

The identification of novel genes required for *Drosophila* anteroposterior axis formation in a germline clone screen using GFP-Staufen

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SUMMARY

The anteroposterior axis of *Drosophila* is defined during oogenesis, when the polarisation of the oocyte microtubule cytoskeleton directs the localisation of *bicoid* and *oskar* mRNAs to the anterior and posterior poles, respectively. Although maternal-effect lethal and female-sterile screens have identified many mutants that disrupt these processes, these screens could not recover mutations in essential genes. Here we describe a genetic screen in germline clones for mutants that disrupt the localisation of GFP-Staufen in living oocytes, which overcomes this limitation. As Staufen localises to the posterior with *oskar* mRNA and to the anterior with *bicoid* mRNA, it acts as a marker for both poles of the oocyte, allowing the identification of mutants that affect the localisation of either mRNA, as well as mutants that disrupt oocyte polarity. Using this approach, we have identified 23 novel complementation groups

on chromosome 3R that disrupt anteroposterior axis formation. Analyses of new alleles of *spn-E* and *orb* show that both SPN-E and ORB proteins are required to organise the microtubule cytoskeleton at stage 9, and to prevent premature cytoplasmic streaming. Furthermore, *yps* mutants partially suppress the premature cytoplasmic streaming of *orb* mutants. As *orb*, *yps* and *spn-E* encode RNA-binding proteins, they may regulate the translation of unidentified RNAs necessary for the polarisation of the microtubule cytoskeleton.

Movies available online

Key words: *oskar*, *bicoid*, Staufen, Polarity, Microtubules, *Drosophila*

INTRODUCTION

Drosophila oogenesis provides an excellent model system in which to study cell polarity and mRNA localisation, because the anteroposterior (AP) axis of the embryo is determined by the localisation of two different mRNAs to the anterior and posterior pole of the oocyte (van Eeden and St Johnston, 1999; Riechmann and Ephrussi, 2001). *bicoid* mRNA is localised at the anterior of the oocyte, and is translated after fertilisation to generate a morphogen gradient of a homeodomain transcription factor that patterns the head and the thorax of the embryo (Driever, 1993). *oskar* mRNA localises to the posterior of the oocyte, where Oskar protein directs the formation of the pole plasm that contains the pole cell and abdominal determinants (Ephrussi et al., 1991; Kim-Ha et al., 1991; Ephrussi and Lehmann, 1992).

The localisation of *bicoid* and *oskar* mRNAs depends on reciprocal signalling between the oocyte and the overlying somatic follicle cells. In early stages of oogenesis, an MTOC is present at the posterior of the oocyte and nucleates microtubules that extend through the nurse cells (Theurkauf et

al., 1992). During stages 5–6, Gurken protein signals from the oocyte to induce the adjacent somatic follicle cells to adopt a posterior fate (Gonzalez-Reyes et al., 1995; Roth et al., 1995). These posterior cells are then thought to signal back to the oocyte to induce the disassembly of the posterior MTOC, which leads to the formation of an AP gradient of microtubules, in which most minus ends lie at the anterior of the oocyte, with the plus ends extending towards the posterior pole (Theurkauf et al., 1992; Clark et al., 1994; Clark et al., 1997). This polarised microtubule cytoskeleton defines the destinations of *bicoid* and *oskar* mRNAs, and also directs the migration of the oocyte nucleus and *gurken* mRNA from the posterior of the oocyte to the anterior margin, where Gurken signals a second time to define the dorsal-ventral axis (Neuman-Silberberg and Schüpbach, 1993).

As the localisation of *bicoid* and *oskar* mRNAs depends on the polarised microtubule cytoskeleton, a simple model is that these mRNAs are transported to the anterior and posterior by minus- and plus-end-directed microtubule motors, respectively (Clark et al., 1994; Pokrywka and Stephenson, 1995). In support of this model, *oskar* mRNA localisation requires the

plus-end-directed motor, Kinesin 1 (Brendza et al., 2000). However, it is unclear whether Kinesin directly transports *oskar* mRNA to the posterior, and if so, how it is coupled to its mRNA cargo (Glötzer et al., 1997; Cha et al., 2002; Palacios and St Johnston, 2002). The actin cytoskeleton may also be important in this process, as some alleles of the actin-binding protein Tropomyosin II (TmII; Tp2 - FlyBase) disrupt *oskar* mRNA localization (Erdelyi et al., 1995). How *bicoid* mRNA is directed to the anterior of the oocyte is even less well understood, because the minus ends of microtubules are found along the lateral cortex as well as the anterior (Cha et al., 2001). Indeed, *bicoid* mRNA localises in a microtubule-dependent manner to both the anterior and lateral cortex when it is injected into the oocyte, and only localises specifically to the anterior if it has been exposed to nurse cell cytoplasm. This has led to the proposal that *bicoid* RNA is transported to the anterior along a specific subpopulation of microtubules, and that nurse cell factors render it competent to distinguish these microtubules from those nucleated laterally.

Very little is known about how the oocyte microtubule cytoskeleton becomes polarised, but three classes of mutants disrupt this organisation.

(1) In mutants that abolish the follicle cell signal, such as *gurken* mutants, the posterior MTOC persists after stage 7, leading to the formation of a symmetric microtubule cytoskeleton, which directs the localisation of *bicoid* RNA to both poles of the oocyte and *oskar* mRNA to the centre (Gonzalez-Reyes et al., 1995; Roth et al., 1995). Germline clones of *PKA*, *mago nashi* and *Y14* (*tsunagi*) mutants cause similar phenotypes in the oocyte without affecting the follicle cells, suggesting that they are required for the transduction of the polarising signal in the oocyte (Lane and Kalderon, 1994; Micklem et al., 1997; Newmark et al., 1997; Hachet and Ephrussi, 2001; Mohr et al., 2001). However, Mago and Y14 are components of the exon-exon junction complex that marks where introns have been removed from mRNAs, and they presumably act on microtubules indirectly (Le Hir et al., 2001).

(2) In *par-1* and *14-3-3E* mutants, the posterior MTOC disassembles normally, but the microtubule plus ends and *oskar* mRNA become focussed in the centre of the oocyte (Shulman et al., 2000; Tomancak et al., 2000; Benton et al., 2002). These proteins may therefore play a role in recruiting plus ends to the posterior.

(3) Mutants in the actin-binding proteins, Cappuccino, Spire and Profilin (Chickadee) disrupt *oskar* mRNA localisation by causing the formation of large cortical bundles of microtubules that drive premature cytoplasmic streaming (Theurkauf, 1994b; Manseau et al., 1996). This suggests that the actin cytoskeleton plays an important role in regulating the formation of the polarised microtubule array.

Genetic screens have identified a number of genes that play a role in the localisation of *bicoid* and/or *oskar* mRNAs once the microtubule network has been organised. Amongst these, *staufer* is unique, as it is required for both *bicoid* and *oskar* mRNA localisation. *Staufen* plays a central role in the regulation of *oskar* mRNA, as it is required not only for its transport from the anterior to the posterior of the oocyte, but also for the anchoring and translation of the RNA once it has reached the posterior pole (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991; Rongo et al., 1995; Micklem et al., 2000). Furthermore, *Staufen* co-localises with *oskar*

mRNA throughout oogenesis, in both wild-type and all mutant conditions examined so far, and this localisation requires *oskar* mRNA (St Johnston et al., 1991; Ferrandon et al., 1994). Because *Staufen* is a dsRNA-binding protein, these results indicate that it binds directly and stably to *oskar* mRNA (St Johnston et al., 1992; Ramos et al., 2000). In freshly laid eggs, *Staufen* is also localised at the anterior, where it is required to anchor *bicoid* mRNA (St Johnston et al., 1989; Ferrandon et al., 1994). The anterior localisations of *Staufen* and *bicoid* mRNA are mutually-dependent, again suggesting that *Staufen* interacts directly with the RNA. However, it is unclear when *Staufen* first associates with *bicoid* mRNA, as it is not enriched at the anterior of the oocyte at stage 10a, and the later stages of oogenesis cannot be examined by antibody staining because of the impermeable vitelline membrane that is deposited around the oocyte.

Several other genes are necessary for the anterior localisation of *bicoid* mRNA at earlier stages. *exuperantia* is required for the anterior accumulation of *bicoid* RNA from stage 7 onwards, and appears to function in the nurse cells to render the RNA competent to localise in the oocyte (Berleth et al., 1988; Cha et al., 2001). In *swallow* mutants, the initial localisation of *bicoid* mRNA is normal, but it is not anchored at the anterior of the oocyte from stage 10b (Berleth et al., 1988; St Johnston et al., 1989). Swallow protein physically interacts with the Dynein light chain, and localises to the anterior of the oocyte, but it is unclear whether it plays a direct role in the anchoring of *bicoid* mRNA or functions indirectly in the organisation of the microtubule cytoskeleton (Schnorrer et al., 2000). In support of the latter view, Swallow interacts with γ Tub37C and Grip75, which are components of the γ Tubulin ring complex that nucleates microtubules; mutants in both of these genes also disrupt *bicoid* mRNA anchoring (Schnorrer et al., 2002).

The localisation of *oskar* mRNA is also affected by mutations in a number of other known genes. In *barentsz* mutants and hypomorphic alleles of *mago nashi* and *Y14*, *oskar* mRNA fails to localise to the posterior of the oocyte and accumulates along the anterior margin instead (Newmark and Boswell, 1994; Micklem et al., 1997; Hachet and Ephrussi, 2001). All three proteins transiently localise with *oskar* mRNA at the posterior of stage 9 oocytes, suggesting that they are directly involved in its transport (Newmark et al., 1997; Hachet and Ephrussi, 2001; Mohr et al., 2001). Once *oskar* mRNA reaches the posterior of the oocyte, it needs to be securely anchored. The actin cytoskeleton may be important in this process as two actin-binding proteins, TmII and Dmoesin (Moesin-like - FlyBase), are necessary for the anchoring of *oskar* mRNA to the posterior cortex (Tetzlaff et al., 1996; Jankovics et al., 2002; Polesello et al., 2002). *Oskar* protein also plays an important role in anchoring, as *oskar* mRNA is not maintained at the posterior in *oskar* protein null mutants (Ephrussi et al., 1991; Kim-Ha et al., 1991). Mutations in genes necessary for *oskar* translation, such as *aubergine*, therefore result in similar defects in *oskar* mRNA anchoring (Harris and Macdonald, 2001). In addition, the homologue of the *Xenopus* cytoplasmic polyadenylation element-binding (CPEB) protein, ORB, binds *oskar* mRNA, and is required for its localisation and translation (Lantz et al., 1992; Christerson and McKearin, 1994; Hake and Richter, 1994; Chang et al., 1999).

The majority of the mutants described above were identified

in genetic screens for maternal-effect lethal and female-sterile mutations (Nüsslein-Volhard et al., 1987; Schüpbach and Wieschaus, 1989; Schüpbach and Wieschaus, 1991). Although these screens were very successful at identifying germline-specific factors required for oocyte polarisation and mRNA localisation, they could only recover homozygous viable mutations, and therefore missed many of the essential genes that also play a role in polarity and mRNA localisation in other cell types. This problem can be overcome by using the FLP/FRT/DFS system to perform screens in germline clones (Xu and Rubin, 1993; Chou and Perrimon, 1996). This system allows the recovery of lethal mutations in essential genes, because it selects for germline clones, while most somatic cells remain heterozygous. Perrimon et al. successfully used this technique to screen a sample of 500 lethal P-elements recombined onto FRT autosomes, and identified maternal-effect lethal factors involved in proper egg shell formation and patterning of the cuticle (Perrimon et al., 1996).

A limitation of all of these screens is their use of the embryonic cuticle as a readout for AP patterning, as this precludes the identification of any mutants that affect AP axis formation in the oocyte but block development before cuticle formation. To circumvent this problem, we have designed a novel genetic screen in germline clones for mutations that alter the distribution of GFP-Staufen in living oocytes. Here, we report the results of this screen on chromosome arm 3R, and the characterisation of 23 new complementation groups that play a role in AP axis formation.

MATERIALS AND METHODS

Fly stocks

The following fly stocks were used: w; P[ry⁺; hsp70:neo; FRT]82B, ry⁵⁰⁶ (Xu and Rubin, 1993); w; P[ry⁺; hsp70:neo; FRT]82B, ry⁵⁰⁶ P[w⁺; ovo^{D1-18}]3R1, P[w⁺; ovo^{D1-18}]3R2/TM3, Sb (Chou and Perrimon, 1996); y, w, P[w⁺; mat-tub-alpha4:GFP Staufen], [ry⁺; hs:FLP]; Pr, Dr/TM6B, Tb – this line contains a GFP-Staufen transgene driven from the $\alpha 4$ tubulin promoter and a FLPase transgene under control of the heat-shock promoter (Golic and Lindquist, 1989; Schuldt et al., 1998).

The following alleles were used for complementation tests: *osk*⁵⁴ (Lehmann and Nüsslein-Volhard, 1991), *cnc*⁰³⁹²¹ (Guichet et al., 2001), *spn*^{Ehls Δ 157} (Gillespie and Berg, 1995), *orb*^{F343} (Lantz et al., 1994), *orb*^{mel} (Christerson and McKearin, 1994), *Ets97D*^{me-4} (Schulz et al., 1993), *tml*^{lgs1} (Erdelyi et al., 1995), *btz*² (van Eeden et al., 2001). The *yps*^{JM2} allele and the *yps*^{JM2} *orb* recombinant chromosomes were obtained from Dr Hazelrigg (Mansfield et al., 2002).

Mutagenesis and complementation

w; FRT82B males were starved for 6 hours before they were treated with 25 mM EMS (Sigma) in 1% sucrose for 18–24 hours to induce an average of one lethal hit per chromosome arm. The number of lethal hits was estimated by monitoring the number of X-linked lethals. Mutagenised males were mated with w; Pr, Dr/TM3 virgin females. Single w; FRT82B, */TM3 virgin females (where the asterisk indicates the mutagenised chromosome) were mated with y, w, hs-FLP, GFP-Staufen; FRT82B, ovo^{D1}/TM6B males. The progeny were heat-shocked three times for 2 hours in a 37°C incubator during the third larval instar and pupal stages. Ovaries from 3–5 females of the genotype y, w, hs-FLP, GFP-Staufen/w; FRT82B, */FRT82B, ovo^{D1} were dissected and screened for defects in GFP-Staufen localisation, using an inverted fluorescence microscope (see Fig. 2). If a phenotype

was observed, w; FRT82B, */TM6B males were mated with w; Pr, Dr/TM3 virgin females and balanced stocks were established. Mutants identified in the primary screen were re-screened under the confocal microscope.

Induction of the FLPase in females containing a non-mutagenised FRT chromosome produced germline clones in over 90% of the females. As we dissected 3–5 females for each mutant chromosome, the likelihood of getting clones in at least one female was higher than 99.9%. The mutagenised chromosomes for which no clones were recovered (about 25%) carried either a cell lethal mutation or a mutation arresting oogenesis at early stages, and were indistinguishable from the ovo^{D1} egg chambers. These mutants were discarded.

Two lines were considered allelic if no trans-heterozygous progeny were recovered, or if the trans-heterozygous females were sterile or maternal-effect lethal. We did not routinely test for weaker maternal-effect defects, such as a grandchildless phenotype.

Stains and microscopy

The primary screen was performed on a Leica inverted fluorescence microscope, using a broadband filter (Leica 13 513828) in which the GFP fluorescence appears green, and yolk autofluorescence yellow. For live observation, the ovaries were dissected in Voltalef 3S (screen) or 10S (movies) oil (Elf Atochem) on a coverslip. The time-lapse movies were obtained by collecting z series of five sections at 1 μ m intervals every 30 seconds for 20 minutes on a BioRad confocal MRC1024 microscope. The moving particles were visualised by excitation with 568 nm light, and collection of the emission through an OG515 filter (Palacios and St Johnston, 2002). The Kalman images in Fig. 7E–H were obtained using the Kalman averaging function of the BioRad software to merge eight consecutive scans taken at 3 second intervals. Static particles form dots in these images, whereas moving particles appear as lines that represent the direction and speed of movement. Actin was visualised by fixing ovaries in 4% paraformaldehyde and staining with rhodamine-phalloidin (1:500; Molecular Probes). In situ hybridisations were carried out using RNA probes labelled with Digoxigenin-UTP (Roche). Immunohistochemical detection was performed with alkaline phosphatase-conjugated anti-DIG (1:5000; Roche). For microtubule staining, samples were fixed for 10 minutes in 8% paraformaldehyde and stained with a FITC-conjugated monoclonal anti- α -tubulin antibody (1:400; Sigma) (Theurkauf, 1994a).

RESULTS

To develop a marker for mRNA localisation in living oocytes, we analysed a fusion between green fluorescent protein (GFP) and Staufen that is specifically expressed in the female germline (Schuldt et al., 1998). The GFP-Staufen fusion is fully functional, as it rescues both the *oskar* and *bicoid* mRNA localisation defects of a *staufen* null mutant (data not shown). Like endogenous Staufen, GFP-Staufen accumulates in the oocyte during stages 2–8 (Fig. 1A), and co-localises with *oskar* mRNA at the posterior from stage 9 onwards (Fig. 1B,C). Furthermore, GFP-Staufen localisation parallels that of *oskar* mRNA in mutants in which *oskar* mRNA is mislocalised: it accumulates along the anterior margin in stage 9 *barentsz* mutant oocytes, and concentrates in the middle of the oocyte in *grk* mutants (Fig. 1D–F) (Gonzalez-Reyes et al., 1995; Roth et al., 1995; van Eeden et al., 2001). Because the fusion protein does not need to be stained, it can also be visualised during the later stages of oogenesis, when the oocyte is impermeable to antibodies. At stage 10b, GFP-Staufen accumulates at the anterior cortex of the oocyte, where it remains for the rest of

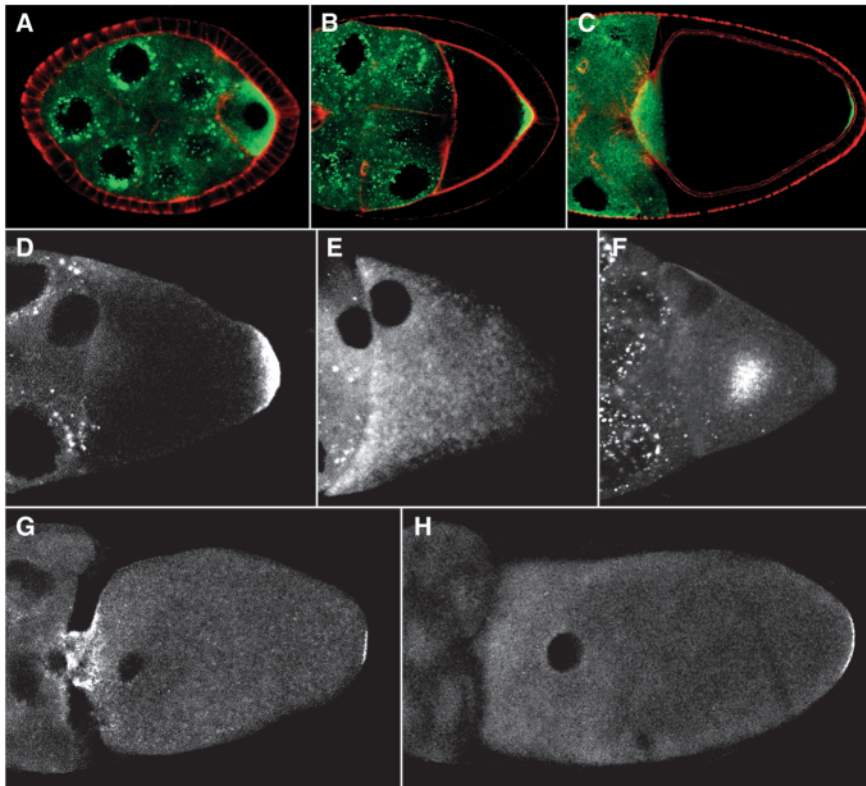


Fig. 1. Localisation of GFP-Staufen.

(A-C) Expression of GFP-Staufen (green) in fixed egg chambers counterstained with rhodamine-phalloidin, which labels F-actin (red). (A) Stage 6 egg chamber, showing the accumulation of GFP-Staufen in the oocyte. (B) Stage 9 egg chamber, showing the localisation of GFP-Staufen to the posterior of the oocyte. (C) Stage 10b egg chamber, in which GFP-Staufen localises both to the anterior and the posterior pole of the oocyte. (D-F) Localisation of GFP-Staufen in stage 9 egg chambers. (D) Wild type. (E) *btz²*. (F) *grk^{2B6}/grk^{2E12}*. (G,H) Localisation of GFP-Staufen in stage 11 egg chambers. (G) Wild type. (H) *exu^{VL}/exu^{QR}*. Note that the posterior localisation of GFP-Staufen is unaffected. Anterior is to the left, posterior to the right in this and all subsequent Figures.

oogenesis (Fig. 1C,G). This anterior localisation is abolished in *exu* or *swa* mutants, which disrupt the localisation of *bicoid* mRNA at an earlier stage, and is increased in females that overexpress *bicoid* mRNA (Fig. 1H and data not shown) (Berleth et al., 1988). Thus, Staufen associates with *bicoid* mRNA at the anterior of the oocyte from stage 10b, where it functions to anchor the RNA during the final stages of oogenesis.

A germline clone screen for mutants defective in GFP-Staufen localisation

Because GFP-Staufen co-localises with *oskar* and *bicoid* mRNAs at opposite poles of the oocyte, and at different stages of oogenesis, it provides an *in vivo* marker for the localisation of both transcripts, and hence the polarity of the AP axis. To identify new genes required for axis formation, we performed a genetic screen in living oocytes for mutants that affect the localisation of GFP-Staufen. We mutagenised flies carrying the FRT82B chromosome with ethyl methyl sulphonate (EMS), and used the FRT/FLP-ovo^{D1} system to generate germline clones of chromosome arm 3R, using the crossing scheme outlined in Fig. 2. From the primary screen of 5023 independent mutagenised lines, we recovered 400 lines with defects in GFP-Staufen localisation. These were then re-screened in fixed samples, which were counterstained with rhodamine-phalloidin to reveal the morphology of the egg chambers. This identified 141 lines that showed reproducible and penetrant phenotypes. We performed *in situ* hybridisations for *bicoid* or *oskar* mRNAs on most of these lines to assay their localisation directly, and this correlated with the localisation of GFP-Staufen in all cases.

We first crossed the mutants to alleles of all of the known

genes on 3R that affect *oskar* or *bicoid* mRNA localisation, and identified two new alleles of *oskar*, four of *orb*, six of *spn-E*, one of *D-elg* (*Ets97D*) and three of *cnc*, but no alleles of *tmlI* or *barentsz* were identified (Ephrussi et al., 1991; Kim-Ha et al., 1991; Christerson and McKearin, 1994; Erdelyi et al., 1995; Gajewski and Schulz, 1995; Gillespie and Berg, 1995; Tetzlaff et al., 1996; Guichet et al., 2001; van Eeden et al., 2001). As expected, all of these mutants affect *oskar* mRNA localisation, and the three new alleles of *cnc* also affect the localisation of *bicoid* mRNA. This indicates that the screen was efficient, and achieved a reasonable degree of saturation.

We classified the remaining mutants on the basis of their phenotypes and used this as a guideline in the complementation tests (Table 1). As expected, we recovered mutants that affect only *oskar* mRNA localisation (74), only *bicoid* mRNA localisation (18), or the localisation of both mRNAs (40). To identify complementation groups, mutants within each phenotypic class were crossed to each other, and the progeny assayed for lethality, female-sterility and maternal-effect lethal phenotypes. In addition, mutants that affect both *bicoid* and *oskar* mRNAs were crossed to mutants that affect the localisation of only one mRNA. With this approach, we identified 23 novel complementation groups (Table 2).

Mutants affecting *oskar* mRNA localisation

The majority of the mutants affect the posterior localisation of GFP-Staufen and *oskar* mRNA, but have little or no effect on the anterior localisation of GFP-Staufen and *bicoid* mRNA (Fig. 3). These mutants display three distinct classes of phenotypes that indicate defects in the three processes required for *oskar* mRNA localisation: microtubule organisation, mRNA transport and mRNA anchoring. The first two classes

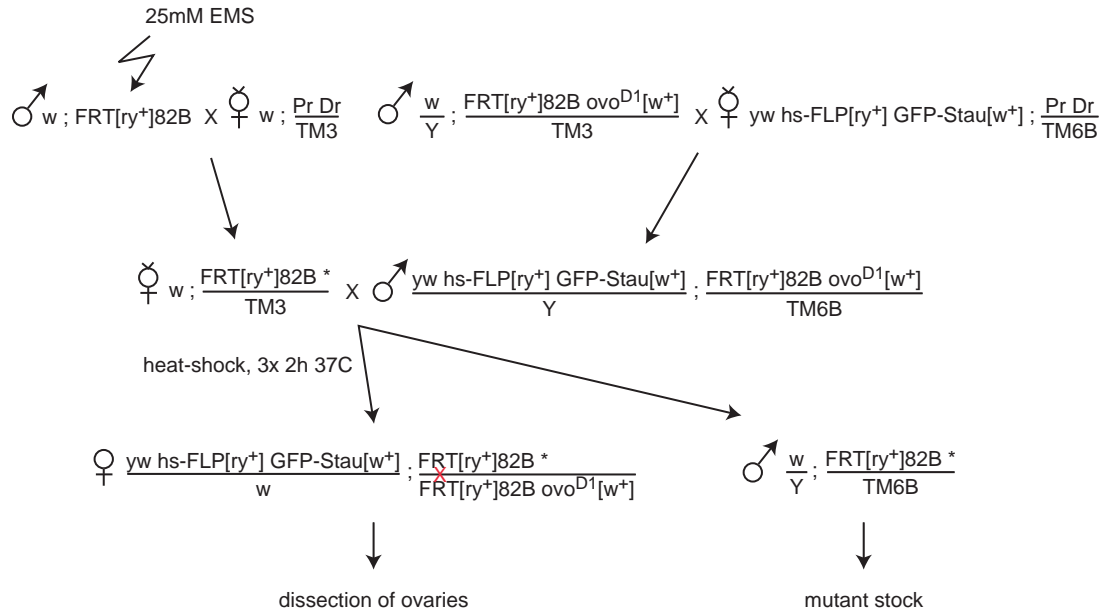


Fig. 2. Crossing scheme for isolating mutants defective in GFP-Staufen localisation. The asterisk indicates the mutagenised chromosome. The red X shows a mitotic recombination event between the two FRT sequences, which occurs after induction of FLP expression in response to heat-shock. *ovoD1* is a dominant female-sterile mutation that blocks oogenesis at early stages. Within a clonal ovary, the only germline cysts that develop to later stages are those that are homozygous for the mutagenised chromosome.

are represented by 55 mutants, in which GFP-Staufen and *oskar* mRNA accumulate in the oocyte but fail to localise to the posterior in a significant proportion of oocytes, and either remain diffuse throughout the cell or localise to its centre. These mutants comprise five *spn-E* alleles, the new alleles of *orb* and *D-elg*, and nine novel complementation groups (see Table 2).

Mutations in *errance*, *sverdrup*, *poulpe*, *strindberg*, *fraenkel* and *lkb1* display phenotypes indicative of a defect in oocyte polarisation. The oocyte nucleus is very occasionally mislocalised in *errance*, *sverdrup*, *poulpe* and *strindberg* mutants. In addition, GFP-Staufen and *oskar* mRNA often localise in an ectopic central dot in *poulpe* and *fraenkel* mutants, as they do in *par-1* and *14-3-3ε* mutants (Fig. 3E,F) (Shulman et al., 2000; Tomancak et al., 2000; Benton et al.,

2002). Examination of the microtubule cytoskeleton with Tau-GFP reveals that *poulpe* mutants display a disorganised microtubule network (data not shown), as do mutants of *lkb1*, which encodes the *Drosophila* homologue of *C. elegans par-4* (Martin and St Johnston, 2003). Thus, these six complementation groups probably affect mRNA localisation indirectly, by regulating microtubule organisation.

vagabond is the only complementation group in this class that gives the characteristic phenotype of mutants that disrupt *oskar* mRNA transport, in which the mRNA remains at the anterior of oocyte (Fig. 3C,D) (van Eeden et al., 2001). *vagabond* mutant oocytes display a range of additional phenotypes, including a mislocalised nucleus and aberrant organisation of the follicle cells, suggesting that the gene has other functions in addition to its role in *oskar* mRNA transport.

Table 1. Classification of the mutant lines according to their phenotype

Phenotypic class*	Number of mutants	Number of single alleles	Number of alleles in complementation groups (number of novel groups)
<i>oskar</i> diffuse/central	55	21	34 (9)
<i>oskar</i> falling off/diffuse at posterior	19	10	9 (3)
<i>bicoid</i> affected early	5	5	—
<i>bicoid</i> affected late	13	7	6 (3)
polarity phenotype†	5‡	2	3 (1)
novel phenotype, both affected	21	6	15 (4)
<i>oskar</i> and <i>bicoid</i> diffuse	14	8	6 (2)
other phenotype	9	7	2 (1)
total	141	66	75 (23)

*The different classes of mutants are described in the text.

†The 'polarity phenotype' is defined by the presence of *bicoid* mRNA at the posterior of the oocyte, whether the oocyte nucleus is mislocalised or not.

‡The second *boussole* allele (8B8-9) is included in this class, although *bicoid* mRNA was not detected at the posterior of the oocyte.

Table 2. Description of all the complementation groups

Mutant class	Name [†]	Number of alleles	Allele names	Germline clone phenotype	Trans-heterozygous phenotype	Penetrance*
<i>oskar</i> mRNA absent from the posterior of the oocyte	<i>franklin (frk)</i>	2	5E7-2, 6F2-18	<i>osk</i> diffuse. 6F2-18 shows additional defects including nurse cells in the oocyte and a weak dumpless phenotype.	lethal	+
	<i>lkb1</i>	2	4A4-2, 4B1-11	<i>osk</i> mainly diffuse, <i>bcd</i> detected around the whole oocyte cortex in some oocytes.	lethal	++
	<i>vagabond (vag)</i>	2	7A1-3, 8D4-17	<i>osk</i> anterior and diffuse, follicle cells disorganised, accumulation of actin in the oocyte, very few late stages, oocyte nucleus occasionally mispositioned.	female-sterile: a few ventralised eggs are laid	+++
	<i>errance (era)</i>	7	3A3-1, 4A4-18, 4E8-5, 5C5-8, 6D1-9, 7G2-9, 9B3-15	<i>osk</i> diffuse or in reduced amounts at the P, oocyte nucleus can be mislocalised, nurse cells fused or present in the oocyte, ectopic accumulation of actin, small eggs.	lethal	+++; allele-dependent
	<i>strindberg (sbg)</i>	2	5D3-3, 6B9-4	<i>osk</i> diffuse (in 5D3-3, <i>osk</i> accumulates in the centre at stage 9), oocyte nucleus can be mislocalised.	female-sterile: eggs either unfertilised or arrested early in development	+++
	<i>fraenkel (fkl)</i>	2	4B2-7, 5B3-7	<i>osk</i> in a central dot near the P, more diffuse in later stages.	lethal	++
	<i>sverdrup (sve)</i>	2	6F6-9, 8D1-7	<i>osk</i> central or diffuse, some eggs small or with fused dorsal appendages. In 8D1-7, very rare oocytes with aberrant <i>bcd</i> localisation.	lethal	+++
	<i>poulpe (pup)</i>	3	4B4-10, 4F2-4, 6C3-11	<i>osk</i> in a central dot or diffuse. In 4B4-10, <i>bcd</i> and the nucleus very rarely detected at the posterior. In 6C3-11, nurse cells in the oocyte in late stages.	lethal	+++
	<i>wellman (wel)</i>	2	8A6-5, 9H4-17	<i>osk</i> central, follicle cells disorganised and migrate between the oocyte and the nurse cells, oocyte too small. In 9H4-17, no late stages.	lethal	+++
	<i>orb</i>	4	7D1-16, 7D4-11, 7E4-5, 9D6-3	<i>osk</i> diffuse, premature cytoplasmic streaming.	female-sterile	+++
	<i>spindle-E (spn-E)</i>	6	2A9-14, 4E2-14, 7G2-5, 8D4-11, 9A2-17, 9A9-18	<i>osk</i> diffuse or in the centre of the oocyte, <i>bcd</i> mRNA seen at the posterior at stage 8 in 2A9-14. 9A9-18 is a weaker allele. Premature cytoplasmic streaming.	female-sterile	+++; allele-dependent
	<i>D-elg</i>	1	8C5-5	<i>osk</i> diffuse, dumpless.	female-sterile	+++
<i>oskar</i> mRNA present at the posterior of the oocyte, but not in a wild-type manner	<i>glissade (gls)</i>	3	7B7-18, 7F1-16, 9C9-3	<i>osk</i> at the P but diffuse, nurse cell fusions, dumpless.	lethal	+++
	<i>sedov (sdv)</i>	2	7D2-10, 9C1-2	<i>osk</i> diffuses from the P, nurse cell fusions, follicle cells disorganised. In rare cases, extra follicle cells are found between the oocyte and the nurse cells.	lethal	+++
	<i>koldewey (kol)</i>	2	4A2-13, 9B6-2	reduced amounts of <i>osk</i> at the P. In 4A2-13, additional defects such as nurse cell fusions and small eggs.	lethal	+++
	<i>oskar (osk)</i>	2	6B3-12, 8B1-2	In 8B1-2, <i>osk</i> at the posterior until stage 10, and then diffuse. In 6B3-12, <i>osk</i> is sometimes mislocalised even earlier.	maternal-effect lethal	+++
<i>bicoid</i> mRNA diffuse late	<i>larsen (lsn)</i>	2	2B6-3, 5F3-8	<i>bcd</i> diffuse (late), <i>osk</i> localisation wild-type but might be in reduced amounts.	lethal	+++
	<i>gerlache (grl)</i>	2	1A7-16, 8A7-15	very reduced anterior <i>bcd</i> localisation, GFP-Staufen retained at ring canals, dumpless.	female-sterile: very small unfertilised eggs	+++
	<i>sorcière (sorc)</i>	2	4E5-2, 7B6-14	very reduced anterior <i>bcd</i> localisation, GFP-Staufen retained at ring canals, dumpless.	female-sterile: very small unfertilised eggs	+++
both <i>oskar</i> and <i>bicoid</i> mRNA localisations affected	<i>boussole (bsl)</i>	2	8B8-9, 8F8-6	<i>osk</i> central or diffuse. In 8F8-6, <i>bcd</i> and the nucleus are occasionally detected at the posterior.	lethal	+
	<i>nansen (nan)</i>	3	4E7-9, 7G2-18, 8A4-1	<i>osk</i> diffuse or central, nucleus occasionally mislocalised, <i>bcd</i> sometimes at the P or lateral near nucleus, ectopic actin in oocyte.	lethal	+++
	<i>abruzzo (abz)</i>	2	7C3-10, 8B1-12	<i>osk</i> in dots near the P, nucleus mislocalised, <i>bcd</i> near the nucleus, dumpless eggs, some actin in oocyte.	lethal	+++

Table 2. Continued

Mutant class	Name [†]	Number of alleles	Allele names	Germline clone phenotype	Trans-heterozygous phenotype	Penetrance*
both <i>oskar</i> and <i>bicoid</i> mRNA localisations affected	<i>weyprecht (wey)</i>	2	9B5-5, 9C1-1	<i>osk</i> in broad cloud at P or central, <i>bcd</i> along lateral cortex. In 9B5-5, some follicle cell defects.	female-sterile: early oogenic arrest at about stage 5	+
	<i>trou</i>	5	1A2-4, 7E4-3, 9A5-5, 9C8-7, 9E4-15	frequent gap between oocyte and posterior follicle cells, variable <i>osk</i> and <i>bcd</i> localisation: <i>osk</i> in general diffuse with stronger P localisation, <i>bcd</i> cortical or diffuse.	semi-lethal and female-sterile: very small unfertilised eggs	+++
	<i>mertz (mez)</i>	2	7E2-8, 8D7-7	<i>bcd</i> initially wild type, diffuse from stage 11-12. Reduced amounts of <i>osk</i> at the P, small eggs. 7E2-8 is a weaker allele.	lethal	++
	<i>ellsworth (els)</i>	4	7B2-16, 7F5-9, 9D5-4, 9G8-14	<i>osk</i> at the anterior or diffuse or in reduced amounts at the P, <i>bcd</i> diffuse in late stages, nurse cell-oocyte fusions.	female-sterile: unfertilised eggs, escapers often show embryonic head defects	++
	<i>cap-n-collar (cnc)</i>	3	5F8-5, 7G2-10, 8A1-14	<i>osk</i> largely diffuse, nucleus mislocalised, <i>bcd</i> near the nucleus.	lethal	+++
other phenotypes	<i>nain</i>	2	4A3-6, 6A2-4	tiny oocyte, which can be slightly misplaced. Rare 4A3-6 late stage escapers.	lethal	+++

*Penetrance of the germline clone phenotype: + indicates that the phenotype was visible in 50% of the oocytes or less; ++ in 50-80%; +++ in 80% or more. However, these estimates should be treated with caution as the sample size was generally smaller than 50.

[†]To reflect the fact most of these mutants disrupt GFP-Staufen localisation to the anterior and/or posterior pole of the oocyte, many complementation groups were named after polar explorers who failed to reach the North or South Pole.

P, posterior; *osk*, *oskar*; *bcd*, *bicoid*.

In this respect, it may be similar to *mago nashi* and *Y14* (Micklem et al., 1997; Newmark et al., 1997; Hachet and Ephrussi, 2001; Mohr et al., 2001).

The third class of mutants consists of those in which *oskar* mRNA localises to the posterior, but fails to adopt a wild-type pattern (19 mutants, of which nine fall into four groups). Mutants in this group are unlikely to affect the transport of *oskar* mRNA or the polarisation of the oocyte, as *oskar* mRNA can reach the posterior. Instead, they probably disrupt the anchoring of *oskar* mRNA to the posterior cortex. In most cases, *oskar* mRNA and GFP-Staufen initially localise to the posterior normally (stage 9; Fig. 3G,H) but fail to be maintained there in later stages (Fig. 3I,J). In *glissade* (Fig. 3K,L) and *sedov*, GFP-Staufen and *oskar* mRNA localise to the posterior part of the oocyte but fail to restrict to a cortical crescent, a phenotype very similar to that of the *oskar* protein-null allele *osk*⁵⁴, which suggests that mutants in this group could also affect *oskar* translation (Ephrussi et al., 1991; Kim-Ha et al., 1991). In agreement with this, we found two novel *oskar* alleles in this class. Western blot analysis failed to detect Oskar protein in these mutants, suggesting that they are protein nulls (data not shown). Thus, most mutants in this group probably affect the translation or anchoring of *oskar* mRNA.

Mutants affecting *bicoid* mRNA localisation

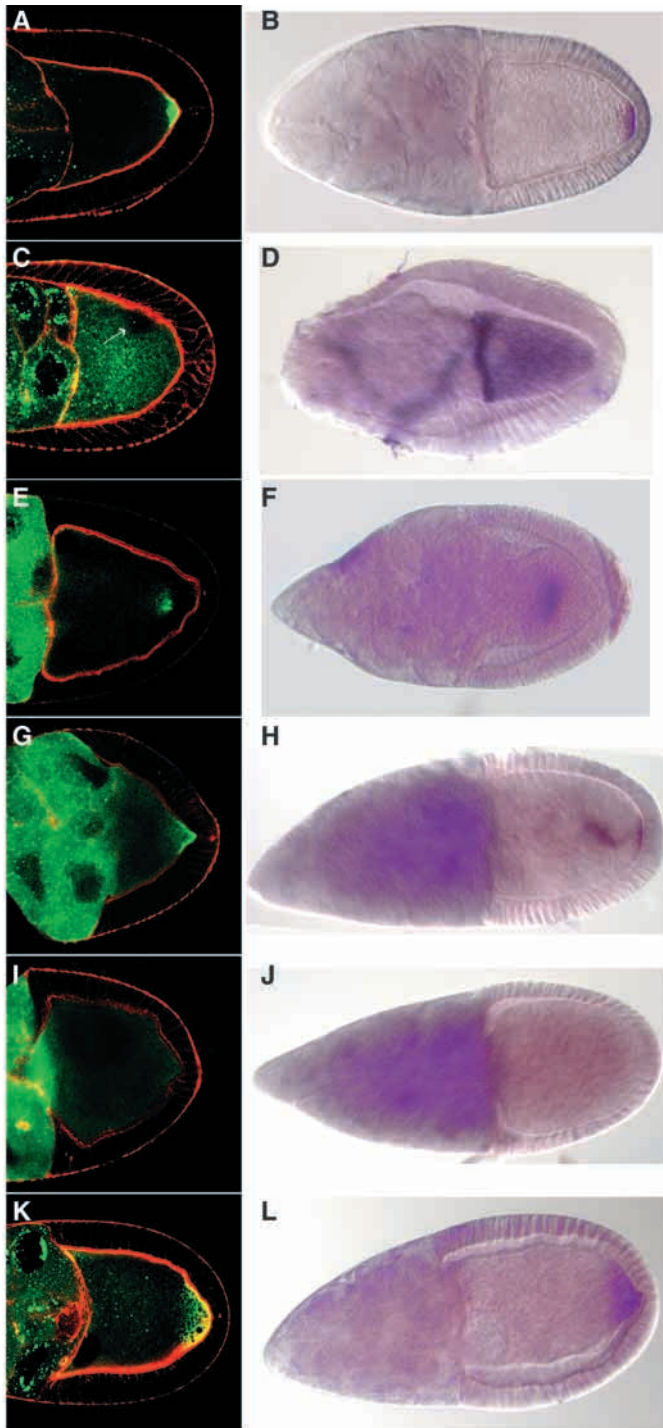
Several mutants disrupt the anterior localisation of GFP-Staufen but have little or no effect on posterior localisation, suggesting that they specifically affect the localisation of *bicoid* mRNA (Fig. 4). These mutants can be separated into two distinct classes based on when the defect in GFP-Staufen localisation is first observed. In the first class (five single alleles), GFP-Staufen never concentrates at the anterior of late stage oocytes and *bicoid* mRNA fails to accumulate at the

anterior of the oocyte at any stage (Fig. 4C,D), indicating that these mutants affect an early step in the localisation of *bicoid* mRNA. In the second class (13 mutants, six of which form three complementation groups), *bicoid* mRNA localises to the anterior of the oocyte in stage 8-10 egg chambers, but GFP-Staufen fails to accumulate at the anterior of late stage oocytes (Fig. 4E,F). These mutants therefore affect a later event in the anterior restriction of *bicoid* mRNA. In agreement with this, *bicoid* mRNA is not concentrated at the anterior of these mutants in late stage oocytes or freshly laid eggs (data not shown; U. Irion, personal communication).

Mutants affecting both *oskar* and *bicoid* mRNA

Forty mutants affect the localisation of both *oskar* and *bicoid* mRNAs (Fig. 5). Amongst these, we recovered four mutants (including one new *spn-E* allele) in which the oocyte nucleus could be mispositioned and *bicoid* mRNA was occasionally localised at the posterior of the oocyte. These included one allele of the lethal complementation group *boussole* (Fig. 5C,D). *oskar* mRNA localises to the centre of the oocyte in this mutant, and *bicoid* mRNA can occasionally be seen at the posterior of the oocyte, along with the nucleus. This phenotype is reminiscent to that of *mago* or *grk* and suggests that this gene may be required in the oocyte to receive the polarising signal back from the posterior follicle cells (Gonzalez-Reyes et al., 1995; Roth et al., 1995; Micklem et al., 1997). However, this defect often correlates with morphological changes in the posterior follicle cells, and may reflect the presence of mutant follicle cell clones.

Twenty-one mutants affect all markers of polarity within the oocyte: *oskar* mRNA usually fails to localise to a posterior crescent, the oocyte nucleus is mislocalised, and *bicoid* mRNA associates strongly with the cortex above the nucleus. This



class contains the 3 novel alleles of *cnc*. We also recovered 3 other complementation groups that give this phenotype: *abruzzi*, *nansen* and *weyprecht*. Of these, *abruzzi* displays the most striking phenotype (Fig. 5E,F): *bicoid* mRNA localises in a cortical ring at the level of the nucleus and is absent from the anterior cortex. In *nansen* and *weyprecht*, *bicoid* mRNA localises next to the mislocalised nucleus but is also present along the anterior cortex of the oocyte. To investigate whether this *bicoid* mRNA localisation phenotype is due to a defect in the organisation of the microtubule cytoskeleton, we stained germline clones of a subset of mutants from this class with an

Fig. 3. Classes of mutants affecting the localisation of *oskar* mRNA. GFP-Staufen (green) and rhodamine-phalloidin staining (red) are shown in the left panels (A,C,E,G,I,K). *oskar* in situ hybridisations are shown on the right (B,D,F,H,J,L). (A,B) Wild-type egg chambers. (C,D) *vagabond*^{7A1-3}. In this mutant, GFP-Staufen and *oskar* mRNA fail to localise to the posterior and *oskar* mRNA accumulates at the anterior of stage 9 oocytes. In C, the oocyte nucleus is also mislocalised to the lateral oocyte cortex (arrow). (E,F) *fraenkel*^{4B2-7}, a member of the class of mutants in which GFP-Staufen and *oskar* mRNA localise ectopically to the centre of the oocyte. (G-J) 2B1-8 egg chambers. In this mutant, GFP-Staufen and *oskar* mRNA localise to the posterior of the oocyte in stage 9 (G), but fail to be maintained there in later stages (I,J). In the stage 10A oocyte shown in H, *oskar* mRNA seems to be falling off from the posterior cortex. (K,L) *glissade*^{9E9-3} exemplifies the class of mutants in which GFP-Staufen and *oskar* mRNA localise to the posterior part of the oocyte, but not in a cortical crescent.

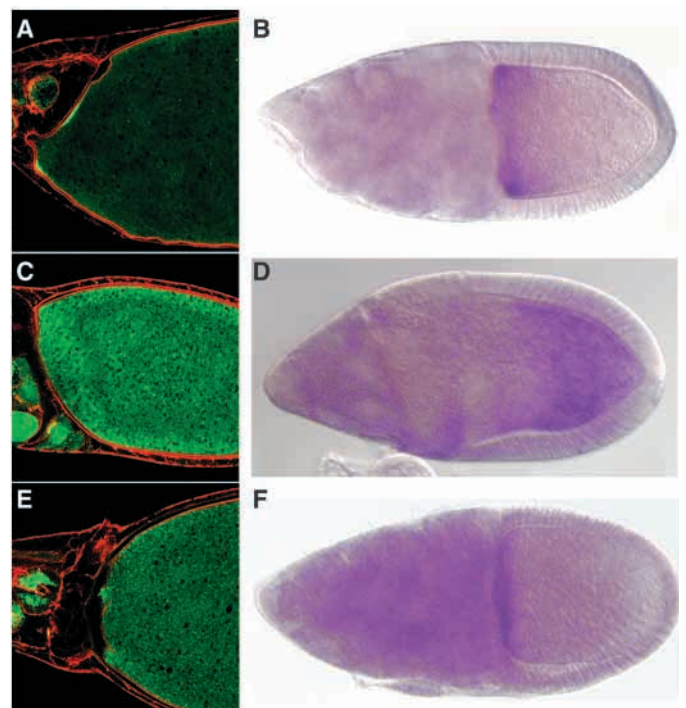


Fig. 4. Classes of mutants affecting the localisation of *bicoid* mRNA. GFP-Staufen (green) and rhodamine-phalloidin staining (red) are shown in the left panels (A,C,E). *bicoid* in situ hybridisations are shown on the right (B,D,F). (A,B) Wild-type egg chambers. (C,D) 7D8-15 egg chambers, an example of the class of mutants in which *bicoid* mRNA fails to concentrate along the anterior cortex in early stages of oogenesis. (E,F) *larsen*^{2B6-3}, a member of the second class of *bicoid*-specific mutants, in which *bicoid* mRNA localisation in early stages is indistinguishable from wild type, but GFP-Staufen fails to concentrate at the anterior cortex of stage 12 oocytes.

anti- α -tubulin antibody. All the mutants studied showed defects in the organisation of microtubules between stage 8 and 10 (data not shown).

Two further complementation groups, *mertz* (Fig. 5G,H) and *ellsworth*, affect the localisation of both *oskar* and *bicoid* mRNAs. In these mutants, the mRNAs localise to their respective poles in much reduced amounts, or are diffusely distributed throughout the oocyte. However, the oocyte nucleus

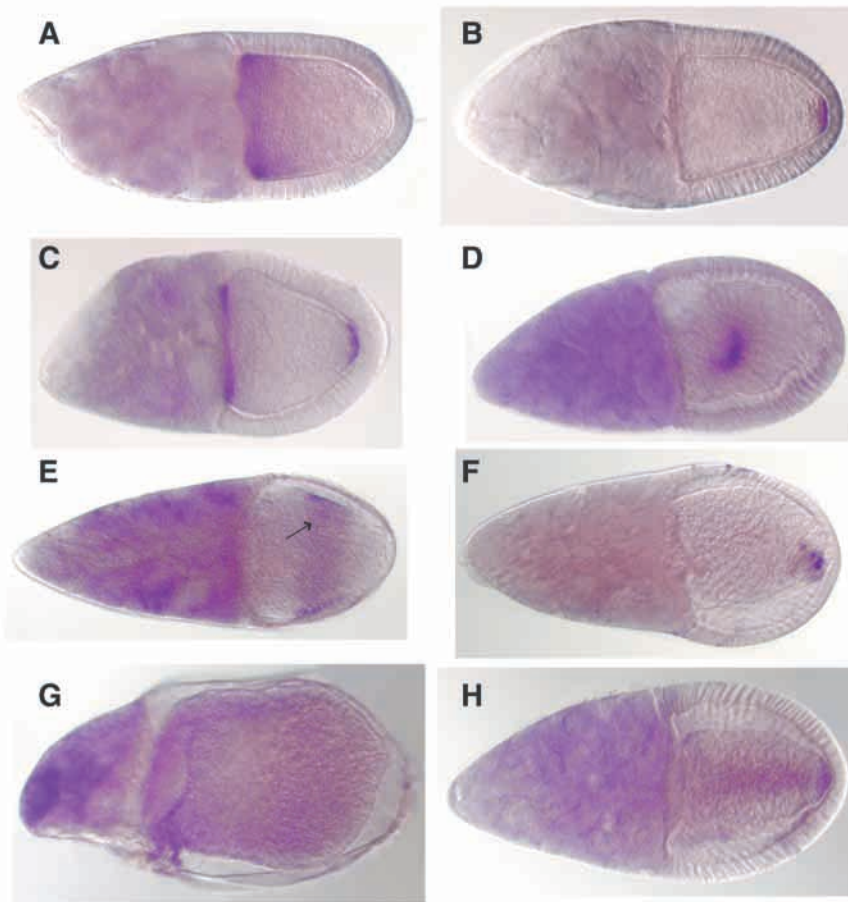


Fig. 5. Classes of mutants affecting the localisation of both *oskar* and *bicoid* mRNA. In situ hybridisations for *bicoid* are shown on the left (A,C,E,G) and hybridisations for *oskar* are shown on the right (B,D,F,H). (A,B) Wild-type egg chambers. (C,D) *boussole*^{8F8-6}. This mutant shows ectopic localisation of *oskar* mRNA to the centre of the oocyte, and posterior accumulation of *bicoid* mRNA in a subset of oocytes. (E,F) *abruzzo*^{7C3-10} is a member of a large class of mutants, in which the oocyte nucleus is frequently mislocalised (see arrow in panel E) and *bicoid* mRNA associates with the cortex proximal to the nucleus. In these mutants, *oskar* mRNA also fails to adopt a wild-type localisation pattern. (G,H) *mertz*⁸⁰⁷⁷, an example of the class of mutants in which both *oskar* and *bicoid* mRNAs fail to localise to their respective poles and are diffuse in the oocyte.

is always positioned normally, suggesting that the oocyte has been polarised correctly. These mutants may therefore affect factors, such as *staufen*, that are necessary for the transport of both *oskar* and *bicoid* mRNAs (Ferrandon et al., 1994). In agreement with this, *oskar* mRNA persists at the anterior of the oocyte in *ellsworth* mutants, as it does in other mutants that specifically disrupt *oskar* mRNA transport.

Other mutants

A number of mutants recovered for their defect in GFP-Staufen localisation also display unexpected additional phenotypes. *sorcière* (Fig. 6A) and *gerlache* are dumpless mutants in which the bulk of GFP-Staufen remains in the nurse cells, in or just anterior to the ring canals. Although this phenotype suggests a defect in ring canal growth, the morphology and size of the ring canals is indistinguishable from wild type, as assayed by rhodamine-phalloidin staining. In *trou* mutants, the posterior of the oocyte detaches from the follicle cells (Fig. 6B). This detachment may interfere with signalling from the posterior follicle cells, which could account for the variable defects in *oskar* and *bicoid* mRNA localisation in these mutants. In *sedov*, *vagabond* and *wellman* mutant egg chambers, excess follicle cells are sometimes found between the oocyte and the nurse cells, indicating a possible defect in the control of follicle cell migration or proliferation (Fig. 6C). We also found mutants that affect the growth of the oocyte (seven mutants, of which two form the complementation group *nain*). As GFP-Staufen

still accumulates in one cell in these mutants, the oocyte appears to be correctly determined, but fails to grow larger than the nurse cells. Finally, we found two mutants that affect the number of germ cell divisions, as the egg chambers contain more than 16 cells, and an oocyte with five or six ring canals (Fig. 6E).

Novel *orb* and *spn-E* alleles reveal a new phenotype

As mentioned above, we recovered six new alleles of *spn-E* and four of *orb*: two genes that encode RNA-binding proteins required for the polarisation of both AP and dorsoventral (DV) axes (Lantz et al., 1992; Christerson and McKearin, 1994; Lantz et al., 1994; Gillespie and Berg, 1995). SPN-E is an RNA helicase required at multiple steps during oocyte development; notably, *spn-E* mutants disrupt *oskar* and *gurken* mRNA localisation, and have an abnormal microtubule cytoskeleton at stage 9 (Gillespie and Berg, 1995; Gonzalez-Reyes et al., 1997). Interestingly, one of our *spn-E* alleles displays a novel phenotype in which *bicoid* mRNA is occasionally localised to the posterior of the oocyte at stage 8-9, suggesting that this may be a stronger allele than those previously studied.

We examined the organisation of the microtubule network in three of the new *spn-E* alleles (*spn-E*^{2A9-14}, *spn-E*^{4E2-14} and *spn-E*^{8D4-11}) and found that all display thick microtubule bundles at stage 9 (Fig. 7A,B). This microtubule organisation resembles that observed in *capu*, *spir* or *chic* mutants, which cause similar defects in *oskar* and *gurken* mRNA localisation

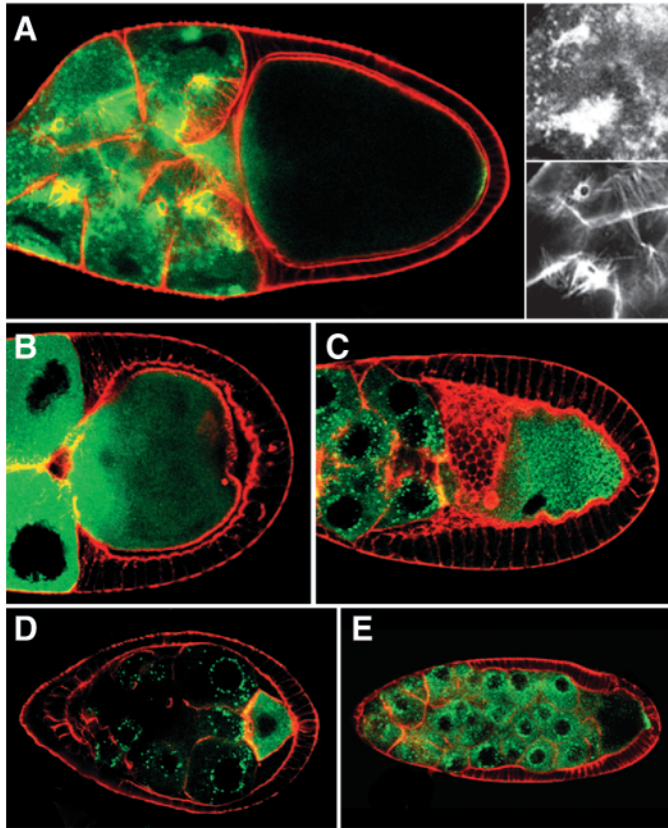


Fig. 6. Other phenotypes of mutants found in the screen.

(A) *sorcière*^{4ES-2}. In this mutant, the bulk of GFP-Staufen (green; top inset) is blocked in the nurse cells and co-localises with the ring canals stained with rhodamine-phalloidin (red; bottom inset). GFP-Staufen also fails to accumulate to the anterior of the oocyte in later stages. (B) *trou*^{9E4-15} shows a defect of adhesion between the posterior follicle cells and the oocyte. This mutant is also defective in *bicoid* and *oskar* mRNA localisation. (C) In *sedov*^{9E9-3}, a member of the class of mutants in which *oskar* mRNA is diffusely localised in the posterior part of the oocyte, the organisation of the follicle cells is aberrant. This phenotype may reflect a premature migration of 'centripetal' follicle cells between the oocyte and the nurse cells. Alternatively, it may indicate an overproliferation of follicle cells. A similar phenotype was observed in *wellman* and *vagabond* mutants. (D) *nain*^{4A3-6} is a member of the class of mutants in which the oocyte fails to acquire wild-type size. The rare oocyte escapers that grow fail to localise GFP-Staufen in a wild-type manner. (E) 4B7-11, one of the two mutants in which the egg chamber contains more than 16 germ cells.

(Neuman-Silberberg and Schüpbach, 1993; Theurkauf, 1994b; Manseau et al., 1996). As these mutants also cause premature cytoplasmic streaming, we studied the movement of auto-fluorescent vesicles in *spn-E* mutant oocytes. Whereas wild-type oocytes show slow and chaotic cytoplasmic movements at stage 9, 40-60% of *spn-E* mutant oocytes show a very fast circular movement (Fig. 7E,F; see Movie 1 at <http://dev.biologists.org/supplemental/>). Thus, *spn-E* is required for the formation of the polarised microtubule network at stage 9 and for the regulation of cytoplasmic streaming.

The role of ORB during mid-oogenesis has previously been studied using a single hypomorphic allele, *orb*^{mel}, in which

oskar mRNA fails to localise to the posterior and *grk* mRNA fails to localise to the dorsoanterior corner of the oocyte (Christerson and McKearin, 1994). As ORB binds *oskar* mRNA, these phenotypes have been interpreted as a function of ORB in RNA transport and translation (Christerson and McKearin, 1994; Chang et al., 1999). ORB is also necessary for the determination of the oocyte, as strong (*orb*^{F303}) and null (*orb*^{F343}) alleles arrest oogenesis at early stages (Lantz et al., 1994). We ordered the new *orb* alleles into an allelic series by analysing the strength of their phenotype in trans-heterozygous combinations over *orb*^{F343} (Fig. 7I). Whereas our stronger allele arrests at early stages of oogenesis (*orb*^{9D6-3}), the weaker alleles show similar defects to *orb*^{mel}: trans-heterozygous females lay ventralised eggs with fused or no dorsal appendages, and the rare eggs that are fertilised have a reduced number of abdominal denticle belts, indicating a defect in pole plasm assembly.

Because *orb* mutants cause a similar defect in both AP and DV patterning to *spn-E* mutants, we studied the organisation of the microtubule cytoskeleton. Both anti- α -tubulin antibodies and Tau-GFP reveal large cortical bundles of microtubules in over 90% of stage 9 *orb* mutant oocytes (Fig. 7A,C; data not shown). This phenotype was observed in germline clones of all four new alleles, as well as in trans-heterozygous combinations of these alleles and *orb*^{mel}, over *orb*^{F343}. In addition, *orb* mutant oocytes display a very fast circular cytoplasmic movement at stage 9 that is characteristic of premature cytoplasmic streaming (Fig. 7E,G; see Movie 2 at <http://dev.biologists.org/supplemental/>). Thus, *orb* hypomorphs have the same effect on the microtubules and cytoplasmic streaming as *capu*, *spir*, *chic* or *spn-E* mutants, suggesting that this is the primary cause of the defect in *oskar* mRNA localisation. Interestingly, ORB protein levels are dramatically reduced in *spn-E* mutants, suggesting that SPN-E may be involved in the post-transcriptional regulation of *orb* mRNA (Fig. 7J).

Ypsilon Schachtel (YPS) is another RNA-binding protein that co-localises with *oskar* mRNA at the posterior, and has been shown to act antagonistically to ORB in *oskar* mRNA localisation and translation (Mansfield et al., 2002). Whereas *oskar* mRNA fails to localise to the posterior in over 90% of *orb*^{mel}/*orb*^{F303} oocytes, about 50% of *yps*^{JM2} *orb*^{mel}/*yps*^{JM2} *orb*^{F303} have wild-type amounts of *oskar* mRNA at the posterior (Mansfield et al., 2002) (data not shown). This has been interpreted as a direct antagonism between YPS and ORB in the transport and translation of *oskar* mRNA (Mansfield et al., 2002). However, we do not detect significant changes in the level of OSK protein in *orb yps* versus *orb* mutants, indicating that YPS does not counteract the translational activation of *oskar* by ORB (Fig. 7J). By contrast, half of *yps orb* double-mutant oocytes have a wild-type microtubule organisation and a normal pattern of cytoplasmic streaming, which indicates that YPS acts antagonistically to ORB in the organisation of the microtubule cytoskeleton (Fig. 7D,H; see movies at <http://dev.biologists.org/supplemental/>). This observation suggests that the presence of *oskar* mRNA at the posterior of *yps orb* double mutants reflects the restoration of a wild-type microtubule network, rather than a direct effect of YPS on *oskar* mRNA. YPS does not appear to exert its effect by altering the levels of ORB protein, as ORB protein levels are not increased in *yps* mutants (Fig. 7J).

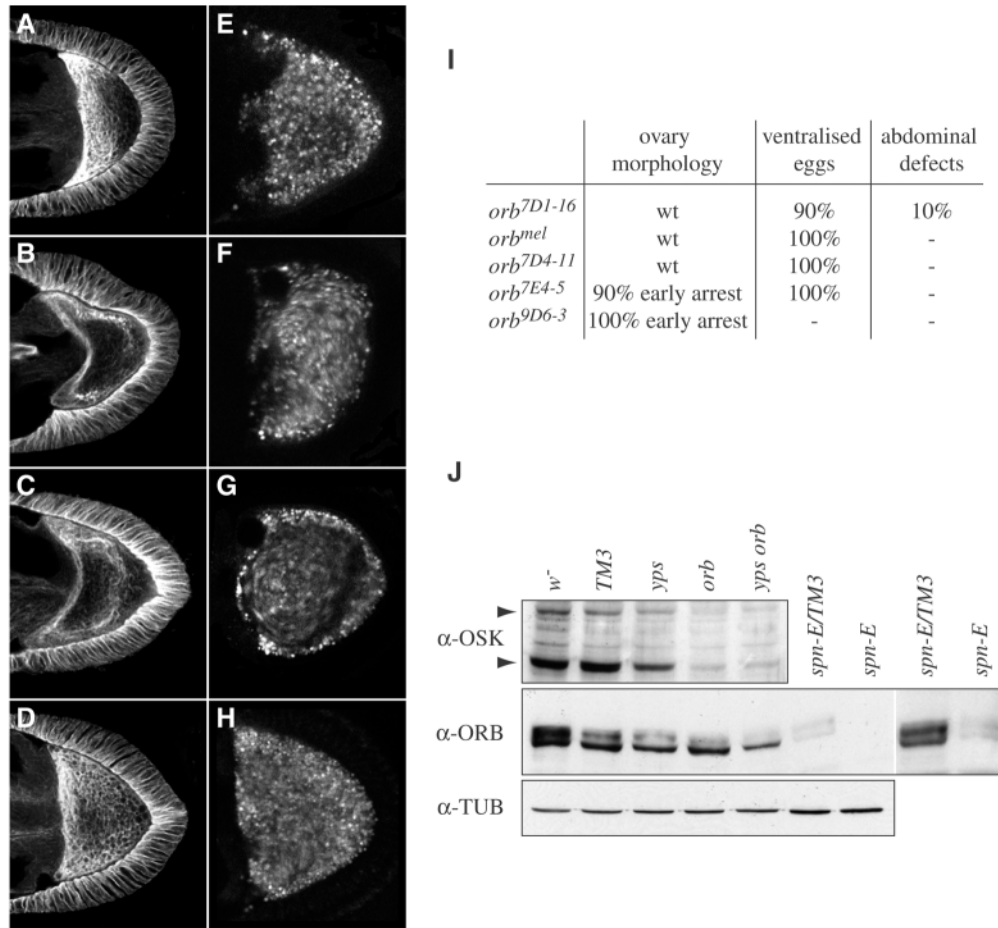


Fig. 7. *orb* and *spn-E* are required for the organisation of the microtubule cytoskeleton at stage 9. (A-D) α -tubulin staining. (E-H) Merge of eight consecutive frames of a time-lapse movie of autofluorescent particles. A static particle shows as a dot, whereas a moving particle forms a line. Wild-type oocytes (A,E) show a characteristic anterior to posterior gradient of microtubules and very little cytoplasmic movement. By contrast, *spn-E*^{4E2-14}/*spn-E*^{hlsΔ157} (B,F) and *orb*^{7E4-5} (C,G) oocytes display thick microtubule bundles and rapid circular cytoplasmic movements. Similar observations were made in *spn-E*^{2A9-14} and *spn-E*^{8D4-11}, and in all *orb* alleles described in panel I. (D,H) Wild-type patterns of microtubule distribution and cytoplasmic streaming are restored in half of *orb yps* double-mutant oocytes (*yps*^{JM2} *orb*^{mel}/*yps*^{JM2} *orb*^{F303}). (I) Allelic series of *orb* alleles. The alleles mentioned in the table were crossed to *orb*^{F343} and classified according to the phenotype of trans-heterozygous females. The ventralised eggs laid by these females were unfertilised. 'early arrest' indicates that egg chambers in these females arrested development during early oogenesis and failed to reach vitellogenic stages. (J) Western blot analysis of Oskar and ORB in *orb*, *orb yps* and *spn-E* ovaries. α -Tubulin (TUB) was used as a loading control. Longer exposure (on the right) shows the presence of low levels of ORB in *spn-E* mutant ovaries. Similar observations were made with *spn-E*^{2A9-14}, *spn-E*^{4E2-14} and *spn-E*^{8D4-11} alleles. Exact genotypes are as follows: TM3, *yps*^{JM2} *orb*^{F303}/TM3; *yps*, *yps*^{JM2}/*yps*^{JM2} *orb*^{F303}; *orb*, *orb*^{mel}/*yps*^{JM2} *orb*^{F303}; *orb yps*, *yps*^{JM2} *orb*^{mel}/*yps*^{JM2} *orb*^{F303}; *spn-E*/TM3, *spn-E*^{hlsΔ157}/TM3; *spn-E*, *spn-E*^{4E2-14}/*spn-E*^{hlsΔ157}.

DISCUSSION

Advantages of GFP clonal screens

We describe here a genetic screen for mutants affecting the AP polarisation of the *Drosophila* oocyte that has two major advantages over screens performed previously. First, by using GFP-Staufen as an in vivo marker for the localisation of *bicoid* and *oskar* mRNAs, we screened directly for defects in oocyte polarity and did not rely on the resulting maternal-effect phenotypes in the embryonic cuticle. To compare these two approaches, we examined whether the embryos derived from germline clones of 51 of our mutants showed a cuticle phenotype. Only 3 produced a clear AP defect in the embryonic cuticle (data not shown), whereas the majority of mutants

failed to produce eggs, or laid eggs that were unfertilised. These mutants would therefore not have been identified in any of the previous screens.

Second, we performed the screen in germline clones, which allowed us to recover lethal mutations. Indeed, 17 of the 23 new complementation groups are essential for viability and would have been missed in female-sterile or maternal-effect lethal screens. This suggests that about 70% of the genes necessary for AP axis formation have essential functions in other cell types. In the one case that we have examined in detail, we found that *lkb1* is also required for epithelial polarity (Martin and St Johnston, 2003). Thus, a proportion of the mutants identified in this screen may be general factors regulating polarity in multiple cell types.

Surprisingly, four mutants that display clear defects in the localisation of GFP-Staufen produce eggs that hatch and develop into normal larvae. Two of these are only partially penetrant, but the others produce similar phenotypes to *barentsz* mutants, in which the posterior localisation of GFP-Staufen and *oskar* mRNA is strongly reduced. It has been suggested that the defect in *oskar* mRNA localisation in *barentsz* mutants is partially rescued by localised translation, resulting in a weak grandchildless phenotype, and this may also be the case for these alleles (van Eeden et al., 2001). This illustrates another advantage of a direct visual screen that allows the identification of mutations with subtle phenotypes that do not cause lethality. The large size of the oocyte makes it a particularly good system in which to detect defects in cellular organisation, and we recovered mutations with a variety of polarity phenotypes that would have been very difficult to identify in smaller cells.

Degree of saturation

One disadvantage of germline clone screens is that they are laborious, which limits the number of lines that can be screened. We screened over 5000 mutagenised chromosomes and recovered new alleles for five of the seven known genes on chromosome 3R required for *oskar* mRNA localisation (*oskar*, *spn-E*, *Ets97D*, *orb* and *cnc*). However, we failed to recover new alleles of *barentsz* and *tmlI*. One reason for this may be that these genes are not easily mutable by EMS, because all of the existing alleles that disrupt *oskar* mRNA localisation are either P-element insertions or deletions (Erdelyi et al., 1995; Tetzlaff et al., 1996; van Eeden et al., 2001). Alternatively, hypomorphic alleles of these genes may exist in our collection of mutants but have been overlooked in the complementation tests, because they only cause a non-penetrant grandchildless phenotype, as is the case for *btz¹* (van Eeden et al., 2001). Thus, the screen was efficient in finding mutants affecting the posterior localisation of GFP-Staufen but did not find mutants in all genes that can be mutated to give this phenotype.

Another indication that the screen did not reach saturation is that 66 mutants (47%) could not be attributed to any complementation group. However, the degree of saturation varies considerably between the different phenotypic classes. It is likely that we missed many mutants that only affect the anterior localisation of GFP-Staufen, as this localisation is more difficult to score in living oocytes than the posterior localisation because of the strong yolk autofluorescence at late stages of oogenesis. In addition, GFP-Staufen only localises to the anterior in late oocytes and is therefore visible in fewer egg chambers per ovary. Another class of genes for which we probably did not reach saturation are those required at multiple stages of oogenesis. *orb*, *par-1* and *14-3-3ε*, for example, not only function to polarise the oocyte during mid-oogenesis, but are also necessary for the determination of the oocyte in the germlarium (Lantz and Schedl, 1994; Cox et al., 2001; Huynh et al., 2001; Benton et al., 2002). As germline clones of null mutations in these genes produce no late egg chambers, one can only recover hypomorphic mutations in a screen of this type. It is therefore likely that many of the single alleles correspond to hypomorphic mutants in such loci. However, it is now relatively straightforward to clone genes for which only one mutant allele is available by meiotic mapping with single

nucleotide polymorphisms (Berger et al., 2001; Martin et al., 2001).

Although we may have missed a number of genes for these reasons, the screen was very successful in identifying new mutants that affect the posterior localisation of GFP-Staufen and *oskar* mRNA. For example, over 70% of the mutants with phenotypes similar to *cnc* belong to complementation groups, which suggests that we are approaching saturation for this phenotype. *oskar* mRNA localisation depends on the polarisation of the oocyte microtubule cytoskeleton, the transport of the mRNA to the posterior and its anchoring at the posterior cortex, and we identified new complementation groups that are required for each of these steps. The cloning of these genes should therefore provide valuable insights into the molecular mechanisms that underlie these processes.

Mutants affecting oocyte polarity

One of the surprising results of the screen was the large number of mutants that appear to affect the polarity of the oocyte. These produce a range of different phenotypes that can be classified into at least four classes.

(1) We recovered only a few mutants in which *bicoid* mRNA localises to both poles of the oocyte, which is the phenotype expected for a defect in the transduction of the polarising signal (Lane and Kalderon, 1994; Micklem et al., 1997; Newmark et al., 1997). All of these are either single alleles, or are the only mutants in a complementation group that give this phenotype, suggesting that at least some are not simple loss-of-function mutations. Thus, there do not appear to be many genes that are specifically required for the transduction of the polarising signal.

(2) Most alleles of *spn-E* and *orb*, and *capu*, *spir* and *chic* mutants, cause the formation of large cortical bundles of microtubules that correlate with premature cytoplasmic streaming, and this disrupts the localisation of *oskar* but not *bicoid* mRNA (Theurkauf, 1994b; Manseau et al., 1996).

(3) *poulpe* and *fraenkel* mutants give a similar phenotype to *par-1* and *14-3-3ε*, in which Staufen and *oskar* mRNA localise to a dot in the centre of the oocyte (Shulman et al., 2000; Tomancak et al., 2000; Benton et al., 2002). These genes may therefore be required to recruit the plus ends of microtubules to the posterior. A related phenotype is produced by *lkb1*, *strindberg* and *sverdrup* mutations, but in these mutants Staufen and *oskar* mRNA are usually found in a diffuse cloud in the centre of the oocyte instead of in a tight 'dot'. A more detailed analysis of *lkb1* indicates that it also disrupts the recruitment of plus ends to the posterior (Martin and St Johnston, 2003). The difference between the central dots and clouds is unclear, but may reflect the degree to which microtubule plus ends are focussed in the centre of the oocyte. As PAR-1 and LKB1 are protein kinases that function in the same signalling pathway, the other genes in this group may encode novel components of this pathway (Shulman et al., 2000; Martin and St Johnston, 2003).

(4) *abruzzo*, *nansen*, *weyprecht* and *cnc* mutants disrupt the localisation of all three markers of oocyte polarity: the oocyte nucleus, *bicoid* mRNA and *oskar* mRNA (Guichet et al., 2001). It is particularly striking that some or all *bicoid* mRNA localises around the cortex adjacent to the misplaced nuclei in these mutants. As both the nucleus and *bicoid* mRNA are believed to localise to microtubule minus-ends, this phenotype

may reflect a defect in the nucleation of microtubules at the anterior of the oocyte. Nothing is known about how the new diffuse anterior MTOC forms at stage 7, but this occurs normally in *grk* mutants and is therefore independent of the posterior follicle cell signal. The mutants in this group may therefore provide an insight into how the microtubules are nucleated from the anterior cortex.

The diversity of phenotypes described above indicates that the polarisation of the microtubule cytoskeleton is a complex process that can be disrupted in several different ways. It is therefore likely that the signal from the posterior follicle cells is not transduced in a simple linear manner, but impinges on several parallel pathways that act together to generate the polarised microtubule array.

The role of *orb*, *yps* and *spn-E* in the localisation of *oskar* mRNA

ORB protein has been shown to bind *oskar* mRNA, and is required for its localisation and translation at the posterior of the oocyte (Christerson and McKearin, 1994; Lantz et al., 1994; Chang et al., 1999). Although this suggests that ORB plays a direct role in both processes, our results indicate that its effects on mRNA localisation are indirect. All hypomorphic *orb* alleles disrupt the organisation of the microtubule cytoskeleton and cause premature cytoplasmic streaming, and this can account for the dramatic reduction in the amount of localised *oskar* mRNA. Indeed, other mutants that cause premature cytoplasmic streaming, such as *capu* and *spir*, cause an identical *oskar* mRNA localisation phenotype (Theurkauf, 1994b; Manseau et al., 1996). Thus, ORB presumably controls the organisation of the microtubules by regulating the translation of some other mRNA(s), and the principal function of its interaction with *oskar* mRNA is to regulate its translation by promoting the formation of a long polyA tail (Chang et al., 1999; Castagnetti and Ephrussi, 2003). This function is very similar to that of its *Xenopus* homologue, CPEB, which binds cytoplasmic polyadenylation elements (CPE) in the 3'UTRs of several maternal mRNAs to stimulate their polyadenylation and translation (Lantz et al., 1992; Hake and Richter, 1994).

The RNA-binding protein YPS has also been proposed to play a direct role in the localisation and translation of *oskar* mRNA, because it co-localises with the mRNA to the posterior of the oocyte and antagonises the effects of ORB (Mansfield et al., 2002). Our results again suggest that this effect is indirect. *yps* mutants partially rescue the microtubule and premature streaming phenotypes of *orb* hypomorphs but have little or no effect on the levels of Oskar protein. Thus, YPS presumably counteracts the effects of ORB on the translation of RNAs that regulate the microtubule organisation. This is consistent with the biochemical characterisation of YPS as a component of a multi-protein complex, containing EXU and ME31B, that has been proposed to repress the translation of many oocyte mRNAs (Wilhelm et al., 2000; Nakamura et al., 2001).

Mutants in *spn-E* produce very similar microtubule and premature streaming phenotypes to *orb* mutants, and have strongly reduced ORB protein levels. SPN-E is an RNA-helicase, raising the possibility that it exerts its effect by regulating the processing of *orb* mRNA (Gillespie, 1995). Alternatively, as ORB is known to regulate its own translation, ORB and SPN-E may function together to regulate the

translation of common target RNAs, including that of *orb* itself (Tan et al., 2001). These results reveal a novel regulation of the microtubule network at the level of RNA translation, and it will be important in future to identify the RNA substrates of ORB, YPS and SPN-E.

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