

# The *Drosophila* CPEB Protein Orb2 Has a Novel Expression Pattern and Is Important for Asymmetric Cell Division and Nervous System Function

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**ABSTRACT** Cytoplasmic polyadenylation element binding (CPEB) proteins bind mRNAs to regulate their localization and translation. While the first CPEBs discovered were germline specific, subsequent studies indicate that CPEBs also function in many somatic tissues including the nervous system. *Drosophila* has two CPEB family members. One of these, *orb*, plays a key role in the establishment of polarity axes in the developing egg and early embryo, but has no known somatic functions or expression outside of the germline. Here we characterize the other *Drosophila* CPEB, *orb2*. Unlike *orb*, *orb2* mRNA and protein are found throughout development in many different somatic tissues. While *orb2* mRNA and protein of maternal origin are distributed uniformly in early embryos, this pattern changes as development proceeds and by midembryogenesis the highest levels are found in the CNS and PNS. In the embryonic CNS, Orb2 appears to be concentrated in cell bodies and mostly absent from the longitudinal and commissural axon tracts. In contrast, in the adult brain, the protein is seen in axonal and dendritic terminals. Lethal effects are observed for both RNAi knockdowns and *orb2* mutant alleles while surviving adults display locomotion and behavioral defects. We also show that *orb2* functions in asymmetric division of stem cells and precursor cells during the development of the embryonic nervous system and mesoderm.

**C**YTOPLASMIC polyadenylation element binding (CPEB) family proteins bind to target sequences in the 3'-UTR of mRNAs and control their localization and translation. CPEB proteins function in many different biological contexts including oogenesis in *Xenopus* and *Drosophila* (Lantz *et al.* 1992, 1994; Christerson and McKearin 1994; Hake and Richter 1994), synaptic plasticity in the rat hippocampus (Wu *et al.* 1998), and long-term memory in *Aplysia* (Si *et al.* 2003, 2010). In *Xenopus* oocytes, CPEB regulates oocyte maturation. Prior to progesterone stimulation, it functions to repress target mRNAs in conjunction with proteins such as Maskin. After hormone stimulation, CPEB is phosphorylated, and the phosphorylated isoform recruits factors that stimulate poly(A) addition and translational activation of mRNAs that mediate the maturation process. In *Drosophila*,

the CPEB protein Orb is required for oocyte determination and the establishment of the anterior–posterior and dorsal–ventral axes of the developing egg chamber. It functions by promoting the on-site translation of mRNAs that are localized in the developing oocyte, and like the *Xenopus* oocyte CPEB, its activity is regulated by phosphorylation.

Most animals have two or more CPEB genes. Completed genome sequences reveal that humans, mice, and *Caenorhabditis elegans* have four CPEB genes, but only two CPEBs in *Drosophila*. The homology between the CPEB proteins is limited to the C-terminal half of the protein, which has two RNA-recognition motif (RRM) domains and a zinc finger domain, while the N-terminal half is highly divergent. Phylogenetic trees indicate that the CPEB genes fall into two different subgroups (Luitjens *et al.* 2000; Mendez and Richter 2001; Huang *et al.* 2006). One subgroup includes *Drosophila orb*, mouse CPEB1, and the canonical *Xenopus* CPEB, while the other subgroup contains the second *Drosophila* CPEB gene, *orb2*, as well as mammalian CPEB2–4.

In the subgroup that contains *orb* and the *Xenopus* CPEB, most of the proteins are expressed in the germline and have important functions in this tissue, but are not essential for viability. This is the case with strong loss-of-function alleles

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of *Drosophila orb* (Christerson and McKearin 1994; Lantz *et al.* 1994) and the mouse knockout of *cpeb1* (Tay and Richter 2001). In *Drosophila*, *orb* expression is only observed in the germline of larvae and adults and in pole cells of the early embryo. Strong loss-of-function alleles are adult viable, but female sterile. The mouse knockout for *cpeb1* has no apparent defect on viability, but is both male and female sterile (Tay and Richter 2001). When *cpeb1* mice are examined for nervous system defects, only minor deficiencies in learning and memory are observed (Alarcon *et al.* 2004; Berger-Sweeney *et al.* 2006). CPEB proteins from this subgroup are also found in other organisms such as clams and zebrafish, but only their function in female oogenesis has been studied to date (Bally-Cuif *et al.* 1998; Minshall *et al.* 1999; Walker *et al.* 1999).

The second subgroup of CPEBs is generally expressed more broadly and is often found in the nervous system as well as in the germline. For instance, CPEB2 in mice is abundantly expressed in male germ cells and the brain (Kurihara *et al.* 2003). CPEB3 and CPEB4 in mice are expressed in the brain and a number of other tissues (Theis *et al.* 2003). The two human genes that fall into this subgroup, CPEB3 and -4, were identified in a cDNA library from brain tissue (Kikuno *et al.* 2004). A human-specific polymorphism in CPEB3 has recently been associated with decreased episodic memory performance (Vogler *et al.* 2009). While the *Drosophila* Orb2 protein is a member of the second subgroup, little is known about its expression or functions. In the studies reported here we have examined the expression pattern of *orb2* mRNA and protein during development and used RNAi and mutations to learn about its functions.

## Materials and Methods

### Generating the UAS-Orb2 double-stranded RNAi fly stocks

cDNA sequence (clone ATO4101 equivalent to the *orb2B* RNA species from *Drosophila* Genomic Research Center) was digested with *Bam*HI and *Cla*I restriction enzymes and ligated into a UASi-GFP $\phi$  plasmid (a gift from Amin Ghabrial). This construct expresses a double-stranded hairpin RNA that inhibits expression of the targeted sequence by RNA-mediated interference. This sequence encodes amino acids 134–459 of the 75-kDa isoform of Orb2. The first 28 amino acids (134–161) are unique to the 75-kDa isoform, while the rest of the sequence is common to both forms of Orb2 protein. A BLAST search to the DNA sequence used in this construct reveals no significant similarity to any sequence except *orb2*. The plasmid was transformed into *Drosophila* embryos according to published methods. Two independent insertion lines were isolated and maintained: 137A (on X) and 39A (on 3).

### Generating Orb2 monoclonal antibodies

DNA sequence corresponding to 1729–2499 bp (443–699 aa of the 75-kDa isoform of Orb2) of the clone ATO4101 was amplified by PCR and cloned into the pGEX-4T3 vector

(Amersham) to generate a GST-Orb2 RRM fusion protein. The fusion protein was purified and injected into mice (Princeton Monoclonal Facility). Clones were screened for immunoreactivity to the purified RRM protein fragment by ELISA and verified by Western blotting to 0- to 24-hr embryo extract and the purified RRM protein fragment. Three positive hybridoma cell lines, 2D11, 4G8, and 7C3, were identified and saved.

### Western blotting

We used the standard Western blotting protocols for extraction of proteins and blotting procedures. Primary antibodies were used as follows: mouse anti-Orb2 2D11 (1:25), mouse anti-Orb2 4G8 (1:25), mouse anti-Snf 4G3 (1:2000), mouse anti-Orb 4H8 (1:60), mouse anti-Orb 6H4 (1:60), and mouse antiactin JLA20 (Developmental Studies Hybridoma Bank, 1:100).

### In situ hybridization

Ovaries were dissected in cold PBS and then fixed in 4% paraformaldehyde for 20 min at room temperature. Samples were washed in PBST and then incubated in 50 mg/ml proteinase K for 4 min. The proteinase treatment was stopped with 2 mg/ml glycine, and the samples were refixed in 4% paraformaldehyde. Samples were washed and then hybridized overnight to an antisense probe or *orb2*. Samples were washed and then blocked in 1% BSA in PBST for 30 min and then incubated for 90 min with 1:5000 AP anti-DIG (Roche). Samples were washed and detection was carried out with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolylphosphate (Roche) until color was readily visible. For embryos, a 0- to 24-hr embryo collection was treated for 2 min in bleach and then fixed for 20 min in 4% paraformaldehyde/heptane and dechorionated with methanol. Embryos were rehydrated into PBST and then treated with 10 mg/ml proteinase K for 2 min. All other steps are similar to the ovary protocol.

### Whole mount staining

Ovary, embryo, and larvae staining was done essentially as described by Costa *et al.* (2005). Primary antibodies used were: mouse anti-Orb2 2D11 IgG<sub>1</sub> and mouse anti-Orb2 4G8 IgG<sub>1</sub> (undiluted), mouse anti-Orb 6H4 IgG<sub>2a</sub> (1:30), mouse anti-BP102 IgG<sub>2A</sub> (1:20), rabbit anti-Eve (1:2000; a gift from Manfred Frasch, University of Erlangen-Nuremberg), DNA was stained with Hoescht (1:2000). Secondary antibodies used were goat antimouse IgG<sub>1</sub> Alexa 546, goat antimouse IgG<sub>2a</sub> Alexa 488, goat antirabbit Alexa 488 (Molecular Probes). For adult brains the procedure described in Gazi *et al.* (2009) was followed. Briefly, adult brains were dissected in cold PBS and then treated for 15–17 min with 1 mg/ml collagenase type VII (Sigma). Samples were fixed in 4% paraformaldehyde for 1 hr at room temperature, washed, and then blocked in 1% normal goat serum for 3 hr at 4°. Primary antibody was added in 10% goat serum and incubated for 2 days at 4°. Mouse anti-Orb2 was used

undiluted. Samples were washed 6 × 20 min in PBST and then blocked again in 10% goat serum for 1 hr at 4°. Fluorescent antibodies were added at 1:500 dilution and incubated for 2 days at 4°. Samples were washed in PBST as above and mounted in Vectashield mounting medium.

### Analysis of *orb2* and CG43113 transcripts

RNAs were extracted with RNAspin kit (cat. no. 25-0500-70). cDNA synthesis was performed with oligo-dT primers. RT products were PCR amplified and analyzed on a 2% agarose gel. For PCR primers we used the following: *orb2* common exon among RA, RB, RC, and RD: CAACAGTGC CACCAGCAGTGC and GCGCAGACTAACTTCGTCTGTT; RA primers: TTGTGTGTGATTGTGAGTGTCCGT and GCGCATC TCCGCCACCAGTT; RB: AATAAGCTTCGCCGTGTAGCAGGC and CGCCCGCACCAACTTTCTACA; RC: ATCACAGTCGT TGCTGACGAG and CGCCCGCACCAACTTTCTACA; RD: AGTAATAAGGGCATAAGG and CGCCCGCACCAACTTTCTACA; *Orb2*-CG43113 hybrid: TGCAAGCCGGTGGTTTCTT CAATC and GATGGTTTGTGTGACCTTGCCCTT; and CG43113 only: ATGAGCAAAGCTCCGTTGAAAGCC and TATCCGGATTA ACCGTGTTCCGCA.

### Locomotion assays

**Climbing assay:** Flies were knocked down to the bottom of a vial and the number of flies that climb 6 cm in 10 s were recorded.

**Bang-sensitivity assay:** Flies were vortexed for 10 sec and those that return to standing position in 10 sec/20 sec were counted.

**Heat-shock assay:** Flies grown at room temperature were heat shocked at 37° and the number of flies that were still standing after a 10-min heat shock was recorded.

## Results

### The *orb2* gene structure and homology to CPEB

The *orb2* gene is located in the cytogenetic interval 66E4 on the left arm of the third chromosome (Hoskins *et al.* 2000). According to FlyBase, the *orb2* locus is predicted to produce two sets of transcripts and proteins (Figure 1A). One set of transcripts, the *orb2* RNAs, RA, RB, RC, and RD encode the Orb2 CPEB protein. The other set of predicted transcripts are the *orb2*-CG43113 hybrid RNAs, RE, RF, and RG, which encode a composite protein of unknown function.

The *orb2* transcripts, RB, RC, and RD, are expected to encode the same 75-kDa CPEB protein and differ only in their 5'-UTRs and/or 3'-UTRs (Figure 1A). The differences in the UTRs are due to the use of two different promoters (*orb2-1* and *orb2-2*), slightly different transcription start sites, alternative splicing, and polyadenylation. The fourth *orb2* transcript, RA, is expressed from a downstream promoter, *orb2-3*, that is close to the major *orb2* exons and encodes a protein of 60 kDa. The 75-kDa and 60-kDa

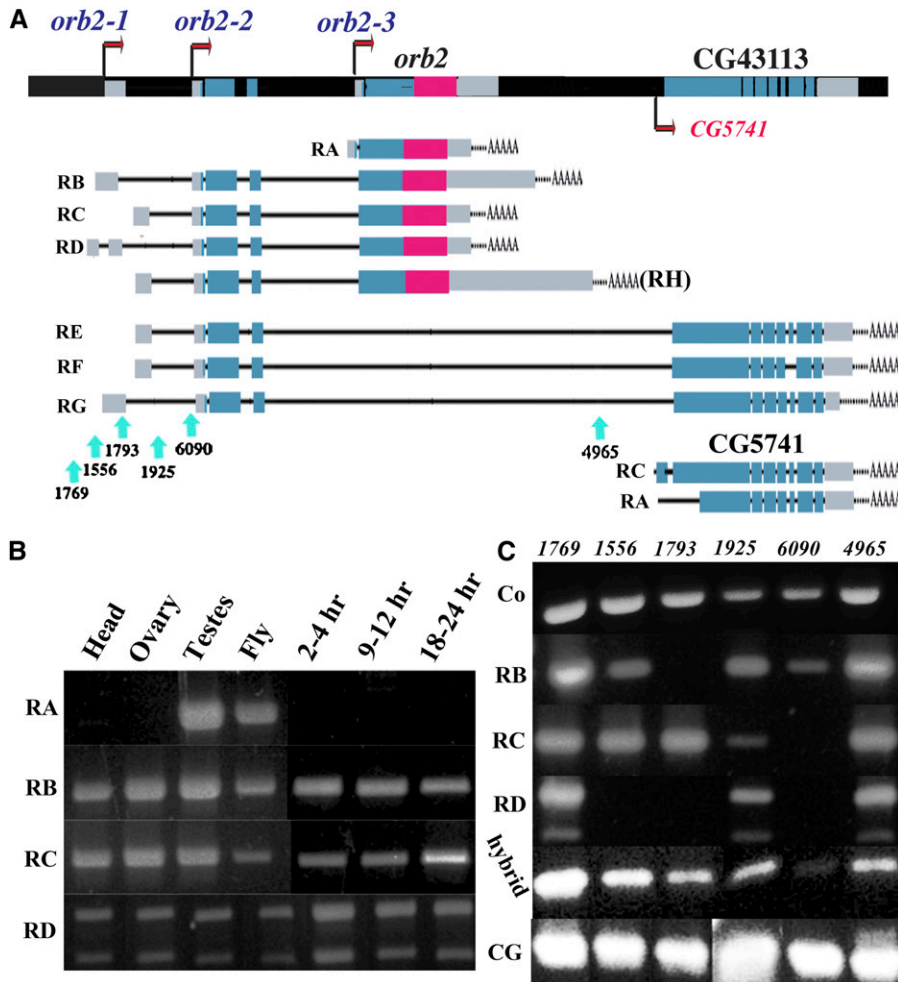
Orb2 CPEB proteins share a common C-terminal region of 542 amino acids, but have different sequences at the N terminus (see Figure 1). The larger isoform has 162 unique amino acids at its N terminus, while the smaller has only nine unique amino acids. While the sequence in the N-terminal half of Orb2 is poorly conserved, it resembles Orb in that it is characterized by many polyglutamine and polyglycine repeats and is rich in histidine and serine. The sequence in the C-terminal half of the Orb2 protein is highly conserved especially for proteins within the same CPEB subgroup, and it contains the two 90-amino-acid RRM domains and a zinc finger domain.

The second set of predicted transcripts is the hybrid *orb2*-CG43113 RNAs, RE, RF, and RG (Figure 1). These RNAs are derived from the upstream *orb2-1* and *orb2-2* promoters, and share 5'-UTR sequences and sequences encoding the first 162 amino acids with the larger *orb2* RNAs. The remainder of the 1221-amino-acid CG43113 protein, which is derived from exons located downstream of the Orb2 coding sequences, bears no resemblance to the Orb2 CPEB proteins. Database searches reveal that this part of the *orb2*-CG43113 gene is found in other flies. However, it is a completely independent transcription unit and does not include N-terminal protein coding sequences derived from the 75-kDa Orb2 isoform. In addition, as is in other *Drosophila* species, *melanogaster* has an independent transcription unit, CG5741, which expresses the CG43113-specific protein coding sequences. The CG5741 promoter is located downstream of the *orb2* 3'-UTR and polyadenylation signals, and it generates five distinct mRNAs (two of which are shown in Figure 1) that differ in their splicing patterns. These mRNAs are predicted to encode proteins ranging from 887 to 1093 amino acids and overlap all of the hybrid *orb2*-CG43113 sequences except for those derived from the *orb2* N terminus.

### *Orb2* is expressed throughout development in both the soma and germline

***orb2* gene activity:** We used RT-PCR with different primer combinations to examine the activity of the *orb2* promoters during development. The predicted *orb2* transcripts (RB, RC, and RD) derived from the two upstream *orb2* promoters, *orb2-1* and *orb2-2*, are detected in 2- to 4-hr, 9- to 12-hr, and 18- to 24-hr embryos, adult flies, heads, ovaries, and testes (Figure 1). In contrast, transcripts from the *orb2-3* promoter, which generates the *orb2*-RA mRNA, are only observed in testes. We also found that transcripts containing the major *orb2* (not shown) and CG5741 (CG43113) (Figure 1) specific protein coding exons are present in all stages and tissues examined. The relationship between *orb2* and the hybrid *orb2*-CG43113/CG5741 genes will be considered further in a subsequent section, while we focus below on the products specific to the *orb2* gene.

**Early embryo:** We used a combination of *in situ* hybridization (with *orb2*-specific probes), whole mount staining, and Western blots to examine *orb2* expression during



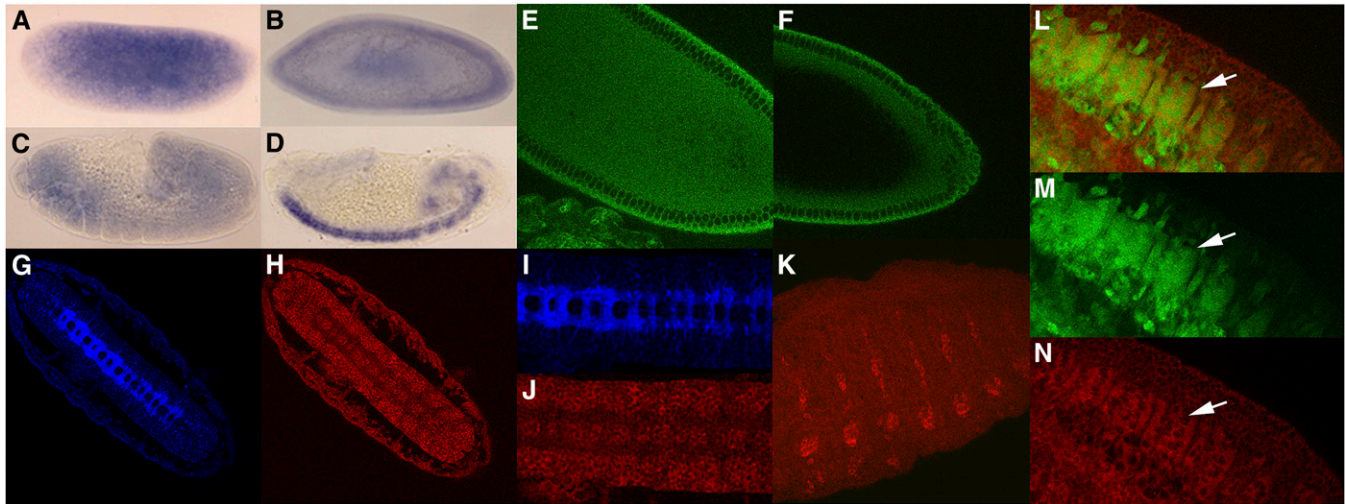
**Figure 1** The *orb2* transcription unit. (A) *orb2* locus is predicted to encode two distinct gene products. One is the CPEB homolog Orb2 with four predicted *orb2* mRNAs. RB, RC, and RD (and also RH, which has a larger 3'-UTR) are transcribed from the *orb2-1* and *orb2-2* promoters and encode the same 701-amino-acid (75 kDa) Orb2 isoform. RA is transcribed from the *orb2-3* promoter, and encodes a smaller, 550-amino-acid (60 kDa) isoform (see text for details). The other predicted *orb2* gene products are hybrid *orb2*-CG43113 mRNAs. The hybrid mRNAs are generated by the *orb2-1* and *orb2-2* promoters and share 5'-UTR sequences and sequences encoding the N-terminal 162 amino acids with the *orb2* transcripts generated by these two promoters (see text). CG5741 has its own promoter and produces at least five different RNA species. These mRNAs encode proteins ranging in size from 887 to 1093 amino acids, depending upon the splicing pattern and translation start codon. Also indicated are the locations of the transposon insertions. (B) RT-PCR of RNAs from heads, ovaries, testes, whole flies, and from 2- to 4-hr, 9- to 12-hr, and 18- to 24-hr embryos. The PCR reactions were done with primers specific to the *orb2* RNAs listed on the left. (C) RT-PCR of RNA from transposon insertions: *orb2*<sup>1769</sup>, 1769; *orb2*<sup>1556</sup>, 1556; *orb2*<sup>1793</sup>, 1793; *orb2*<sup>1925</sup>, 1925; *orb2*<sup>6090</sup>, 6090; and *orb2*<sup>4965</sup>, 4965. The PCR reactions were done with primers specific to the RNAs listed on the left. Co, common to *orb2* RA, RB, RC, RD, and RH mRNAs; hybrid, *orb2*-CG43113 mRNAs; CG, sequences common to the hybrid *orb2*-CG43113 and CG5741 mRNAs.

embryogenesis. We found that *orb2* transcripts are deposited maternally and are distributed uniformly throughout the embryo until the extended germband stage (Figure 2 A-C). In contrast, *orb* mRNA is not detected in Northern blots by 2-4 hr and is seen only in the pole cells by RNA *in situ* (Lantz *et al.* 1992). Whole mount staining with Orb2 monoclonal antibodies shows that the protein is distributed rather uniformly throughout the precellular blastoderm embryo (Figure 2E). However, there appears to be an apical concentration of the protein, just above the nuclei as the blastoderm cellularizes. Consistent with a maternal deposition, a protein of the expected size (75 kDa) for the RB, RC, and RD transcripts is found in extracts from unfertilized eggs. A protein of the same size is found in precellular blastoderm embryos (0-1, 1-2, and 2-3 hr) and early gastrula embryos as well (3-4 hr, Figure 3A). The 60-kDa protein predicted for the RA transcript is not detected at these stages and is only found much later in development (see below). In contrast to Orb2, Orb is not seen in Western blots of unfertilized eggs or in 0- to 4-hr embryos (Figure 3A).

**Mid-to-late embryogenesis:** During germband extension, the *orb2* message appears to turnover and there is a drop in

the *in situ* hybridization signal in most regions of the embryo. Consistent with this decrease, the level of Orb2 protein also drops as can be seen in the Western blots of 0- to 4-hr and 4- to 8-hr embryo extracts (Figure 3B). Expression is upregulated in 8- to 16-hr embryos and there is an increase in the amount of Orb2 protein (Figure 3B). After germband retraction commences, the highest levels of *orb2* mRNA are found in the developing CNS (Figure 2D) and PNS, and there are lower levels elsewhere in the embryo. While it seems likely that mRNA seen at earlier stages is largely of maternal origin, much of the message present in the CNS and PNS in mid-to-late embryogenesis is likely due to *de novo* transcription.

Like the mRNA, the highest levels of Orb2 protein are in germband retracted embryos in the CNS (Figure 2, H and J) and PNS (Figure 2K). Interestingly, the protein in the CNS is concentrated in the cell bodies, while it is largely excluded from the longitudinal and commissural axon tracts. This is evident from the fact that there is little overlap between Orb2 (Figure 2, H and J) and BP102 (Figure 2, G and I), which is a marker for the longitudinal connectives and commissural axon tracts in the CNS. We also found evidence for Orb2 expression in the cells derived from specific



**Figure 2** *orb2* expression in embryos. (A–D) *In situ* hybridization using a probe common to all *orb2* transcripts. Maternally deposited *orb2* message is uniform throughout syncytial (A) and blastoderm (B) stage embryos and (C) germband extended stage. By late in embryogenesis, *orb2* message is primarily concentrated in the brain and central nervous system (D). (E and F) Orb2 protein expression during early embryogenesis. Shallow confocal image through a blastoderm-stage embryo shows cytoplasmic staining around the cortical nuclei (E). Deeper confocal section showing cytoplasmic staining in the posterior half of the embryo, including the pole cells (F). (G–J) Orb2 is largely restricted to the cell bodies of neurons during embryogenesis. Compare the expression pattern of Orb2 (H and J, red) to that of BP102 (G and I, blue), a marker for axons of the central nervous system. (K) Orb2 expression in the PNS of a germband retracted embryo. (L–N) Orb2 (red) is also expressed in the mesoderm. (L and M) Twist-GFP. (N) Orb2. Twist (green) accumulates in the cytoplasm and nucleus, while Orb2 is only observed in the cytoplasm.

neuronal lineages. One such neuronal lineage is the Even-skipped (Eve)-positive neuroblast (NB)4-2 → ganglion mother cell (GMC)4-2a → RP2/sib lineage. In stage 11 embryos (Figure 4, A and B), Orb2 can be seen in Eve-positive GMC4-2a cells (see arrows; GMC4-2a is also known as GMC-1), while in stage 14–15 embryos (Figure 4, C and D) Orb2 is found in the Eve-positive RP2 cells (see arrows). Orb2 is also detected in the Eve-positive aCC/pCC neurons (see arrowhead).

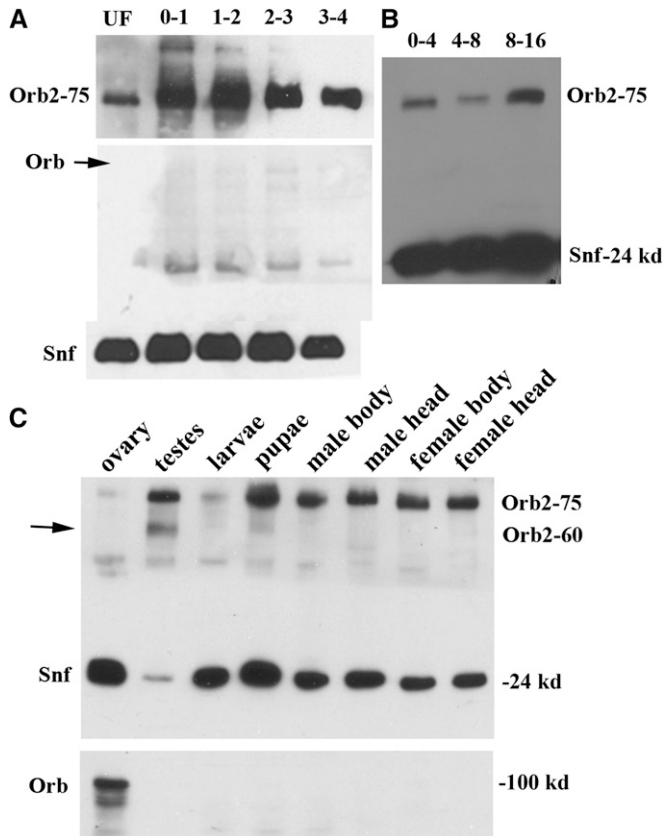
Our antibody staining indicates that Orb2 is not restricted to the nervous system and lower levels of the protein can be detected in the ectoderm and mesoderm. For example, Figure 2, L–N shows that Orb2 is expressed in at least a subset of the Twist-positive mesodermal cells. Note that the GFP marker seems to accumulate in both nucleus and cytoplasm of these mesodermal cells, while Orb2 is restricted to the cytoplasm (Figure 2N).

**Larval stage:** Western blots (Figure 3C) indicate that the amount of *orb2* is relatively low during the larval stage. The lowest levels are seen in the first and second instars, while the level begins to increase during the third instar (data not shown). To examine the distribution of Orb2 protein in the nervous system at this stage, we dissected the brain and ventral nerve cord. Figure 5, A–C shows the condensed/retracted nerve cord and two successive images of a lobe from the brain of a third instar larva. As was observed in the embryo, Orb2 is found primarily in the cell bodies of neurons. While labeling of cell bodies of different neurons is evident in each focal plane, Orb2 appears to be largely excluded from axonal projections. Also, Orb2 is expressed at

high levels in only a subset of the neurons (see Figure 5, B and C). As in embryos it is largely restricted to cell bodies of neurons in the ventral nerve cord as well.

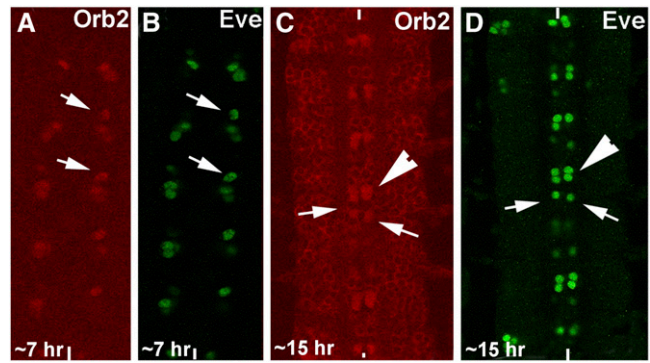
**Adults:** Orb2 levels increase substantially during the pupal stage and remain relatively high in adults (Figure 3C). To determine where Orb2 is expressed in adults, we probed Western blots of extracts from the heads, bodies, and gonads of males and females. For comparison, we also probed the same blots with Orb antibody. As shown in Figure 3C, the overall distribution of Orb2 differs substantially from that of Orb. There is little, if any, Orb in somatic tissues from the beginning of embryogenesis to the adult stage (Lantz *et al.* 1994) and as illustrated in this figure, it is not detected in the head or bodies of adult flies. In contrast, high levels of the 75-kDa Orb2 protein are present in the head and bodies of both sexes. There are also differences in the expression of the two CPEB proteins in the adult germline. Although both are found in male and female gonads, Orb2 is much more abundant in the testes than in the ovaries. Figure 3C shows that the 75-kDa Orb2 isoform is barely detected in the slightly overloaded sample from ovaries. In contrast, both the 75-kDa and 60-kDa Orb2 isoforms are readily observed in the greatly underloaded extract from testes. Note that while the 60-kDa RA Orb2 is present in testes, it is not found in extracts from heads or bodies of adult flies. This is consistent with our RT-PCR data, which indicates that the *orb2-3* promoter is only active in testes.

**Orb2 expression in the adult CNS:** Whole mount staining of dissected brains reveals that Orb2 is expressed throughout



**Figure 3** Western blot analysis of Orb2 during development. (A) Orb2, Orb, and the loading control Snf in unfertilized eggs (UF) and in very early stages of embryogenesis (0- to 1-hr, 1- to 2-hr, 2- to 3-hr, and 3- to 4-hr embryos). The 75-kDa isoform is deposited maternally and like the Snf loading control, it is detected in unfertilized eggs and in fertilized preblastoderm and blastoderm-stage embryos. The 60-kDa Orb2 isoform or Orb are not detected in these embryos. (B) Orb2 protein is present throughout embryogenesis. (C) Orb2 expression in larvae, pupae, and various adult tissues. The 75-kDa isoform of Orb2 is detected in larvae and pupae. In adult flies it is found in the heads, bodies, and gonads of both sexes. Note that very high levels of the 75-kDa isoform are found in male testes (see Snf loading control), while there is less in larvae and in ovaries. The 60-kDa isoform is found in testes, while it is not readily detected in adult heads or bodies. There may be a small amount of the 60-kDa isoform in pupae. (Bottom) Orb is not found outside of the germline. In this experiment, Orb is also not seen in the testes lane because this lane was intentionally underloaded because of the very high levels of Orb2 in the male gonad (compare levels of Snf, a loading control).

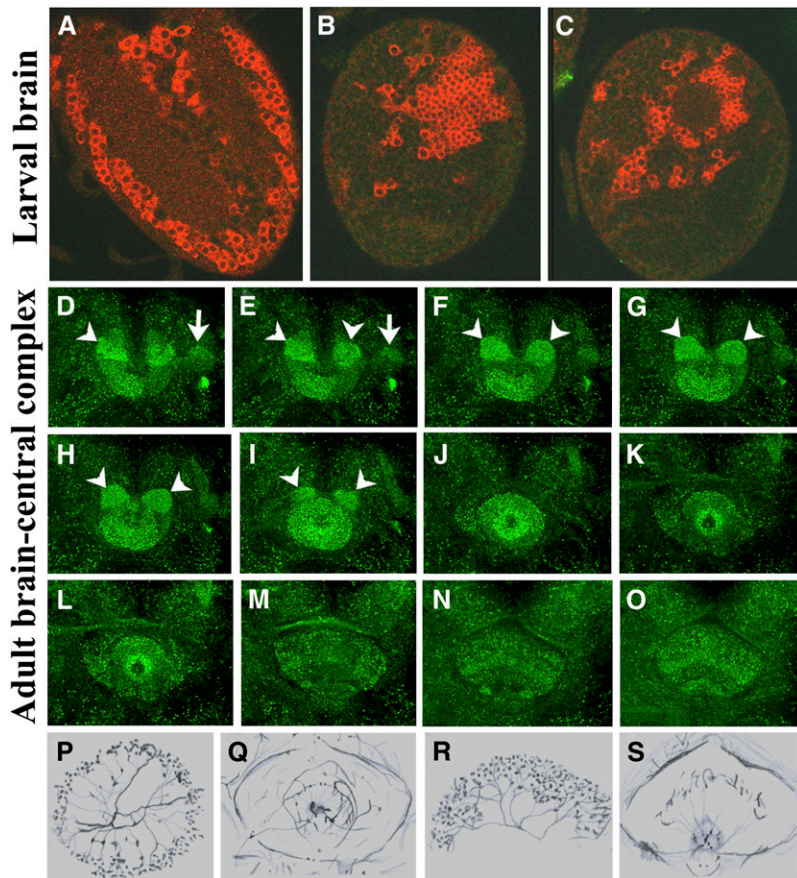
the brain. However, it is also greatly enriched in certain specialized structures. This is shown in serial confocal sections in Figure 5. There is a high level of Orb2 present in the ellipsoid body (Figure 5, D–L), which begins as a discrete posterior structure (Figure 5, D–H), then becomes a ring-like structure (Figure 5, I–L, shown also in Figure 5, P and Q as line drawings). Moreover, two Orb2-positive discrete structures above the ellipsoid body can also be observed (marked by arrowheads in Figure 5, D–I); both of these are not part of the ellipsoid body, but just above it (see Figure 5, D–I). There is also another Orb2-positive structure (indicated by an arrow in Figure 5, D and E),



**Figure 4** Orb2 protein is expressed in several different neuronal lineages. Embryos were double stained with Orb2 (red) and Eve (green). Anterior end is up, midline is marked by vertical lines. Arrowhead indicates aCC/pCC neurons and arrows indicate GMC4-2a1/RP2 cells. In the RP2 lineage, Orb2 is present in GMC4-2a (A) and continues to be present in RP2 neurons (C).

but visible only on the left side of the brain, suggesting that there might be asymmetry in the left–right Orb2 expression pattern in adult brains. It is possible that this novel structure is present only on the left side of the brain, as opposed to Orb2 expression being limited to only one of the two structures. Orb2 is also found in the Fan-shaped body (Figure 5, L–O, R, and S). The staining pattern suggests that Orb2 is localized to the synaptic terminals in a fashion similar to the localization of Synaptotagmin (see Gazi *et al.* 2009). These structures are part of the central complex within the central brain area and are believed to function as higher order processing sites for locomotion control (Strauss 2002). These structures are rich in axonal and dendritic terminals and have efferents from the protocerebral bridge. In this context it is interesting to note that the intracellular distribution of Orb2 in neurons of adult brains differs from that seen at earlier stages. Whereas most of the protein localized in the cell bodies of neurons at earlier stages, in the adult brain, the protein appears to be localized to synaptic terminals.

**Orb2 accumulation in ovaries:** Since the expression of *orb* mRNA and protein in developing egg chambers has been extensively characterized, it was of interest to compare it with that of *orb2*. *orb* mRNA and protein are first detected in the newly formed 16-cell cysts in the germarium where both are concentrated primarily in the presumptive oocyte. Between stages 1 and 7, *orb* mRNA and protein are concentrated in the region near the posterior pole of the oocyte. After the onset of vitellogenesis, *orb* mRNA is concentrated along the anterior margin of the oocyte, while Orb protein is localized around the entire oocyte cortex (Figure 6G). A very different expression pattern is observed for *orb2*. There is little *orb2* mRNA in either the germarium or in early previtellogenic stage chambers (Figure 6, A and B). Midway through oogenesis, the level of *orb2* mRNA begins to increase and it then remains high through at least stage 10; however, *orb2* differs from *orb* in that the message is largely



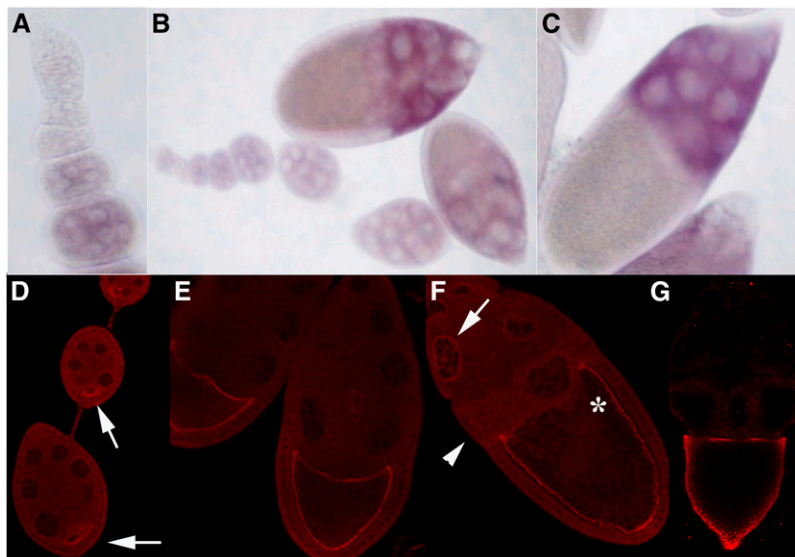
**Figure 5** Orb2 expression in the larval CNS and adult brain. (A–C) Orb2 protein staining in the larval CNS. (A) Expression in the ventral nerve cord. (B and C) Two different optic sections of a single brain hemisphere. Orb2 staining is observed in the cytoplasm of numerous cells in a complex pattern. (D–O) Orb2 expression in the adult brain, thin serial confocal sections, anterior to poster. (D–L) Ellipsoid body and two unidentified identical structures (arrowheads) occurring on both sides of the midline of the brain and a third Orb2-positive structure in only one of the brain hemispheres (arrows). This asymmetric pattern was seen consistently in each of the brains examined (at least six brains were examined). (K–O) Fan-shaped body. In panels P and Q, line drawings of the ellipsoid body are shown. In panels R and S, line drawings of the Fan-shaped body are shown.

restricted to the nurse cells (Figure 6, B and C). The distribution of Orb2 protein also differs from that of Orb. While Orb is restricted to the germline, Orb2 is present in both germ cells and follicle cells (Figure 6D). In the germline nurse cells, it is most heavily concentrated in a ring around the nuclei (see arrows in Figure 6F) and in the cytoplasm of nurse cells close to the oocyte (see arrowhead in Figure 6F). Even though there is little *orb2* mRNA in the oocyte, Orb2 protein is present and its localization pattern resembles that seen for Orb. In previtellogenic chambers, it forms a cap posterior to the oocyte nucleus (Figure 6D). At later stages, most of the Orb2 protein appears to be localized along the cortex of the oocyte just like Orb (compare Figure 6, F and G).

#### **Orb2 RNAi causes lethality**

We used two different approaches to learn more about the biological functions of the *orb2* gene. In the first, we generated a double-stranded RNA specific to a region of the *orb2* mRNAs common to the 60-kDa and 75-kDa Orb2 isoforms, which does not contain the conserved CPEB RRM domains (see *Materials and Methods*). We used the Gal4-UAS system to express the *orb2* dsRNA in a tissue-specific manner (Brand and Perrimon 1993). Flies carrying two independent *UAS-orb2RNAi* inserts were crossed to different Gal4 lines to drive expression in distinct tissue-specific patterns. Since high levels of *orb2* mRNA and protein are present in the

CNS and PNS, we reasoned that expression of *orb2* dsRNA using nervous system Gal4 drivers might have deleterious effects. However, there were no obvious effects on viability using the *elav*-GAL4 driver, which drives expression mostly in postmitotic neurons (Berger *et al.* 2007). While this could mean that *orb2* has no essential function in the fly nervous system, it is also possible that the amount of dsRNA produced by the *elav* driver is not sufficient and/or the timing of its production is not appropriate to compromise a vital *orb2* function in the nervous system. This possibility is supported by the fact that lethal effects are observed for many other nervous system drivers. Altogether we tested 12 other Gal4 drivers that have been reported to be expressed in the CNS and/or PNS of embryos and/or larva and found that all had lethal effects when combined with *UAS-orb2-RNAi* transgenes (Ward *et al.* 2002). As shown in *Supporting Information, Table S1*, the severity of the lethal effects varied depending upon the driver and the *UAS-orb2-RNAi* insert. Further supporting the idea that *orb2* has a vital function during the development of the nervous system, we found that expression of *orb2* RNAi using a *scabrous* Gal4 driver also had lethal effects (not shown). *scabrous* is activated earlier in development than *elav*. It initially comes on in the late blastoderm stage in the neuroectoderm and is further upregulated in neuroblasts after they delaminate from neuroectoderm; however, it is not active in postmitotic neurons (Mlodzik *et al.* 1990).



**Figure 6** Orb2 expression in the ovary. *In situ* hybridization using a probe specific for all *orb2* transcripts. Message is detected throughout most of oogenesis, particularly in the cytoplasm of the nurse cells. (A) Close-up magnification of the germarium and early-stage egg chambers. mRNA is first detected in region 2 of the germarium, and in early-stage egg chambers *orb2* transcripts are detected in the cytoplasm of every germline cell in the cyst. (B and C) Later in oogenesis, *orb2* message is only detected in the nurse cell cytoplasm. (D–F) Orb2 protein staining. Orb2 is detected throughout the egg chamber and is enriched in the oocyte (D, arrows). In late stages, Orb2 is enriched in a cortical ring around the oocyte, but is also detected throughout the cytoplasm of the remaining cells in the cyst (E). In addition to localizing in the oocyte cortex, Orb2 also accumulates in a ring around the nurse cell nuclei (F, arrow). A graded distribution of Orb2 is seen in this image. In the 15 nurse cells, the highest levels of protein are found in the posteriormost nurse cells (arrowhead). Within the oocyte a stream of Orb2 can be seen emanating from ring canals of the posterior nurse cells (\*). (G) Orb accumulation in a late-stage egg chamber is

shown for comparison. In the oocyte, the localization patterns of Orb and Orb2 are similar in that both proteins accumulate along the oocyte cortex. Comparatively lower levels of Orb are found in the nurse cells, while there is no Orb in the follicle cells.

### **Piggybac insertions in the *orb2* locus disrupt expression of *orb2* gene products**

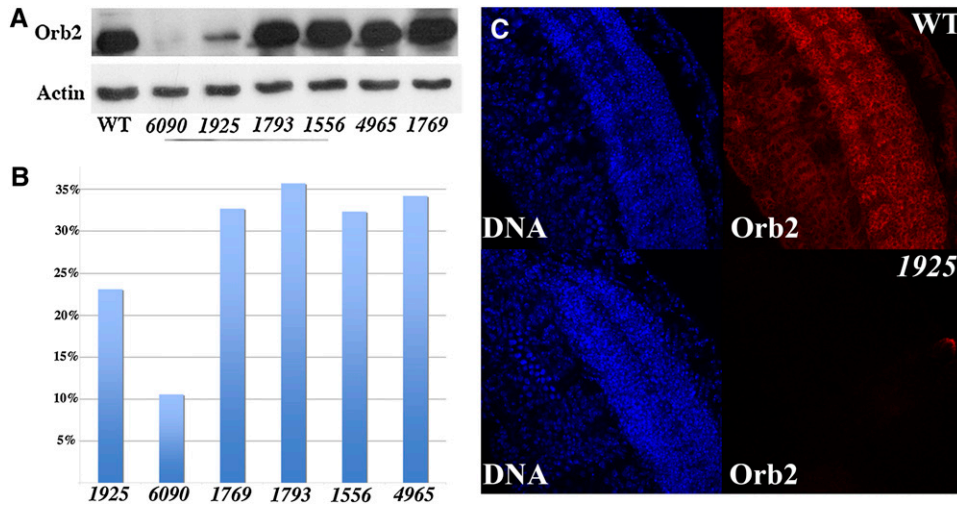
In the second approach, we characterized a collection of five *piggybac* transposons and one *P*-element transposon inserted within or near the *orb2*/CG43113/CG5741 transcription units. The *piggybac* transposon *orb2*<sup>1769</sup> is inserted upstream of the *orb2-1* promoter, while the *piggybac* transposon *orb2*<sup>1556</sup> and the *P*-element *orb2*<sup>1793</sup> are inserted in the first (or second) exon for the *orb2-1* promoter. These three insertions should only affect transcripts expressed from the *orb2-1* promoter. The *piggybac* transposons *orb2*<sup>1925</sup> and *orb2*<sup>6090</sup> are located in the first intron downstream of the *orb2-2* promoter and could potentially affect transcripts from both this promoter and the *orb2-1* promoter. The final *piggybac* insertion, *orb2*<sup>4965</sup>, is located in a large intron downstream of the *orb2* protein coding sequences, but upstream of the protein coding sequences unique to CG43113/CG5741. This transposon would not be expected to have any effect on the expression of the various *orb2* or CG5741 mRNAs, but could disrupt expression of the hybrid *orb2*-CG43113 transcripts originating from the *orb2-1* and *orb2-2* promoters. We used RT-PCR, Western blots, and whole mount antibody staining to examine the effects of these transposons on the expression of *orb2* and CG43113/CG5741 gene products in adults.

With respect to the different *orb2* and hybrid *orb2*-CG43113 transcripts, we used semiquantitative analysis and found that the *piggybac* 1769 insertion, which is upstream of the *orb2-1* promoter, had no apparent effect on any of the *orb2* or the hybrid *orb2*-CG43113 RNAs (Figure 1) and the levels of the different RNAs were approximately equivalent to that in wild type (data not shown). Similarly, the *orb2*<sup>4965</sup> transposon, which is inserted downstream of the *orb2* mRNA coding sequences, did not affect any of the

*orb2* mRNAs. However, expression of the hybrid *orb2*-CG43113 mRNAs originating from the *orb2* promoters upstream of the insertion was substantially reduced in 4965 compared to the control (1769 in the experiment shown in Figure 1). Significantly, when we used RT-PCR primers complementary to sequences located entirely within the CG5741 protein-coding region, we found that the level of the CG5741 RNAs in 4965 is close to that in the 1769 (or wild type) control. Moreover, a similar pattern is seen for some of the other transposon insertions: they have reduced levels of the hybrid *orb2*-CG43113 RNAs generated by the *orb2* promoters, but nevertheless have substantial amounts of the CG5741 specific RNAs. These results suggest that sequences critical for CG5741 promoter activity are located downstream of the 4965 transposon insertion and that this promoter is fully active in the 4965 allele and in all of the other transposon insertions. It would also appear that a substantial fraction of the CG5741/CG43113 transcripts are derived from the CG5741 promoter rather than from the upstream *orb2* promoters.

When we used a primer set specific for the 75-kDa *orb2* protein-coding sequence, only two transposons, 6090 and 1925, showed a significant reduction in the levels of *orb2* mRNA. Both mutants also showed reduced amounts of the *orb2* RB, RC, and RD transcripts when primer sets specific for these RNAs were used (Figure 1). The insertions in 1556 and 1793 are upstream of the RC *orb2-2* promoter, and the levels of RC transcripts in both these alleles were similar to the control. On the other hand, both had reduced levels of the *orb2* RD and RB transcripts (Figure 1). As expected, we found that none of the transposon insertions had any effect on the expression of either the 60-kDa Orb2 RA protein or the *orb2* RA mRNA (data not shown). Consistent with our RT-PCR experiments, only two, 6090 and 1925, had major





**Figure 7** Orb2 protein expression and viability in the transposon insertions. (A) Expression of the 75-kDa Orb2 isoform in wild type (WT) and the transposon mutants. (B) Percentage of adult flies homozygous for the different transposon mutations. In these crosses, 33% should be homozygous for the transposon if it has no lethal effects. The viability of 1925 and 6090 is reduced. (C) Whole mount embryos showing Orb2 expression in the CNS, PNS, and other tissues in wild type (top) and 1925 (bottom) mutants. DNA is in blue, Orb2 in red. Identity of the transposon insertions is the same as in legend to Figure 1.

effects on the expression of the 75-kDa protein. As shown in the Western blot of adult heads in Figure 7A, the levels of the 75-kDa protein are markedly reduced in both mutants. By contrast, there is no obvious change in the expression of this protein in the remaining transposon alleles. We also examined the expression of Orb2 in whole mounts of *orb2*<sup>1925</sup> and *orb2*<sup>6090</sup> embryos. As shown in Figure 7C, the expression of Orb2 in the embryonic CNS, PNS, and in other tissues of homozygous *orb2*<sup>1925</sup> embryos is reduced compared to wild type. Similar results were obtained for *orb2*<sup>6090</sup>.

#### Two of the piggybac insertions cause lethality

Since the RNAi knockdowns of *orb2* had lethal effects, we tested whether any of the piggybac or P-element insertions also had lethal effects. As shown in Figure 7B, 1769, 1556, 1793, and 4965 flies are fully viable. In the case of 1769, this is not surprising since this allele expresses wild-type levels of *orb2* RNA and protein. While there are reductions in the levels of several *orb2* transcripts in 1556 and 1793, the reductions are either not sufficient to cause lethality or are not in tissues in which *orb2* activity is needed for full viability. Similarly, the disruptions in the expression of the hybrid *orb2*-CG43113 transcripts in 4965 showed no adverse effects on viability.

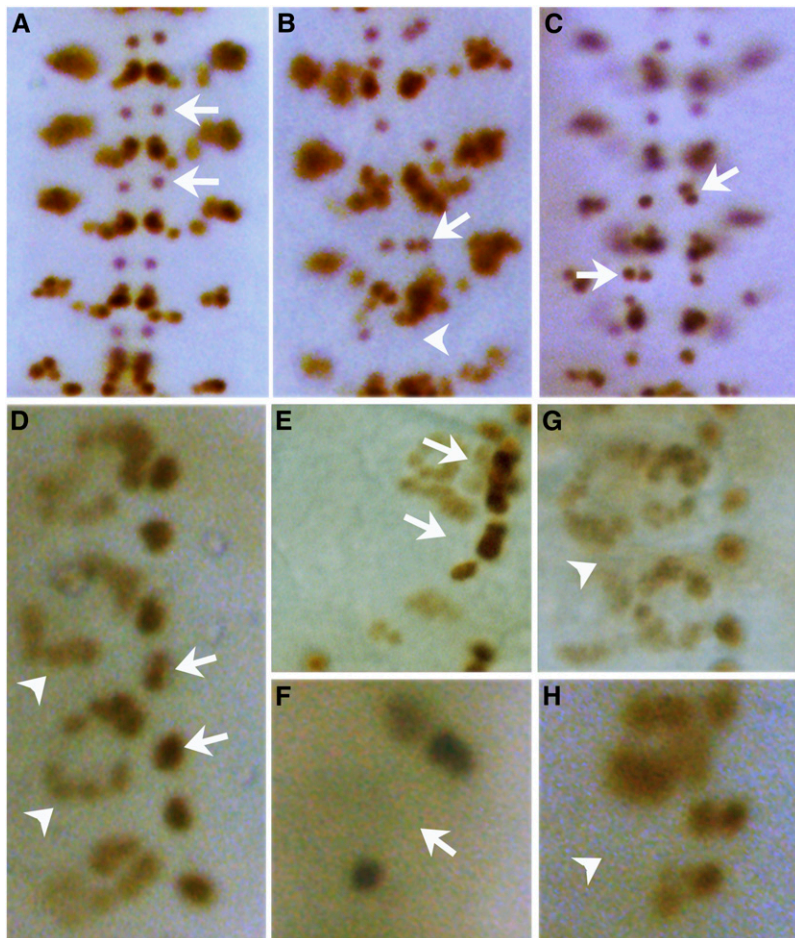
On other hand, lethal effects are observed for both 6090 and 1925. These results are consistent with studies of a null allele of *orb2* (*orb2*<sup>Δ</sup>) generated by homologous recombination and described by Keleman *et al.* (2007). For 6090, only about one-third of the homozygous flies survive to adulthood, while about two-thirds of the 1925 homozygotes survive. While some mutant animals die during embryogenesis, most appear to die during the pupal stage. Arguing against potential background effects, we found that when 6090 flies are *trans* to *Df(3L)ED4421*, which uncovers the *orb2* gene, only ~10% ( $n = 351$ ) of the expected number of the *trans*-heterozygous flies survived to adulthood. Similar results were obtained for 1925. We also found that the lethality of 1925 and 6090 can be reverted by precise excision of

the piggybac transposons. Finally, we found that the *orb2*<sup>6090</sup> allele showed a reduction in life span (see Figure S1).

#### *orb2* functions in asymmetric cell division in the CNS

Since Orb2 appears to specifically accumulate in the cell bodies but not in their axons during embryogenesis, we wondered whether it has any role in the specification of these neurons. To explore this question, we examined the specification of RP2 neurons, a well-studied neuronal lineage (reviewed in Bhat 1999; Gaziova and Bhat 2007), under conditions where *orb2* activity is compromised. During embryogenesis, each neuroblast (NB) undergoes a series of asymmetric divisions generating a self-renewing NB and a daughter GMC. Each GMC divides, producing two daughter cells, which typically differentiate into two different neurons. The Eve-positive RP2 neuron arises from the NB4-2 → GMC4-2a lineage (Bhat and Schedl 1994; Buescher *et al.* 1998; Skeath and Doe 1998; Wai *et al.* 1999). In wild-type embryos, GMC4-2a (also known as GMC-1) divides asymmetrically, producing two daughter neurons: RP2 and sib. The sib cell is smaller in size compared to RP2. While Eve is expressed in both daughter cells initially, it eventually disappears from the sib (Frasch *et al.* 1987; Patel *et al.* 1989; Fujioka *et al.* 2003). In wild-type stage 14 embryos, a single RP2 neuron is observed in each hemisegment (arrows in Figure 8A). However, if the asymmetric division of a GMC4-2a is disrupted, two cells with the same identity (RP2 or sib) can be formed, which can be determined by the Eve-staining pattern (Bhat and Schedl 1994; Bhat *et al.* 1995; Buescher *et al.* 1998; Wai *et al.* 1999).

As shown in Figure 8, A–D, in 11% of the *orb2*<sup>1925</sup> and 25% of the *orb2*<sup>6090</sup> embryos (Figure 9A), we observed RP2 neuron specification defects. In most instances (~70%) the RP2 neurons are duplicated and there are two equal sized Eve-positive cells per hemisegment (arrows in Figure 8, B and C). However, we also observed hemisegments that lacked Eve-positive RP2 neurons (arrowhead in Figure 8B), an indication that such hemisegments had either no RP2 lineage formed or that the GMC divided into two sib



**Figure 8** Specification defects are observed in neuronal and mesodermal lineages in *orb2*<sup>1925</sup> and *orb2*<sup>6090</sup> mutants. Wild-type (A) and mutant (B and C) embryos stained for Eve. Arrows in A show properly specified and localized RP2 neurons in several consecutive hemisegments. Arrows in B and C show hemisegments in which the RP2 neuron is duplicated. Note that the duplicated cells are approximately of the same size. Arrowhead in B points to a hemisegment in which RP2 is missing. Eve-positive EPC cells (arrows) and multinucleated DA1 muscle cells (arrowheads) in wild-type (D) and *orb2* mutant (E–H) embryos. Normally there are two Eve-positive EPC cells per hemisegment (arrows, D). In *orb2* mutants some hemisegments have extra (arrows, E) or are missing EPC cells (arrow, F). Each wild-type hemisegment has a multinucleate DA1 muscle cell (arrowheads in D). In *orb2* mutants, the DA1 muscle cell can be duplicated (arrowhead in G) or missing (arrowhead in H).

cells. These findings argue that *orb2* is required for the proper elaboration of this lineage. Consistent with this conclusion, RP2 specification defects were also observed when *orb2* activity is knocked down by RNAi in the nervous system using Gal4 drivers such as *cb43*, *scabrous* (Figure 9A), and *cb36*.

#### ***orb2* may have a general role in asymmetric cell division**

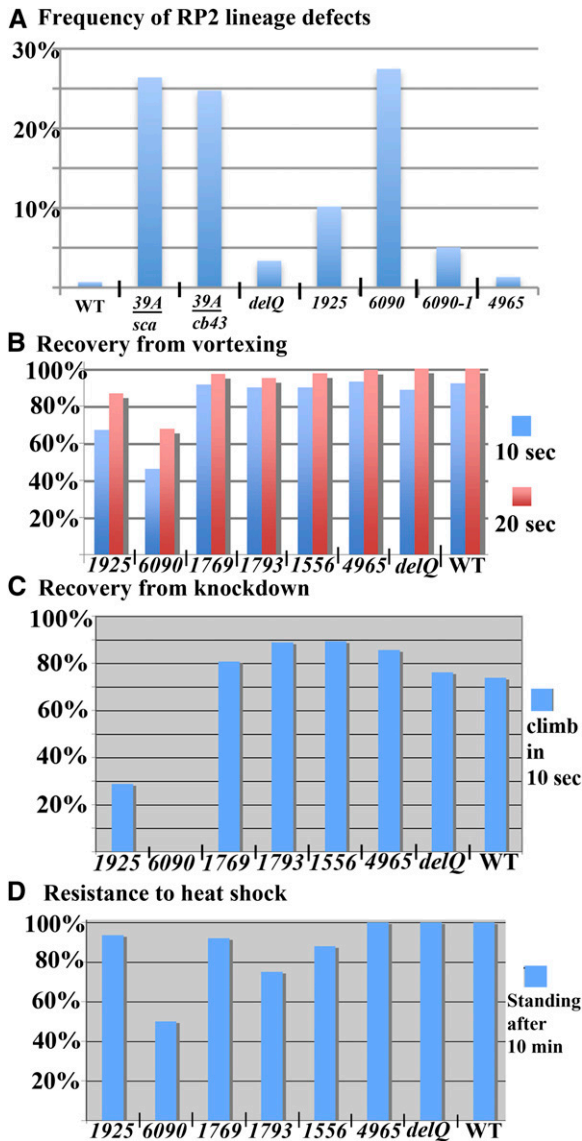
Many of the genes implicated in the asymmetric NB4 → GMC-1 → RP2/sib cell division pathway are known to function in the asymmetric division of other lineages (Ruiz Gomez and Bate 1997; Carmena *et al.* 1998; Paululat *et al.* 1999; Tio *et al.* 2001). To test whether *orb2* also has a more general role in asymmetric cell division, we examined cell division patterns in the mesoderm. In each hemisegment there are two Eve-expressing mesodermal lineages that undergo asymmetric divisions. In one of these lineages, the P2 cell divides asymmetrically to generate the founder of the Eve-positive pericardial cells (FEPCs) and FEPC sib cells. The FEPC daughter then divides symmetrically to form a pair of Eve-positive pericardial cells (EPCs) in each hemisegment (arrow in Figure 8D). In the other lineage, the P15 cell divides asymmetrically to produce the Eve-positive DA1 muscle founder cell FDA1 and the FDA1 sib cell. The

FDA1 daughter then generates the multinucleate DA1 muscle (arrowhead in Figure 8D).

As was observed for the RP2 lineage, *orb2* is required for asymmetric cell division in the P2 → EPC and P15 → DA1 lineages. In both *orb2*<sup>1925</sup> and *orb2*<sup>6090</sup> mutant embryos, we observe abnormalities in these two lineages. Figure 8E shows a hemisegment in which the P2 cell divided symmetrically to produce an extra Eve-positive EPC cell (arrow), while Figure 8F shows a hemisegment in which the P2 or FEPC asymmetric division was abnormal and no Eve-positive EPC cell was produced (arrow). Extra and missing Eve-positive cells are also seen in the P15 → DA1 lineage. Figure 8G (arrowhead) shows a hemisegment in which P15 divided symmetrically to produce two DA1 muscles, while Figure 8H shows a hemisegment in which the DA1 muscle is absent (arrowhead).

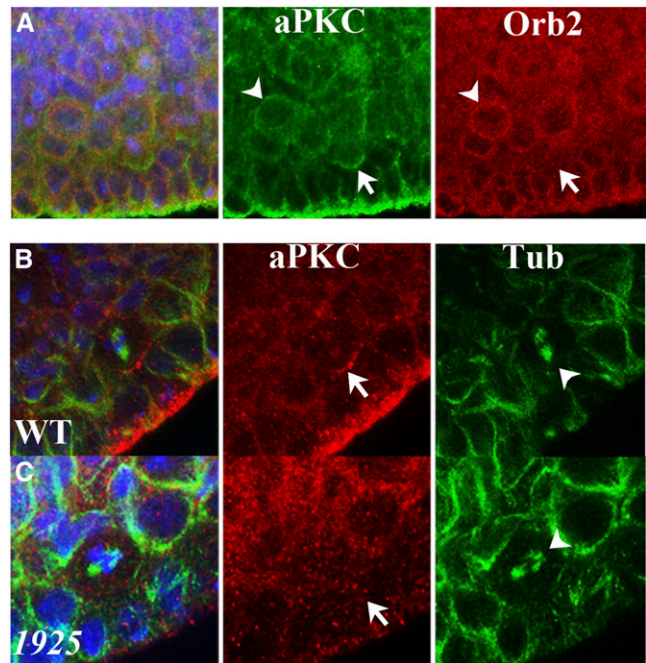
#### **Alignment of the mitotic spindle is disrupted in *orb2* mutants**

Mastushita-Saki *et al.* (2010) have shown that one of the mRNAs associated with Orb2 in the adult CNS encodes *Drosophila* atypical protein kinase C (aPKC). Since this kinase is known to play a central role in asymmetric cell division, we wondered whether there were any effects on aPKC in *orb2*



**Figure 9** RP2 lineage, locomotion, and activity defects in the transposon insertions. (A) Frequency of embryos with RP2 lineage defects when *orb2* function is compromised. With the exception of *Gal4 39A/UAS cb43*, >130 embryos were examined in each case. WT, wild type; *39A/sca*, *UAS-39A/Gal4-sca*; *39A/cb43*, *UAS-39A/Gal4-cb43*; *delQ*, *orb2<sup>delQ</sup>*; *1925*, *orb2<sup>1925</sup>*; *6090*, *orb2<sup>6090</sup>*; *6090-1*, *orb2<sup>6090-1</sup>*; and *4965*, *orb2<sup>4965</sup>*. *6090-1* is a precise excision of the *6090 piggybac* insertion. (B) Percentage of flies that have recovered from vortex after 10 sec (blue) or 20 sec (red). Genotypes are as indicated. (C) Percentage of flies climbing a defined distance after being knocked down to the bottom of the vial in 10 sec. (D) Percentage of flies still standing after a 10-min heat shock.

mutant neuroblasts. In nondividing wild-type neuroblasts, aPKC is distributed around the cortex of the cell (Figure 10A, cell with arrowhead), while in neuroblasts about to undergo asymmetric division (Figure 10A, arrow) it concentrates on the apical side of the cell. Orb2 is also in a cortical ring in nondividing neuroblasts (Figure 10A, arrowhead). However, the Orb2 ring does not appear to overlap with aPKC (Figure 10A, left). Instead, it is localized in a slightly more interior region of the cell. In dividing neuroblasts,



**Figure 10** Neuroblast cell division is altered in *orb2* mutants. (A) Distribution of aPKC (green) and Orb2 (red) in nondividing (arrowhead) and dividing (arrow) neuroblasts. Both aPKC and Orb2 are cortical in nondividing neuroblasts, but do not colocalize. In dividing neuroblasts, aPKC concentrates apically, while Orb2 becomes diffusely distributed. (B) Mitosis in WT and *1925 (orb2<sup>1925</sup>)* neuroblasts. In wild type, aPKC (red) is localized on the apical surface (arrow), while the mitotic spindle (visualized with tubulin staining in green) has a basal–apical orientation (arrowhead). In *1925*, apical localization of aPKC is lost (arrow), while the mitotic spindle is oriented along anterior–posterior embryo axis.

Orb2 differs from aPKC in that it disappears from the cortex and becomes largely unlocalized (Figure 10A, arrow). When wild-type neuroblasts divide, the mitotic spindle (arrowhead in Figure 10B) is oriented along the apical–basal axis. After division, the apical daughter cell that inherits the cap of aPKC (arrow in Figure 10B) retains neuroblast identity, while the basal daughter becomes a GMC. In *orb2* mutants, aPKC can be detected in nondividing neuroblasts; however, it seems to be somewhat less tightly linked to the cortex than in wild type. In dividing mutant neuroblasts, the mitotic spindle orientation is often randomized with respect to the apical–basal axis (arrowhead in Figure 10C). Interestingly, in these neuroblasts, the apical cap of aPKC is also missing. These findings further support the conclusion that *orb2* plays a role in asymmetric cell division and suggest that it may function by promoting the localized accumulation of aPKC.

#### Behavioral deficiencies in *orb2* adults

In the adult CNS, high levels of Orb2 are found in the ellipsoid and fan shaped bodies. As these structures have been implicated in sensing distance, orientation, and walking, we tested different *orb2* alleles in behavioral assays that measure locomotion and activity of adult flies. These assays

were recovery from vortexing (bang assay) and recovery from knockdown (climbing assay). We also tested recovery from heat shock. As shown in Figure 9, B and C, the three *orb2* mutants, 1769, 1793, and 1556, that had no apparent effects on Orb2 protein expression in adult brains recover from vortexing and knockdown as rapidly as wild type. This is also true for an *orb2* mutant, *orb2<sup>delQ</sup>*, lacking the N-terminal glutamine-rich region, which has previously been implicated in learning and memory (Keleman *et al.* 2007). The 4965 allele, which disrupts the *orb2* promoter-dependent CG43113 mRNAs, but not the *orb2* mRNAs, also resembles wild type. In contrast, 6090, and to a lesser extent 1925, take longer than wild type to recover from vortexing. Even more pronounced defects are evident in the knockdown experiment. Finally, we also found that 6090, but not 1925, recovers more slowly from heat shock than wild type (Figure 9D).

## Discussion

### Sequence organization of *Orb2* and the neighboring CG43113 transcription units

The sequence organization and coding properties of the *orb2* transcription unit is quite complex. There are at least three *orb2* promoters and they generate two quite different sets of mRNAs. One set of mRNAs, the *orb2* mRNAs, encode the CPEB protein Orb2, while the other set of mRNAs, the hybrid *orb2*-CG43113 mRNAs, encode a conserved protein of unknown function (Figure 1).

Three *orb2* mRNAs, RB, RC, and RD, are expressed from the *orb2-1* and *orb2-2* promoters and encode the same 75-kDa Orb2 isoform. The fourth mRNA, RA, is expressed from the *orb2-3* promoter and encodes a smaller 60-kDa protein. These two Orb2 isoforms share a common 542-amino-acid C-terminal region, which includes the two CPEB RRM domains and the zinc finger, but have unique N termini of 162 and 9 amino acids, respectively. The other set of transcripts is the hybrid *orb2*-CG43113 mRNAs. They are generated by the *orb2-1* and *orb2-2* promoters and share several 5' exons with the *orb2* RB, RC, and RD mRNAs. These 5' exons include the translation start signal and the "unique" 162-amino-acid N terminus of the 75-kDa Orb2 isoform. However, instead of being spliced to the downstream sequences encoding Orb2, the CG43113 mRNAs are spliced to a conserved 1059-amino-acid open reading frame, which is located ~7 kb beyond the end of *orb2*. This downstream open reading frame is also part of an independent transcription unit, CG5741, which has its own promoter and generates five different mRNAs. These mRNAs encode variants of the same conserved open reading frame depending on their splicing pattern and the location of the AUG codon.

An important issue is the relationship between *orb2*, *orb2*-CG43113, and CG5741. In most other fly species *orb2* and CG5741 (CG43113) appear to be distinct genes. For example, the *pseudoobscura* CG43113 gene, GA19098, is

also located downstream (12 kb) of the *orb2* (GA1909) open reading frame and like CG5741, it is predicted to have its own promoter. Our data would suggest that in spite of the fact that some of the CG571/CG43113 transcripts are derived from *orb2* promoters, the *orb2* and CG5741 genes are probably distinct in *melanogaster* as well. In particular, we found that a *piggybac* insertion, *orb2<sup>4965</sup>*, located downstream of the *orb2* open reading frame interrupts CG43113 transcripts emanating from the two *orb2* promoters, and greatly reduces the level of the hybrid *orb2*-CG43113 mRNAs. However, there were no obvious reductions in the level of mRNA specific for the CG5741/CG43113 protein coding sequences. We also found that insertions in the upstream *orb2* introns, which disrupt transcripts from the *orb2-1* and *orb2-2* promoters, reduce the levels of *orb2* and *orb2*-CG43113 hybrid mRNAs but do not alter the level of mRNAs specific for the CG5741/CG43113 protein coding sequences.

### *Orb2* expression pattern is different from that of *Orb* during development

*Drosophila* has two CPEB genes, *orb* and *orb2*. While the expression of *orb* is restricted to the germline and its only essential functions are in the female ovary where it plays a key role in the development of the egg, *orb2* is expressed not only in the germline, but also in a wide range of somatic tissues in the embryo, larvae, and adult. While *orb* and *orb2* mRNAs and proteins are deposited in the developing egg during oogenesis, their fate in embryos is quite different. With the exception of the *orb* gene products that are incorporated into the germline pole cells, all maternal *orb* mRNAs and proteins turnover by the midblastula transition. In contrast, maternally derived *orb2* mRNA is translated in pre-cellular blastoderm embryos, and high levels of the 75-kDa Orb2 protein are distributed throughout the early pre-cellular and cellular blastoderm embryo. After the onset of gastrulation, there is a general reduction in the levels of *orb2* message and protein, which likely reflects the turnover of the maternal gene products in the absence of significant *de novo* synthesis. However, once the embryonic CNS and PNS begin to differentiate, *orb* mRNA and the 75-kDa Orb2 protein isoform are expressed at high levels in these tissues. The Orb2 protein in the embryonic CNS is found predominantly in the cell bodies and is largely excluded from axon tracts. Orb2 protein is also detected in other differentiating tissues like the mesoderm, though the levels are lower than in the CNS or PNS.

High levels of Orb2 persist in the nervous system during the larval stages, and as in the embryos, the protein is predominantly localized in cell bodies, and not in axons or dendrites. Interestingly, in the adult brain, Orb2 is found mostly in axon/dendritic terminals. As *orb2* has been implicated in learning and memory (Keleman *et al.* 2007; Si *et al.* 2010), this apparent shift in distribution in the adult CNS could reflect a change in the functional role of the Orb2 protein. Orb2 is also found in adult gonads. Recently, the smaller *Drosophila* Orb2 60-kDa isoform was shown to

assemble into large aggregates in response to synaptic stimulation when ectopically expressed in *Aplysia* sensory neurons (Si *et al.* 2010). Once formed, these large aggregates were proposed to have self-perpetuating properties that would contribute to long-term facilitation. It was argued that the special prion-like properties of the N-terminal domain in the 60-kDa isoform was responsible for multimerization in response to synaptic stimulation. In contrast, when the larger *Drosophila* Orb2 75-kDa isoform was ectopically expressed in *Aplysia* neurons it was unable to form these self-propagating bodies either with or without stimulation (Si *et al.* 2010). As this study seemed to directly implicate the Orb2 60-kDa isoform in long-term facilitation, it was of interest to examine its distribution in different fly tissues. Strikingly, there is little if any of the 60-kDa isoform in Westerns of adult heads, even when overloaded, and only the larger Orb2 75-kDa isoform seems to be expressed in this tissue. In contrast, both isoforms are found in testes, though even in this tissue the 60-kDa isoform is less abundant than the 75-kDa isoform. Thus, it seems quite unlikely that the Orb2 60-kDa isoform plays a central role in nervous system function in adult *Drosophila*. If the assembly of Orb2 into prion-like aggregates is critical for synaptic stimulation-dependent long-term facilitation, then the experiments of Si *et al.* (2010) would imply that there must be special ancillary factors in the fly CNS, but not in *Aplysia* sensory neurons, that are able to promote the assembly of the 75-kDa isoform into prion-like aggregates upon synaptic stimulation. Since all of the smaller isoforms are included in the larger isoform except for nine amino acids at the very N terminus, one could imagine that Orb2 75 kDa should also be capable of forming prion-like aggregates under certain condition(s).

### Function of Orb2 during development

Though *orb2* function has been implicated in learning and memory (Keleman *et al.* 2007; Mastushita-Saki *et al.* 2010), what other functions it might have, if any, during the *Drosophila* life cycle are largely unknown. In our work, we have attempted to identify some of these other activities using RNAi knockdowns and transposon insertion mutations. This task has been complicated by the fact that the *orb2* locus generates mRNAs encoding not only the Orb2 CPEB protein (s) but also a hybrid Orb2-CG43113 protein.

While not entirely definitive, the properties of the *orb2* transposon insertions argue that the observed phenotypes are due to an effect on *orb2* gene activity and not on the activity of either the hybrid *orb2*-CG43113 or the CG5741 gene. First, with the exception of 1769, which is inserted upstream of the *orb2-1* promoter, all of the transposon insertions reduce expression of the hybrid *orb2*-CG43113 mRNAs. However, only two of these, 1925 and 6090, have obvious phenotypic effects. While 6090 has little of the hybrid *orb2*-CG43113 mRNA, the amount of hybrid mRNA in 1925 is close to that seen in the three other transposon insertions (1556, 1793, and 4965) that have no phenotype.

Second, 4965 differs from the other *orb2* transposons in that it is downstream of the *orb2* coding sequences. It has no effect on *orb2* mRNAs, but does reduce expression of the hybrid mRNAs. However, it has none of the phenotypes evident in the 1925, 6090, or *orb2* RNAi animals. Third, while all but one of the transposon insertions reduce expression of the hybrid *orb2*-CG43113 mRNAs, the total level of the CG5741 (CG43113) transcripts appears to be unaffected in all of the mutants. This is likely due to the fact that the bulk of the CG5741/CG43113 transcripts are derived from the CG5741 promoter and not the *orb2* promoters. Since the level of the hybrid *orb2*-CG43113 mRNAs can be reduced without any phenotypic effects, this would argue that CG5741 may be able to substitute, perhaps even fully, for whatever function the hybrid *orb2*-CG43113 protein might have. The suggestion that the 1925 and 6090 phenotypes are attributable to *orb2* rather than the hybrid *orb2*-CG43113 gene product is supported by the fact that they are also observed in *orb2* RNAi experiments. Less than 10% of the ~1 kb double-stranded RNA expressed by *orb2* RNAi vector is from the common region and thus the *orb2* RNAi would be expected to have a much greater effect on *orb2* than on the hybrid mRNAs.

We found that both the *orb2* RNAi knockdowns and the 1925 and 6090 *piggybac* insertions reduce viability. As might be expected from the high levels of *orb2* mRNA and protein in the CNS/PNS, *Gal4* drivers that were expressed in the nervous system generally reduced viability. Also consistent with important functions in the nervous system, surviving 1925 and 6090 adults exhibit behavioral defects in assays that measure fly locomotion and activity. One of the *piggybac* mutants was also found to have a reduced life span. Moreover, the studies of Mastushita-Saki *et al.* (2010) on potential targets for *orb2* regulation showed that mRNAs encoding factors involved in neuronal growth and synapse formation are associated with Orb2 in the adult brain.

One of the important nervous system functions appears to be in asymmetric cell division. In the embryonic CNS, Orb2 protein can be detected in the cell bodies of specific neurons such as the Eve-positive RP2 and aCC/pCC neurons. However, it appears to be needed prior to the formation of these neurons as we found that the proper elaboration of the NB4-2 → GMC4-2a → RP2/sib lineage depends upon *orb2* activity. There are defects in the specification of the RP2 and sib cells in two different *orb2 piggybac* alleles, and also in *orb2* RNAi knockdowns. The most common phenotype is the duplication of RP2. This duplication appears to be due to the loss of asymmetric division of the parent GMC to generate two RP2s. Since we also observe hemisegments with no Eve-positive RP2 cells, either the parent GMC generates two sibs (or some other neurons) or the defect occurs at even earlier stages. The effects of *orb2* mutations on asymmetric cell division are not limited to the embryonic CNS, since a similar fate transformation is seen in two asymmetrically dividing mesodermal lineages that give rise to the Eve-positive pericardial EPCs and to the DA1 muscle.

The genes that have been implicated in asymmetric cell division fall into two general categories, those responsible for the asymmetry and those responsible for specifying the fate of the daughter cells. In the former category are genes like *inscuteable*, *bazooka*, and *atypical protein kinase C* (Schaefer and Knoblich 2001; Bhalerao *et al.* 2005; Gaziouva and Bhat 2007). Mutations in these genes disrupt the process of asymmetric cell division and give rise to two identical cells. In the latter category are genes like *numb*, the transcription factor *prospero*, and various components of the *Notch* signaling pathway. Cells mutant in these genes still divide asymmetrically; however, though the daughter cells are unequal, they still assume the same fate. It seems likely that *orb2* falls into the former category as the duplicated RP2 cells are equal in size. This is also observed for mutations in *inscuteable*, but not for mutations in the *Notch* pathway, where the two daughter cells are still unequal in size (Buescher *et al.* 1998; Wai *et al.* 1999).

Our analysis of neuroblast cell division in *orb2* mutants points to roles in localizing aPKC and orienting the mitotic spindle. In wild-type nondividing neuroblasts, aPKC is localized around the cortex. Orb2 is also concentrated around the cortex, but localized just inside the cortical layer that contains aPKC. When neuroblasts divide asymmetrically, aPKC relocates to the apical cortex, and during mitosis, the spindle is oriented along the apical–basal axis. At this point, Orb2 is distributed uniformly in the cytoplasm. In *orb2* mutants, aPKC is cortical in nondividing neuroblasts but appears to be somewhat more diffusely localized than in wild type. During cell division, aPKC does not concentrate at the apical cortex and the spindles are not properly aligned relative to the apical–basal axis. Since Orb2 is known to bind to *apkc* mRNA (Mastushita-Saki *et al.* 2010), a plausible mechanism is that *orb2* helps promote the apical accumulation of aPKC by activating the localized translation of *apkc* mRNA.

While these findings point to a role in polarizing the cell during asymmetric cell division, the two *orb2* mutants (and RNAi knockdowns) we have examined differ from mutations in genes like *apkc* and *inscuteable* in that only a relatively small percentage of the mutant embryos exhibit defects in the RP2 lineage. This could be due to the fact that we have not fully eliminated *orb2* activity. Alternatively, *orb2* might have only an ancillary role in generating asymmetry. Further studies will be required to determine how *orb2* fits into the asymmetry pathway and why the phenotypic effects of *orb2* mutations are relatively modest.

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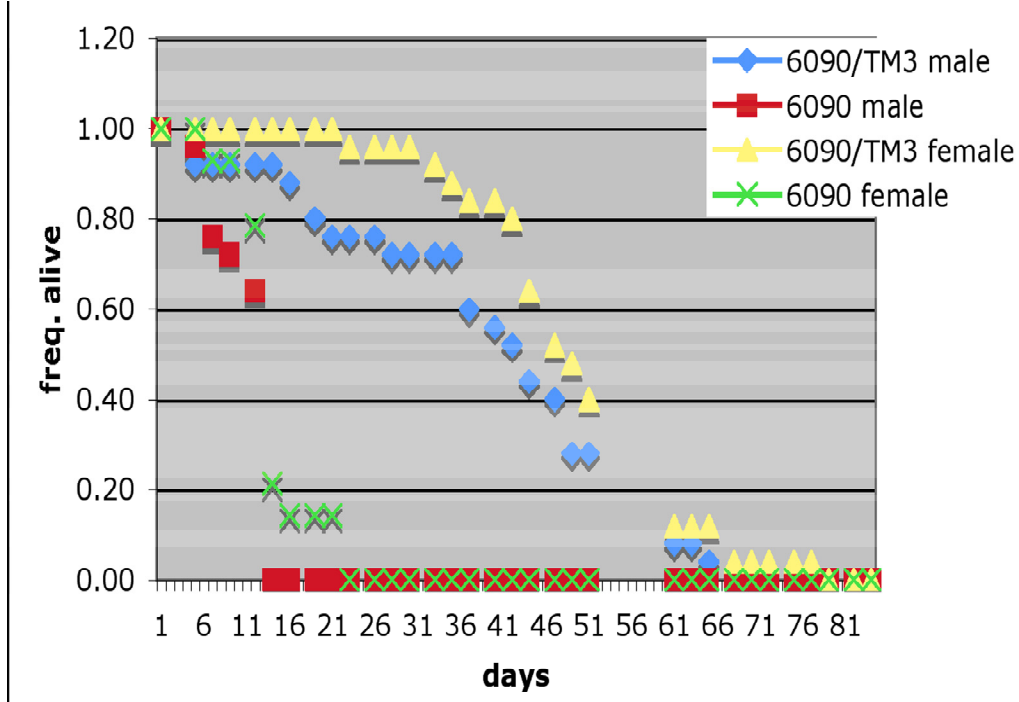
Supporting Information

<http://www.genetics.org/content/suppl/2011/09/07/genetics.110.123646.DC1>

## **The *Drosophila* CPEB Protein Orb2 Has a Novel Expression Pattern and Is Important for Asymmetric Cell Division and Nervous System Function**

Nathaniel Hafer, Shuwa Xu, Krishna Moorthi Bhat, and Paul Schedl





**Figure S1 *orb2<sup>6090</sup>* flies have a reduced lifespan.** Lifespan of *orb<sup>6090</sup>* homozygous males and females and their *orb2<sup>6090</sup>/TM3* sibs was measured as described in Tan *et al.*, 2008, Average lifespan of surviving homozygous *6090* flies is about 10-12 days while the average lifespan of their heterozygous sibs is close to 40 days.

Tan L, Schedl P, Song HJ, Garza D, Konsolaki M. (2008). The Toll-->NFkappaB signaling pathway mediates the neuropathological effects of the human Alzheimer's Abeta42 polypeptide in *Drosophila*. PLoS One.3 :e3966.

**Table S1 UAS orb2RNAi in combination with certain Gal4 drivers is lethal.**

| P female genotype | P male genotype | F1 genotype            | total F1 | F1 observed (% of expected) | P female genotype | P male genotype | F1 genotype      | total F1 | F1 observed (% of expected) |
|-------------------|-----------------|------------------------|----------|-----------------------------|-------------------|-----------------|------------------|----------|-----------------------------|
| 137A/137A         | cb06/Y          | 137A/cb06              | 64       | 18 (56)                     | 39A/TM3Ser        | w, cb06/Y       | cb06/+;<br>39A/+ | 180      | 1 (4)                       |
| 137A/137A         | w, cb09/Y       | 137A/cb09              | 91       | 39 (86)                     | 39A/TM3Ser        | w, cb09/Y       | cb09/+;<br>39A/+ | 479      | 66 (55)                     |
| 137A/137A         | cb14/Y          | 137A/cb14              | 47       | 0 (0)                       | 39A/TM3Ser        | w, cb14/Y       | cb14/+;<br>39A/+ | 180      | 0 (0)                       |
| 137A/137A         | Pin/cb20        | 137A/+ or Y;<br>cb20/+ | 157      | 65 (82)                     | 39A/TM3Ser        | Pin/cb20        | cb20/+;<br>39A/+ | 411      | 41 (40)                     |
| 137A/137A         | Pin/cb29        | 137A/+ or Y;<br>cb29/+ | 206      | 72 (70)                     | 39A/TM3Ser        | Pin/cb29        | cb29/+;<br>39A/+ | 401      | 7 (7)                       |
| 137A/137A         | Pin/cb36        | 137A/+ or Y;<br>cb36/+ | 217      | 0 (0)                       | 39A/TM3Ser        | Pin/cb36        | cb36/+;<br>39A/+ | 265      | 0 (0)                       |

“F1 genotype” refers to the progeny that inherited both the UAS-orb2RNAi transgene and the Gal4 driver. The total number of progeny for each male/female cross is listed in the “Total F1” column. “F1 observed” refers to the number of flies carrying both the driver and the RNAi transgenes. The percentage of survivors of this genotype is calculated relative to that expected from the cross.