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Miranda couples *oskar* mRNA/Staufen complexes to the *bicoid* mRNA localization pathway

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Abstract

The double-stranded RNA binding protein Staufen is required for the microtubule-dependent localization of *bicoid* and *oskar* mRNAs to opposite poles of the *Drosophila* oocyte and also mediates the actin-dependent localization of *prospero* mRNA during the asymmetric neuroblast divisions. The posterior localization of *oskar* mRNA requires Staufen RNA binding domain 2, whereas *prospero* mRNA localization mediated the binding of Miranda to RNA binding domain 5, suggesting that different Staufen domains couple mRNAs to distinct localization pathways. Here, we show that the expression of Miranda during mid-oogenesis targets Staufen/*oskar* mRNA complexes to the anterior of the oocyte, resulting in bicaudal embryos that develop an abdomen and pole cells instead of the head and thorax. Anterior Miranda localization requires microtubules, rather than actin, and depends on the function of Exuperantia and Swallow, indicating that Miranda links Staufen/*oskar* mRNA complexes to the *bicoid* mRNA localization pathway. Since Miranda is expressed in late oocytes and *bicoid* mRNA localization requires the Miranda-binding domain of Staufen, Miranda may play a redundant role in the final step of *bicoid* mRNA localization. Our results demonstrate that different Staufen-interacting proteins couple Staufen/mRNA complexes to distinct localization pathways and reveal that Miranda mediates both actin- and microtubule-dependent mRNA localization.

Keywords: mRNA transport; Axis formation; Bicaudal; Pole plasm; Translational control

Introduction

Asymmetric localization of mRNAs is a common mechanism for targeting proteins to the regions of the cell where they are required (St Johnston, 2005; Tekotte and Davis, 2002). This process is particularly important in the developing oocytes of many organisms, where localized mRNAs function as cytoplasmic determinants (Kloc et al., 2002). This has been best characterized in *Drosophila*, where the localization of *bicoid* (*bcd*) and *oskar* (*osk*) mRNAs to the anterior and posterior poles of the oocyte defines the primary axis of the embryo (Johnstone and Lasko, 2001). *bcd* mRNA is translated after fertilization to produce a morphogen that patterns the head and thorax of the embryo, whereas *osk* mRNA is translated when it reaches the posterior of the oocyte, where Oskar protein nucleates the assembly of the pole plasm, which contains the abdominal determinant *nanos* mRNA, as well as the germ line determinants. Localized mRNAs can also function as determinants during asymmetric cell divisions. For example, the asymmetric inheritance of mating type switching in budding yeast is controlled by the localization of Ash1 mRNA to the bud tip, which segregates the repressor ASH1p into only the daughter cell at mitosis (Long et al., 1997; Takizawa et al., 1997). Similarly, *prospero* (*pros*) mRNA localizes to the basal side of *Drosophila* embryonic neuroblasts and is inherited by only the smaller daughter cell of this asymmetric cell division, where Prospero protein acts as a determinant of ganglion mother cell fate (Broadus et al., 1998; Li et al., 1997).

To be localized, an mRNA must contain *cis*-acting localization elements that are recognized by RNA-binding proteins, which couple the mRNA to the localization machinery.

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This process is only well understood for ASH1 mRNA, which contains four localization elements that are recognized by She3p, which then links the mRNA to the myosin motor complex Myo4p/She2p so that it can be transported along actin cables to the bud tip (Bohl et al., 2000; Gonsalvez et al., 2005; Munchow et al., 1999). Biochemical and genetic approaches have led to the identification of a number of RNA-binding proteins that associate with localized mRNAs in higher eukaryotes, but it is not known how these interactions target the mRNA to the correct region of the cell.

One of the best candidates for an RNA-binding protein that plays a direct role in mRNA localization is the dsRNA-binding protein Staufen (Stau). Staufen was first identified because it is required for the localization of *osk* mRNA to the posterior of the oocyte and co-localizes with it at the posterior pole (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991). This localization depends on the polarized microtubule cytoskeleton and the plus end-directed microtubule motor kinesin, suggesting that Staufen may play a role in coupling *osk* mRNA to kinesin, which then transports the *osk* mRNA complex along microtubules (Brendza et al., 2000). The posterior localization of *osk* mRNA also requires the exon junction complex components Mago nashi (Mago), Y14, eIF4AIII and Barentsz (Btz), as well as HRP48, which is needed for the formation of Staufen/*osk* mRNA particles (Hachet and Ephrussi, 2001; Huynh et al., 2004; Mohr et al., 2001; Newmark and Boswell, 1994; Palacios et al., 2004; van Eeden et al., 2001; Yano et al., 2004).

Staufen homologues seem to play a similar role in the microtubule-dependent localization in vertebrates. GFP-Stau particles have been observed to move along microtubules in cultured neurons, and the protein is a component of large ribonucleo-protein complexes that contain kinesin and dendritically localized mRNAs (Kanai et al., 2004; Köhrmann et al., 1999). In addition, a *Xenopus* Staufen homologue associates with Vg1 mRNA and is required for its microtubule-dependent localization to the vegetal pole of the oocyte, which is also thought to be mediated by a kinesin (Yoon and Mowry, 2004).

As well as this possible conserved role in kinesin-dependent transport, *Drosophila* Staufen is also required for the last phase of *bcd* mRNA localization and co-localizes with the mRNA at the anterior of the oocyte from stage 10B onwards (Martin et al., 2003; St Johnston et al., 1989). Furthermore, when the *bcd* 3′ UTR is injected into the early embryo, it recruits Staufen into particles that move in a microtubule-dependent manner to the poles of the mitotic spindles, consistent with minus end-directed microtubule transport (Bullock and Ish-Horowicz, 2001; Ferrandon et al., 1994).

Staufen also binds to *prospero* mRNA and is required for its localization to the basal side of the embryonic neuroblasts (Broadus et al., 1998; Li et al., 1997). In contrast to the other examples of Staufen-dependent mRNA localization, this process depends on the actin cytoskeleton and the adapter protein Miranda (Mira) (Barros et al., 2003; Matsuzaki et al., 1998; Petritsch et al., 2003; Schuldt et al., 1998; Shen et al., 1998).

The varied functions of Staufen raise the question of how the same protein can function in both actin- and microtubule-dependent mRNA localization, as well as in the targeting of *osk* and *bcd* mRNAs to opposite ends of the same cell. Some insight into this comes from the analysis of Staufen protein, which contains five conserved dsRNA-binding domains (dsRBDs) (St Johnston et al., 1992). In all Staufen homologues, dsRBD2 is split by a proline-rich insertion in one of the RNA-binding loops, and deletion of this insertion disrupts the localization of *osk* mRNA, but not that of *prospero* mRNA, leading to the proposal that this domain couples Staufen/mRNA complexes to a kinesin-dependent posterior localization pathway (Micklem et al., 2000). In contrast, removal of dsRBD5 prevents the localization of *prospero* mRNA, whereas *osk* mRNA localizes normally but is not translated at the posterior of the oocyte. Indeed, dsRBD5 binds directly to Miranda to couple Staufen/*prospero* mRNA complexes to the actin-based localization pathway (Fuerstenberg et al., 1998; Schuldt et al., 1998). The localization of *bcd* mRNA also requires dsRBD5, although the loss of the insert in dsRBD2 also affects its localization slightly.

The results above suggest that different domains of Staufen couple mRNAs to distinct localization pathways, raising the possibility that the fate of Staufen mRNA complexes may depend on which Staufen-interacting proteins are present in the cell. To test this hypothesis, we have examined the effects of expressing Miranda during oogenesis to determine whether it can influence the localization of *bcd* or *osk* mRNAs.

Materials and methods

Molecular biology

For the *mira-GFP* transgenes, either the entire coding region of *mira* or the region corresponding to amino acids 1–727 was amplified by PCR and cloned via *Xba*I/*Avr*II and *Not*I into a vector containing the α-Tub67C promoter and mGFP6 (Micklem et al., 1997). Transgenic flies were generated using standard transformation techniques (Rubin and Spradling, 1982; Spradling and Rubin, 1982) and Δ2–3 turbo as a helper plasmid.

Drosophila genetics

Flies carrying the *mira-GFP* transgenes on one of the autosomes were kept as heterozygous males with *w f* females; males carrying insertions on the X-chromosome were kept with *C(1)DX,y w f* females. Flies with the *miraΔ103-GFP* transgene on the 2nd chromosome were also kept as a stock with the following genotypes: w^{-} *P{*w^{+}*, miraΔ103-GFP}/T(1;2)OR64/SM6a*.

Other mutants stocks used were: $stau^{D3}$*, P{*w^{+}*, stauΔRBD5}/CyO;* $stau^{D3}$*, P{*w^{+}*, stauΔloop2},sp/CyO* (Micklem et al., 2000); *Df(3R)pXT103,ru st e ca/TM3*; osk^{54}*, st e/TM3* (Kim-Ha et al., 1991); *P{*w^{+}*, osk-LacZ}* (Gunkel et al., 1998); exu^{SC}*, cn bw/CyO*; exu^{VL}*, pr cn bw/CyO* (Schüpbach and Wieschaus, 1989); swa^{14}*, ct v/FM7c*; swa^{99}*, w cv/FM7c* (Frohnhöfer and Nüsslein-Volhard, 1987); *sry-*δ^{14}*/TM3*; *P{*ry^{+}*, sryDB56}, Df(3R)X3F, e/TM3* (Payre et al., 1994); *Df(2R)F36, cn bw/SM5*; $mago^{1}$*, cn bw sp/SM1* (Boswell et al., 1991).

Stainings and microscopy

Antibody stainings, in situ hybridizations and cuticle preparations were performed according to standard protocols (Nüsslein-Volhard et al., 1984; Palacios and St Johnston, 2002). The following antibodies were used: rabbit anti-Staufen (1:1000) (St Johnston et al., 1991), rabbit anti-Vasa (1:1000) (Lasko and Ashburner, 1990). Digoxigenin labeled anti-sense RNA probes for in situ hybridization were synthesized using the RNA labeling mix from Roche.

For the drug treatments, flies were starved for 6 h and then allowed to feed on yeast containing approximately 100 μg/ml Colcemid (Demecolcine,

Sigma) or 250 μg/ml Latrunculin A (Sigma) for 18 h. The ovaries were dissected and either fixed and stained with TRITC-Phalloidin (0.5 μg/ml) (Sigma), or cytoplasmic movements were recorded using a BioRad 1024 Inverted Confocal Microscope (Palacios and St Johnston, 2002).

The stainings for β-Galactosidase activity were carried out on fixed ovaries in the following buffer: 100 mM Na-Phosphate pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.1% Triton X-100, 0.15% X-Gal (5-Bromo-4-Chloro-3-Indoyl-β-D-Galactoside).

Results

Ectopic Miranda produces a bicaudal phenotype

To examine whether Miranda plays any role in oogenesis, we generated germ line clones of a null mutation *mira*^{L44} (Matsuzaki et al., 1998). The homozygous mutant clones were completely wild type, however, and produced hatching first instar larvae if fertilized by wild type sperm. Antibody stainings in wild type ovaries detected no Miranda protein during stages 1–10B of oogenesis, which is the latest stage that antibodies can reliably penetrate the egg chamber before the impermeable vitelline membrane and chorion are deposited. The protein is probably expressed in late oocytes, however, because it is detected in western blots of ovary extracts and is deposited maternally in the early embryo (Mollinari et al., 2002).

To ectopically express Miranda in the female germ line, we fused the full-length Miranda coding region with a C-terminal GFP tag to the maternal-specific α4-tubulin promoter, which drives expression from stage 2 of oogenesis onwards. The C-terminal region of Miranda is not required for its localization in neuroblasts or for Staufen binding but triggers its degradation in the Ganglion mother cells (Fuerstenberg et al., 1998; Ikeshima-Kataoka et al., 1997). We therefore generated a second transgene in which the C-terminal 103 amino acids are deleted (*miraΔ103-GFP*, equivalent to the *mira*^{RR127} allele) to ensure the stable expression of the protein in the germ line. Surprisingly, transgenic lines for both constructs show a dominant maternal effect bicaudal phenotype, in which the embryos develop an anterior abdomen in place of the head and thorax (Figs. 1A, B). 100% of the embryos from mothers expressing the *miraΔ103-GFP* construct show a fully symmetric bicaudal phenotype, whereas the phenotype produced by the wild type construct was more variable (20–75% bicaudal). This difference correlates with the amount of Miranda protein produced by each construct since western blots of ovaries show that wild type Mira-GFP is present at lower levels than MiraΔ103-GFP (Supplementary Fig. 1).

A bicaudal phenotype can arise in at least three different ways: (1) mutants such as the dominant alleles of *Bicaudal-D* cause the partial mis-localization of *osk* mRNA to the anterior of the oocyte (Ephrussi et al., 1991; Wharton and Struhl, 1989). This leads to a bicaudal phenotype because Oskar protein recruits pole plasm components to the anterior, including *nanos* mRNA, leading to the translational repression of the anterior determinants *bcd* and *hunchback* mRNAs by Nanos protein (Wharton and Struhl, 1991). (2) A similar phenotype is produced by the over-expression of *osk* mRNA or mutants that cause the premature translation of the mRNA, such as *Bi-caudal-C* (Cinnamon et al., 2004; Mahone et al., 1995; Smith et al., 1992). In these cases, *osk* mRNA is still localized normally to the posterior pole, but the protein levels are increased. (3) Finally, embryos that are mutant for both maternal and zygotic *hunchback* also display a bicaudal phenotype, without any effect on *osk* mRNA or protein (Lehmann and Nüsslein-Volhard, 1987).

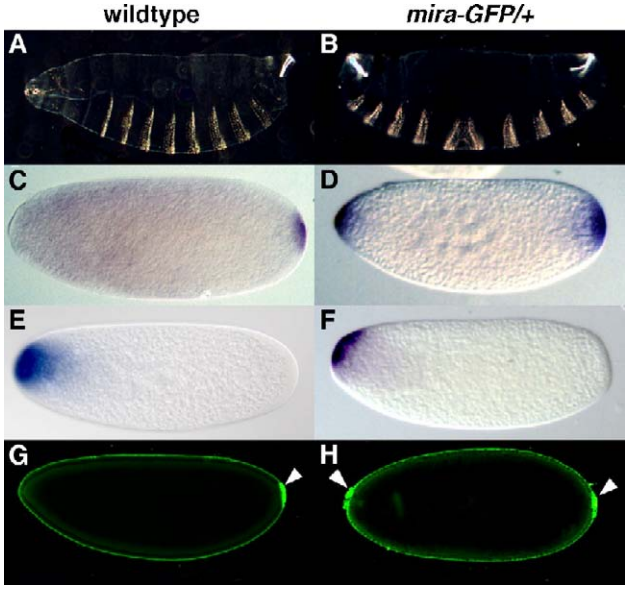

Fig. 1. *mira-GFP* expression in the female germ line results in a bicaudal phenotype. (A, B) Cuticle preparations of a wild type embryo (A) and a bicaudal embryo from a *mira-GFP* expressing female (B), in which an anterior abdomen replaces the head and thorax. (C, D) *osk* mRNA localization in a wild type (C) and a *mira-GFP* embryo (D). *osk* mRNA is ectopically localized to the anterior pole in the *mira-GFP* embryo, in addition to its normal localization to the posterior pole. (E, F) *bcd* mRNA localization in wild type (E) and *mira-GFP* (F). (G, H) Vasa staining to mark the pole cells (indicated by white arrowheads) in wild type (G) and *mira-GFP* (H) syncytial blastoderm embryos. *mira-GFP* embryos develop ectopic anterior pole cells.

To investigate the mechanism by which Miranda induces an anterior abdomen, we examined the localization of *osk* and *bcd* mRNAs in the eggs laid by Miranda-expressing females. These show a clear anterior accumulation of *osk* mRNA, as well as the wild type crescent at the posterior pole (Figs. 1C, D). In contrast, *bcd* mRNA is localized normally to the anterior of these eggs but disappears prematurely by the syncytial blastoderm stage (Figs. 1E, F). Thus, Miranda causes a bicaudal phenotype by a similar mechanism to the dominant alleles of *Bicaudal-D*, in which anterior *osk* mRNA induces the anterior localization and translation of *nanos* mRNA. The early disappearance of *bcd* mRNA is consistent with this since the repression of *bcd* mRNA translation by Nanos results in its degradation.

Although Miranda induces a similar bicaudal phenotype to Bic-D^{Dom}, it is significantly stronger because the embryos not only develop a second abdomen, but also form ectopic anterior pole cells, which are marked by the accumulation of Vasa protein (Figs. 1G, H). This difference may reflect the fact that *osk* mRNA is more tightly localized to the anterior pole of the former, and the diffuse anterior localization in Bic-D^{Dom} does

not produce a high enough local concentration of pole plasm to induce pole cell formation. Indeed, the fully penetrant symmetric bicaudal phenotype induced by Miranda has only been previously observed in embryos derived from females expressing an *osk-bcd* 3′ UTR transgene, in which *osk* mRNA is targeted to the anterior by the *bcd* localization signal (Ephrussi and Lehmann, 1992).

Miranda targets Staufen/osk mRNA complexes to the anterior of the oocyte

To investigate how Miranda causes the mis-localization of *osk* mRNA, we examined the distribution of *osk* mRNA during oogenesis. The mRNA accumulates normally in the oocyte up until stages 7–8, but from stage 9 onwards, *osk* mRNA is found at the anterior of the oocyte, as well as in the normal crescent at the posterior pole (Figs. 2A–D). Furthermore, Mira-GFP also localizes to the anterior and posterior of the oocyte from stage 9 onwards (Figs. 2E, F). To visualize the localization of Miranda and the *osk* mRNA complex in the same egg chambers, we stained *mira-GFP* expressing ovaries for Staufen protein, which associates with *osk* mRNA throughout oogenesis. Staufen and Miranda become concentrated in the oocyte from germarial stages onwards and co-localize perfectly at both the anterior and posterior of the oocyte after stage 8 (Figs. 2G, I, K). Thus, Miranda appears to associate with the *osk* mRNA localization complex and diverts a proportion of it to the anterior of the oocyte, instead of the posterior pole.

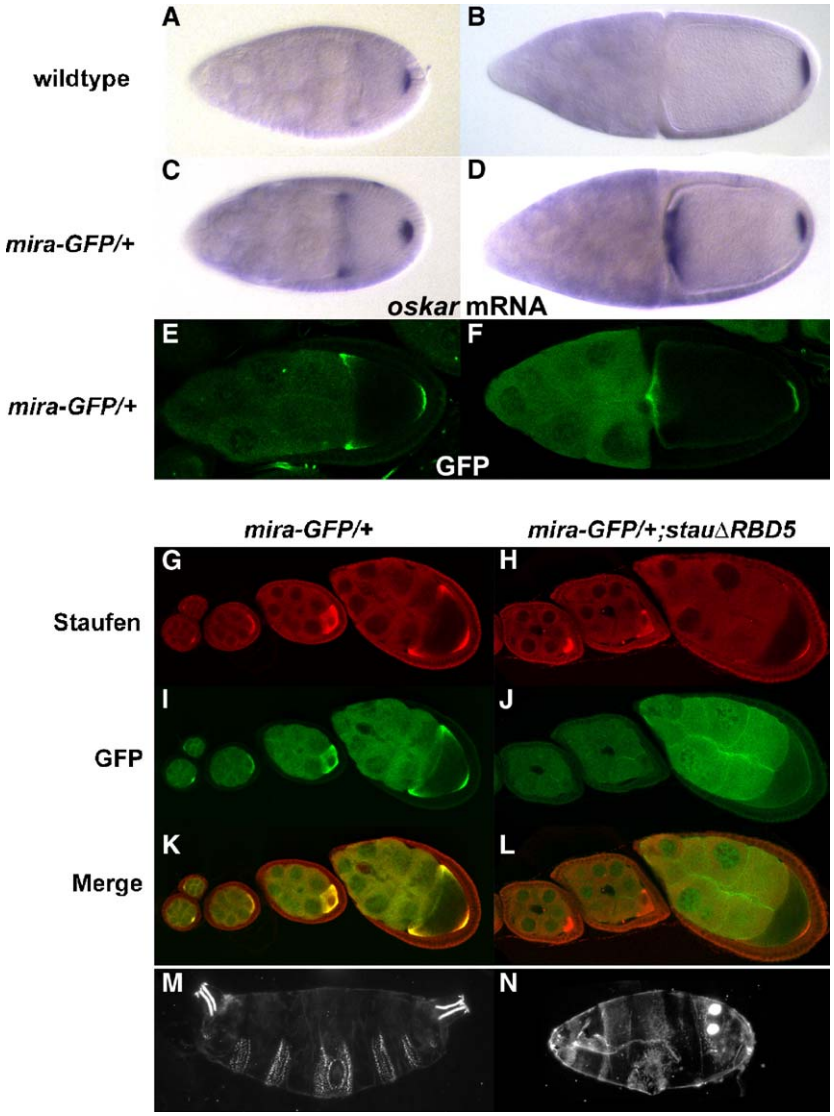

Fig. 2. Miranda targets *osk* mRNA and Staufen to the anterior of the oocyte. (A, B) *osk* mRNA localization in wild type oocytes at stage 9 (A) and stage 10B (B). (C, D) *osk* mRNA localization in *mira-GFP* oocytes at stage 9 (A) and stage 10B (D). The mRNA localizes to the anterior as well as to the posterior pole. (E, F) Mira-GFP in stage 9 (E) and stage 10B (F) oocytes. Miranda co-localizes with *osk* mRNA at both poles. (G–L) The localization of Staufen (G, H) and Mira-GFP (I, J) in *mira-GFP* (G, I) and *mira-GFP; stau^{D3}, P{w^{+},stauΔRBD5}* egg chambers. Mira-GFP does not co-localize with Staufen that lacks dsRBD5 and fails to accumulate in the oocyte. Panel K shows the merges of panels G and I, and panel L the merge of panels H and J. (M, N) Cuticle preparations of embryos laid by a *mira-GFP* (M) and a *mira-GFP; stau^{D3}, P{w^{+},stauΔRBD5}* female (N). The removal of dsRBD5 from Staufen suppresses the *mira-GFP* bicaudal phenotype, resulting in embryos that show the typical posterior group phenotype of *stauΔRBD5*.

Miranda binds directly to Staufen, which in turn binds to *osk* mRNA, suggesting that Miranda may mis-target *osk* mRNA to the anterior through its interaction with Staufen protein. We therefore examined the effects of removing the Miranda-binding domain of Staufen, dsRBD5, on the localization of Staufen and Miranda. The truncated Staufen protein no longer localizes to the anterior in the presence of Miranda, but still localizes to the posterior normally as this domain is not required for the kinesin-dependent transport to the posterior pole (Fig. 2H). In contrast, Miranda protein fails to accumulate in the oocyte (Figs. 2J, L). As a consequence, StaufenΔdsRBD5 completely suppresses the Miranda bicaudal phenotype, and the resulting embryos develop a normal head and thorax, but lack the abdomen, because Staufen dsRBD5 is required for the translation of *osk* mRNA at the posterior pole (Figs. 2M, N) (Micklem et al., 2000). These results reveal that Miranda associates with the *osk* mRNA complex through dsRBD5 of Staufen and then hitchhikes with it into the oocyte. Miranda then targets some of the Miranda/Staufen/*osk* mRNA complexes to the anterior cortex, while the rest are carried to the posterior of the oocyte by the normal localization pathway.

Translation of osk mRNA at the anterior of the oocyte

The translation of *osk* mRNA is normally very tightly regulated, so that it is repressed before it is localized and is only activated once it reaches the posterior pole (Gunkel et al., 1998; Kim-Ha et al., 1995; Rongo et al., 1995). To produce a bicaudal phenotype, this translational repression must be overridden to express Oskar protein at the anterior. Miranda binds to the domain of Staufen that is required for the de-repression of *osk* mRNA translation, raising the possibility that Miranda inappropriately activates *osk* mRNA translation. Indeed, precocious translation could account for the mis-localization of *osk* mRNA to the anterior since Oskar protein anchors its own mRNA to the cortex, and *osk* mRNA and Staufen normally show a transient accumulation at the anterior before they localize to the posterior pole (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991; Vanzo and Ephrussi, 2002). We therefore examined the localization of Miranda and *osk* mRNA in *miraΔ103-GFP, osk^{54}/Df* females, which carry a protein null mutation in *oskar*. Although neither Mira-GFP nor *osk* mRNA accumulate at the posterior of these oocytes because of the lack of anchoring by Oskar protein, they both still localize to the anterior cortex (Figs. 3A, C). Thus, the mis-localization of *osk* mRNA does not require Oskar protein, suggesting that Miranda targets Staufen/*osk* mRNA complexes to the anterior by another mechanism.

To test directly whether Miranda activates *osk* mRNA translation at the anterior, we examined the distribution of Oskar protein. Although there is always a clear crescent of Oskar protein at the posterior of Mira-GFP expressing oocytes, no protein is detectable at the anterior (data not shown). Since Oskar protein must be produced at some stage to generate a bicaudal phenotype and antibodies do not reliably penetrate into the oocyte after stage 10B, we crossed an *osk-LacZ* construct into *miraΔ103-GFP* females as a reporter for *osk* mRNA translation (Gunkel et al., 1998). As seen with the antibody, β-galactosidase activity is detected at just the posterior of the oocyte at stages 9–10, despite the symmetrical localization of the mRNA to the anterior and posterior poles (Fig. 3B). However, β-galactosidase is expressed at the anterior in stage 14 oocytes (Fig. 3D). Thus, the mis-localized *osk* mRNA is subject to the normal translational repression during stages 8–13 of oogenesis and is only translated at the anterior in mature oocytes.

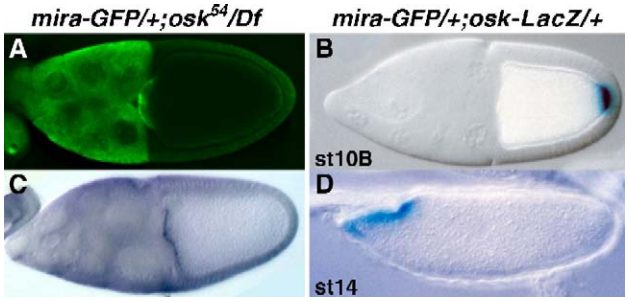

Fig. 3. *osk* mRNA is not translated at the anterior of *mira-GFP* egg chambers until stage 14. (A, C) Mira-GFP (A) and *osk* mRNA (C) in *mira-GFP; osk^{54}/Df(3R) pXT103* egg chambers (Oskar protein null mutant). Both Mira-GFP and *osk* mRNA still localize to the anterior, indicating that Oskar protein is not required for transport and anchoring at the anterior cortex. Neither localizes to the posterior of these egg chambers because Oskar is required to anchor its own RNA. (B, D) β-galactosidase staining in stage 10B (B) and stage 14 (D) *mira-GFP; osk-LacZ* egg chambers. The *osk-LacZ* construct is a reporter for Oskar translation and reveals that the mRNA that is targeted to the anterior by Mira-GFP is only translated at stage 14, whereas the posterior mRNA is translated at stage 9 as in wild type.

Miranda couples Staufen/osk mRNA complexes to a microtubule-dependent localization pathway

Since the localization of Miranda and Staufen to the basal side of the neuroblast is an actin-dependent process, we tested whether this is also the case for their localization to the anterior of the oocyte. However, both Mira-GFP and Staufen still accumulate at the anterior of oocytes treated with the actin-depolymerizing drug Latrunculin A, although this localization is slightly more diffuse than in the ethanol-treated controls (Figs. 4A, B). This subtle change in localization is not specific to Miranda as the anterior ring of *bcd* mRNA also becomes more diffuse after Latrunculin A treatment (data not shown). In contrast, the posterior crescents of Miranda and Staufen disappear, consistent with the requirement for actin for the anchoring of the *osk* mRNA complex (Babu et al., 2004). To confirm the efficacy of the Latrunculin A, we also made time-lapse films of the treated oocytes and observed the expected premature cytoplasmic streaming caused by the depolymerization of the actin cytoskeleton (data not shown) (Wellington et al., 1999).

The lack of a requirement for actin suggested that the anterior localization of Miranda might instead depend on the microtubule cytoskeleton. This is indeed the case as, this Miranda localization is abolished by the microtubule-depolymerizing drug colcemid, which also disrupts its posterior localization with Staufen and *osk* mRNA and the positioning of the nucleus at the dorsal/anterior corner of the oocyte (Figs. 4C, D).

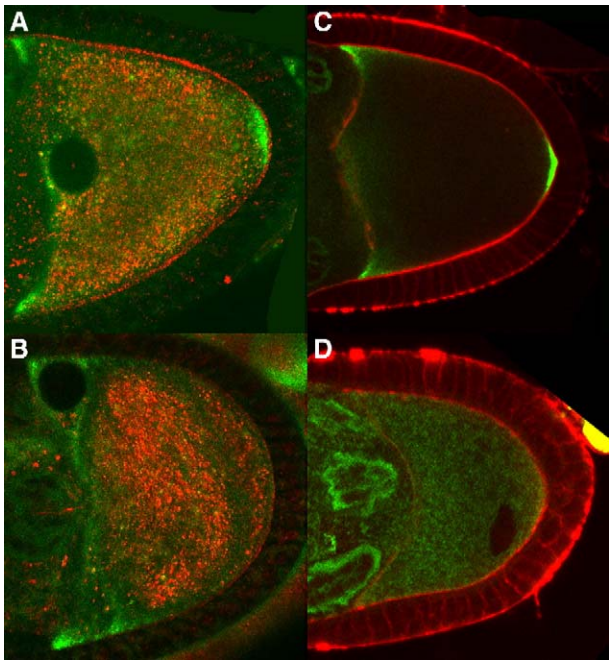

Fig. 4. The anterior localization of Mira-GFP is microtubule-dependent. (A) Mira-GFP localization in a control stage 9 egg chamber treated with buffer alone. (B) Miranda-GFP localization in a stage 9 oocyte after treatment with the actin-depolymerizing drug Latrunculin A. The red in panels A and B shows autofluorescent particles that act as markers for the cytoplasmic movements. Actin depolymerization triggers premature cytoplasmic streaming in the oocyte and disrupts the posterior localization of Mira-GFP. Mira-GFP still localizes to the anterior of these oocytes but is more diffuse than in the control. (C, D) Mira-GFP (green) in a control egg chamber (C) and an egg chamber treated with the microtubule depolymerizing drug colcemid (D). Actin is stained with TRITC-Phalloidin (red) to mark the outlines of the cells. Microtubule depolymerization disrupts the localization of Mira-GFP to both the anterior and posterior of the oocyte and results in the mis-positioning of the oocyte nucleus (dark disc near the posterior pole in (D)).

The anterior localization of Miranda/Staufen/*osk* mRNA complexes is very similar to that of *bcd* mRNA, which also localizes to the anterior of the oocyte in a microtubule-dependent manner from stage 8 to 9 to the end of oogenesis. We therefore examined whether mutants that disrupt *bcd* mRNA localization also affect the Miranda-dependent bicaudal phenotype caused by the mis-localization of *osk* mRNA. *exuperantia* (*exu*) mutants abolish the localization of *bcd* mRNA from stage 9 of oogenesis onwards, resulting in embryos that lack anterior head structures (Frohnhöfer and Nüsslein-Volhard, 1987; St Johnston et al., 1989). *mira*Δ*103-GFP/+; exu* females produce embryos with a typical *exu* phenotype, in which the thorax and abdomen develop normally, but the head is absent (Figs. 5A, D). Thus, *exu* suppresses the Miranda gain-of-function bicaudal phenotype. Consistent with this, *osk* mRNA is not mis-localized to the anterior of the oocyte or embryo, while its localization to the posterior is unaffected (Figs. 5B, C, E, F).

bcd mRNA localizes normally to the anterior of *swallow* (*swa*) mutants at stage 9 but is then released at stage 10B to form a shallow anterior to posterior gradient in the embryo, and this results in a milder phenotype, in which the head is reduced but not lost (Frohnhöfer and Nüsslein-Volhard, 1987; St Johnston et al., 1989). Embryos from *swa; mira*Δ*103-GFP/+* females show the same phenotype as *swallow* alone, indicating that *swallow* is also epistatic to Miranda gain-of-function. Like *bcd* mRNA, *osk* mRNA and Miranda initially localize to the anterior of the oocyte, but they are released at stage 10B and diffuse to form a gradient in the embryo (Figs. 5G–I). The localization of *osk* mRNA and Miranda at the posterior of the embryo is also more diffuse than in wild type, as has been previously observed for Staufen protein (Ferrandon et al., 1994). Thus, two mutants that specifically disrupt the anterior localization of *bcd* mRNA have an identical effect on the anterior localization of *osk* mRNA by Miranda, strongly suggesting that Miranda targets Staufen/*osk* mRNA complexes to the anterior through the same localization pathway as *bcd* mRNA.

Staufen associates with *bcd* mRNA, as well as *osk* mRNA, albeit at a later stage of oogenesis and can also dimerize in vitro (Ferrandon et al., 1994) (J.A. and D.St J., unpublished results). This suggests that one way that Miranda might couple Staufen and *osk* mRNA to the *bcd* pathway is by inducing the precocious formation of Staufen/*bcd* mRNA complexes, which then dimerize with Staufen/*osk* mRNA complexes, so that the latter can hitchhike with *bcd* mRNA to the anterior. If this is the case, the anterior localization of *osk* mRNA by Miranda should depend on *bcd* mRNA, and this can be tested by using a viable combination of a *serendipity-δ* mutant and a *serendipity-δ/β* transgene in which *bcd* mRNA is not transcribed (*sry-δ*^{14}*/DfX3F,P{ry*$^{+}$*,sryDB56}*) (Payre et al., 1994). However, *osk* mRNA is still localized to both the anterior and posterior poles of the oocyte in *mira*Δ*103-GFP; sry-δ*^{14}*/DfX3F,P{ry*$^{+}$*, sryDB56}*, and the resulting embryos display a fully penetrant bicaudal phenotype (Fig. 5J). *bcd* mRNA is therefore not required for the anterior localization of *osk* mRNA by Miranda, indicating that Miranda couples Staufen/*osk* mRNA complexes to the *bcd* localization pathway, but not to *bcd* mRNA itself.

Requirements for the interaction between Staufen and osk mRNA

Since Staufen contains four functional dsRNA-binding domains, it is thought to bind to *osk* mRNA by recognizing multiple stems in its 3′ UTR. It has been difficult to determine which domains are necessary for this interaction, however, because there is no vitro assay for binding, and it is hard to distinguish in vivo between mutations that abrogate RNA binding and those that still recognize the RNA, but cannot mediate its localization to the posterior pole. The anterior localization of Staufen/*osk* mRNA complexes by Miranda provides a convenient way to separate these steps since it is independent of posterior localization and thus only measures the ability of Staufen to bind stably to *osk* mRNA. We therefore crossed *mira*Δ*103-GFP* into the two mutant Staufen constructs that have previously been shown to block the localization of Staufen and *osk* mRNA to the posterior: deletion of the insertion in dsRBD2 (StauΔloop2) and mutations in the five amino acids in dsRBD3 that are required for its binding to dsRNA (Micklem et al., 2000; Ramos et al., 2000). In both cases, MiraΔ103-GFP and *osk* mRNA localize to just the anterior of the oocyte, resulting in embryos that

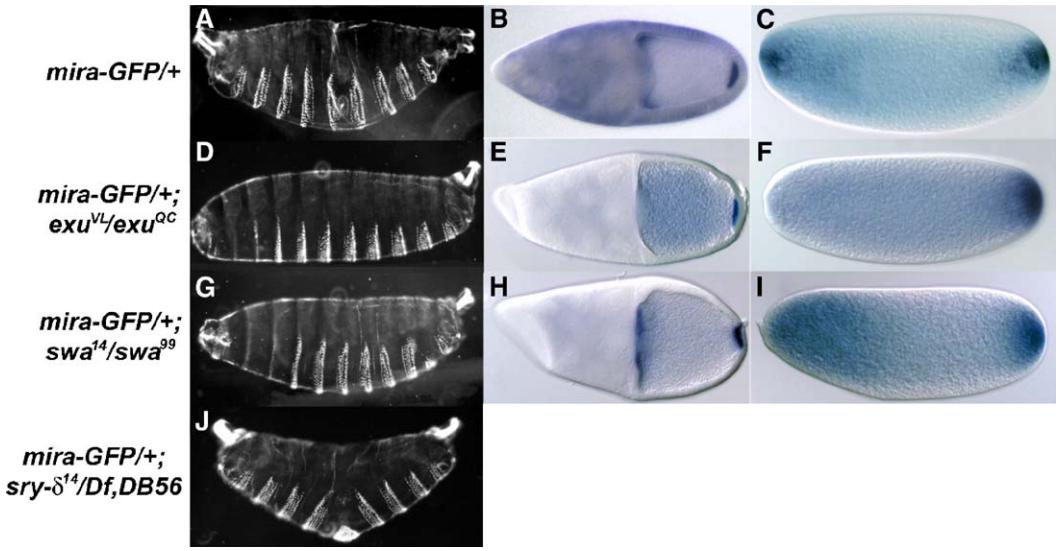

Fig. 5. The anterior localization *osk* mRNA by Miranda requires Exu and Swallow, but not *bcd* mRNA. (A–C) *mira-GFP.* Embryonic cuticle preparation (A); *osk* mRNA localization at stage 10A (B); *osk* mRNA localization in a freshly laid egg (C). (D–F) *mira-GFP; exu^{VL}/exu^{QC}*. The *mira-GFP* bicaudal phenotype is suppressed, and the embryos display only the *exu* phenotype in which the head is absent (D). *osk* mRNA localizes normally to the posterior of the oocyte but is not mis-localized to the anterior (E, F). (G–I) *swa^{14}/swa^{99}, mira-GFP.* The embryos have a reduced head as seen in *swa* alone (G). *osk* mRNA localizes to the anterior at stage 10A (H). The mRNA is not maintained at the anterior after stage 10B and is enriched only at the posterior of the freshly laid egg (I). (J) *mira-GFP; sry-δ^{14}/Df(3R) X3F,P{ry^{+},sryDB56}*. Mira-GFP still produces a bicaudal phenotype in the absence of *bcd* mRNA, indicating that Miranda/Staufen/*osk* mRNA complexes do not hitchhike to the anterior on *bcd* mRNA particles.

develop a complete abdomen with reverse polarity at their anterior, but no posterior abdomen (Figs. 6A–D and data not shown). This reveals that both mutant forms of Staufen still bind *osk* mRNA to couple it to Miranda, indicating that they specifically disrupt transport to the posterior.

The *osk* mRNA localization complex assembles in a stepwise manner, in which some components essential for its localization to the posterior of the oocyte are recruited in the nurse cell nuclei and others in the cytoplasm. HRP48 is thought to bind to the mRNA co-transcriptionally, the exon junction complex (EJC: Mago, Y14 and eIF4AIII) associates with the RNA when it is spliced, Barentsz protein is then recruited to the EJC as it is exported from the nucleus, while Staufen binds in the cytoplasm (reviewed in St Johnston, 2005). This raises the possibility that the binding of Staufen to *osk* mRNA might depend on some of these other factors, either because they interact with Staufen directly or because they help to fold the RNA to form double-stranded stems in the correct positions. We were not able to test *hrp48* mutants since these are homozygous lethal, and the dominant female sterility caused by Miranda expression makes it impossible to use the ovoD/FLP/FRT system to generate germ line clones. However, MiraΔ103-GFP still directs the localization of Staufen and *osk* mRNA to the anterior of *mago* and *btz* mutant oocytes, resulting in embryos with an anterior abdomen with reverse polarity and no posterior abdomen (Figs. 6E, F and data not shown). The exon junction complex is therefore not required for the interaction between Staufen and *osk* mRNA. Finally, we crossed *miraΔ103-GFP* into a *tropomyosin II* mutant (Erdélyi et al., 1995), which also disrupts the posterior localization of Staufen and *osk* mRNA, and observed an identical reversed polarity phenotype. Thus, the anterior localization of Staufen/*osk* mRNA complexes by Miranda is independent of all of the other factors required for transport to the posterior pole that we have analyzed.

Discussion

Although Miranda is not required during oogenesis, its ectopic expression causes a striking defect in anterior–posterior

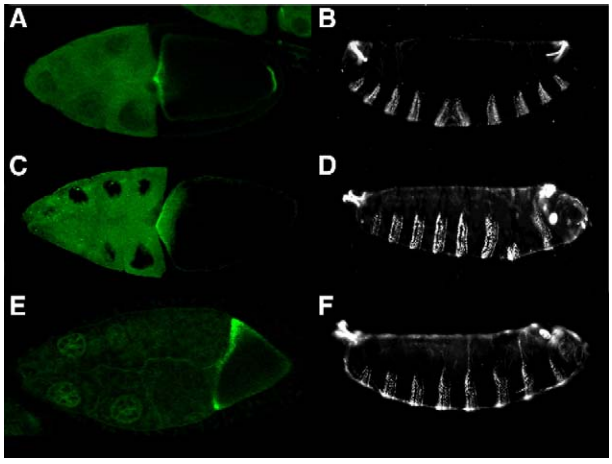

Fig. 6. The binding of Staufen to *osk* mRNA does not require dsRBD2 or 3 or the Exon junction complex. (A, B) *mira-GFP.* Mira-GFP localizes to the anterior and posterior poles of the oocyte (A) and gives rise to bicaudal embryos (B). (C, D) *mira-GFP; stau^{D3}, P{w^{+},StauΔloop2}*. The removal of the insert in Staufen dsRBD2 prevents the posterior localization of Mira-GFP (C) and *osk* mRNA, resulting in embryos that form a complete reverse polarity abdomen at the anterior with no posterior abdomen (D). (E, F) *mira-GFP; mago^{1}/Df(2R)F36*. Mira-GFP (E), Staufen and *osk* mRNA still localize to the anterior in *mago* mutants to give rise to embryos with anterior abdomens (F), indicating that Mago is not required for the binding of Staufen to *osk* mRNA.

axis formation that reveals several important features of the mechanisms that control the targeting and translation of localized mRNAs. Firstly, our results provide strong support for the idea that the destination of Staufen/mRNA complexes is determined by the Stau-interacting factors that are present in the cell (Fig. 7). During wild type oogenesis, Staufen associates with *osk* mRNA to mediate its kinesin-dependent localization to the posterior of the oocyte at stage 9, and this requires the insertion in Staufen dsRBD2, suggesting that this domain couples Staufen/*osk* mRNA complexes to the posterior localization pathway (Fig. 7A). However, the expression of Miranda is sufficient to target a proportion of these complexes to the anterior. This localization is mediated through the binding of Miranda to dsRBD5 of Staufen because deletion of this domain abolishes anterior localization without affecting the transport to the posterior pole (Figs. 7B, C). By contrast, deletion of the insert in dsRBD2 in the presence of Miranda results in the localization of all Staufen/*osk* mRNA complexes to the anterior pole (Fig. 7D). Thus, these two pathways act through different domains of Staufen to direct localization to opposite ends of the same cell. These pathways compete with each other, resulting in the partitioning of the Miranda/Staufen/*osk* mRNA complexes to the anterior and posterior poles, but each is capable of localizing all of the complexes when the other pathway is compromised. *exu* and *swa* mutants abolish the Miranda-dependent anterior localization, and *osk* mRNA now localizes exclusively to the posterior, whereas *btz*, *mago* and *TmII* mutants block the posterior localization pathway, resulting in the localization of all *osk* mRNA at the anterior cortex and the formation of reverse polarity embryos.

Since dsRBD5, which is not an RNA-binding domain (Micklem et al., 2000), is necessary and sufficient for the interaction of Staufen with Miranda, the anterior localization of *osk* mRNA by Miranda provides a simple in vivo assay for the binding of Staufen to *osk* mRNA. This reveals that neither the insert of dsRBD2 nor the RNA-binding residues of dsRBD3 are required for the stable association of Staufen with the RNA. The lack of a requirement for the insert in dsRBD2 is consistent with the observation that dsRBD2Δloop binds dsRNA in vitro when expressed on its own, whereas the full-length dsRBD2 does not (Micklem et al., 2000). It is more surprising, however, that the mutations in dsRBD3 have no effect on Staufen binding to *osk* mRNA since this domain binds to dsRNA with the highest affinity in vitro, and these mutations in the five key amino acids that contact the RNA abolish the domain's RNA-binding activity in vitro (Ramos et al., 2000). The two other functional dsRNA-binding domains in Staufen (dsRBD1 and 4) must therefore be sufficient to form a stable complex with *osk* mRNA.

The specific effect of the quintuple mutant in dsRBD3 on posterior localization, but not on RNA binding of full-length Staufen, further suggests that these five amino acids play a role in coupling Staufen/*osk* mRNA complexes to the posterior localization pathway. Although it is possible that these residues are required for an interaction with a *trans*-acting factor, it seems more likely that it is the association of dsRBD3 with the RNA that is important because this affects either the folding of the RNA or the conformation of Staufen protein. For example, it has been suggested that the binding of Staufen dsRBDs1, 3 and 4 to *osk* mRNA presents a double-stranded region of the RNA

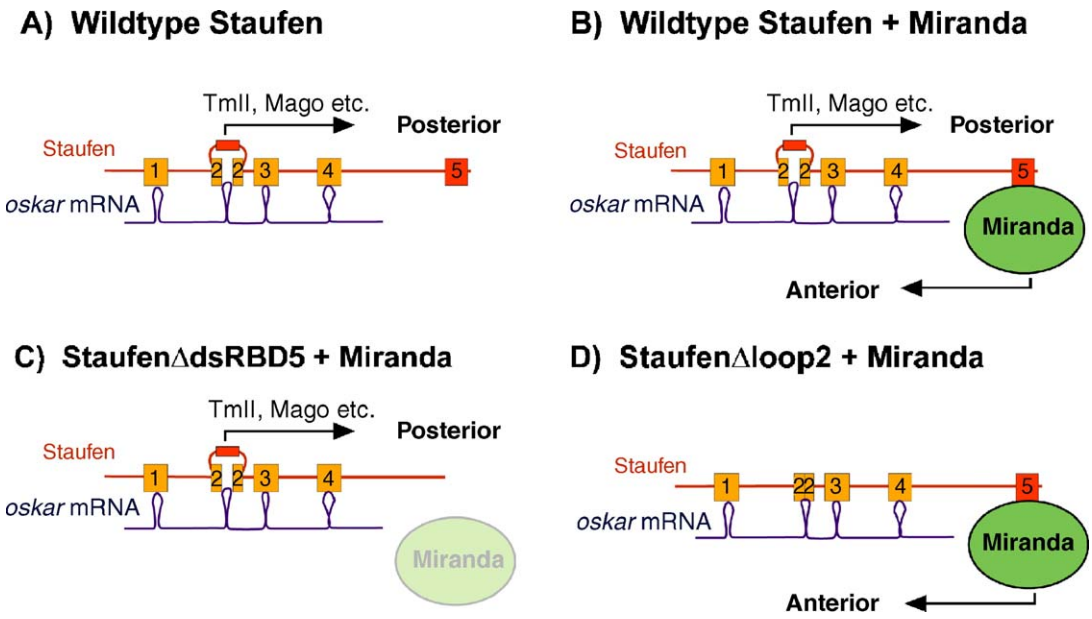

Fig. 7. A model for the targeting of Staufen/*osk* mRNA complexes in wild type and Miranda-expressing oocytes. (A) Wild type Staufen. The binding of *osk* mRNA to dsRBDs1, 3 and 4 of Staufen induces dsRBD2 to adopt an RNA-binding conformation, which loops out the insertion in the domain required to couple Staufen/*osk* mRNA to the posterior localization pathway. (B) Wild type Staufen + Miranda. Miranda binds to Staufen dsRBD5 to couple Staufen/*osk* mRNA complexes to the anterior localization pathway. This pathway competes with the normal posterior localization pathway, resulting in the partitioning of Miranda, Stau and *osk* mRNA between the anterior and posterior poles. (C) StaufenΔdsRBD5 + Miranda. Miranda cannot bind to Staufen lacking dsRBD5, and Staufen and *osk* mRNA therefore only localize to the posterior. (D) StaufenΔloop2 + Miranda. Deletion of the loop in Staufen dsRBD2 prevents the posterior localization of Staufen/*osk* mRNA complexes and allows Miranda to target all complexes to the anterior.

to dsRBD2, which induces a conformational change in dsRBD2 that brings together the two RNA-binding regions of the domain and loops out the large insertion, which is then exposed to interact with the transport machinery (Micklem et al., 2000). The effect of the point mutations in dRBD3 is consistent with this model and the idea that dsRBD2 functions as an RNA-binding sensor that couples Staufen/*osk* mRNA complexes to factors that target it to the posterior.

Although all mRNAs that accumulate in the oocyte localize at least transiently to the anterior, several lines of evidence indicate that Miranda links Staufen and *osk* mRNA specifically to the *bcd* localization pathway. Firstly, all other anterior mRNAs, except *bcd* and *hu li tai shao* (*hts*), only localize to the anterior during stages 9–10A and become delocalized at stage 10B when rapid cytoplasmic streaming begins. In contrast, Miranda maintains *osk* mRNA at the anterior throughout oogenesis, so that it is still localized in a tight anterior cap in the freshly laid egg. Secondly, Miranda, Staufen and *oskar* undergo the same change in their anterior localization at stage 10B as *bcd* mRNA: they initially localize as a ring around the anterior cortex and then move towards the middle of the anterior when the centripetal follicle cells start to migrate inwards (Figs. 2C, D; St Johnston et al., 1989). Finally, like *bcd*, the anterior localization of *osk* mRNA by Miranda requires Exu, Swallow and Staufen, whereas *hts* mRNA localization is independent of Exu and Staufen (Ding et al., 1993; St Johnston et al., 1989; Whittaker et al., 1999). Since the anterior localization does not require *bcd* mRNA itself, Miranda cannot simply hitchhike on the *bcd* mRNA localization complex, and it therefore presumably links *osk* mRNA to the same microtubule-dependent anterior transport pathway used by *bcd* mRNA.

In addition to its role in *osk* mRNA localization, Staufen associates with *bcd* mRNA during the late stages of oogenesis to mediate the final steps in its localization to the anterior cortex of the oocyte. Since this localization requires the Miranda-binding domain of Staufen and Miranda couples Staufen/mRNA complexes to the *bcd* localization pathway, it is attractive to propose that Miranda normally mediates the late anterior localization of *bcd* mRNA. *mira* mutants have no phenotype during oogenesis, however, although the protein is expressed in late oocytes. Thus, if Miranda does play a role in *bcd* mRNA localization, it must function redundantly with another unidentified factor. This is perhaps to be expected given the previous evidence for redundancy in the localization of *bcd* mRNA. For example, none of the small deletions within the *bicoid* localization signal abolishes its anterior localization, indicating that it contains redundant localization elements (Macdonald and Kerr, 1997), and two distinct *bcd* mRNA recognition complexes have been purified biochemically from ovarian extracts (Arn et al., 2003).

The elucidation of the role of Miranda in *bicoid* mRNA localization will require the identification of other factors that couple Staufen/*bicoid* mRNA complexes to the anterior localization pathway, which may function redundantly with Miranda. There are no obvious candidates for these factors, however, since Staufen is the only known protein that is specifically required for the final step of *bicoid* mRNA localization. Indeed, one reason why such factors may have been missed in genetic screens for mutants that disrupt *bicoid* mRNA localization is because they are redundant with Miranda and have no phenotype on their own. For these reasons, it is hard to address the question of redundancy using a genetic approach, but further analysis of how Miranda targets Staufen/mRNA complexes to the anterior may help resolve this issue. For example, mapping the Miranda domains that direct anterior localization may provide a clue as to the molecular nature of the unidentified factors that also fulfil this function, while screens for proteins that interact with this domain could identify other components of the anterior localization pathway.

Our results reveal that Miranda, like Staufen, has the capacity to mediate both microtubule- and actin-dependent localization, raising the question whether the former plays any role in its well-characterized function during the asymmetric divisions of the embryonic neuroblasts. The localization of Miranda to the basal side of the neuroblast is actin-dependent. However, the protein also accumulates at the apical centrosome during both embryonic and larval neuroblast divisions, and this localization is even more prominent in *l(2)gl* or *dlg* mutants (Mollinari et al., 2002; Peng et al., 2000; Schuldt et al., 1998). Furthermore, Miranda was independently identified as a component of the pericentriolar matrix and co-localizes with γ-tubulin on all of the centrosomes at syncytial blastoderm stage (Mollinari et al., 2002). Although the centrosomes disappear in the female germ line, the anterior cortex is the major site for microtubule nucleation and γ-tubulin localization in the oocyte (Schnorrer et al., 2002; Theurkauf et al., 1992). Thus, Miranda may localize to the anterior of the oocyte by the same mechanism as it localizes to centrosomes.

osk mRNA translation and pole plasm formation

The phenotype of *mira-GFP* also provides insights into the translational control of *osk* mRNA. In wild type ovaries, *osk* mRNA is translationally repressed before it is localized, and this repression is then specifically relieved once the mRNA reaches the posterior pole (Wilhelm and Smibert, 2005). In principle, translational activation of *osk* mRNA could occur by a specific signal at the posterior, but it could also be due to some other consequence of localization, such as the concentration of the RNA in a small region or its association with the oocyte cortex. Evidence in favor of a specific posterior signal comes from an experiment in which a *LacZ* reporter gene under the control of the *oskar* 5′ region and the first 370 nt of the 3′ UTR was targeted to the anterior by the *bcd* localization element (Gunkel et al., 1998). Since this anterior RNA was not translated, concentration at the cortex appeared to be insufficient to relieve BRE mediated repression. However, it has recently emerged that this reporter RNA lacks the two clusters of IMP binding elements in the distal *oskar* 3′ UTR that are essential for *oskar* translational activation at the posterior, making it hard to draw any conclusions from the lack of translation of this reporter RNA at the anterior (Munro et al., 2006). Mira-GFP provides an alternative way to test this hypothesis because it directs the anterior localization of wild type *osk* mRNA, with all of its

translational control elements intact. This anterior mRNA is not translated during stages 9–13, despite being efficiently localized to the cortex, whereas the *osk* mRNA at the posterior of the same oocytes is translated normally. Thus, concentration at the cortex is not sufficient to de-repress translation, strongly supporting the idea that activation depends on a specific posterior signal.

Although the anterior *osk* mRNA is not translated at the normal time, the repression system breaks down at the very end of oogenesis, and the mRNA is very efficiently translated in mature oocytes. This suggests that some key component of the repression system disappears at this stage, and a good candidate is the BRE-binding protein Bruno. Bruno is highly expressed during oogenesis but is not detectable in embryos (Webster et al., 1997). Furthermore, the addition of Bruno is sufficient to cause the repression of exogenous *osk* mRNA in an embryonic translation system (Castagnetti et al., 2000; Lie and Macdonald, 1999). These results indicate that Bruno is degraded at the end of oogenesis, whereas all other components necessary for translational repression of *osk* mRNA are still present in the embryo. Thus, the translation of anterior *osk* mRNA in *mira-GFP* oocytes is most probably triggered by the disappearance of Bruno.

Once it is translated at the posterior of the oocyte, Oskar protein nucleates the formation of the pole plasm with its characteristic electron-dense polar granules, which gradually assemble during stages 9–14 of oogenesis. This appears to be a stepwise process, in which Oskar protein recruits some polar granule components as soon as it is translated at stage 9, such as Vasa and Fat facets, while other components are added in sequence during the rest of oogenesis (Breitwieser et al., 1996; Fischer-Vize et al., 1992). For example, Tudor, Capsuleen and Valois are recruited during stage 10A, whereas *nanos*, *Pgc* and *gcl* mRNAs only become enriched at the posterior at stages 10B–11 (Anne and Mechler, 2005; Bardsley et al., 1993; Cavey et al., 2005; Forrest and Gavis, 2003; Nakamura et al., 1996). It is therefore surprising that the anterior Oskar protein, which is only synthesized in stage 14 oocytes, can still nucleate fully functional pole plasm that induces the formation of anterior pole cells. Thus, although the pole plasm normally assembles in an ordered fashion over the last 5 stages of oogenesis, this whole process can still occur once oogenesis is complete. This indicates that the assembly of the pole plasm does not depend on the order of addition of its components, all of which must still be present and freely diffusible in mature oocytes.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.05.029.

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