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Zebrafish Staufen1 and Staufen2 are required for the survival and migration of primordial germ cells

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Abstract

In sexually reproducing organisms, primordial germ cells (PGCs) give rise to the cells of the germ line, the gametes. In many animals, PGCs are set apart from somatic cells early during embryogenesis. Work in *Drosophila*, *C. elegans*, *Xenopus*, and zebrafish has shown that maternally provided localized cytoplasmic determinants specify the germ line in these organisms (Raz, E., 2003. Primordial germ-cell development: the zebrafish perspective. Nat. Rev., Genet. 4, 690–700; Santos, A.C., Lehmann, R., 2004. Germ cell specification and migration in *Drosophila* and beyond. Curr. Biol. 14, R578–R589). The *Drosophila* RNA-binding protein, Staufen is required for germ cell formation, and mutations in *stau* result in a maternal effect grandchild-less phenotype (Schupbach, T., Wieschaus, E., 1989. Female sterile mutations on the second chromosome of *Drosophila melanogaster*: I. Maternal effect mutations. Genetics 121, 101–17). Here we describe the functions of two zebrafish Staufen-related proteins, Stau1 and Stau2. When Stau1 or Stau2 functions are compromised in embryos by injecting antisense morpholino modified oligonucleotides or dominant-negative Stau peptides, germ layer patterning is not affected. However, expression of the PGC marker *vasa* is not maintained. Furthermore, expression of a green fluorescent protein (GFP):nanos 3′UTR fusion protein in germ cells shows that PGC migration is aberrant, and the mis-migrating PGCs do not survive in Stau-compromised embryos. Stau2 is also required for survival of neurons in the central nervous system (CNS). These phenotypes are rescued by co-injection of *Drosophila stau* mRNA. Thus, *staufen* has an evolutionarily conserved function in germ cells. In addition, we have identified a function for Stau proteins in PGC migration.

Keywords: Antisense morpholino oligonucleotides; Cell death; Dominant negative proteins; Germ line; Double stranded RNA binding domain; Cell migration; nanos1; Neurons; Primordial germ cells; staufen; stau1; stau2; vasa; Zebrafish

Introduction

The cells of the germ line populate the gonads and are essential for maintenance of species. Development of PGCs is regulated at several steps, which include specification of these cells, maintenance of their fate, and finally, their appropriate migration to their final destination, the gonads, where they differentiate into the male and female gametes. In many organisms, PGCs are set aside from somatic cells very early in development.

The molecules that function in PGC specification in the invertebrates, *Drosophila* and *C. elegans*, were identified via genetic screens (reviewed in Santos and Lehmann, 2004). Some of the key genes that regulate PGC development in these organisms appear to be conserved in vertebrates as well (reviewed in Raz, 2003). For instance, the germ line-specific DEAD-box RNA helicase gene, *vasa*, is expressed in the germ line of many species, including zebrafish (Yoon et al., 1997). The germ plasm or nuage, an electron dense structure containing RNA, mitochondria, and proteins, is important for specification of the germ line in *C. elegans*, *Drosophila*, and zebrafish. However, unlike *C. elegans* and *Drosophila* where Vasa protein is an important component of the germ plasm, in

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zebrafish, only vasa RNA but not Vasa protein, is a component of the germ plasm (Knaut et al., 2000). Localization of transcripts and other determinants to the germ plasm is an important step in the specification of these cells.

Staufen, a double-stranded RNA binding protein, was first identified in *Drosophila* as an essential factor required for germ line specification and the anterior–posterior axis formation (St Johnston et al., 1991). Stau protein is required for *oskar* RNA localization to the posterior pole plasm and its translational control (Micklem et al., 2000). Staufen and staufen-related proteins have five conserved double stranded RNA binding domains (dsRBDs), and a tubulin-binding domain (Micklem et al., 2000; Wickham et al., 1999). Staufen is thought to attach to microtubule adaptor proteins through its tubulin binding domain and the motors, which interact with adaptor proteins, transport cargo transcripts to various sub-cellular domains. During *Drosophila* oogenesis, the various Staufen RNA binding domains play distinct roles in *oskar* mRNA localization and translation. For instance, dsRBD2 is required for microtubule dependent localization of *oskar* mRNA, whereas dsRBD5 functions in translational de-repression (Micklem et al., 2000). The dsRBDs-1, -3, and -4 bind double-stranded RNA in vitro but RBDs-2 and -5 do not bind RNA (Micklem et al., 2000). In *Drosophila* embryos, the asymmetric localization of *prospero* RNA during neuroblast division is also dependent on Staufen (Li et al., 1997; Broadus et al., 1998).

Mammals have several *staufen* homologs, some of which undergo alternative splicing to generate multiple isoforms (Kiebler et al., 1999; Marion et al., 1999; Saunders et al., 2000; Monshausen et al., 2001). Mouse *staufen1* and *staufen2* are expressed in germ cells during oogenesis and embryogenesis (Saunders et al., 2000). It is thought that, in neurons, localized RNAs are required to elicit a spatially restricted response to a local stimulus, and Stau particles have been found in RNA-containing granules that co-localize with microtubules in dendrites of hippocampal neurons (Kohrmann et al., 1999). The Stau1 protein is expressed in intestinal epithelial cells, partly co-localized with the endoplasmic reticulum. Localization to the apical region of intestinal epithelial cells suggests that Stau1 may be involved in RNA localization to the apical domain in these cells as well (Gautrey et al., 2005). In *Xenopus*, Stau proteins have been found localized in the vegetal cortex in a complex containing Vg1 RNA, and disruption of Stau function blocks localization of Vg1 RNA (Allison et al., 2004; Yoon and Mowry, 2004). Thus, Stau and Stau-related proteins play key roles in cell fate specification and establishment of polarity during development.

To examine the function of Stau-related genes in zebrafish, we isolated two homologs. Although germ layer patterning is unaffected in embryos where Stau function is compromised, depletion of or interference with Stau1 or Stau2 function results in aberrant migration of germ cells. The PGCs do not survive, nor do neurons in the dorsal central nervous system throughout the length of the embryo. Thus, the zebrafish Stau proteins have evolutionarily conserved functions in germ cells and in neurons.

Materials and methods

Zebrafish strains

Embryos were obtained from natural mating of zebrafish of the AB strain (Johnson et al., 1994) or a local strain and maintained as described (Kimmel et al., 1995).

Table 1
RT-PCR primers

Gene	Primer sequence	Predicted product (bp)
oskar	F 5′ TGGAAGAATCCCTTCAAACG 3′ R 5′ CTGGTTAAAATCGTTGGCGT 3′	500
gurken	F 5′ GCCTCCTCTTTGTTGGATGGCAAA 3′ R 5′ TCGACTCGAGTCCCAATCCTCTTCT 3′	500
globin	F 5′ TGTTCTTTTTGCAGAAGCTCAG 3′ R 5′ GAAACAAACAGTGCTAGAATGTG 3′	220
actin	F 5′ GGCTACAGCTTCACCACCA 3′ R 5′ TGCTGATCCACATCTGCTG 3′	520
cyclinB	F 5′ CGCTTCCTTCAGGATCATCCAG 3′ R 5′ TTGGCAATATGCTGCATCACAG 3′	450
nanos1	F 5′ CCGGGAGATTTGAAGAAACA 3′ R 5′ AGCGACATGAAAATATGGCG 3′	450
stau1, Exon1–2	F1 5′ AAGCTGCTCAGTGAAGAAGGACCA 3′ R1 5′ TTGTTTGGGCGAGAGCACTTGATG 3′	110 (spliced) 210 (unspliced)
stau1, Exon1–Intron1	F2 5′ AGCCCGTTACAACAAGATCC 3′ R2 5′ CTGACCAACACACACCGTCA 3′	155 (unspliced)
stau2, Exon2–3	F1 5′ AGCCCGTTTCAACAGGATTCAACC 3′ R1 5′ TGCCTTCAGACTCCCATACC 3′	110 (spliced)
stau2, Exon2–Intron2	R2 5′ CTGCACGCACACAAACAGTCCAAC 3′	350 (unspliced)
stau2, Exon4–5	F1 5′ TTTACGTGCAGCTGACAGTG 3′ R1 5′ AGGGCAATCTCATAGACCAG 3′	220 (spliced)
stau2, Exon4–Intron4	R2 5′ GGACAACACATCACATCCATG 3′	550 (unspliced)

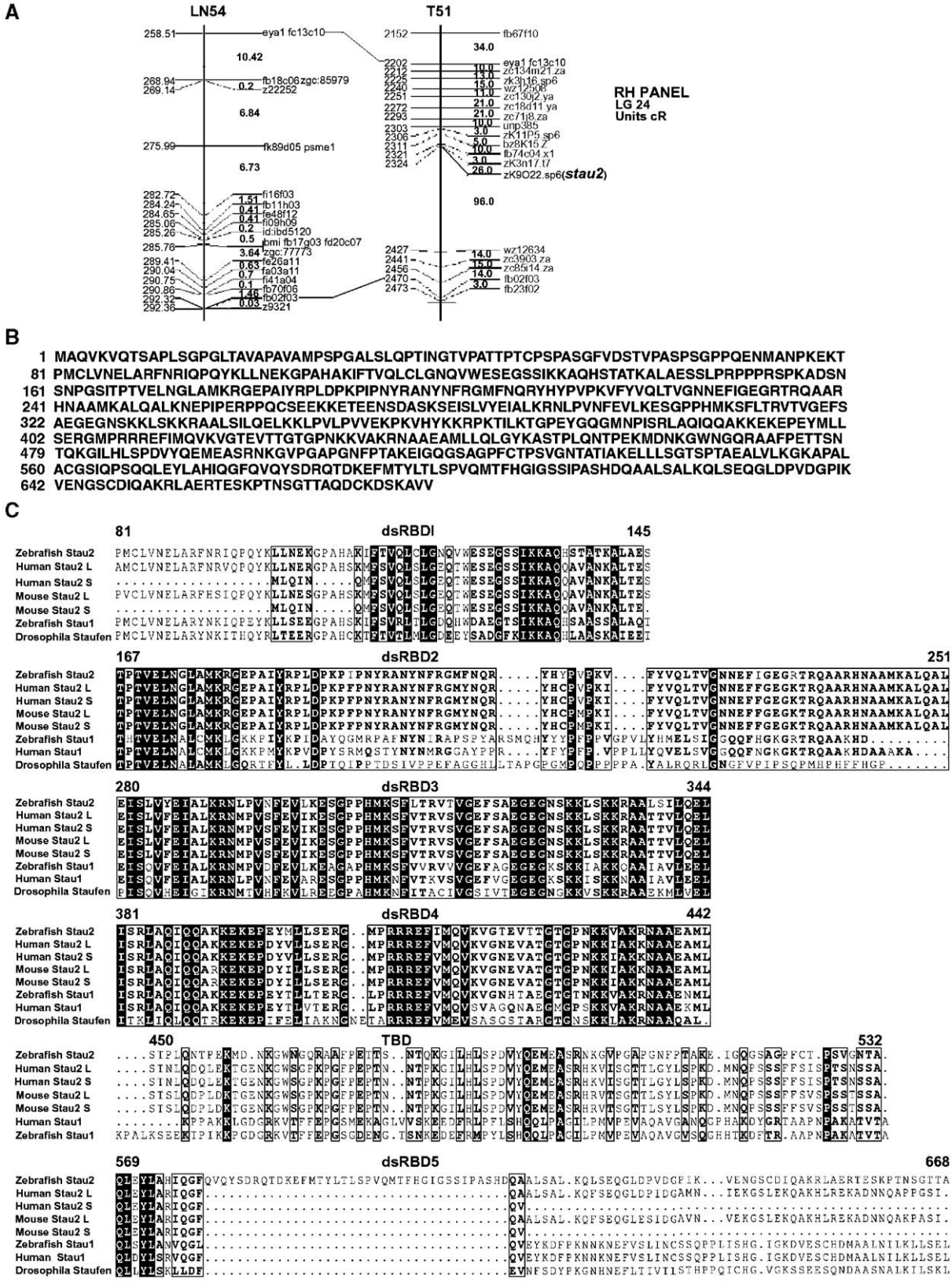


Fig. 1. Mapping and sequence analysis of zebrafish *stau2*. (A) *stau2* maps to LG 24. Schematic representation of the genomic location of *stau2* on the T51 and LN54 radiation hybrid maps. (B) Amino acid sequence of Stau2 protein. (C) Sequence alignment of zebrafish, human, mouse Stau1, and Stau 2 proteins with *Drosophila* Staufen. The long and short isoforms of human and mouse Stau2 proteins are indicated as L or S. Conserved amino acids are shaded in black or boxed. Numbers indicate amino acid residue in zebrafish Stau2. dsRBD1-5 indicates the 5 double-strand RNA Binding Domains; TBD, Tubulin binding domain.

Cloning of stau2

The human and mouse *staufen2* (*stau2*) sequences (Duchaine et al., 2002) were used to identify homologous zebrafish EST sequences by BLAST. The zebrafish *stau2* cDNA was amplified by PCR with the primers 5′ TGCCAATGTTGGAGGTGTAACTG 3′, 5′ TTAGCATGTGCAGGTCCTTTCTC3′ and 5′ TGAGAAAGGACCTGCACATG 3′, and 5′ CCACCATATTGCATGTTGAC 3′, respectively, from plasmids containing the ESTs AL924155 and NM_200925.

Radiation hybrid mapping

Radiation hybrid mapping was performed as described (Hukriede et al., 1999). PCR was performed on the zebrafish T51 RH panel in duplicates for each of the 96 hybrid DNA samples, using the primers 5′ CTGCAAGGATTCAAAGGCTGTCGT 3′ and 5′ GCCTGTTTAAATCCACTTGGAGGC 3′. PCR results were analyzed, scores were assigned according to (http://zfrhmaps.tch.harvard.edu/ZonRHmapper/) and the map position was obtained.

Expression of recombinant fusion proteins

The RNA binding domains Stau1 RBD4 and Stau2 RBD5 were amplified with the primers 5′ CGGAATTCCGGGCATGAACCCCATCAGCAG 3′, 5′ CCAAGCTTGGGGTTTCTCATCAGTTTTCAGTGC 3′, 5′ GAAGAAGCTTGTCCAGTCCTTGTTCTGACAG 3′, and 5′ GAAGGAATTCATCCAGCCTTCCCAACAGC 3′, respectively.

The amplified products were cloned in the plasmid pGexKG for expression in *E. coli* (Guan and Dixon, 1991). The GST fusion proteins were expressed in *E. coli* BL21(DE3 plysS) cells at 18°C and cells were lysed in buffer containing 50 mM NaH_2PO_4, 50 mM Na_2HPO_4, 300 mM NaCl, 0.1% TritonX-100, 0.2 M EDTA, and 100 μM PMSF. Fusion proteins were purified using glutathione sepharose beads and eluted in 10 mM glutathione.

Embryo injections

The following antisense morpholino-modified oligonucleotides were obtained from Gene tools LLC:

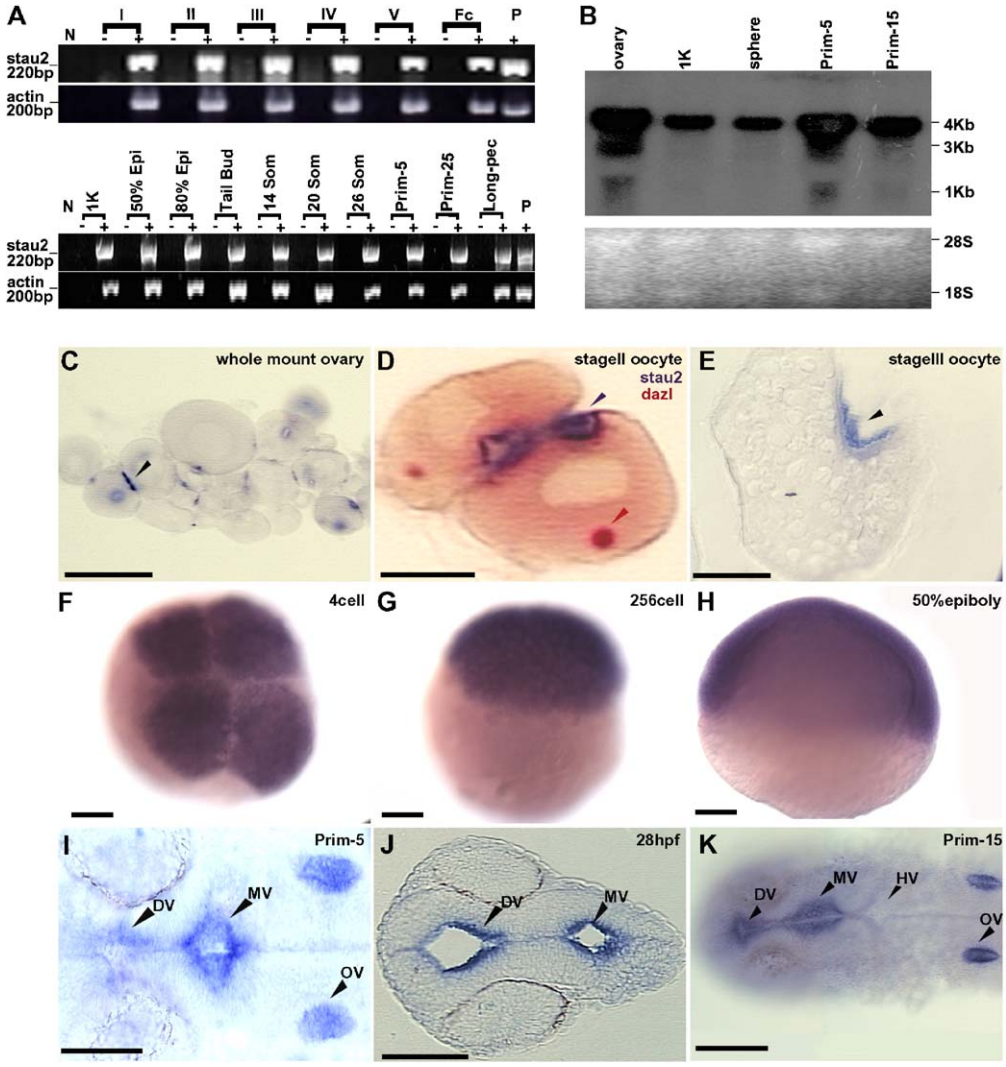


Fig. 2. Expression of *stau2* in oocytes and embryos. (A) RT-PCR analysis of *stau2* expression in comparison to actin in oocytes (top panel) and embryos (bottom panel) (stages indicated above lanes). Abbreviations: fc, follicle cells; epi, epiboly; som, somites; N, no template control; P, plasmid control; − indicates RT-control for each stage. (B) Northern Blot analysis of *stau2* expression. A predominant 4 kb transcript is detected in oocytes and embryos. In addition, a shorter 3 kb transcript is seen in oocytes and prim-5 embryos. Lower panel, Ethidium bromide stained 28S and 18S rRNA loading controls. (C–K) Spatiotemporal expression of *stau2* by whole mount (C, F–I, K) or section (D, E, J) in situ hybridizations on oocytes (C–E) and embryos (F–K); black arrowheads, stau2; red arrowhead, dazl; stau2 is expressed in 2–3 cortical patches in oocytes (C–E), is uniform in early cleavage stage embryos (F–H), and is localized in embryos from late segmentation stages (I–K). Abbreviations: DV, diencephalic ventricle; MV, midbrain ventricle; HV, hindbrain ventricle; OV, otic vesicle. Scale bars, 100 μm.

stau2 MO1 5′ TAACAGTACCTTAGCATG 3′,
stau2 MO2 5′ TAGTGAAGGTTACCTGAGGTGGTCT 3′,
stau2 MO3 5′ GGCTGATGTCTGCACTTTCACTTGA 3′
mismatch stau2 MO (mismatched nucleotides are underlined) 5′ GGCTCATCTCTCCACTTTGACTTCA 3′,
stau1 MO1: 5′ AGGTCTTACCTTGGAGTG 3′,
standard control MO: 5′-CCTCTTACCTCAGTTACAATTTATA 3′.

Morpholinos were resuspended in sterile water and injected at a final concentration of 10 ng per embryo. For *stau2*, although all three morpholinos gave similar phenotypes, stau2 MO2 was the most robust and was used in all the experiments described. To detect germ cells, RNA encoding GFP-nos1 3′UTR (Koprunner et al., 2001) was synthesized using the mMessage mMachine kit (Ambion), and 40-pg doses were injected with either control, stau1 or stau2 morpholinos, GST peptide, or the Stau1 RBD4 or Stau2 RBD5 peptides, at the 1-cell stage. GFP expression in PGCs was examined at 24 hpf using a Leica MZ FL-III microscope. Images were obtained using a Zeiss Axioplan2 microscope equipped with a Nikon DXM 1220F camera. The plasmid pCS2 + Dmstau was generated by amplifying the *Drosophila melanogaster staufen* cDNA using the primers 5′ GAATTCGAAATGCAGCACAACGTTC 3′ and 5′ CAATCTA-GATTATTTCATGGCATTG 3′, and cloning in the plasmid pCS2+ (Turner and Weintraub, 1994). To generate pCS2 + stau:nos3′UTR, the *Drosophila melanogaster stau* coding sequence was amplified with the primers 5′ TCCCCCGGGATGCAGCACAACGTTCATG 3′ and 5′ CGACGTCGACT-TATTTCATGGCATTGTTGAG 3′, and cloned in the plasmid pSP64 nos3′UTR (Koprunner et al., 2001).

To rescue the Stau peptide or morpholino injection phenotypes, capped mRNA was prepared from pCS2 + Dm*stau* (*Not*I, SP6 RNA Polymerase) using the mMessage mMachine kit (Ambion) and 40-pg aliquots were injected into 1-cell stage embryos.

To rescue the Stau peptide or morpholino injection phenotypes, capped mRNA was prepared from pSP64 Dmstau:nos3′UTR (*Not*I, SP6 RNA polymerase) using the mMessage mMachine kit (Ambion) and 20-pg to 150-pg aliquots were injected into 1-cell stage embryos.

In situ hybridization

In situ hybridizations were performed as described (Tian et al., 2003). The following plasmids were linearized and digoxigenin UTP-labeled antisense probes were synthesized by in vitro transcription: pBS*ntl* (*Xho*I, T7 RNA polymerase) (Schulte-Merker et al., 1994b), pSK*pax2* (Krauss et al., 1991), pBS*gsc* (*Eco*RI, T7 RNA polymerase) (Schulte-Merker et al., 1994a), pKS *krox20* (*Pst*I, T3 RNA polymerase), pKS*vasa* (*Xho*I, T7 RNA polymerase) (Yoon et al., 1997), and pGEM*stau2* (*Spe*I, T7 RNA polymerase for sense, and *Sph*I, SP6 RNA polymerase for antisense RNA). To detect the hybridized probes, BM purple precipitating substrate (Roche) was used in the color reaction.

Time lapse video microscopy

Embryos were grown at 28°C until the tail bud stage, dechorionated manually, and mounted in 0.3–0.5% low-melting temperature agarose (Sigma) and embryo medium on cover slip-bottomed dishes. Movement of PGCs in live embryos was observed using a Zeiss LSM Meta 510 confocal laser-scanning microscope with a 20× water immersion objective lens. Optical sections were obtained at 5-μ intervals. We recorded 30–50 consecutive time points at 120-s intervals. Captured images were processed using the Zeiss LSM image browser software package.

RNA binding assays

Sense RNA was transcribed in vitro from the plasmids pKS *oskar* (*Not*I, T3 RNA polymerase) (Ephrussi and Lehmann, 1992), pKS *gurken* (*Not*I, T3 RNA polymerase) (Neuman-Silberberg and Schupbach, 1993), and pSP64T *Xenopus globin* 3′UTR (*Xba*I, SP6 RNA polymerase) (Krieg and Melton, 1984). Total RNA from zebrafish ovary was extracted using the Trizol reagent (Invitrogen), and 2.5- or 5-μg aliquots of total RNA were used. The Stau RBD GST fusion proteins bound to glutathione-sepharose beads were washed with native buffer containing 50 mM NaH_2PO_4, 50 mM Na_2HPO_4, 300 mM NaCl, 0.1% TritonX-100, 0.2 M EDTA, and 100 μM PMSF. Binding buffer (50 mM Tris pH 7.5, 150 mM NaCl, 20 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.05% NP40, 0.125 mg/ml BSA, 40 units/ml RNasin) was added to the bead–protein mixture with in vitro transcribed *Drosophila oskar*, *Drosophila gurken*, control Xenopus *globin* RNA, or zebrafish whole ovary RNA for 30 min at 4°C. The beads were washed in buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 0.05% NP40, 0.5 M urea, and 40 units/ml RNasin. RNA bound to the beads was extracted using phenol/chloroform, and used for RT-PCR to detect *oskar*, *gurken*, *globin*, zebrafish *actin*, zebrafish *cyclinB* (Kondo et al., 2001), and zebrafish *nanos* cDNAs.

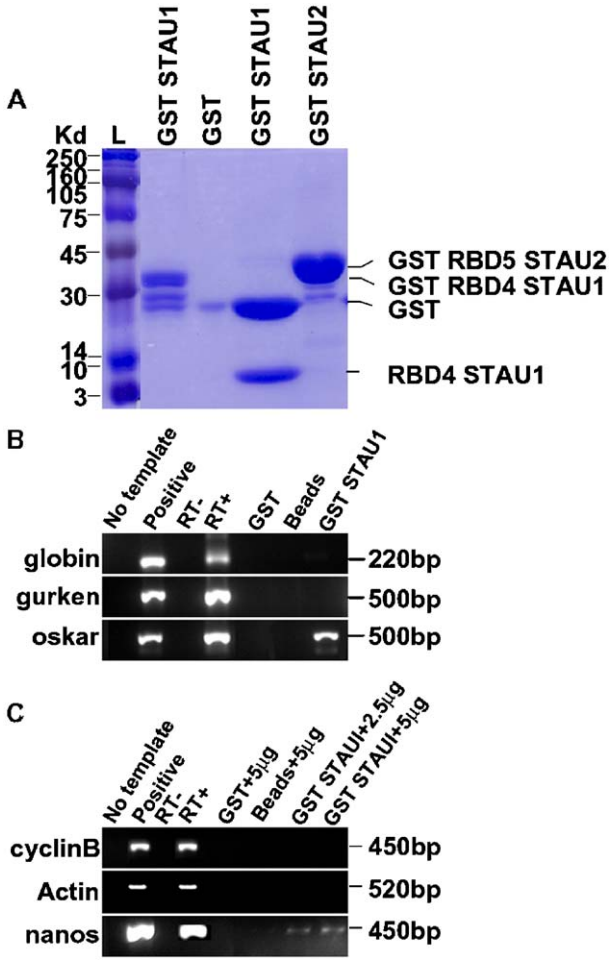


Fig. 3. GST-Stau1 fusion protein binds to *oskar* and *nanos1* RNA. (A) SDS-PAGE analysis of purified GST fusion proteins. L, Rainbow ladder, GST Stau1 RBD4, GST, GST Stau1 RBD4 digested with thrombin protease to release GST (28 kDa) and Stau1 RBD4 (7 kDa), GST Stau2 RBD5. (B) RT-PCR of *globin*, *gurken*, or *oskar* cDNAs from GST Stau1 RBD4 beads. No template control, positive *globin*, *gurken*, or *oskar* plasmid controls, RT−, RT+, GST (1 μg) incubated with 250 ng of *globin*, *gurken*, or *oskar* RNA, Control Glutathione beads incubated with 250 ng of *globin*, *gurken*, or *oskar* RNA, and GST Stau1(1 μg) beads incubated with 250 ng of *globin*, *gurken*, or *oskar* RNA. (C) RT-PCR from GST Stau1 RBD4 beads incubated with zebrafish ovary RNA. No template control, positive *cyclinB*, *actin*, or *nanos* plasmid controls, RT−, RT+, GST protein (1 μg) incubated with 5 μg of zebrafish ovary RNA, Glutathione beads incubated with 5 μg of zebrafish ovary RNA, GST Stau1 beads (1 μg) incubated with 2.5 μg of zebrafish ovary RNA, and GST Stau1 beads (1 μg) incubated with 5 μg of zebrafish ovary RNA.

RT-PCR analysis

RNA eluted from the beads was used to synthesize first strand cDNA by reverse transcription using oligo dT or p(dN)$_6$ primers (Roche) and Superscript II (GIBCO Life Sciences) enzyme, according to the manufacturer's instructions. To detect unspliced or incorrectly spliced products in *stau* morpholino or control morpholino-injected embryos, total RNA was extracted and cDNA was synthesized using p(dN)$_6$ primers (Roche) and Superscript II enzyme (GIBCO Life Sciences). PCR was performed with the primers discussed in Table 1.

TUNEL labeling

Morpholino and peptide-injected embryos were fixed at 4°C overnight in PBS containing 4% paraformaldehyde. Fixed embryos were washed in PBST, de-chorionated, dehydrated in an ethanol series, and stored in 100% ethanol until use. Embryos were hydrated, washed several times with PBST, and rinsed for 15 min in 0.1% sodium citrate in PBST. The in situ cell death detection kit (Roche) was used according to the manufacturer's instructions to detect dead cells. Stained embryos were cleared and mounted in glycerol, and images captured using a Zeiss Axioplan2 microscope equipped with a Nikon DXM 1200 color camera.

Immunohistochemistry

Embryos injected with control GST or Stau peptides were fixed in PBS containing 4% paraformaldehyde at 4°C overnight. Fixed embryos were de-chorionated, washed in PBST, and blocked in 2% blocking agent (Roche) in maleic acid buffer (pH 7.5). Blocked embryos were incubated with mouse antibodies to detect acetylated-tubulin (Sigma), followed by rabbit anti-mouse secondary antibodies conjugated with Alexa 488 (Molecular Probes). Fluorescence was detected using a Zeiss LSM 510 Meta confocal laser-scanning microscope, and captured images were processed with the Zeiss LSM image browser software package.

Results

Identification of zebrafish staufen homologs

Based upon homology to *Drosophila* and mammalian staufen genes, we identified several staufen-related genes in the zebrafish genome (http://www.sanger.ac.uk/Projects/D_rerio/Zv5_assembly_information.shtml). Two of these, which we hereafter refer to as zebrafish *staufen1* (*stau1*) and *staufen2* (*stau2*), are 67% and 74% similar to the mammalian counterparts (Fig. 1C). The predicted Stau1 and Stau2 proteins are 44% identical to each other at the amino acid level. Zebrafish Stau1 and Stau2 are similar to the mammalian Staufen homologs in the presence of 5 RNA-binding domains (RBDs) and 1 tubulin-binding domain (TBD) (Fig. 1C). In addition, protein alignment programs (Corpet, 1988; Gouet et al., 1999; Thompson et al., 1994) show that zebrafish Stau2 is more similar to the long isoform of mouse and human Stau2 (Duchaine et al., 2002; Kiebler et al., 1999; Wickham et al., 1999) (Fig. 1C). Radiation hybrid mapping using the T51 panel shows that whereas zebrafish *stau1* maps to LG 6 (Bateman et al., 2004), *stau2* maps to LG 24, at 23 cR from the marker zk3n17.T7 (Fig. 1A), to the BAC marker zk9O22.SP6. RT-PCR (Fig. 2A) and Northern hybridization (Fig. 2B) show that similar to *stau1* (Bateman et al., 2004), *stau2* is expressed in oocytes and embryos. We detect a major *stau2* transcript of 4 kb in oocytes and embryos, and a smaller 3 kb transcript in oocytes and in prim-5 stage (24 hpf) embryos. In situ hybridization shows that, in oocytes, *stau2* transcripts are present

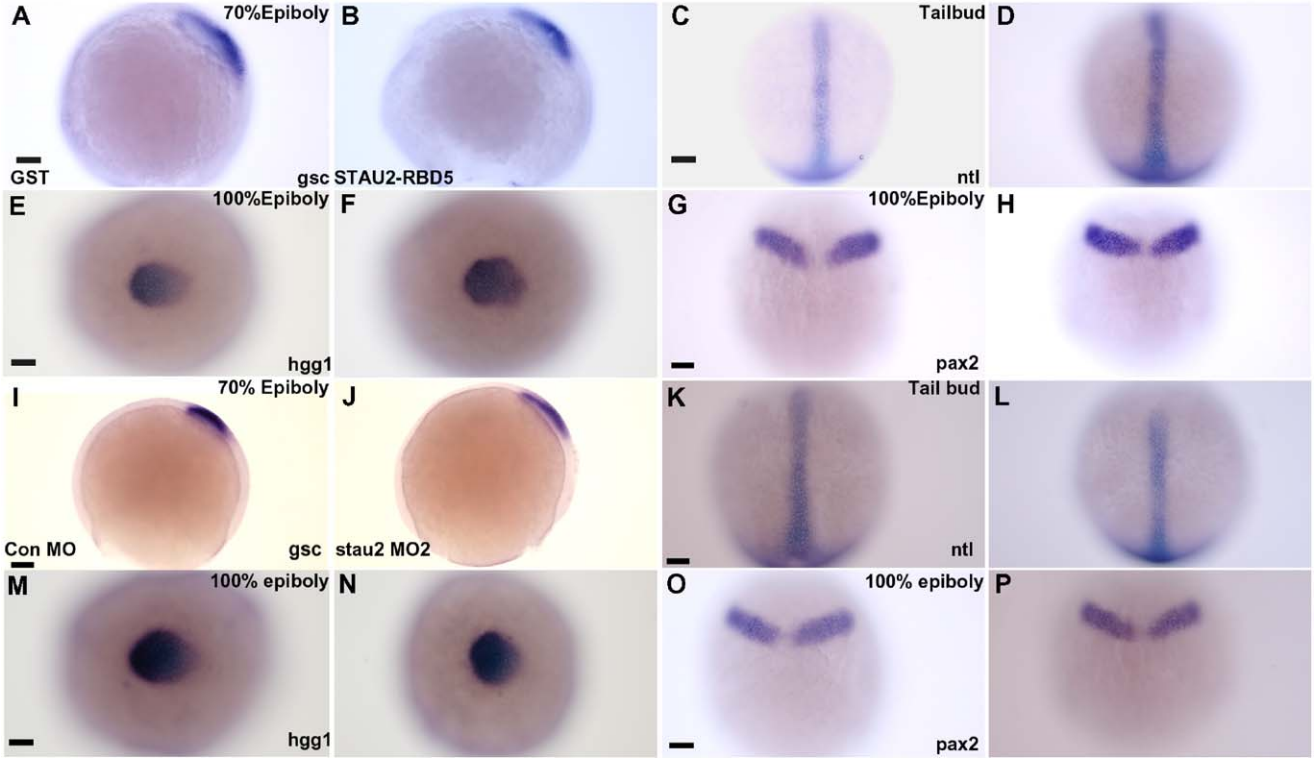


Fig. 4. Germ layer patterning is not affected by disruption of Stau2 function. Expression of *gsc* (A, B, I, J), *hgg* (E, F, M, N), *ntl* (C, D, K, L), and *pax2* (G, H, O, P) in embryos injected with GST (A, E, C, G), Stau2 RBD5 (B, F, D, H), Control morpholino (I, M, K, O), or stau2 morpholino2 (J, N, L, P); Dorsal views with the exception of panels A, B, I, and J (lateral views). Scale bars, 100 μm.

in 2–3 cortical patches (Figs. 2C–E), and in early cleavage and gastrula stage embryos, similar to *stau1* (Bateman et al., 2004), stau2 RNA is ubiquitous (Figs. 2F–H). In 24 hpf embryos, stau2 expression is restricted to periventricular neurons of the diencephalon and midbrain regions, and to the otic vesicles (Figs. 2I–K). However, unlike stau1, stau2 RNA is not detected in the hindbrain. Thus, *stau1* and *stau2* have overlapping, but distinct expression domains in developing zebrafish embryos.

RNA binding by zebrafish Stau proteins

Since Stau and Stau-related proteins harbor double-stranded RNA binding domains, we tested some domains of the zebrafish Stau proteins for RNA binding activity. The predicted RBD4 sequence of Stau1 and the RBD5 sequence of Stau2 were fused with glutathione-S transferase, expressed in *E. coli* (Fig. 3A). The purified Stau1 RBD4 peptide was tested for its ability to bind in vitro a known target of *Drosophila* Staufen, *oskar* mRNA (St Johnston et al., 1991; Ephrussi and Lehmann, 1992). RT-PCR analysis of cDNA extracted from beads incubated with either *oskar* RNA, *gurken* RNA (Neuman-Silberberg and Schupbach, 1993), or control *globin* RNA shows amplification of *oskar* cDNA from GST-Stau1 beads incubated with synthetic *oskar* RNA (Fig. 3B), but not those incubated with control *globin* RNA or *Drosophila melanogaster gurken* RNA. RT-PCR on control GST protein beads incubated with *oskar* RNA also shows no amplification of *oskar*. Therefore, zebrafish Stau1 can bind *oskar* RNA in vitro, but not *gurken* or *globin* sequences. We also incubated GST-Stau1 beads with total RNA extracted from zebrafish ovary, and tested for binding of a number of known localized transcripts. RT-PCR of cDNA synthesized from the GST-Stau1 beads incubated with ovary RNA shows amplification of *nanos1* fragments, whereas *actin* and *cyclinB*, which both encode localized transcripts (Kondo et al., 2001; Lawrence and Singer, 1986), fail to amplify (Fig. 3C). At the same doses as used with Stau1, GST-Stau2 RBD5 does not bind *oskar* RNA (data not shown), consistent with previous observations that *Drosophila* Stau RBD5 does not bind RNA (Micklem et al., 2000). Therefore, the GST-Stau1 fusion peptide does not indiscriminately bind any RNA, and specifically binds *Drosophila oskar* RNA in vitro, and to zebrafish *nanos1* in whole ovary RNA samples.

Germ layer patterning is not affected by disruption of Stau function

Since the Stau1 RBD4 peptide binds oskar RNA in vitro, and to *nanos1* RNA in whole ovary RNA samples, we tested if it could function as a dominant–negative inhibitor of Stau protein function in zebrafish embryos. The Stau1 RBD4 and Stau2 RBD5 peptides were injected at the 1-cell stage, and the effect on patterning of the primary germ layers was examined. Expression of *goosecoid* (*gsc*) in the organizer is

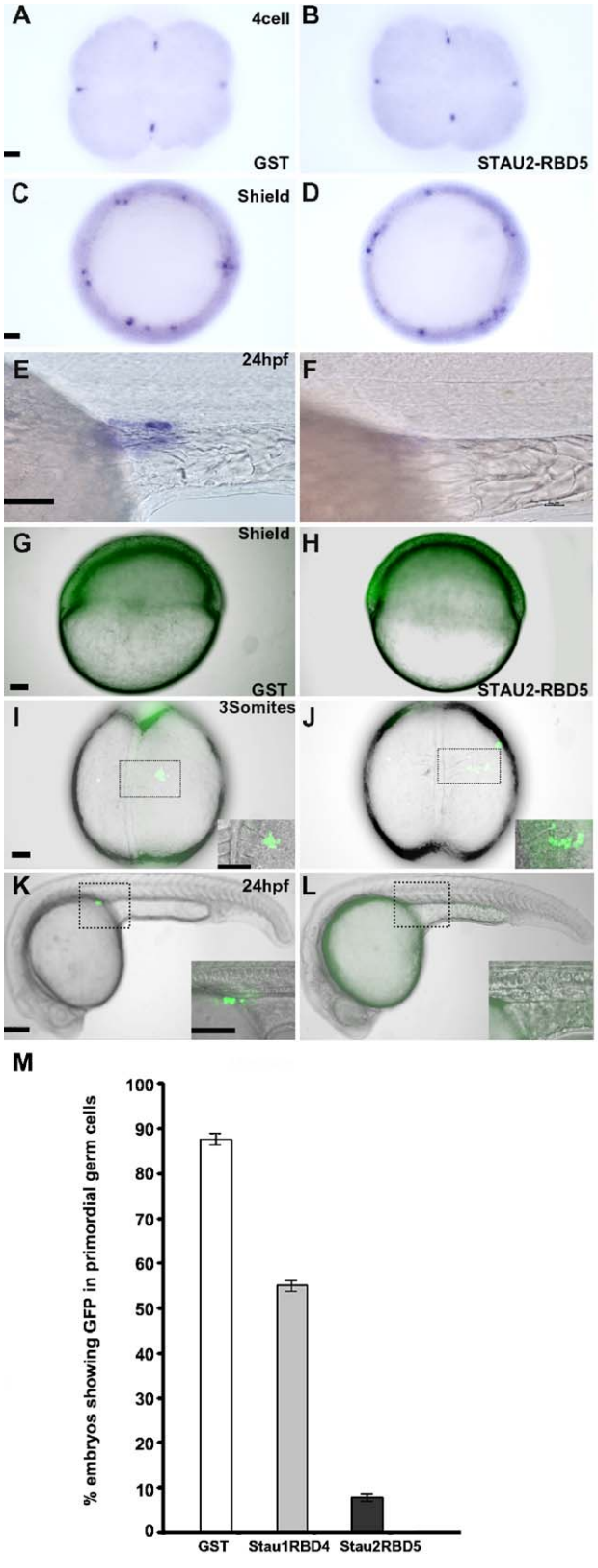
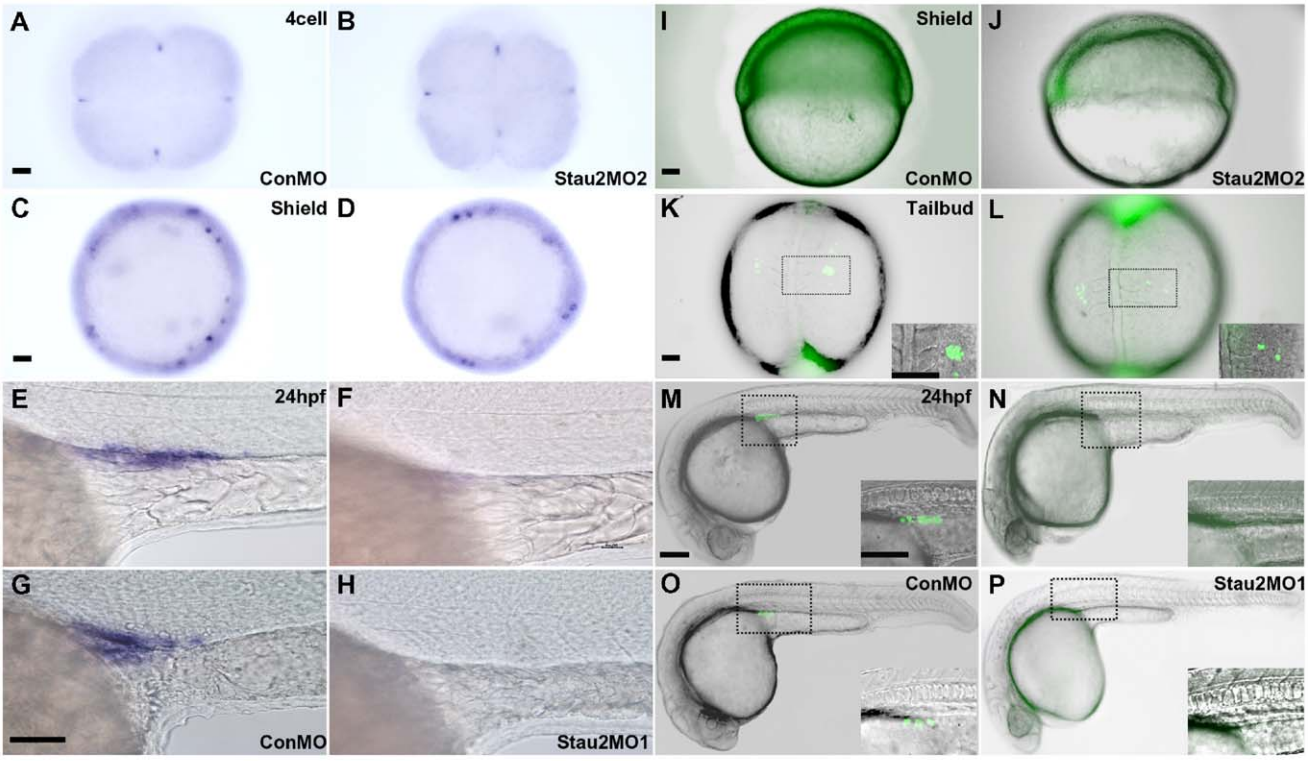


Fig. 5. Stau1 and Stau2 peptides overexpression abolishes PGCs. (A–F) Expression of the germ cell specific gene, *vasa*, in 4 cell (A, B), shield stage (C, D), and 24 hpf (E, F) embryos injected with GST (A, C, E), Stau2 RBD5 (B, D, F); GFP:nos1 expression in shield stage (G, H), 3-somite stage (I, J), and 24 hpf (K, L) embryos injected with GST (G, I, K), Stau2 RBD5 (H, J, L), histogram in M shows % embryos with GFP expression in PGCs. In panels I–L, dotted boxes outline the areas shown at higher magnification in the insets. A–D, animal pole views; E–H and K, L, lateral views; I, J dorsal views. Scale bars, 50 μm for panels A, C, G, I and 100 μm for panels E, K, and insets.


A
4cell
ConMO
B
Stau2MO2
I
Shield
ConMO
J
Stau2MO2
C
Shield
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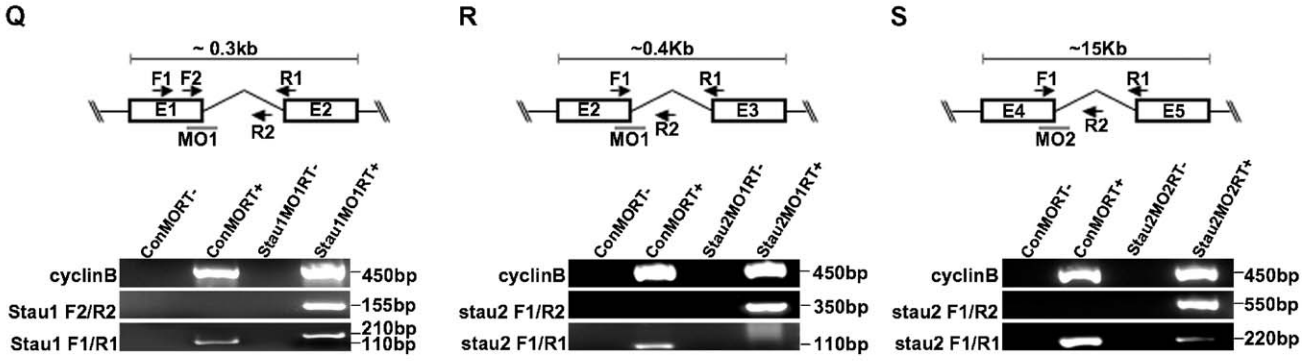
Stau2MO1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450bp
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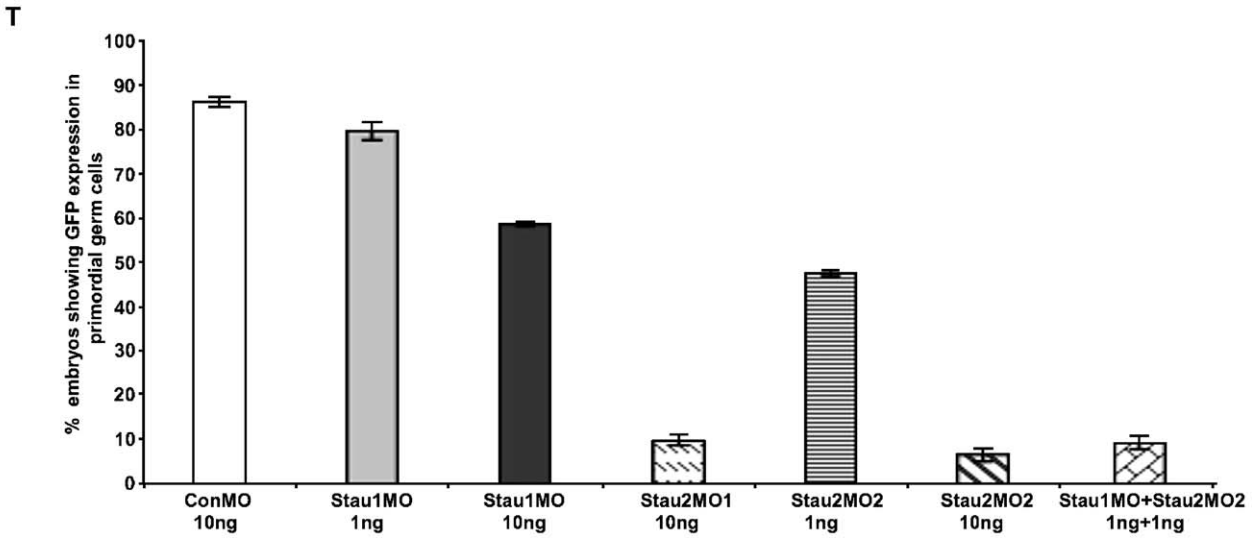
T
% embryos showing GFP expression in primordial germ cells
100
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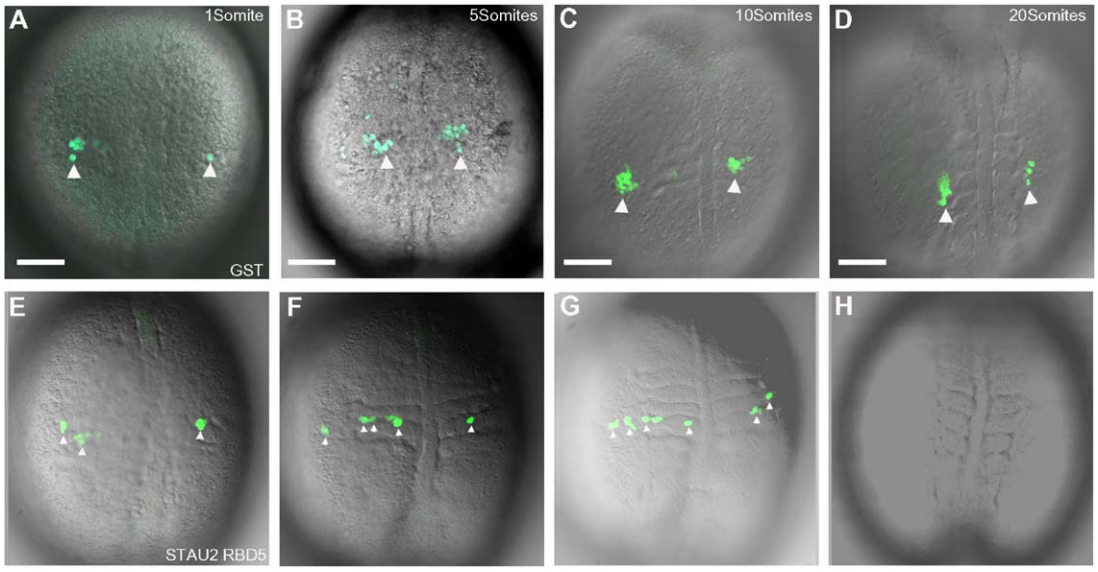


Fig. 7. PGC migration is aberrant when Stau2 function is disrupted. Time-lapse microscopy on GST-injected embryos (A–D) shows clusters of GFP:nos expressing cells (large white arrowheads) that migrate to the gonadal ridge. In contrast, in embryos injected with Stau2 RBD5 (E–H), GFP expressing cells fail to form clusters, remain dispersed (small white arrowheads in panels E, F, G), do not migrate to the gonadal ridge, and eventually disappear (H). Dorsal views of 1 somite stage (A, E), 5 somite stage (B, F), 10 somite stage (C, G), and 20 somite stage (D, H) embryos. Scale bars, 100 μm.

not affected in Stau2 RBD5-injected embryos (93%, n = 60) in comparison to control GST protein-injected embryos (96%, n = 74, data not shown). Expression of *gsc* in the prechordal plate mesendoderm and anterior neurectoderm in midgastrula embryos is also not altered (Figs. 4A, B). The prechordal plate marker, *hgg1*, also does not show any difference in Stau2 RBD5-injected embryos (95%, n = 71; Figs. 4E, F). Expression of *no tail* (*ntl*) in the notochord and *pax2* in the midhindbrain boundary indicates that these cell types are also not affected (93%, n = 55 for *ntl* and 93%, n = 77 for *pax2*) (Figs. 4C, D, G, H). Similar phenotypes were observed in embryos injected with three independent *stau2* antisense morpholinos (93% of stau2 MO2-injected embryos, n > 60 for each marker; Figs. 4I–P), *stau1* morpholino (94% of stau1 MO1-injected embryos, n > 60 for each marker), and the Stau1 RBD4 peptide (93%, n > 55 for each marker). Therefore, interfering with Stau1 or Stau2 function in embryos does not affect germ layer patterning.

Stau function is required for primordial germ cells

Although germ layer patterning is not disrupted by injection of Stau GST fusion proteins or morpholinos targeting Stau1 and Stau2, expression of the primordial germ cell (PGC) specific gene, *vasa*, is affected. The initial expression of *vasa* RNA in cleavage stage and gastrula stage embryos in Stau peptide-injected embryos is similar to that of control GST (92%, n = 42 for Stau2 RBD5; 92% n = 43 for GST; Figs. 5A–D). However, by 24 hpf, *vasa* expression in the gonadal anlagen is not detected in 94% of embryos injected with Stau2 RBD5 (n = 37; Figs. 5E, F). 46% of Stau1 RBD4-injected embryos (n = 96) lack *vasa* expression at 24 hpf.

Expression of a GFP-nanos1 3′UTR fusion protein in PGCs is detected in embryos at gastrula and early segmentation stages of Stau2 RBD5 (81%, n = 77; Figs. 5G–J), but is not detectable in 92% of Stau2 RBD5 (n = 87; Figs. 5K–M) at 24 hpf. 45% (n = 153) of embryos injected with the Stau1 RBD4 peptide lack GFP:nos3′UTR expression in PGCs (Fig. 5M). In contrast, 88% of embryos injected with control GST protein (n = 184; Figs. 5K, M) have GFP expression in PGCs.

Similarly, the initial expression of *vasa* in stau splice junction morpholino-injected embryos is the same as that in control morpholino-injected embryos (n = 80 for stau1 MO1; n = 44 for stau2 MO2; Figs. 6A–D). However, by 24 hpf, *vasa* expression is not detected in stau morphant embryos (47%, n = 80 for stau1 MO1; 93%, n = 39 for stau2 MO2; Figs. 6E–H). Expression of GFP:nos3′UTR in stau morpholino-injected embryos is also present at early stages (Figs. 6I–L), but is abolished by 24 hpf (95% of stau2 MO2-injected embryos, n = 101; 45% of embryos injected with stau1 morpholinos, n = 153; Figs. 6M–P, T). In addition, 90% of embryos co-injected with low doses of stau1 and stau2 morpholinos (n = 63) show loss of GFP:nanos 3′UTR expression in PGCs (Fig. 6T). In contrast, >85% of embryos injected with control morpholinos (n = 115; Fig. 6T)

Fig. 6. Injection of stau1 and stau2 morpholinos causes loss of germ cells. (A–H) Expression of the germ cell specific gene, *vasa*, in 4 cell (A, B), shield stage (C, D), and 24 hpf (E–H) embryos injected with control morpholinos (A, C, E, G), Stau2MO1 (H), or Stau2 MO2 (B, D, F). GFP:nos1 expression in shield stage (I, J), 3-somite stage (K, L) and 24 hpf (M–P) embryos injected with control morpholinos (I–O), Stau2 MO1 (P), or Stau2 MO2 (J–N). Schematic diagrams in panels Q, R, and S show the position of morpholinos targeting exon junctions in stau1 (Q) or stau2 (R, S), with primer positions indicated. RT-PCRs show amplification of *cyclinB* control cDNA, *stau1* (Q) or *stau2* (R, S) products in control or morpholino-injected embryos. Histogram in panel T shows % embryos with GFP expression in PGCs. Scale bars, 50 μm in panels A, C, I, K and 100 μm in panels G, M, and insets.

have GFP expression in PGCs. Similar phenotypes were detected by examining the expression of the germ cell-specific Histone H1 M transcript ($n = 44$; data not shown). The efficacy of the morpholinos was tested by RT-PCR using primers to detect the spliced products (Figs. 6Q–S). Whereas control morpholino-injected embryos show the correctly spliced

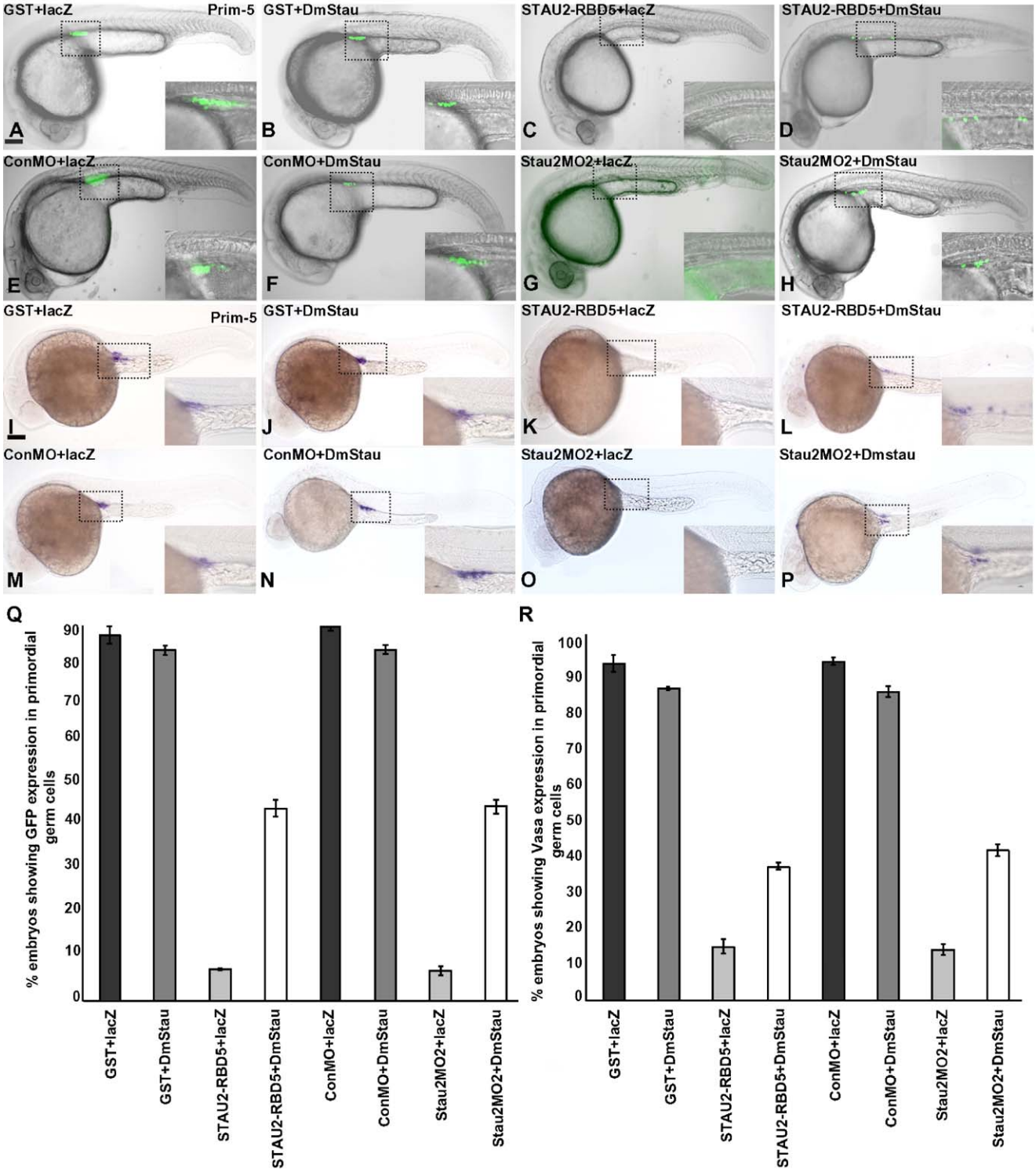


Fig. 8. Loss of PGCs in Stau disrupted embryos is rescued by co-injection of *Drosophila staufen*. GFPnos1 expression (A–H, Q) or *vasa* expression (I–P, R) in embryos (24 hpf) injected with GST protein + lacZ RNA (A, I), GST protein + *Drosophila melanogaster staufen* RNA (B, J), Stau2 RBD5 peptide + lacZ RNA (C, K), Stau2 RBD5 peptide + *Drosophila melanogaster staufen* RNA (D, L), control morpholinos + lacZ RNA (E, M), control morpholino + *Drosophila melanogaster staufen* RNA (F, N), Staufen2 morpholino2 + lacZ RNA (G, O), and staufen2 morpholino2 + *Drosophila melanogaster staufen* RNA (H, P). Dotted boxes in panels A–P outline the areas shown at higher magnification in the insets. Lateral views, scale bar, 100 μm. (Q) % embryos with GFPnos expression in PGCs, (R) % embryos with *vasa* RNA expression at 24 hpf.

products, the *stau1* (Fig. 6Q) and *stau2* (Fig. 6R) morpholino-injected embryos show aberrantly spliced products. These morpholinos are predicted to block splicing and the unspliced introns contain stop codons (13 codons after the exon junction for Stau1 MO, 12 codons after the exon junction for Stau2MO1, and 26 codons after the exon junction for Stau2 MO2). In stau2 MO2-injected embryos, although the majority of the RNA remains unspliced, we can also detect a small proportion of correctly spliced product (Fig. 6S). Primers to detect cyclinB control amplify the correct fragment in control and *stau* morpholino-injected embryos. Thus, the morpholinos appear to be specific, and Stau1 and Stau2 are required in primordial germ cells.

Since *vasa* as well as GFP:nos1 expression is present during gastrulation and early segmentation, but is not detected at 24 hpf in Stau1 and Stau2-depleted embryos, we examined the dynamics of the GFP-expressing cells by time-lapse video-microscopy through the first day of development. In control embryos, PGCs migrate and form clusters on both sides of the midline (Figs. 7A–D and Weidinger et al., 2002). In contrast, PGCs in Stau2 RBD5 (or stau2 MO2; data not shown)-injected embryos do not migrate properly, fail to form clusters, and eventually disappear (Figs. 7E–H). Therefore, Stau2 is required for the migration and survival of PGCs.

Since *Drosophila stau* RNA is not complementary to the morpholinos towards zebrafish stau1 or stau2, we tested if the loss of PGCs in Stau-depleted embryos is rescued by co-injection of mRNA encoding *Drosophila melanogaster* Staufen. Examination of GFP-nos1 protein expression (Figs. 8A–H, Q) as well as that of *vasa* RNA (Figs. 8I–P, R) shows that PGCs are present in embryos injected with Stau2 RBD/stau2 MO2 and fly *staufen* mRNA (42%, n = 101 for Stau RBD5 + fly *stau* RNA; 45%, n = 95 for stau2 MO2 + fly *stau* RNA), but not in embryos injected with Stau2 RBD5/stau2 MO2 and lacZ mRNA (7%, n = 96 for *stau* RBD5 + lacZ RNA and 8%, n = 88 for stau2 MO2 + lacZ RNA). In addition, co-injection of *Drosophila stau* results in proper migration of the PGCs to the gonadal ridge (Figs. 8D, H). Thus, *Drosophila* Staufen is sufficient for PGC survival as well as PGC migration in the absence of zebrafish Stau1 or Stau2 function.

We also tested if *Drosophila stau* coding sequence fused to the zebrafish *nanos1* 3′UTR could rescue the loss of germ cells seen upon blocking or interfering with Stau function. Co-injection of Stau2 RBD5 peptide with Dmstau:nos3′UTR did not cause rescue GFP expression in the PGCs at 20 pg (n = 88), 100 pg (n = 99) or 150 pg (n = 32) doses of stau:nos RNA.

Stau function in the central nervous system

Although initial patterning of the mesendoderm as well as neurectoderm is unaffected in Stau2 disrupted embryos, we observed that by 24 hpf, a significant number of cells in dorsal regions throughout the length of the embryo undergo cell death (Figs. 9A, B, F, G, K). Co-localization of TUNEL labeling (Figs. 9D, I) with an antibody towards acetylated

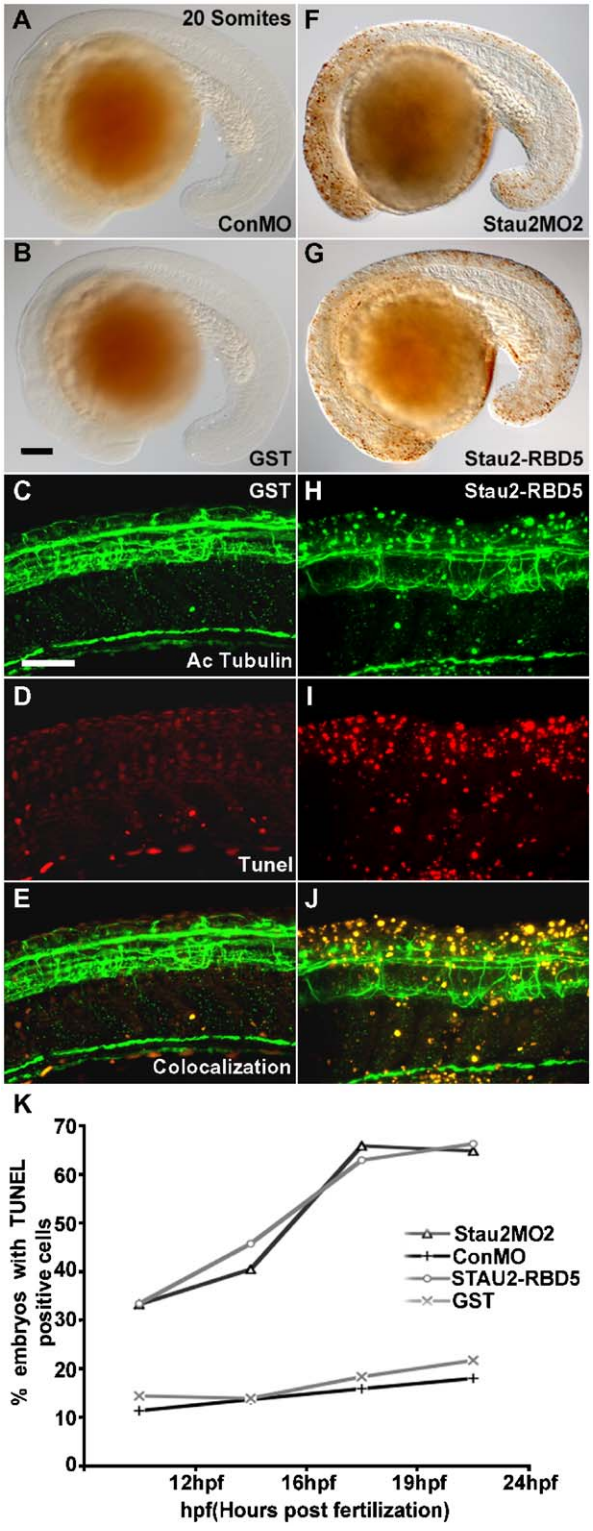
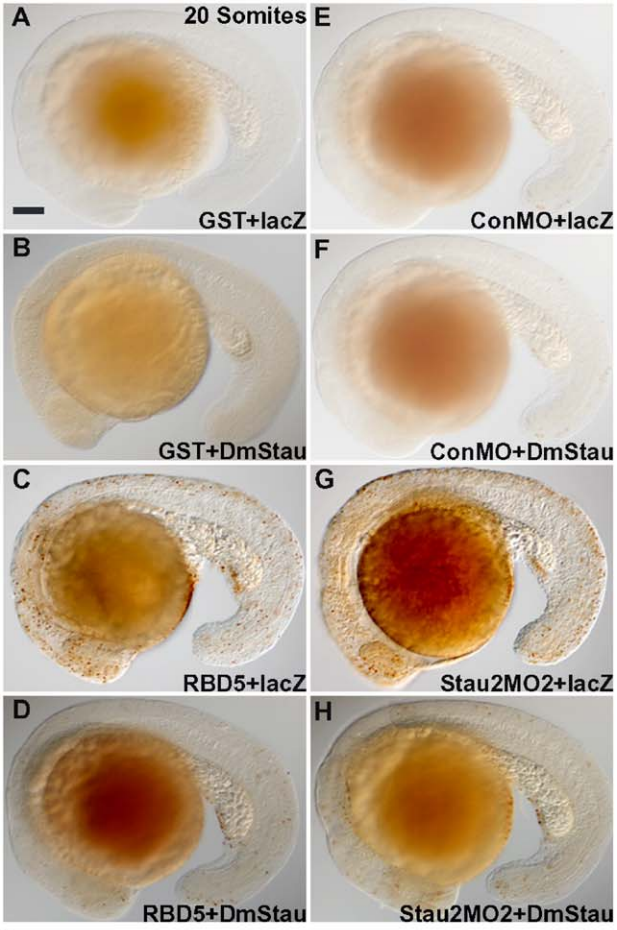
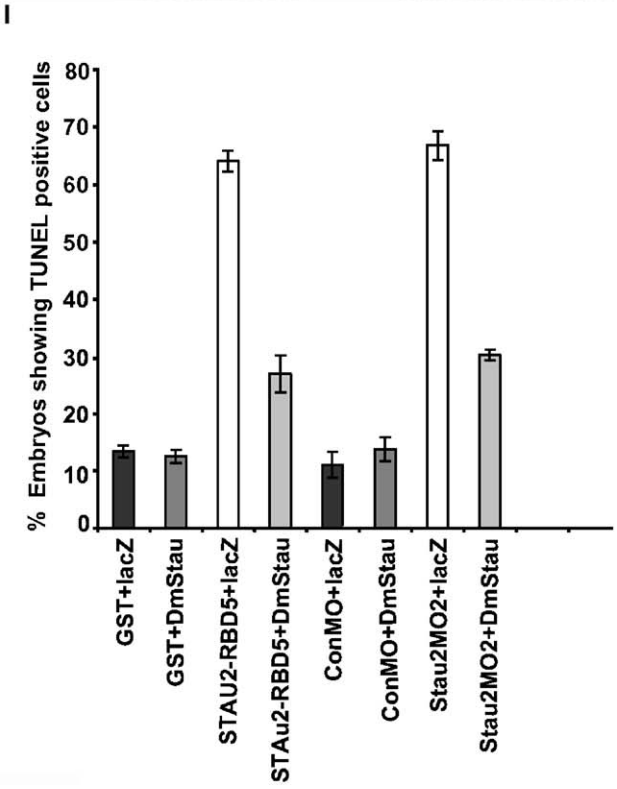


Fig. 9. Cell death in the CNS of Stau2-depleted embryos. (A–D) TUNEL labeling showing dead cells in 20 somite stage embryos that were injected with control morpholino (A), GST (B, D), Stau2 MO2 (F), or Stau2 RBD5 (G, I). Immunostaining with an antibody towards acetylated tubulin to detect neurons (C, H) in embryos injected with GST (C–E) or Stau2 RBD5 peptide (H–J), and co-localization of acetylated tubulin with TUNEL positive cells (E, J). Lateral views, scale bar in panel B for panels A, B, F, G, 100 μm; panels C for panels C–E and H–J, 200 μm. (K) % embryos with TUNEL labeling at 12 hpf, 16 hpf, 19 hpf, and 24 hpf upon injection of control morpholino, stau2 morpholinos, GST peptide, or Stau2 RBD5 peptide.





tubulin to detect neurons (Figs. 9C, H) indicates that the majority of the dead cells are neurons in the dorsal neural tube (Figs. 9C–E, H–J).

By the end of gastrulation, the number of dead cells determined by TUNEL labeling is ~2 times higher in Stau2 RBD5 or Stau2 MO2-injected embryos in comparison to GST or control morpholino-injected embryos (Fig. 9K). This number increases to 4–5 times by 24 hpf (Fig. 9K).

Co-injection of mRNA encoding *Drosophila melanogaster* Staufen rescues cell death in the CNS of embryos depleted of Stau2 function (Figs. 10D, H), whereas co-injection of *lacZ* RNA with either the stau2 morpholinos or the Stau2 RBD5 peptide does not rescue cell death in the CNS (Figs. 10C, G). Therefore, in addition to its role in migration and survival of PGCs, Stau2 is also required for survival of neurons in the embryonic CNS.

Discussion

Germ cell specification in *Drosophila* requires the function of Staufen protein. Our identification of a function for the zebrafish Stau1 and Stau2 proteins in survival and maintenance of germ cells indicates an evolutionarily conserved function for Stau proteins in the germ line. Interestingly, although germ cells are affected by disrupting Stau function in both *Drosophila* and zebrafish, the stages at which they function are different. Furthermore, whereas *Drosophila* Stau affects RNA localization causing the defects in germ line formation, in zebrafish, the function of the Stau proteins seems to be performed in preformed germ cells. This may reflect, in part, the differences in the establishment of the germ line in these organisms. An alternate possibility is that Stau may have other additional maternal functions in establishment of the germ line, which our experiments do not uncover.

Drosophila Stau binds and localizes *bicoid* RNA to the anterior and *oskar* RNA to the posterior pole of oocytes, respectively, and plays a critical role in establishment of the anterior–posterior axis (St Johnston et al., 1991). In *Drosophila*, Stau RBD4 binds target RNAs in vitro, whereas RBD5 cannot bind in vitro. We find that zebrafish Stau1 RBD4 can bind to *oskar* RNA and zebrafish *nanos1* RNA in vitro. The transcripts bound by the endogenous zebrafish Stau proteins are not known. Identification of the targets to which the Stau proteins bind will help understand the mechanisms by which these proteins function in cell polarity and specification in vertebrates.

We did not detect binding of Stau2 RBD5 to *oskar* RNA at the same doses as used for Stau1 RBD4. This is consistent with previous observations that the *Drosophila* Stau RBD5 does not bind RNA, but is required for translational de-repression of *oskar* in and the actin-dependent localization of *prospero*

Fig. 10. *Drosophila melanogaster staufen* rescues CNS cell death in Stau2-disrupted embryos. TUNEL labeling in embryos co-injected with GST (A, B), Stau2 RBD5 (C, D), control morpholino (E, F), or stau2 morpholino2 (G, H) and either lacZ RNA (A, C, E, G) or *Drosophila melanogaster staufen* RNA (B, D, F, H). Lateral views of 20 somite stage embryos. (I) % embryos with TUNEL-labeled cells.

mRNA (Matsuzaki et al., 1998; Micklem et al., 2000). Stau RBD5 is presumed to interact with other proteins to regulate oskar translation (Micklem et al., 2000). In neuroblasts, Stau RBD5 binds to Miranda to localize *prospero* RNA (Matsuzaki et al., 1998; Schuldt et al., 1998; Shen et al., 1998). Thus, overexpression of RBD5 has the potential to interfere with binding of endogenous Stau2 to other proteins, and cause dominant-negative effects. The proteins that bind Stau2 are not known. Identification of these proteins will help understand the mechanisms by which Stau2 functions in germ cells.

Depletion of Stau1 or Stau2 proteins in embryos does not disrupt germ layer patterning or axis specification. This is in contrast to *Drosophila*, where Stau function is essential for anterior–posterior axis specification (St Johnston et al., 1991). It is likely that our experiments where we inject Stau RBD peptides and morpholinos into embryos only disrupt the zygotic functions of these proteins. Disruption of the function of the Stau proteins in the ovary may uncover any maternal function of these proteins in regulating axis specification or germ layer patterning in zebrafish.

Depletion of zebrafish Stau1 or Stau2 proteins or interference with Stau RBD peptides results in aberrant migration of germ cells. The phenotype of Stau interference and depletion is similar to that of depletion of zebrafish *nanos1* (Koprunner et al., 2001). Given that we find binding of purified Stau1 RBD4 peptide to *nanos1* RNA in a mixture of whole ovary RNA, it is possible that the Stau proteins exert their function in germ cells by binding to nanos1 RNA. The germ cell migration and survival phenotype are also observed when the germ plasm component, Dead end, is depleted (Weidinger et al., 2003). The RNA of *dead end* is a component of the germ plasm, and is expressed in PGCs during embryogenesis. Dead end protein is present in peri-nuclear granules within PGCs. It is possible that Stau proteins function in the same complex that Dead end functions or may cooperate with Dead end in PGC migration and survival.

In mammals, Stau2 is involved in RNA transport in neurons (Kohrmann et al., 1999; Roegiers and Jan, 2000). Stau2 protein localizes to the somatodendritic compartment of neurons and interacts with ribosomes (Duchaine et al., 2002). The *Drosophila* Staufen and Inscuteable proteins co-localize with *prospero* RNA on the apical cortex of interphase neuroblasts. *Drosophila* Stau is required for some aspects of neuroblast asymmetry, and loss of Stau function results in aberrant ganglion mother cell development (Li et al., 1997; Broadus et al., 1998). Our finding that depletion of Stau2 or interference of function with dominant-negative peptides causes neuronal cell death suggests that Stau2 is essential for neuronal cell viability in zebrafish. Thus, in addition to the PGCs, the zebrafish Stau proteins are also required in other cell types in the embryo.

In addition to intracellular transport and localization of RNA, recent reports suggest that Stau1 may function in nonsense-mediated mRNA decay in mammalian cells (Kim et al., 2005; Meyer and Gavis, 2005). It is not known if the zebrafish Stau proteins perform such a function. Understanding the mechanisms by which the zebrafish Stau proteins function can help understand the mechanisms by which these proteins regulate cell fate specification and survival.

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