Anterior-posterior axis specification in *Drosophila* oocytes: Identification of novel *bicoid* and *oskar* mRNA localisation factors.

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A Miranda-GFP suppressor screen

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ABSTRACT

The *Drosophila melanogaster* anterior-posterior axis is established during oogenesis by the localisation of *bicoid* and *oskar* mRNAs to the anterior and posterior poles of the oocyte. Although genetic screens have identified some trans-acting factors required for the localisation of these transcripts, other factors may have been missed because they also function at other stages of oogenesis. To circumvent this problem, we performed a screen for revertants and dominant suppressors of the bicaudal phenotype caused by expressing Miranda-GFP in the female germline. Miranda mislocalises *oskar* mRNA/Staufen complexes to the oocyte anterior by coupling them to the *bicoid* localisation pathway, resulting in the formation of an anterior abdomen in place of the head. In one class of revertants, Miranda still binds Staufen/*oskar* mRNA complexes, but does not localise to the anterior, identifying an anterior targeting domain at the N-terminus of Miranda. This has an almost identical sequence to the N-terminus of vertebrate RHAMM, which is also a large coiled-coil protein, suggesting that it may be a divergent Miranda orthologue. In addition, we recovered 30 dominant suppressors, including multiple alleles of the spectroplakin, *short stop*, a lethal complementation group that prevents *oskar* mRNA anchoring, and a female sterile complementation group that disrupts the anterior localisation of *bicoid* mRNA in late oogenesis. One of the single allele suppressors proved to be a mutation in the actin nucleator, Cappuccino, revealing a previously unrecognised function of Cappuccino in pole plasm anchoring and the induction of actin filaments by Long Oskar protein.
INTRODUCTION

The subcellular localisation of mRNAs is an important mechanism for restricting specific proteins to the region of a cell where they are required, and plays a key role in axis formation, synaptic plasticity and cell polarity (BECALSKA and GAVIS 2009; ST JOHNSTON 2005). Indeed, nearly 70% of all tested mRNAs show some pattern of localisation in the early Drosophila embryo, indicating this is a widespread means for protein targeting (LÉCUYER et al. 2007). mRNAs can be delivered to the correct destination in a variety of ways, such as local protection from degradation, diffusion and anchoring, or active transport along either the actin or microtubule cytoskeletons. One of the best characterised systems for studying the latter mechanism is the formation of the anterior-posterior axis in Drosophila, which is specified by the microtubule-dependent localisation of bicoid mRNA to the anterior of the oocyte and of oskar mRNA to the posterior (BASTOCK and ST JOHNSTON 2008). bicoid mRNA is translated at the anterior after egg activation to produce a protein gradient that acts as a morphogen to pattern the head and thorax of the embryo (EPHRUSSI and JOHNSTON 2004). oskar mRNA, on the other hand, is translated once it is localised to the posterior pole of the oocyte, where Oskar protein defines the site of pole plasm assembly, leading to the posterior recruitment of the abdominal determinant, nanos RNA (EPHRUSSI et al. 1991; KIM-HA et al. 1991).

Mutants that disrupt the localisation of bicoid mRNA produce embryos with defective heads, whereas oskar mRNA localisation mutants result in embryos without pole cells or an abdomen, and this has allowed the identification of a number of trans-acting factors in screens for maternal-effect lethal mutations (NUSSLEIN-VOLHARD et al. 1987; SCHUPBACH and WIESCHAUS 1989). One limitation of these screens is that they
could not identify zygotic lethal mutations in essential genes. This problem can be
circumvented, however, by using the FLP/FRT system to perform screens in germ
line clones, and additional factors have been identified in such screens for mutants
with embryonic patterning phenotypes, as well as more targeted screens for mutations
that disrupt the localisation of GFP-Staufen, an RNA-binding protein that associates
with oskar and bicoid mRNAs (Chou and Perrimon 1996; Luschnig et al. 2004;
Martin et al. 2003).

Analyses of the mutants that affect oskar mRNA localisation have revealed that its
localisation to the posterior of the oocyte occurs in multiple steps. The RNA is
transcribed in the nurse cells of the germline cyst and first moves along microtubules
through the ring canals to the anterior of the oocyte in a process that is probably
mediated by dynein (Clark et al. 2007). The mRNA is then transported by kinesin
along a weakly polarised microtubule cytoskeleton to the oocyte posterior (Brendza
et al. 2000; Zimyanin et al. 2008). This step requires a number of factors that
associate with the RNA, including HRP48, Tropomyosin II, the exon junction
complex components, Mago nashi, Y14, Barentsz and eIF4AIll, and the dsRNA-
binding protein, Staufen (Hachet and Ephrussi 2001; Hachet and Ephrussi 2004;
Mohr et al. 2001; Newmark and Boswell 1994; Palacios et al. 2004).

Once localised, oskar mRNA is translated from two alternative in frame start codons
to produce long and short forms of Oskar that have distinct functions. Long Oskar is
required for the anchoring of oskar mRNA and all other pole plasm components,
whereas Short Oskar nucleates the formation of pole plasm (Vanzo and Ephrussi
2002). Oskar stimulates endocytosis at the posterior pole by recruiting a number of
endocytic factors and also induces the formation of long actin filaments, and both of these activities are thought to be required for anchoring, although the exact mechanisms involved have yet to be resolved. (TANAKA and NAKAMURA 2008; VANZO and EPHRUSSI 2002; VANZO et al. 2007).

The localisation of bicoid mRNA also occurs in multiple steps during oogenesis, although genetic screens have been less successful at identifying the necessary factors (ST JOHNSTON et al. 1989). Like oskar, bicoid mRNA is transcribed in the nurse cells and transported by dynein into the oocyte (CLARK et al. 2007; MISCHE et al. 2007). It is then localised to the anterior cortex of the oocyte in a poorly understood process that requires Exu protein (BERLETH et al. 1988; CHA et al. 2001). Several additional proteins are required to keep bicoid mRNA at the anterior of the oocyte after stage 10a of oogenesis, including Swallow, γ-tubulin 37C, the γ-tubulin ring complex components, GRIP75 and GRIP128 and the dynein light chain (SCHNORRER et al. 2002; ST JOHNSTON et al. 1989; VOGT et al. 2006; WEIL et al. 2006; WEIL et al. 2010).

Although the RNA-binding proteins that recognize bicoid mRNA to mediate early localisation have not been identified, these latter stages require Staufen and the ESCRT II complex, which binds specifically to a region of the bicoid 3’UTR (FERRANDON et al. 1994; IRION and ST JOHNSTON 2007). The localisation of bicoid mRNA changes again in mature oocytes, and the RNA becomes stably anchored to the actin cortex until the egg is activated, which releases bicoid mRNA/Staufen complexes into the egg cytoplasm and activates Bicoid translation (WEIL et al. 2008).
Although many of the factors required for \textit{oskar} and \textit{bicoid} mRNA localisation have already been identified, there are still a number of important gaps in our understanding of these processes. For example, it is not known how \textit{oskar} mRNA translation is activated at the posterior of the oocyte or how increased endocytosis and actin filaments collaborate to anchor the mRNA, while only one of the specific factors required for the first phase of \textit{bicoid} mRNA localisation has been identified so far. There are several reasons why some important localisation factors may have been missed in previous genetic screens. Some mutants might block development before the phenotype could be scored in the oocyte or embryo, or might lie in regions of the genome that have not been screened in germline clone screens. As an alternative approach, we have performed a screen for dominant suppressors of a dominant bicaudal phenotype caused by expressing a Miranda-GFP fusion protein in the female germline that allows us to screen the entire genome at the same time. Because the suppressor mutations are heterozygous, this screen can also identify lethal mutations and mutants with pleiotropic phenotypes that mask an effect on mRNA localisation.

Miranda is a large adaptor protein that mediates the basal targeting of the cell fate determinants, Prospero and Brat during asymmetric neuroblast divisions (\textsc{Ikeshima-Kataoka et al.} 1997; \textsc{Shen et al.} 1997). In addition, Miranda binds directly to the C-terminal domain of Staufen to localise Staufen/prospero mRNA complexes to the basal cortex of the neuroblast (\textsc{Broadus et al.} 1998; \textsc{Matsuzaki et al.} 1998; \textsc{Schuldt et al.} 1998; \textsc{Shen et al.} 1998). Miranda is not normally expressed in the female germline, but binds to Staufen/oskar mRNA complexes when expressed ectopically, and localises with them to both the anterior and posterior of the oocyte (\textsc{Irion et al.} 2006). While the posterior localisation reflects Miranda hitch-hiking on
the normal *oskar* mRNA localisation pathway, the anterior localisation occurs because Miranda couples Staufen/*oskar* mRNA complexes to the *bicoid* mRNA localisation pathway, leading to the anterior formation of pole plasm and the recruitment of *nanos* RNA. This results in the development of embryos with pole cells at both ends and an anterior abdomen in place of the head and thorax because Nanos blocks the translation of *bicoid* and *hunchback* RNAs (EPHRUSI and LEHMANN 1992; WHARTON and STRUHL 1991).

Although the Miranda-GFP bicaudal phenotype is highly penetrant, it seems to be a good system to select for suppressors, because the normal posterior localisation pathway and the Miranda-dependent anterior localisation pathway compete for Staufen/*oskar* mRNA complexes. Any mutants that partially impair the anterior pathway will therefore result in most *oskar* mRNA localising posteriorly, thereby suppressing the phenotype. The phenotype is also very sensitive to the amount of Oskar protein produced anteriorly, and mutants that reduce Oskar translation or anchoring would also be predicted to be suppressors. We therefore performed a larger scale screen for dominant suppressors of the female sterility caused by Miranda-GFP in order to identify novel factors involved in *bicoid* mRNA localisation and *oskar* mRNA translation and anchoring.

**MATERIALS AND METHODS**

**Drosophila stocks:** As the Miranda-GFP stock, we used an insertion of P w+ matα4tub:Miranda-GFP at 18D11 on the X chromosome, which was maintained over the attached X chromosome, *C(1) DX, y w f* (IRION et al. 2006). The stock therefore
contains only Mira-GFP/Y males and X^X/Y females, so that Miranda–GFP transgene is not expressed until the males are out-crossed to normal females. Other stocks used were dp^{ov1}; e^l (Bloomington stock centre); UASp:oskar-bicoid 3’UTR, UASp:oskar(M1L)-bicoid 3’UTR, UASp:oskar(M139L)-bicoid 3’UTR (TANAKA and NAKAMURA 2008), Df(2L)ed^{SZ1} (PRESGRAVES 2003); spire^{BR} (MANSEAU and SCHUPBACH 1989), spire^{2E} (WELLINGTON et al. 1999); capu^{EY13172} (BELLEN et al. 2004); UASp:Capu-GFP and UASp:CapuΔN-GFP (DAHLGAARD et al. 2007), nos-Gal4-VP16 (VAN DOREN et al. 1998); Prospero-GAL4 (SHIGA, 1996); Df(3R)ora^{19} (SHEN et al. 1997); and UASp:Mira, UASp:Mira 1B2-2, UASp: Mira 1A4-1 (generated for this work). The mapping chromosomes wg^{Sp-1} B^l^1 L^{mm} Bc^l Pu^2 Pin^B/SM5 and al^l dp^{ov1} b^l pr^l c^l px^l sp^l were obtained from Bloomington Stock Centre. Germ-line clones were generated by heat shocking hsFLP; FRT G13 Su(Mir)/FRT G13 ovo^{D1} larvae for 2 hr on 3 consecutive days (CHOU and PERRIMON 1996).

**Mutagenesis and screen for Miranda suppressors:** Miranda-GFP males were starved for 6 hours before being fed 30 mM EMS (Sigma) in 1% sucrose for 18 hours to induce an average of one lethal hit per chromosome. Mutagenised males were mated with w^{1118} virgin females. Single w^\prime P[w^+:Miranda-GFP], Su^?/+; Su^?/+; Su^?/+ virgin females were mated with w^{1118} males. Females with hatching rate greater than 10% were selected for further analysis as bearing a potential suppressor mutation. The same selection procedure was repeated for an additional generation. To map suppressors to a chromosome, single w^\prime P[w^+:Miranda-GFP], Su^?/+; Su^?/+; Su^?/+ virgin females were mated with w^\prime; dp; e males and then single w^\prime P[w^+:Miranda-GFP], Su^?/+; Su^?/dp; Su^?/e males were mated to w^\prime; dp; e virgin females. The hatching rate of several females of genotypes dp, e or dp; e were tested.
If suppression was observed, e or dp males from the same cross were mated to virgin females with a corresponding balancer (2\textsuperscript{nd} or 3\textsuperscript{rd} chromosomes) (See Fig.1) and balanced stocks were established. For X chromosome linked suppressors, virgin females from F3 (See Fig.1) were used to establish balanced stocks. Identified suppressors were re-screened to confirm the presence of suppression and chromosomal location.

**Staining and microscopy:** Antibody stainings and *in situ* hybridizations were performed according to standard protocols. Actin was visualised by fixing ovaries in 4\% formaldehyde for 20 min and staining with Rhodamine-phalloidin (1:500; Invitrogen). For better visualization of Long Oskar-induced actin filaments, the actin mesh fixation protocol was used (DAHLGAARD *et al.* 2007). Ovaries were fixed in 10\% formaldehyde for 30 min and stained with Rhodamine-phalloidin overnight. Guinea pig anti-Oskar antibody was raised against amino acid residues 292-606 and used at 1:200; mouse anti-pH3 was used at 1:200 (Cell Signaling), rabbit anti-GFP was used at 1:200 (AMS Biotechnology). Antisense *oskar* RNA probes for *in situ* hybridizations were synthesized using DIG RNA Labeling mix (Roche). Cy3-conjugated IgG mouse anti-DIG antibody (Jackson ImmunoResearch Laboratories) was used at 1:200.

Imaging was performed using a Zeiss LSM510 confocal microscope (Carl Zeiss MicroImaging, Inc.) and LSM510 AIM software. Images were processed using ImageJ and Adobe Photoshop.

**Molecular biology:** *NotI-GFP-Strep-ProtA-SacII* was cloned into pUASp-PL to make UAS-GFP-Strep-ProtA construct. ‘Wild-type’ *mira*\textsuperscript{RR127} (coding region
corresponding to amino acids 1-727), and the \( \text{mira}^{1B2-2} \) and \( \text{mira}^{1A4-1} \) revertants were cloned via \( \text{KpnI/NorI} \) to pUASp-GFP-Strep-ProtA vector to make UAS-mira-GFP-Strep-ProtA constructs.

RESULTS

We initially set out to map the domains of Miranda that mediate its localisation to the anterior of the oocyte by screening for revertants of the dominant bicaudal phenotype caused by Mira-GFP expression. In addition to the expected revertants, we identified a number of dominant suppressors (data not shown). These suppressors must reduce the amount of anterior Nanos activity, and this could occur either by reducing the amount of anterior Miranda-GFP and \( \text{oskar} \) mRNA by impairing the \( \text{bicoid} \) mRNA localisation pathway or by reducing the amount of anterior Oskar protein by impairing its translation or anchoring. Indeed, we found that \( \text{exu} \) and \( \text{sww} \) mutants functioned as moderate suppressors of Miranda-GFP, while heterozygosity for an RNA null allele of \( \text{oskar} \) (\( \text{osk}^{87} \), (JENNY et al. 2006)) almost completely suppressed the formation of an anterior abdomen (99.2%). Although the embryos from \( \text{Mira-GFP/+; osk}^{87}/+ \) females formed normal heads, only very few of them hatched (0.6%). This appeared to be due posterior defects caused by a reduction of Oskar activity at the posterior pole, and can be explained by the fact that Mira-GFP already leads to the mis-localisation of about half of \( \text{oskar} \) mRNA to the anterior, while the \( \text{osk}^{87} \) allele reduces \( \text{oskar} \) mRNA levels by a further 50%. Nevertheless, these results indicated that one could identify novel factors involved in \( \text{bicoid} \) mRNA localisation as dominant suppressors of the dominant maternal-effect lethal phenotype of Mira-GFP, and that one might also identify factors involved in Oskar translation or anchoring,
provided that they are not so strong that they produce posterior defects. We therefore performed a large scale screen for dominant suppressors of the Miranda-GFP maternal effect lethal phenotype (Su (Mir) mutations).

**Screen for revertants and dominant suppressors of Mira-GFP:**

Males of the genotype \( w^{1118} P(Mira-GFP \, w^+)/Y \) were mutagenised with the chemical mutagen, EMS, and were crossed to \( w^{1118} \) stock that had been isogenised for the autosomes. The F1 female progeny were then placed in individual egg laying tubes, and the hatching rate of their progeny was determined. In the genetic background of the screen, the progeny of \( Mira-GFP/+ \) females have a hatching rate of <1%, and we selected any females whose progeny had a hatching rate of >10% as a potential suppressors or revertants. After one generation of backcrossing to remove extraneous mutations, the mutations were mapped to a chromosome using recessive markers (Figure 1). We identified 6 revertants from the pilot and large scale screens, and 144 putative suppressors (from a screen of 7062 females), 82 of which gave fertile progeny. Unfortunately, homozygosity for the \( dp \) chromosome strongly enhanced the bicaudal phenotype, leading to the loss of most suppressors on the 3rd chromosome. Nevertheless, 30 suppressor lines survived the mapping procedure and still showed robust suppression after they had been mapped to a chromosome and retested.

**Analysis of Mira-GFP revertants:**

We were primarily interested in revertants that specifically disrupt the anterior localisation of Mira-GFP rather than mutations that abolish Mira-GFP expression or its ability to bind to Staufen, and we therefore examined the localisation of the GFP fusion protein at mid-oogenesis. Since the GFP is fused to the C-terminus of
Miranda, nonsense mutations will produce no detectable GFP signal, while mutations that disrupt Staufen-binding cannot enter the oocyte by hitch-hiking with Staufen/oskar mRNA complexes. By contrast, Miranda-GFP revertants that specifically disrupt the anterior localisation domain still localise normally to the posterior pole at stage 9, but fail to accumulate at the anterior (Figure 2A and B). Two of the six revertants fell into this category and we therefore sequenced the coding regions of each Miranda transgene to identify the responsible mutations. In each case, the mutation fell in the first 13 amino acids of Miranda (Figure 2C).

Although Miranda is a large coiled coil domain protein with no significant homologies to other proteins over its full-length, BLAST searches revealed that the very N-terminus of the protein show significant similarity with the same N-terminal region of vertebrate RHAMM, and both revertants disrupt amino acids that are identical in Miranda and RHAMM (Figure 2D). Like Miranda, RHAMM is a large coiled coil domain protein, and the NCBI conserved domain database predicts that both proteins contain SMC prok B domains that form an extended helical structure (MARCHLER-BAUER et al. 2009). Furthermore, structural modelling predicts that Miranda and RHAMM form very similar donut shaped structures (Figure 2E and F).

Although there are no well-conserved orthologues of Miranda in vertebrates, these striking similarities raise the possibility that RHAMM represents such an orthologue that has diverged significantly at the sequence level while retaining the same overall structure.

The N-terminal region of Miranda is required for its localisation to the basal cortex of mitotic neuroblasts (FUERSTENBERG et al. 1998). Furthermore, although the localisation of Miranda depends on actin, mutants that disrupt the astral microtubules
of the neuroblast cause a variable defect in Miranda accumulation at the basal cortex, suggesting that microtubules also play a role (BASTO et al. 2006; GIANSANTI et al. 2008; GIANSANTI et al. 2001). To test whether the revertants also affect the basal localisation of Miranda in the neuroblast, we introduced the mutations found in two of the revertants (1A4-1 and 1B2-2) into a UAS-Miranda1-727-GFP construct, as the original Miranda transgene is not expressed in neuroblasts. Miranda forms homodimers, and we therefore examined the ability of each transgene to localise in mira mutant background to preclude the formation of heterodimers between the revertants and wildtype Miranda that might rescue any defects (YOUSEF et al. 2008). Wild-type Miranda-GFP driven by Pros-Gal4 rescued Df(3R)oraI9 (miranda null) homozygotes to pupal stages, whereas Df(3R)oraI9 animals expressing the revertant proteins were embryonic or larval lethal. Furthermore, although both revertant Miranda proteins formed basal crescents in metaphase neuroblasts, they both showed a significantly higher frequency of cells with a diffuse cytoplasmic signal (Figure 2G and H). Thus, the very N-terminal domain of Miranda is not essential for anchoring to the basal cortex, but may contribute to the efficient delivery of Miranda to this region.

**Analysis of Su(Mir) mutations:** The 30 surviving suppressor mutations were crossed to the other suppressors on the same chromosome and tested for lethality or sterility. This identified three complementation groups on the second chromosome, which we named Su(Mir)1-3, two of which are lethal with 3 and 11 alleles respectively and a third that is female sterile with 4 alleles (Table 1).

All alleles of Su(Mir)1 are lethal over Df(2R) CX1, which removes 49C1-4 to 50C23-D2, and the position of the locus was further refined by crossing alleles to smaller
deficiencies in this region, which revealed that Su(Mir)1 falls in the 50C5-C9 region uncovered by Df(2R) Exel71299 (PARKS et al. 2004). Su(Mir)1 alleles failed to complement mutations in short stop (shot), a very large gene that spans most of this interval. Furthermore, a null allele of the locus, shot^3, dominantly suppresses Miranda-GFP as well as the new alleles, giving a hatching rate of 11.2%. The large size of Shot (4100-8800 amino acids, depending on the isoform) and the fact that loss of function alleles suppress the bicaudal phenotype probably explains why we recovered so many more alleles of this complementation group than the others.

short stop encodes a large spectroplakin protein that binds to both actin and microtubules, making it a good candidate for a factor involved in either bicoid or oskar mRNA localisation or anchoring (GREGORY and BROWN 1998; KOLODZIEJ et al. 1995; ROPER et al. 2002). Shot also performs an essential function in stabilising microtubules on the fusome earlier in oogenesis, however, and shot mutant germline clones fail to make an oocyte and arrest development (ROPER and BROWN 2004). Indeed, homozygous germline clones of the shot alleles that we have tested from the screen block oocyte determination in the gerarium, making it impossible to assess their effects on mRNA localisation later in oogenesis. Thus, further investigation of the role of Shot in mRNA localisation will require methods to specifically knock down its activity at later stages of oogenesis.

We have not yet identified the genes affected in the two other complementation groups, but both have been mapped to an approximate region. This has allowed us to cross alleles of the lethal complementation group, Su(Mir)3, onto a FRT G13 chromosome and examine their phenotype in germline clones. While one allele blocks
oogenesis at an early stage, the two other alleles show a defect in the anchoring of oskar mRNA and protein at the posterior of the oocyte (Figure 3 E and F). This suggests that Su(Mir)3 mutants suppress the Miranda-GFP phenotype by reducing the amount of ectopic Oskar anchored at the oocyte anterior. By contrast, females transheterozygous for viable mutant combinations of Su(Mir)2 alleles show normal oskar mRNA localisation, but bicoid mRNA is mislocalised in a ring in the centre of the oocyte next to the misplaced oocyte nucleus at stage 10b (Figure 3C, D). This phenotype is similar to that seen in cap'n collar (cnc) and skittles germline clones (Gervais et al. 2008; Guichet et al. 2001). Su(Mir)2 does not correspond to either of these loci, however, indicating that it represents a new gene required for the anterior anchoring of bicoid mRNA during the later stages of oogenesis.

**Cappuccino and Spire play a role in Oskar anchoring:** We also examined the phenotypes produced by the two single alleles that are homozygous viable. 1E3-2 homozygotes are maternal-effect lethal, but show no obvious defects in bicoid or oskar mRNA localisation. By contrast, 3G3-1 homozygous females are sterile, and their ovaries contain ventralised oocytes that show premature streaming of the oocyte cytoplasm at mid-oogenesis. This phenotype is very similar to that produced by cappuccino and spire mutants (Manseau et al. 1996; Manseau and Schupbach 1989). Complementation tests and sequence analysis indicated that 3G3-1 is a new allele of capu caused by the insertion of the juan element into the first common exon of the Capu coding region. This should result in the expression of a truncated protein with only the first 103 amino acids of Capu-PA followed by 20 amino acids encoded by juan, and removes all of the conserved formin homology domains of the protein. To confirm that this mutation is responsible for the suppression, we first tested
whether other *capu* alleles suppress the Miranda-GFP phenotype. Although heterozygosity for a deletion of *capu* reduced the frequency of bicaudal embryos laid by Mira-GFP/+/ females, it did not give any hatching larvae and *capu*<sup>G7</sup> did not suppress at all (Figure 4A). However, a P-element insertion in the same exon as *capu*<sup>3G3-1</sup>, *capu*<sup>EY12344</sup> also acted as a strong suppressor, suggesting that expression of just the N-terminal 100 amino acids of Capu might exert a dominant negative effect. Capu functions with Spire to assemble a cytoplasmic network of actin in the oocyte, and binds directly to Spire protein (DAHLGAARD et al. 2007; QUINLAN et al. 2007; ROSALES-NIEVES et al. 2006). We therefore also tested two alleles of *spire* for suppression, and both gave similar levels of suppression to the Deficiency for *capu*, suggesting that Spire and Capu act together in this context. To confirm that the suppression was due to a reduction in Capu activity, we examined whether expression of full-length Capu or a constitutively active form of Capu lacking the N-terminal inhibitory domain (CapuΔN) could block the suppression of Miranda-GFP by *capu*<sup>3G3-1</sup> (DAHLGAARD et al. 2007). Although over-expression of either form of Capu had no effect on the phenotype of Miranda-GFP alone, both reversed the suppression by *capu*<sup>3G3-1</sup>, confirming that the suppression is caused by a reduction in Capu activity (Figure 4B).

Capu and Spire are both actin nucleators that work together to assemble an actin mesh in the oocyte cytoplasm from stage 5-10b of oogenesis that limits kinesin-dependent cytoplasmic flows (DAHLGAARD et al. 2007; EMMONS et al. 1995; PRUYNE et al. 2002; QUINLAN et al. 2005). In the absence of the mesh, premature cytoplasmic streaming washes the microtubules to the cortex and the prevents the kinesin-dependent transport of *oskar* mRNA to the oocyte posterior, while *bicoid* mRNA
localisation is unaffected (DAHLGAARD et al. 2007; SERBUS et al. 2005; ZIMYANIN et al. 2008). To investigate the basis for Miranda suppression by \( capu^{3G3-1} \), we examined the localisation of Miranda-GFP, oskar mRNA and Oskar protein in \( Mira-GFP/+; capu^{3G3-1/+} \) oocytes and eggs. Miranda-GFP and oskar mRNA localise to the anterior and posterior poles of the oocyte, as they do in the absence of \( capu^{3G3-1} \) (Figure 4C). Oskar protein is not translated at the anterior of \( Miranda-GFP/+ \) oocytes until the end of oogenesis, and we therefore examined its distribution in freshly-laid eggs (IRION et al. 2006). Oskar is only localised to the posterior pole of wild type and \( capu^{3G3-1/+} \) eggs, but is localised symmetrically at the anterior and posterior poles in \( Mira-GFP/+ \) eggs (Figure 4D). By contrast, no Oskar protein is detectable at the anterior of \( Mira-GFP/+; capu^{3G3-1/+} \) eggs, and Oskar levels at the posterior are also significantly reduced (Figure 4D). Thus, heterozygosity for \( capu^{3G3-1} \) appears to disrupt the anchoring of Oskar protein at the anterior and reduces the anchoring of Oskar at its normal position at the posterior of the oocyte.

These results suggest that Capu and Spire play a role in the anchoring of Oskar protein and the pole plasm that has been obscured by their earlier requirement in the localisation of oskar mRNA to the posterior of the oocyte. To circumvent this problem, we took advantage of a series of oskar-bicoid 3’UTR constructs that express either Long Oskar (required for anchoring), Short Oskar (nucleator of the pole plasm) or both Oskar isoforms at the anterior of the oocyte at mid-oogenesis (Figure 5A-C) (TANAKA and NAKAMURA 2008). Long Oskar remains stably anchored at the anterior cortex of the oocyte in \( capu \) or \( spire \) null mutants, and most Short Oskar also seems to be anchored at the anterior in the presence of Long Osk (Figure 5 D, E, G and H). However, Short Oskar is not stably anchored at the anterior of the oocyte in
capu or spire mutants when it is expressed in the absence of the long isoform (Figure 5F, F’, I and I’). Thus, the anchoring of Short Oskar at the anterior seems to require either Capu and Spire or Long Oskar protein.

Since Capu and Spire are actin nucleators and Oskar has been shown to trigger the formation of long actin filaments, we examined F-actin organization in oocytes expressing the osk-bcd constructs with or without Capu and Spire activity (Figure 5J-S) (TANAKA and NAKAMURA 2008; VANZO et al. 2007). Long Oskar induces the formation of long arrays of actin filaments extending from the anterior cortex, whereas Short Oskar alone does not (Figure 5J-M). These filaments do not form in capu and spire mutants, however, indicating that Capu and Spire act downstream of Long Oskar to nucleate these F-actin structures (Figure 5N-S).

To test directly whether Spire plays a role in the normal anchoring of oskar mRNA at the posterior of the oocyte, we took advantage of the fact that expression of constitutively active CapuΔN3 suppresses the premature streaming phenotype of spire mutants, allowing the posterior localisation of oskar mRNA in the absence of Spire activity (DAHLGAARD et al. 2007). A third of the stage 9 spireRP; CapuΔN3/+ egg chambers show a typical oskar mRNA anchoring defect, in which the RNA is localised to the posterior region of the oocyte, but is not tightly apposed to the posterior cortex (Figure 6 A-D). Once cytoplasmic streaming starts, this mRNA is often washed away from the posterior and shows a diffuse distribution throughout the oocyte cytoplasm (Figure 6C). These observations support the view that Spire plays a similar role in the normal process of Oskar anchoring at the posterior of the oocyte as it does at the anterior in Miranda-GFP and osk-bcd females.
DISCUSSION

The goal of the screen reported here was to identify novel factors involved in *bicoid* and *oskar* mRNA localisation and to isolate revertants of Miranda that define its anterior localisation domain. The screen itself was very simple to perform, as it was actually a selection, with only revertants and suppressors producing viable progeny. On the other hand, the downstream analysis of the new mutants proved challenging, since one cannot follow the mutations in males and must therefore establish and retest multiple lines for each mutant. Furthermore, the extent of the suppression varied with genetic background, and the suppression became too weak to maintain a number of mutant during the crosses to map them to a chromosome. Nevertheless, the screen succeeded in identifying revertants at the expected frequency (~1/1000), as well as three *Su (Mir)* complementation groups and a number of single alleles.

The revertants of the Miranda-GFP transgene identified the very N-terminus of the protein as the region responsible for its anterior localisation. This region showed a short, but significant homology to the very N-terminus of vertebrate RHAMM, which is also a large coiled-coiled domain protein, which is predicted to fold into a remarkably similar structure to Miranda. Thus, RHAMM may represent a very divergent orthologue of Miranda that has conserved its structure, but only retained a very small region of primary sequence similarity. This idea is supported by the similar properties of both proteins. Although RHAMM was originally identified as a cell-surface Hyaluronic Acid Receptor, the protein lacks a signal peptide, and localises to the mitotic spindle and centrosomes, where it plays an essential role in the chromatin-mediated spindle assembly pathway (Groen et al. 2004; Hofmann et al.)
Miranda also localises to centrosomes in the Drosophila embryo, and decorates the mitotic spindle in neuroblasts that are mutant for \textit{lgl}, \textit{dlg} or \textit{scribble} (Albertson and Doe 2003; Ohshiro et al. 2000; Peng et al. 2000). Furthermore, N-terminus of Miranda is essential for its microtubule-dependent localisation to the anterior of the oocyte, as it is disrupted by single amino acid mutations in the short N-terminal region with homology to RHAMM, and the region of RHAMM that associates with microtubules has also been mapped to its N-terminal domain (Irion et al. 2006; Maxwell et al. 2003). It would therefore be interesting to test whether Miranda also plays a role in the chromatin-mediated spindle assembly pathway, which is redundant with the centrosomal pathway under normal conditions and has not been well-characterised in \textit{Drosophila}.

The domain that targets Miranda to the basal cortex of the neuroblast has also been mapped to the N-terminal 290 amino acids of the protein (Fuerstenberg et al. 1998; Matsuzaki et al. 1998; Shen et al. 1998). The mutations that block anterior Miranda localisation in the oocyte do not disrupt its basal localisation in the neuroblast, however, consistent with the data that the latter occurs by a distinct actin-dependent mechanism (Erben et al. 2008; Shen et al. 1998). Nevertheless, these single amino acid changes appear to delay the formation of the Miranda basal crescent, since the protein is more frequently observed to be cytoplasmic. This suggests that the mutations either partially impair the ability of Miranda to associate with the actin-rich cortex, or that they inhibit a redundant localisation pathway that increases the efficiency of the targeting of Miranda to the basal cortex. In support of the second possibility, it has recently been found that there are redundant pathways that localise the key polarity factor, Pins, to the apical cortex: it is directly recruited by
Inscuteable, but is still delivered apically in the absence of Inscuteable by a microtubule-dependent pathway that involves the microtubule motor protein, Khc-73 and Dlg (Schaefer et al. 2000; Siegrist and Doe 2005; Yu et al. 2000). Since mutations that disrupt the astral microtubules impair Miranda localisation, it is possible that a similar microtubule-dependent mechanism plays a redundant role in delivering Miranda to the basal cortex (Basto et al. 2006; Giansanti et al. 2008; Giansanti et al. 2001).

Modifier screens are based on the idea that recessive loss of function mutations can become dominant in a sensitised genetic background in which the process that they affect is limiting (Simon 1994; St Johnston 2002). Because Miranda-GFP links some oskar mRNA to the bicoid mRNA localisation pathway, we expected the suppressors to be dosage sensitive factors involved in the transport of bicoid mRNA to the anterior, or regulators of oskar mRNA anchoring and translation that reduce the amount of anterior pole plasm produced. Although we have only partially analysed the three complementation groups, Su(Mir)2 and 3 appear to fall into each expected class, with the former disrupting bicoid mRNA localisation, and the latter giving an oskar mRNA anchoring phenotype. By far the most frequent class of suppressor, however, was alleles of the giant actin and microtubule-binding protein, Shot. Shot is therefore an excellent candidate to play a role in one or both of these processes, although we have been unable to test this directly because all of the alleles we have tested block oogenesis at an early stage.

In addition to the complementation groups, we also recovered a number of single mutations, including the capu3G3-1 allele. Interestingly, capu3G3-1 and the very similar
allele, *capu<sup>EEY12344</sup>* suppress the Miranda-GFP bicaudal phenotype much more strongly than a deficiency for the locus, indicating that they have an antimorphic effect, and this may explain why we only recovered a single allele at this locus. This revealed an unexpected role for Capu and its binding partner Spire in Oskar anchoring that was not detected previously, because *oskar* mRNA is not localised to the posterior in *capu* and *spire* mutants. *capu<sup>3G3-1</sup>* dominantly disrupts the anchoring of Oskar at the anterior of Miranda-GFP eggs and also reduces the normal anchoring of Oskar at the posterior.

The anchoring of Short Oskar and associated pole plasm depends on F-actin and Long Oskar (BABU et al. 2004; JANKOVICS et al. 2002; POLESELLO et al. 2002; VANZO and EPHRUSSI 2002). In addition, the localisation of Oskar to either the anterior or posterior of the oocyte induces the formation of actin filaments from the adjacent cortex (TANAKA and NAKAMURA 2008; VANZO et al. 2007). Our results demonstrate that it is Long Oskar that induces actin filaments, and that their formation requires both Capu and Spire. Thus, these two actin nucleators act downstream of Long Oskar to nucleate actin filaments. Since, Long Oskar, Capu, Spire and F-actin are all required for the anchoring of Short Oskar, it seems likely that Short Oskar is tethered in someway to this actin structures.

Long Oskar recruits a number of endocytic factors to its site of localisation to locally stimulate endocytosis, and endocytic mutants also disrupt Short Oskar anchoring at the oocyte posterior (TANAKA and NAKAMURA 2008). This raises the question of the relationship between Capu and Spire-dependent actin filament formation and Oskar-dependent endocytosis. One possibility is that the Capu and Spire are activated as a
consequence of endocytosis. However, F-actin is still enriched at the anterior of osk-
bcd 3’ UTR oocytes when endocytosis is disrupted, although the actin forms
aggregates rather than filaments. Thus, Long Oskar induces actin polymerisation in
the absence of increased endocytosis (TANAKA and NAKAMURA 2008). The
interpretation of the relationship between Long Oskar, endocytosis, Capu and Spire
and anchoring is further complicated by the fact that the requirements for Short Oskar
anchoring vary according to stage and position within the oocyte. Capu and Spire are
required for the anterior anchoring of Short Oskar at stage 10b in the absence of Long
Oskar in oskM1L-bcd 3’ UTR oocytes, indicating that they must be able to nucleate
actin filaments that anchor Short Oskar in the absence of Long Oskar. On the other
hand, they are dispensable for the anterior anchoring of Short Oskar at this stage when
Long Oskar is present. There must therefore be another parallel mechanism to hold
Short Oskar at the anterior under these conditions. Similarly, although the induction
of increased endocytosis by Long Oskar is required for anchoring of Short Oskar at
the posterior, this is not the case at the anterior. Thus, Long Oskar-dependent
endocytosis and actin filament formation may act redundantly to anchor Short Oskar
at the anterior in mid-oogenesis. This redundancy does not appear to exist at the
posterior of the oocyte, however, as Long Oskar, Capu and Spire and endocytosis are
all necessary for efficient anchoring, and the same is true at later stages for the
anterior of Miranda-GFP expressing oocytes. This difference may reflect the presence
or absence of cytoplasmic flows at each stage and position, since Short Oskar
anchoring at the posterior is only important after stage 10b when cytoplasmic
streaming begins. As this movement is weaker very close to the anterior cortex, there
may be less force causing Short Oskar to spread away from this position.
Our results show that Capu and Spire play a key role in the normal anchoring of Short Oskar at the posterior, and hence in pole plasm formation, but also indicate the existence of partially redundant anchoring pathways downstream of Long Oskar. These results are consistent with the observations of Babu et al who found that there are two overlapping anchoring pathways for Oskar, an actin-dependent pathway that involves the Bifocal protein, and an actin-independent pathway that requires the Homer protein (BABU et al. 2004). The anchoring of Short Oskar probably therefore depends on a complex network of interactions involving Long Oskar, endocytic vesicles and actin filaments, all of which contribute some anchoring activity. A complete understanding of this process will therefore require deciphering the molecular links between these components.

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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Crossing scheme for isolating and mapping Suppressors of Miranda.

FIGURE 2. Characterization of Miranda revertants.
(A-B) Miranda-GFP localisation in stage 9-10 oocytes. (A) Wild-type. (B) 1A4-1 Miranda-GFP revertant. (C) Schematic representation of the N-terminus of Miranda. White boxes indicate point mutations identified in the two Miranda revertants. (D) Alignment of the N-terminus of Miranda and Xenopus RHAMM. (E-F) Predicted 3D structures for Miranda (E) and Xenopus RHAMM (F). The predictions were generated using the I-TASSER Internet service (Zhang 2008, 2009). (G) Localisation of Miranda-GFP and Miranda-GFP revertants in Df(3R)oraI9 embryonic neuroblasts. (H) Quantification of Miranda-GFP localisation in embryonic neuroblasts.

FIGURE 3. Su(Mir)2 disrupts bcd mRNA localisation and Su(Mir)3 disrupts oskar RNA localisation.
In situ hybridisation to bicoid (A, C, E) and oskar (B, D, F) mRNAs in wild-type (A-B), Su(Mir)2 (C-D) and Su(Mir)3 (E-F) oocytes.

FIGURE 4. A new allele of cappuccino suppresses the Miranda-GFP bicaudal phenotype.
(A) Bar diagram showing the level of suppression of the Miranda-GFP bicaudal phenotype by various capu and spire alleles (Miranda-GFP/+;capu<sup>3G3</sup>/*+, Miranda-GFP/+;Df(2L)ed<sup>S2L</sup>/*+, Miranda-GFP/+;capu<sup>G7</sup>/*+, Miranda-GFP/+;capu<sup>EY13172</sup>/*+, Miranda-GFP/+;spire<sup>Rp</sup>/*+, Miranda-GFP/+;spire<sup>2F</sup>/*+, and Miranda-GFP/*). Suppressed embryos were scored as embryos that did not form abdominal structures.
or posterior spiracles at the anterior, but still failed to hatch. (B) Bar diagram showing the effect of Capu overexpression using a maternal Gal4 driver on the suppression of the Miranda-GFP bicaudal phenotype by capu<sup>3G3</sup>. Capu represents a full–length Capu protein, and Capu ΔN is a truncated form that is constitutively active. (C) Localisation of Miranda-GFP (left panels) and oskar mRNA (right panels) in Miranda-GFP/+ (upper panels) or Miranda-GFP/+; capu<sup>3G3</sup/+ egg chambers (lower panels). (D) Localisation of Oskar protein in wild-type, Miranda-GFP/+, Miranda-GFP/+; capu<sup>3G3</sup>+ or capu<sup>3G3</sup>+ embryos.

FIGURE 5. Cappuccino and Spire are required for the anchoring of Short Oskar and the formation of Long Oskar-dependent F-actin filaments.

(A-I’) Localisation of Oskar protein in oocytes expressing of oskar-bcd 3’UTR (A, D, G), oskar (M139L)-bcd 3’UTR (B, E, H) or oskar (M1L)-bcd 3’UTR (C, F, F’, I, I’) in wild-type (A-C), capu<sup>3G3</sup>/Df(2L)ed<sup>SZ1</sup> (D-F’) or spire<sup>Rp</sup> (G-I’) mutants. (J-S) High magnification views of F-actin staining at the anterior of oocytes expressing oskar-bcd 3’UTR (J, N, Q), oskar (M139L)-bcd 3’UTR (K, O, R) or oskar (M1L)-bcd 3’UTR (L, P, S), in wild-type (J-M), capu<sup>3G3</sup>/Df(2L)ed<sup>SZ1</sup> (N-P) or spire<sup>Rp</sup> (Q-S). (M) High magnification view of F-actin staining at the anterior of a wildtype oocyte.

FIGURE 6. Effect of spire and cappuccino mutants on oskar mRNA anchoring

(A-C) oskar mRNA localisation in wild-type (A) and spire<sup>Rp</sup>; nos-Gal4 UAS-CapuΔN3/+ egg chambers (B-C). (D) Bar diagram showing a quantification of oskar mRNA localisation in stage 9 and stage 10-11 oocytes in wild type, spire<sup>Rp</sup>/+; nos-Gal4 UAS-CapuΔN3/+, and spire<sup>Rp</sup>; nos-Gal4 UAS-CapuΔN3/>. Posterior refers to
wildtype posterior localisation (A), posterior and diffuse refers to some posterior localisation with the mRNA extending away from the posterior pole (B), and diffuse refers to no posterior enrichment (C).
### TABLE 1
Description of the complementation groups

<table>
<thead>
<tr>
<th>Suppressor/ gene name</th>
<th>Alleles</th>
<th>Chromosome location/ map position</th>
<th>Homozygous viability/ fertility</th>
</tr>
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<tbody>
<tr>
<td>Mira-GFP Revertants</td>
<td>Rev(Mir)1A4-1, Rev(Mir)1B2-2</td>
<td>1st</td>
<td>Fertile; Mira-GFP localisation to the anterior is abolished but posterior localisation remains.</td>
</tr>
<tr>
<td>shot</td>
<td>1E2-5, 1J3-2, 1J3-3, 2C2-3, 2A2-4, 2C3-1, 3C2-2, 3F1-1, 3I2-1, 2F2-2, 2B2-8</td>
<td>50C</td>
<td>lethal</td>
</tr>
<tr>
<td>Su(Mir)2</td>
<td>1C2-2, 2H3-1, 1E3-5, 1J3-4b</td>
<td>between b and c</td>
<td>1C2-2, 2H3-1 viable, female sterile. 1E3-5, 1J3-4b lethal. All transheterozgous combinations viable, female sterile.</td>
</tr>
<tr>
<td>Su(Mir)3</td>
<td>1H2-1a, 1A2-1, 1G3-1a</td>
<td>between L and Pin</td>
<td>lethal</td>
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**Single alleles**

<table>
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<th>Allele</th>
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<th>Viability/Fertility</th>
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<td>capu</td>
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<td>24C</td>
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<td></td>
<td>1A2-2</td>
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