medium (EMM) (Moreno et al., 1991). Colony growth and cell viability were monitored using the vital dye Phloxine B (PB) (Sigma), which accumulates in dead cells resulting in a dark pink color.

Cells were fixed in formaldehyde (Moreno et al., 1991), mounted on slides with Vectashield Mounting Medium with DAPI (Vector Laboratories) to visualize the DNA, examined using a Zeiss Axioskop fluorescence microscope and photographed with a DVC 1300 Black and White CCD camera using QED software (Media Cybernetics).

Heat-shock overexpression and immunostaining

A 1.5 kb Spel-EcoRV fragment containing the zebrafish *hsp70* promoter was taken from pzHSP70/4prom, described in Halloran et al. (2000). This was subcloned into the Nhe1-EcoRV sites of Tol2MCS.2, a gift of Maria Dorsett and Steve Johnson (Washington University, St. Louis), and is derived from the Tol2 transposon described in Kawakami et al. (2004). *cdc25a* and *cdc25d* and their phosphatase-inactive mutant forms were cloned into this vector fused to a N-terminal 6-myc tag. Comparing the activity of tagged and untagged protein expressed from mRNA injected during cleavage stages suggested that the Myc-tag does not impair function of either Cdc25 protein (Dalle Nogare et al., manuscript in preparation). DNA was purified before injection by phenol-extraction/ethanol precipitation followed by purification on a QIAQuick PCR-purification column (Qiagen) and diluted to 20ng/μL in ddH_2O/phenol red. This solution was mixed 1:1 with 50ng/μL transposase mRNA to yield a final injection solution of 10ng/μL plasmid with 25ng/μL transposase mRNA. 1nL of this solution was injected into a single blastomere at the 8-cell stage using a Harvard Apparatus PLI-90 microinjector. Embryos were kept at 28°C in E3 media until shield stage, at which point they were moved to a 37°C air incubator for 30 minutes, followed by a 20-minute recovery period at 28°C and fixation in 4% paraformaldehyde in PBS. Immunostaining was carried out using the 9E10 anti-myc monoclonal antibody (Roche, 1:500) and polyclonal anti-phosphohistone H3 antibody (upstate biotech, 1:200). Embryos were incubated with primary antibodies overnight at 4°C followed by extensive washes in PBT. Embryos were then incubated in Alexafluor secondary antibodies (goat anti-mouse 546 and goat anti-rabbit 488, Invitrogen) at a1:500 dilution for 2 hours at room temperature. Embryos were then washed in PBT containing 1:1000 Hoechst 33342 (Invitrogen) and mounted in Vectashield (Vector laboratories).

Mitotic index calculation

Confocal stacks (10 planes at 1μm spacing) were acquired on a Zeiss LSM510 confocal microscope using a 40X objective lens. Images were imported into Imaged (Wayne Rasband, NIH) and quantification of total nuclei in the field was performed using the 3D Object Counter Plugin (Fabrice Cordelières, Institut Curie). A typical field was 200–300 cells, of which 15% were Myc-positive. Phosphohistone and Myc positive cells were manually assigned and counted. The mitotic index of transgene expressing cells was calculated by dividing the number of myc-positive/PH3-positive nuclei by the total number of myc-positive nuclei. The mitotic index of non-expressing cells was calculated by dividing the number of myc-negative/PH3-positive nuclei by the total number of Myc-negative nuclei. To ensure consistency, only PH3-positive nuclei were counted; in the rare cases where mitotic figures were observed that were not positive for the phosphohistone antigen (late telophase), these cells were not considered in mitosis.

Results

Cloning and phylogenetic characterization of two zebrafish cdc25 homologues

In order to clone *cdc25* homologues from *Danio rerio*, a BLAST search of the NCBI NR database, as well as the partially completed zebrafish genome was performed using various vertebrate *Cdc25* genes. These searches revealed the presence of two distinct *cdc25* genes in the zebrafish. To determine the full cDNA sequences of these genes, we conducted a hybridization screen of a mid-gastrula zebrafish cDNA library using probes designed against the *cdc25* fragments found in the database. From this screen we recovered 10 cDNAs representing a first isoform of *cdc25* and three cDNA's representing a second isoform. To confirm that the cDNA sequences recovered were full length, we performed 3′ and 5′ RACE on mRNA from mid-gastrula embryos. The more abundant *cdc25* isoform cDNA maps to chromosome 13 and is 3042bp in length containing a 1692bp (563 amino acid) open reading frame, with a 290bp 5′ and a 1060bp 3′ UTR. A minority of these cDNAs (2/10) contained a truncated 3′ UTR (157bp), however the biological significance of this remains unknown. The second, smaller cDNA maps to chromosome 1 and is 1830bp in length, containing an 1160bp (386 amino acid) open reading frame (Fig. 1a).

To determine the evolutionary relationship of these two zebrafish *cdc25* genes to other known members of this gene family, we conducted phylogenetic reconstruction using the full-length protein sequences of both zebrafish *cdc25* genes along with a variety of *cdc25* genes from both vertebrate and invertebrate lineages (Fig. 1b). The results of this reconstruction suggested that the larger 563 amino acid isoform falls within the *cdc25a* group when compared with mouse, human, rat and Xenopus genes and thus we have designated this isoform *cdc25a.* This placement is supported by the presence of conserved serine residues known to be targets of the Chk1 kinase (S178 and T507 in human *cdc25a,* S213 and T546 in zebrafish *cdc25a,* Chen et al 2003). T546 is unique to mammalian A-type Cdc25 proteins, while S178 is unique to A- and B-type Cdc25 proteins. The conservation of these and other Chk1 phoshorylation sites (Goulodina et al., 2003; Sorensen et al., 2003; see Fig. 1a) suggest that zebrafish Cdc25a may be subject to similar regulatory mechanisms as the human Cdc25a.

The second, less abundant, and more divergent *cdc25* isoform is placed basal to the mammalian and Xenopus *Cdc25ABC* group and the invertebrate Drosophila/echoniderm/mollusc group. Clear orthologues of this gene cannot be found by BLAST in other fish species, including within the completely sequenced genomes of the pufferfish *Takifugu rubripes* and *Tetraodon nigraviridis* or within the partially sequenced medaka (*Oryzias latipes*) genome, all of which only appear to contain a single gene orthologous to zebrafish *cdc25a.* To reflect this divergence from any known vertebrate *cdc25* isoforms, we have designated this gene *cdc25d.*

Interestingly, *cdc25d* contains a 19-residue insertion near the active site that is not seen in other vertebrate *cdc25* genes (see alignment in Fig. 1c). Insertions at this position are present in two described *cdc25* genes from the Basidiomycete fungi *Cryptococcus neoformans* and *Ustilago maydis* and, although shorter in length, *string* from *Drosophila melanogaster.* We have no evidence to suggest that these insertions are homologous.

Cdc25a and Cdc25d have proliferation-promoting activity in *S. pombe*

In order to confirm that both genes are functional *cdc25* homologues, we assayed the ability of each gene to rescue a conditional mutation in the fission yeast *Shizosaccharomyces pombe.* The *S. pombe cdc25-22* mutant is viable and essentially wild-type in growth rate and cell morphology at 25°C, but fails to grow at 34°C (Russell and Nurse, 1986; compare Fig. 2 panels f and I with r). The *cdc25-22* mutant will grow at 34°C when housing a functional *cdc25* gene on a plasmid, and for a positive control cells were transformed with fission yeast

cdc25^{+Sp} (Russell and Nurse, 1986) in plasmid pREP1 (Maundrell, 1993; Fig 2q). We used the pREPSx plasmid, containing the *nmtl* promoter to expresses zebrafish *cdc25* genes at low levels in the presence of thiamine and at much higher levels in the absence of thiamine (Forsburg, 1993). Strains carrying the *cdc25d* gene grew at both the restrictive and permissive temperature (Figs. 2i, o). This rescue is dependent on the phosphatase activity of *cdc25d*, as strains carrying phosphatase-dead *cdc25d*^{C295S} were unable to grow at the restrictive temperature (Fig. 2p). However, neither wild type *cdc25a* (Fig. 2m) nor the phosphatase dead mutant *cdc25a*^{C470S} (Fig. 2n) were able to rescue *cdc25-22*. Interestingly, cells transformed with *cdc25a* plasmid grown at the permissive temperature in the absence of thiamine (high pREP3X promoter activity) also grew poorly (compare Figs. 2a and g), suggesting that expression of *cdc25a* is toxic.

To determine the nature of *cdc25a* toxicity, we examined the morphology of cells at the permissive temperature in the presence and absence of thiamine. Cells expressing *cdc25a* (Fig. 3b) at low levels were shorter than control cells (Fig. 3a). The short size indicates rapid progression through the cell cycle, and is characteristic of *wee* mutants (Nurse, 1975), which are deficient in phosphoinhibition of Cdk1 activity. The phenotype is more severe in the absence of thiamine (compare Figs. 3c and d). Specifically, the presence of small binucleated cells indicates that this strain was entering mitosis prematurely, suggesting that the failure to rescue results from excessive activity of *cdc25a*. Multiple attempts achieve *cdc25a* activity levels sufficient for rescue, but without toxicity were unsuccessful. However, these data are consistent with the conclusion that both zebrafish *cdc25* genes have proliferation-promoting activity in *S. pombe* cells.

cdc25a and *cdc25dare* expressed during early development

To determine the developmental expression of the zebrafish *cdc25* homologues, we performed in situ hybridization on fixed embryos. The results of this analysis are shown in Figures 4 and 5. *cdc25a* is present maternally (Fig. 4a) and very little change in expression is observed at the MBT. Shortly after the MBT, three mitotic domains emerge. The extraembryonic yolk syncitial layer (YSL) and enveloping layer (EVL) cease or delay mitotic proliferation. The deep layer gives rise to all of the embryonic tissue and continues to proliferate rapidly (Kane et al., 1992). *cdc25a* transcripts are observed in all deep cells (Fig. 4b), but not in the YSL (Fig. 4c) or EVL (Fig. 4d), which represent separate mitotic domains. Expression is maintained throughout early epiboly (Fig. 4e, f), decreasing toward the end of epiboly (Fig. 4g). To further test whether maternal *cdc25a* mRNA is degraded at the MBT, we performed semi-quantitative RT-PCR on mRNA extracted from staged pools of embryos around the MBT. The results of this analysis (Fig. 4j and k) suggested that *cdc25a* mRNA is not globally degraded at the MBT as in Drosophila, although we cannot preclude destruction followed by extremely rapid zygotic resynthesis.

Patterned expression is first observed at bud stage in the midline and anterior neural plate (Fig. 4h and i) and expression in the midline and trunk of the embryo continues throughout early development (Fig. 5a–d). We have not observed obvious restriction of mitotic cells, detected by anti-Phosphohistone-H3 immunostaining, to regions expressing *cdc25a* after gastrulation (not shown). We note, however, that expression is absent in the polster (Fig. 5a, arrow), one of the first tissues to exit the cell cycle in Xenopus (Saka and Smith, 2001) and Zebrafish (D.E. Dalle Nogare and M.E. Lane, unpublished observation). Widespread expression of *cdc25a* is maintained throughout early segmentation (Fig. 5e–m), and as development proceeds expression appears to become gradually restricted to the anterior portion of the embryo (Fig. 5n), although transcripts can be detected in the neural tube through 24hpf (Fig. 5r). At 24hpf, patterned anterior expression of *cdc25a* is observed in the rhombomeres and proliferative zones of the hindbrain (Fig. 5o–q), correlating with increased cell proliferation in the latter tissue.

Little *cdc25a* expression is observed after 24hpf, concentrated mostly in the developing CNS (data not shown).

Expression of *cdc25d* appears to be more restricted than *cdc25a.* While RT-PCR data suggest that this gene is present throughout early development (data not shown), the first detectable patterned expression by in situ is observed between 24hpf and 32hpf in a small cluster of bilaterally distributed cells in the ventral mesoderm above the yolk extension (Fig. 5t–v) and in the nasal placodes (Fig. 5s). The number of labeled cells in the ventral mesoderm appears to be variable, although the position of these cells does not change with time (compare Fig. 5t with u). No other patterned expression of *cdc25d* is observed, and the significance of these expression domains remains unclear.

Cdc25a, but not Cdc25d, can rapidly induce cells in gastrulating embryos to enter mitosis

Direct observation of cell cycle lengths demonstrated that cycle length increases progressively during epiboly (Kimmel et al., 1994). Our finding that *cdc25a* expression levels appear to decrease during this same interval suggested the possibility that availability of *cdc25a* is directly regulating cell cycle progression. To test this possibility, we asked whether ecoptic expression of *cdc25a* can promote proliferation in these cells. We first injected mRNA for either *cdc25a* or *cdc25d* into embryos at the one-cell stage, but this resulted in morphologically abnormal embryos (DDN and MEL, unpublished observations). To overcome this early effect of widespread overexpression, we mosaically expressed either *cdc25a, cdc25d* or their phosphatase-inactive mutant forms by injecting single blastomeres at the 8-cell stage with DMA constructs in the ToL2 transposon (Kawakami et al., 2004) containing Myc-tagged versions of these genes under the control of the *hsp70* promoter (Halloran et al., 2000). In Drosophila, such ectopic overexpression of *string* mRNA by a heatshock-inducible transgene during cycle 14, when approximately half of the embryonic cells are in G2, induces these cells to enter a precocious mitotic division. If zebrafish *cdc25a* is the rate-limiting regulator of cell cycle, we expected to see an increase in the number of mitotic cells following overexpression of *cdc25a.*

Injected shield stage embryos were subjected to 37°C heatshock for 30 minutes, allowed to recover at 28°C for 20 minutes then fixed and stained with antibodies against the Myc epitope to mark cells expressing the transgene, and phosphohistone H3 to visualize cells in mitosis. Following heatshock and recovery, cells expressing the transgene (blue cells in Fig. 6) were intermixed with non-expressing cells as a result of cell movement and intercalation and mosaic inheritance of the DMA construct. This allowed us to directly compare the mitotic index (defined at number of mitotic cells/total number of cells) of cells expressing *cdc25* transgenes with non-expressing cells within the same embryo.

The mitotic index of *cdc25a* expressing cells is increased three-fold over non-expressing control cells (Fig. 6a and c, $p < 0.01$, 99% confidence interval), suggesting that expression of *cdc25a* in these cells is sufficient to induce cells to enter mitosis. The increase in the mitotic index of cells expressing *cdc25a* was dependent on phosphatase activity, as the phosphatase-inactive mutant $cdc25a^{C470S}$ had no significant effect on the mitotic index of expressing cells (Fig. 6d, $p > 0.1$). Analysis of mitotic figures in *cdc25a*-expressing cells (Fig. 6g–j) shows that all stages of mitosis are represented, suggesting that these cells are undergoing complete mitotic divisions. We do not see any evidence of mitotic catastrophe in these cells. In contrast, expression of either *cdc25d* (Fig. 6b and e $p > 0.05$) or the catalytically inactive $cdc25d^{C295S}$ (Fig. 6f, $p > 0.1$) was not sufficient to increase the mitotic index of expressing cells. We note, however, that *cdc25d*-expressing cells do undergo mitosis (white arrows in Fig. 6f). These data suggest that availability of *cdc25a,* but not *cdc25d,* is rate-limiting for cell cycle progression during gastrulation.

Discussion

We have identified two non-orthologous genes encoding Cdc25 mitotic activators in zebrafish. Our phylogenetic analysis clearly indicates that the *cdc25a* gene is related to the tetrapod Cdc25A genes, while the *cdc25d* gene is not related to tetrapod genes and is of indeterminate origin. We have demonstrated distinct phenotypic effects of overexpression of the *cdc25* genes in yeast and in zebrafish embryonic cells, and differences in relative expression levels and patterns in developing embryos.

We found a single member of the tetrapod Cdc25 gene family, and the most recent zebrafish genome assembly (Zv7) does not show additional members of this family. The absence of other family members, coupled with the observations of higher expression and apparently stronger activity in both yeast and zebrafish assays suggests that in fish, most or all of the Cdc25 activity may come from the *cdc25a* gene. We also find a single gene in other teleosts where genome data is available, suggesting that the ancestral vertebrate condition is a single Cdc25A-like gene, and that the other tetrapod family members arose after the divergence of teleosts and tetrapods. These observations enhance the prospect for future genetic analysis in zebrafish and Medaka of developmental roles for vertebrate Cdc25 function.

Comparing the dynamics of zebrafish *cdc25a* expression with that of the Drosophila *string* gene reveals both similarities and important differences that are correlated with distinct cell behaviors during and immediately after the MBT/MZT. Whereas *cdc25a* and *string* mRNAs are abundant in early embryos as a result of maternal transcription, *string* mRNA is abruptly degraded at the MZT by factors dependent on zygotic transcription, and transcribed dynamically during gastrulation. We see no evidence for a dramatic alteration in *cdc25a* or mRNA levels at the MBT in the deep cells. In contrast, our observations are consistent with a gradual decay of maternally provided *cdc25a* message throughout gastrulation followed by dynamic spatially restricted expression upon completion of gastrulation. The drop in Cdc25 activity at the Drosophila MZT is required for a long G2 phase during cellularization of the embryo (Edgar and Datar, 1996), and is followed by pulses of *string* transcription that drive mitosis in domains corresponding to cell fate assignments. In zebrafish, only three mitotic domains have been described (Kane et al., 1992), and we see correlation between *cdc25a* expression and these domains. The domains comprise two extraembryonic layers, the YSL and the EVL, and the deep layer, which gives rise to all of the embryonic cells. The YSL and EVL are not actively proliferating during epiboly and gastrulation, and we do not observe *cdc25a* expression in these cells. While cells of the deep layer are actively proliferating, no mitotic subdomains linked to cell fate have been observed within the deep layer (Kane et al., 1992; Kimmel et al., 1994), in stark contrast to what is seen in the Drosophila embryo. Instead, deep cells proceed through up to three cell divisions throughout gastrulation, with no apparent spatial patterning. However cell cycles do progressively increase in length, and this correlates well with our observations that *cdc25a* transcripts are not spatially restricted but appear to decrease in abundance as gastrulation progresses. Our demonstration that overexpression of *cdc25a* during gastrulation forces cells into mitosis supports the proposal that *cdc25a* is limiting for cell cycle progression during gastrulation. Thus, while the spatiotemporal dynamics of cell cycle progression following the MBT/MZT are very different between Drosophila and zebrafish, in both cases, mechanisms that limit abundance and distribution of *cdc25* transcripts may control the timing of entry into mitosis.

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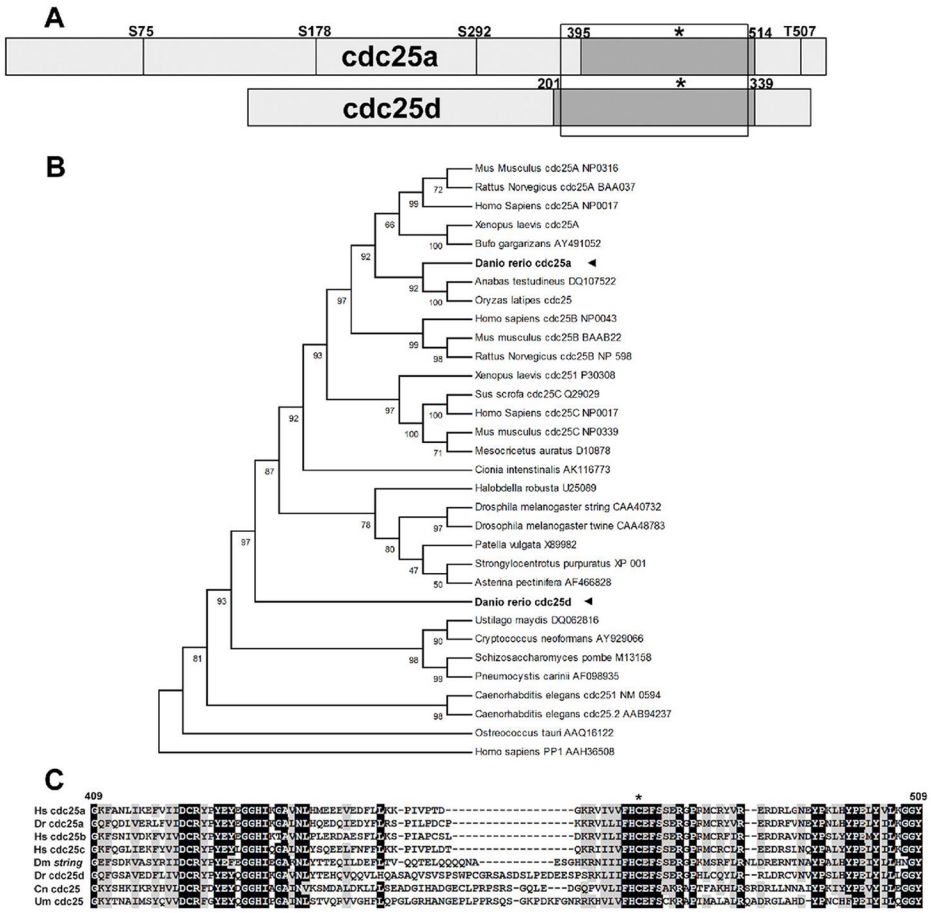

Figure 1.

a: Schematic of *cdc25a* and *cdc25d* from zebrafish, to scale. Conserved Chk1 phosphorylation target sites from human Cdc25A are shown as vertical lines, labeled according to the residue in human Cdc25A. Boxed region shows alignment in figure 1c and dark box delineates cdc25 phosphatase domain as defined by NCBI conserved domain search. Asterisk indicates position of HCXXXXXR catalytic motif. b: UPGMA phylogenetic reconstruction of *cdc25* genes from various species. Accession numbers used are noted in the figure. c: Alignment of the phosphatase domain of Cdc25 proteins: Dr *Danio rerio* Dm *Drosophila melanogaster* Hs *Homo sapiens* Cn *Cryptococcus neoformans* Um *Ustilago maydis*. Accession numbers are as in panel a.

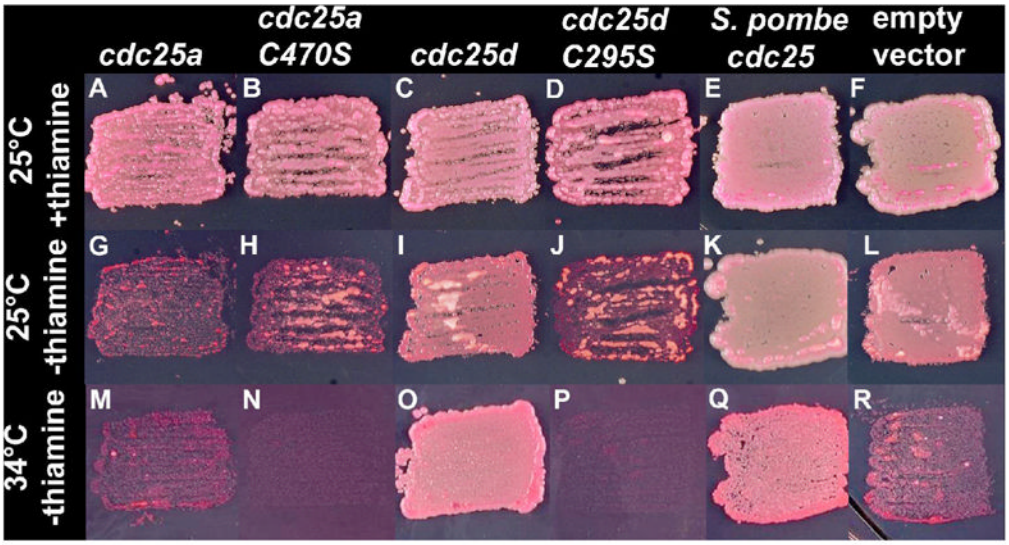

Figure 2.
Rescue of the *cdc25-22* mutation in *S. pombe* by various *cdc25* constructs. All experiments were performed in the *cdc25-22* background. Growth conditions are indicated at left and all genes expressed from the *nmt1* promoter are noted above.

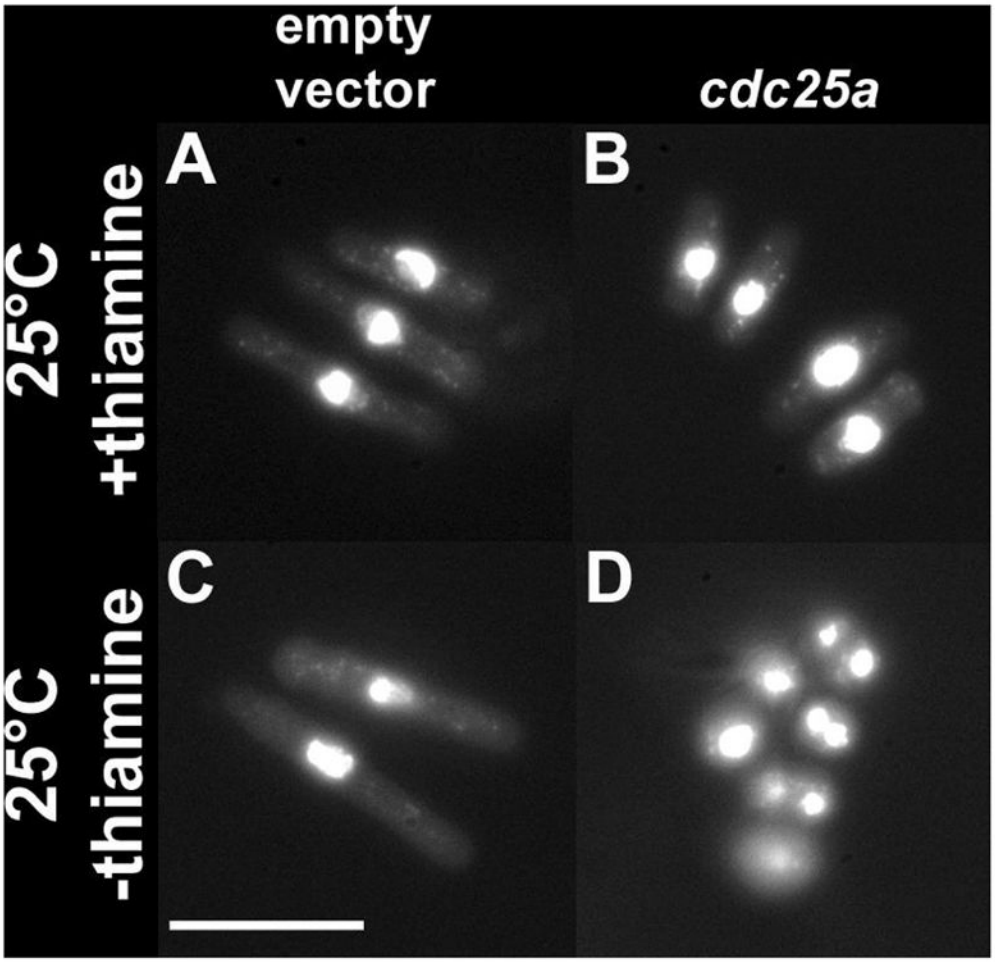

Figure 3.
Morphology of fixed DAPI stained *S. pombe cdc25-22* cells transformed with *cdc25a* or empty vector. Growth conditions are as indicated at left; All panels are to same scale. Scale bars are 10μm.

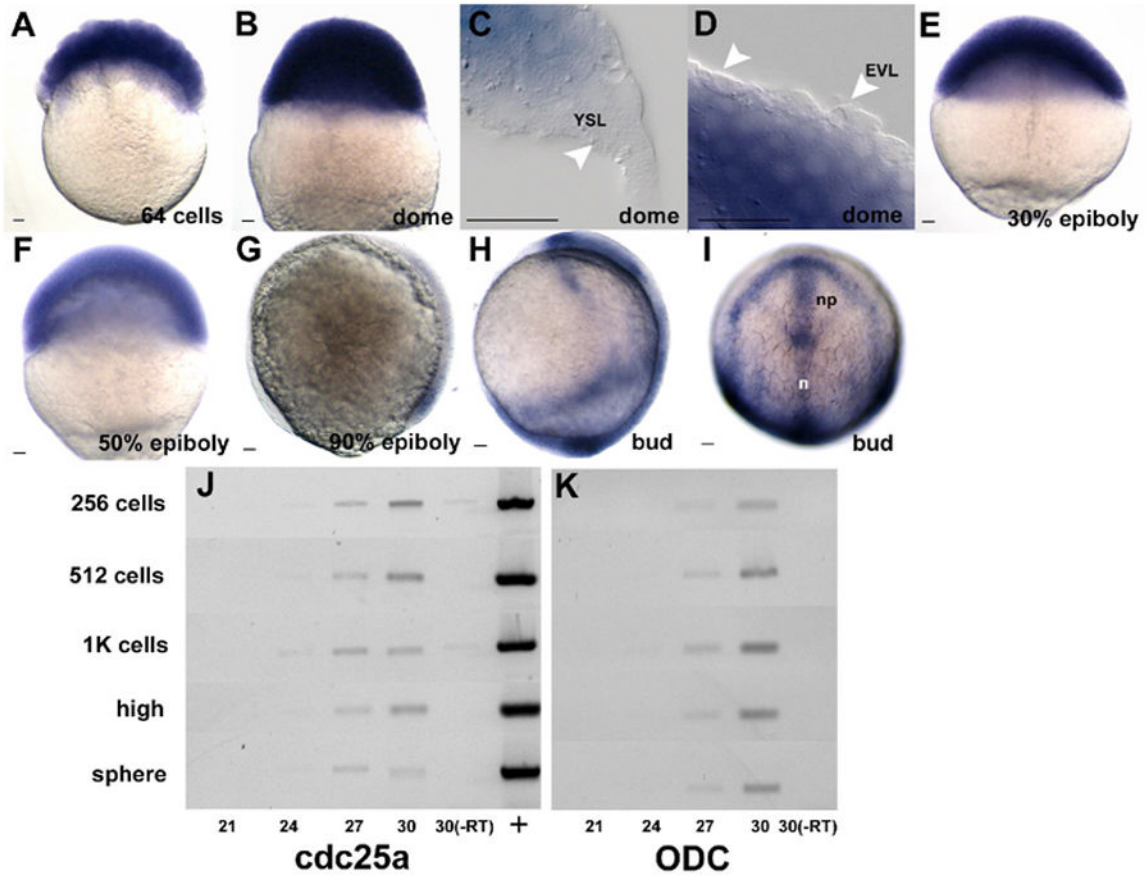

Figure 4.

In situ hybridization using a *cdc25a* antisense probe during epiboly (a–i). Panels represent staged semi-quantiative RT-PCR results using either *cdc25a* (j) or ODC (k) specific primers. Cycle numbers are as indicated below panels, with stages at left. (+) plasmid positive control; (-RT) no RT control reactions. All embryos are oriented animal pole up. g and h are lateral views with the dorsal side to the right and i is a dorsal view. *YSL* is yolk syncitial layer, *EVL* is enveloping layer, *n* is notochord and *np* is neural plate. Scale bar is 20μm.

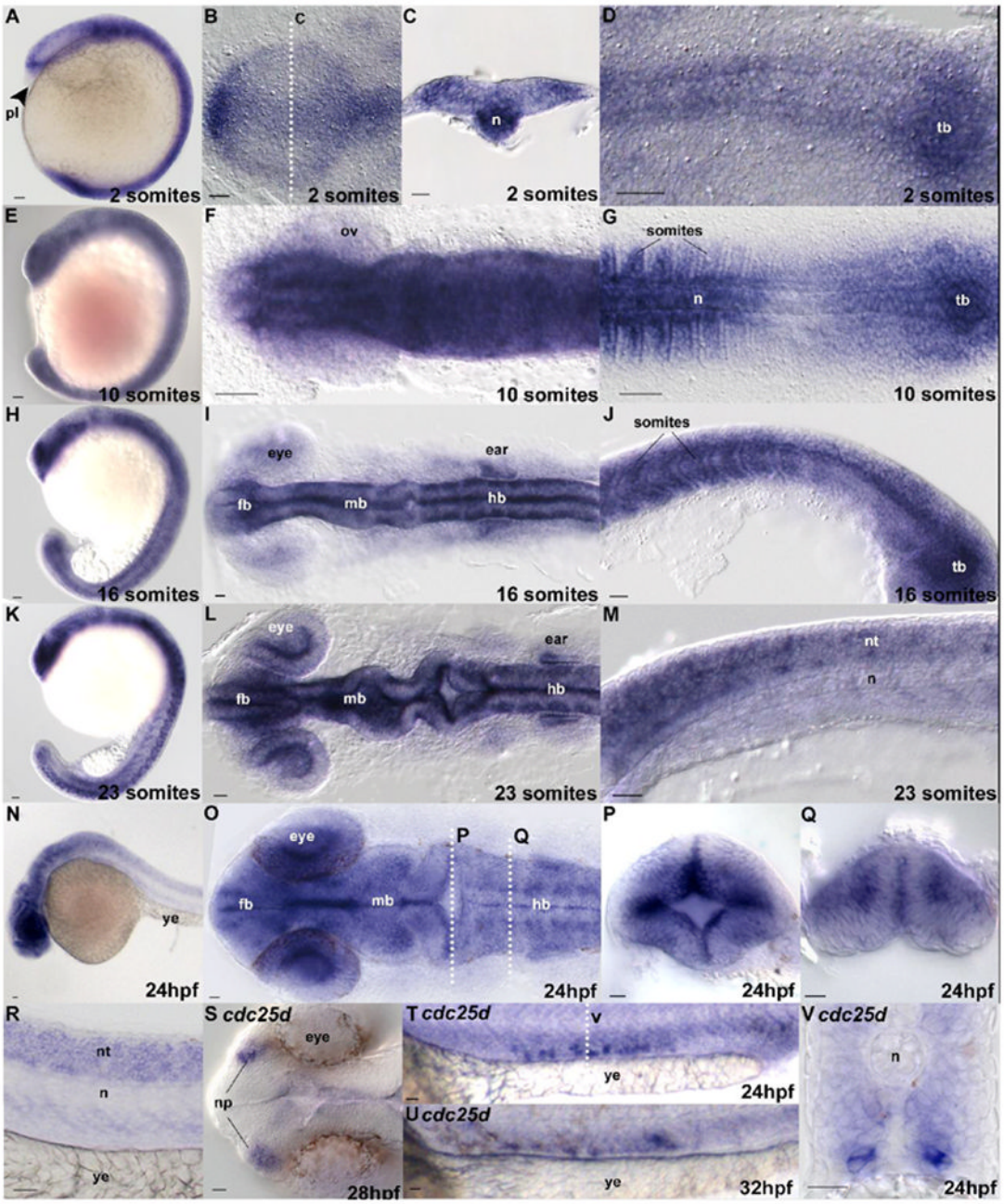

Figure 5.

In situ hybridization using *cdc25a* (a–r) and *cdc25d* (s–v) antisense probes. Stages are indicated in the lower-left of each panel. Panels p and q are cross sections through a 24hpf embryo at the positions labeled in panel o. Panel v is a section as indicated in panel t. Anatomical structures, where appropriate for orientation, are labeled. Embryo orientations are as follows: a, e, h, and k are lateral views, b, f, I, I, o and s are dorsal views of the anterior regions of the embryo, anteriormost to the left. d, g are similarly oriented but focused on the posterior region of the embryo. J, m, n, r, t, and u are lateral views. Cross sections c, p, q and v are dorsal up. *tb* is tailbud; *n* is notochord; *pl* is polster; *fb* is forebrain; *mb* is midbrain; *hb* is hindbrain; *ov*

is optic vesicle; *ye* is yolk extension; *nt* is neural tube; *np* is nasal placodes. Scale bar is 20μm.

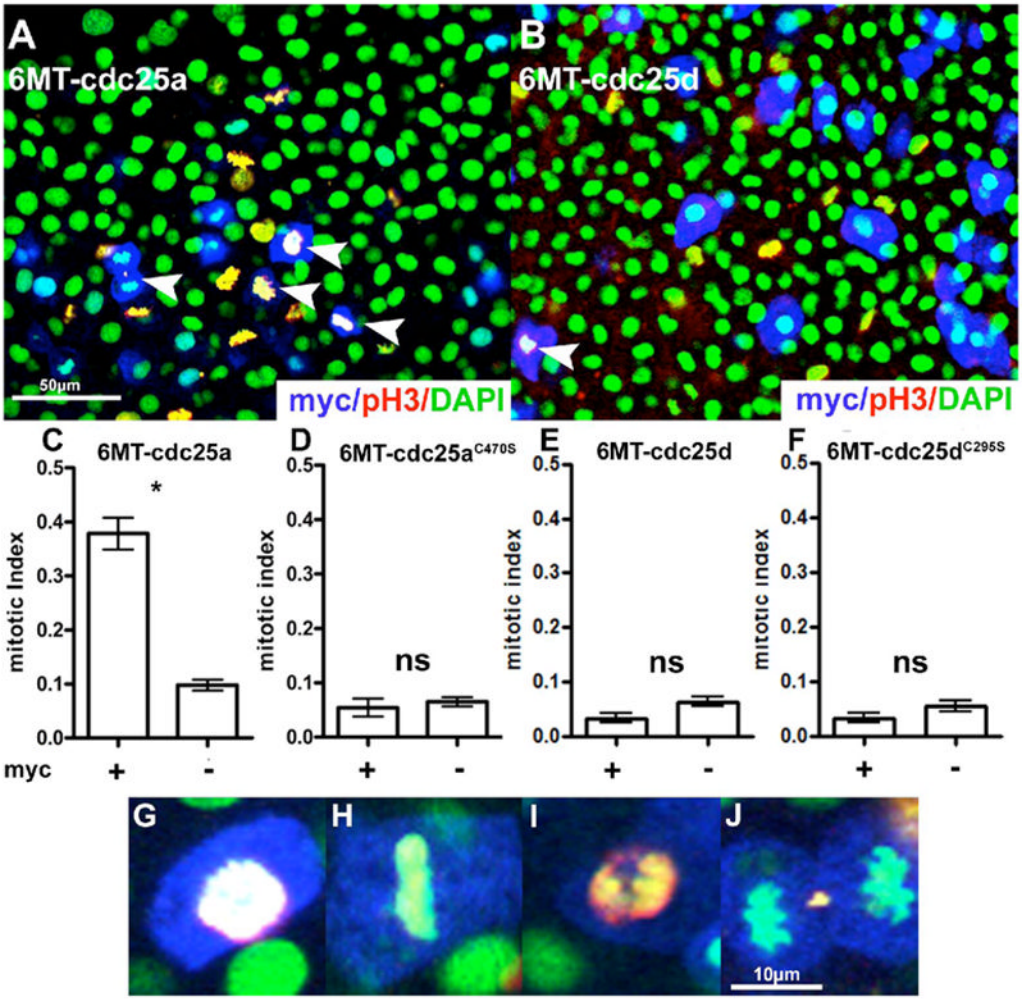

Figure 6.

Overexpression of *cdc25a* and *cdc25d* in gastrulating zebrafish embryos. Panels a and b are example fields from either *cdc25a* or *cdc25d* expressing embryos following heatshock and staining. Mitotic index quantification is shown in panels c–f. (*) $p < 0.01$ (ns) Not Significant. For *cdc25a*, n=14 fields, and for *cdc25d*, n=8 fields. See Materials and Methods for details. Panels g–j are enlarged images of *cdc25a* expressing (myc+) cells in various phases of mitosis. Stains are as indicated in the lower-right of panels a and b. White arrowheads in a and b indicate Myc-positive (therefore cdc25-positive) mitotic figures.