ERRATUM

Oskar anchoring restricts pole plasm to the posterior of the *Drosophila* oocyte

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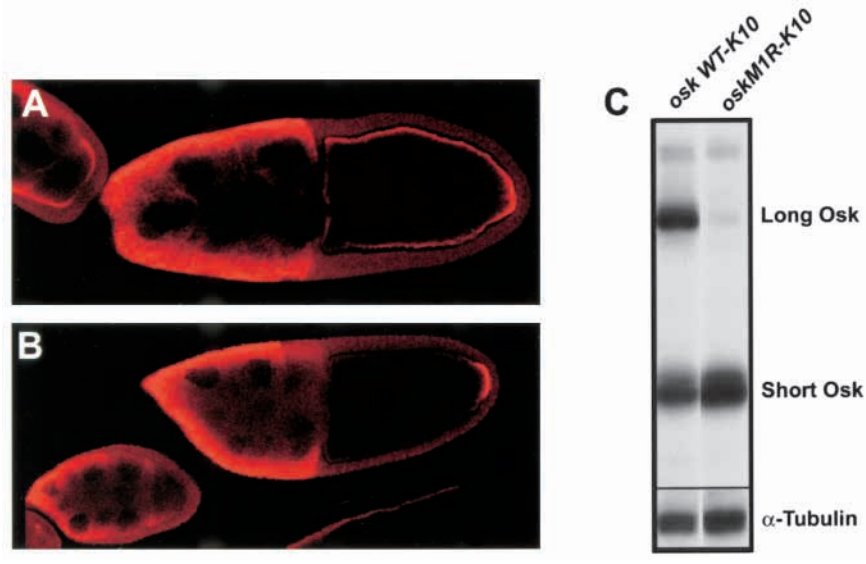
The reproduction of Fig. 6 in this article was innaccurate. The correct version of the figure is given below. We apologise to the authors and readers for the mistake.



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Oskar anchoring restricts pole plasm formation to the posterior of the *Drosophila* oocyte

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SUMMARY

Localization of the maternal determinant Oskar at the posterior pole of *Drosophila melanogaster* oocyte provides the positional information for pole plasm formation. Spatial control of Oskar expression is achieved through the tight coupling of mRNA localization to translational control, such that only posterior-localized *oskar* mRNA is translated, producing the two Oskar isoforms Long Osk and Short Osk. We present evidence that this coupling is not sufficient to restrict Oskar to the posterior pole of the oocyte. We show that Long Osk anchors both *oskar* mRNA and Short Osk, the isoform active in pole plasm assembly, at the posterior pole. In the absence of anchoring by Long Osk, Short Osk disperses into the bulk cytoplasm during late oogenesis, impairing pole cell formation in the embryo. In addition, the pool of untethered Short Osk causes anteroposterior patterning defects, owing to the dispersion of pole plasm and its abdomen-inducing activity throughout the oocyte. We show that the N-terminal extension of Long Osk is necessary but not sufficient for posterior anchoring, arguing for multiple docking elements in Oskar. This study reveals cortical anchoring of the posterior determinant Oskar as a crucial step in pole plasm assembly and restriction, required for proper development of *Drosophila melanogaster*.

Key words: Oskar, Anchoring, Cortex, Pole plasm, Determinants, Germline, Germ cell, *Drosophila*

INTRODUCTION

The asymmetric transport of mRNA to precise subcortical regions of the cell is an evolutionarily conserved mechanism for the segregation of cytoplasmic determinants involved in cell-fate specification or patterning (Bashirullah et al., 1998). For example, in S*accharomyces cerevisiae*, the asymmetric sorting of Ash1p, the repressor of cell-type switching, to the daughter cell relies on actin-based transport of *Ash1* mRNA to the bud tip during mitosis (Takizawa et al., 1997). Here, cytokinesis occurs soon after *Ash1* mRNA transport, ensuring segregation of the localized determinant away from the mother cell. However, in other organisms, an extensive delay can exist between localization of determinants and their ultimate entrapment by membranes. One strategy to achieve durable localization over longer periods is to anchor determinants robustly to their target site.

One prominent example in which a delay of several hours occurs between segregation and subsequent partition of cell-fate determinants into cells concerns the process of germline formation. In *Drosophila melanogaster*, this process begins during mid-oogenesis and progresses over many hours through the successive recruitment of RNAs and proteins, leading to formation of a specialized cytoplasm, the germ plasm, at the posterior tip of the mature oocyte (for a review, see Mahowald, 2001). It is only during cellularization of the embryo that germ plasm determinants are incorporated into pole cells, specifying the primordial germ cells of the fly. Delayed cellularization causes a germ cell-less phenotype due to the delocalization of the determinants before their internalization (Iida and Kobayashi, 2000). This underscores the importance of cortical anchoring of the determinants after localization. Similarly, anchoring most probably ensures maintenance of germ plasm localization at the vegetal cortex of full-grown *Xenopus laevis* oocytes awaiting maturation (Alarcon and Elinson, 2001). Although long-term cortical maintenance of determinants seems decisive for germline formation both in invertebrates and vertebrates, little is known of the mechanisms governing this process.

Germline formation and posterior patterning of the *Drosophila* embryo are regulated by a common genetic pathway. A crucial actor in this pathway is the maternally provided *oskar* (*osk*) mRNA, which localizes to the posterior pole of the oocyte at mid-oogenesis (Ephrussi et al., 1991; Kim-Ha et al., 1991). Posterior localization of *osk* mRNA requires a polarized microtubule network and an intact microfilament-based cytoskeleton, as revealed by mutants in the plus-end motor Kinesin I or in the actin-related proteins Profilin and Tropomyosin II, in which the RNA localization process fails (Brendza et al., 2000; Manseau et al., 1996; Erdélyi et al., 1995). Cis-acting elements located in the *osk* mRNA 3′ untranslated region (3′UTR) mediate RNA transport

(Ephrussi and Lehmann, 1992; Kim-Ha et al., 1993). Two RNA-binding proteins, Staufen and Y14, as well as Mago nashi and Barentsz, are thought to be part of the transport complex (St Johnston et al., 1991; Hachet and Ephrussi, 2001; Mohr et al., 2001; Micklem et al., 1997; van Eeden et al., 2001). Until posterior localization, *osk* mRNA translation is repressed by several trans-acting factors bound to its 3′UTR (Kim-Ha et al., 1995; Gunkel et al., 1998; Lie and Macdonald, 1999). Upon localization, *osk* translation is activated by a still unknown mechanism involving a derepressor element in the 5′ part of *osk* mRNA and Staufen (Gunkel et al., 1998; Micklem et al., 2000). Throughout the rest of oogenesis and until early embryogenesis, both *osk* mRNA and Oskar (Osk) protein remain concentrated at the posterior cell cortex. Here, Osk nucleates formation of the germ plasm, also known as pole plasm because of its location, recruiting the components for both germline formation and posterior patterning. In contrast to germ cell determinants, which are maintained in a narrow subcortical region at the posterior of the egg until their encapsulation into pole cells, the posterior determinant Nanos diffuses towards the anterior, forming a gradient that governs abdominal segmentation of the embryo (for reviews, see Williamson and Lehmann, 1996; Wang et al., 1994).

The decisive role of Osk in induction of pole plasm assembly is revealed by genetic loss- and gain-of-function experiments. Embryos that lack maternal *osk* activity develop neither posterior segments nor pole cells (Lehmann and Nüsslein-Volhard, 1986). Conversely, mis-expression of *osk* at the anterior of the embryo causes formation of an ectopic pole plasm, interfering with normal anterior development, because of production of Nanos in the anterior of the embryo (Ephrussi and Lehmann, 1992; Smith et al., 1992). Observed defects range from loss of head structures to duplication of the abdomen and occasionally germ cells at the anterior, the latter requiring higher doses of Osk.

Despite the obvious contribution of RNA localization in generating a concentrated source of *osk* mRNA at the posterior pole, another mechanism must be responsible for its maintenance. Indeed, the polarized microtubule network, which is established at stages 7/8 and mediates the bulk of *osk* mRNA localization, is disassembled during stage 10, and the vigorous cytoplasmic flow of vesicles, called the oocyte streaming, ensues (Gutzeit and Koppa, 1982; Theurkauf et al., 1992). From then onwards, maintenance of *osk* localization requires Osk protein, Staufen and BicD (Kim-Ha et al., 1991; Ephrussi et al., 1991; Rongo et al., 1995; Swan and Suter, 1996). Of the two Osk isoforms translated from alternative in-frame initiation codons, Long Osk has been proposed to mediate *osk* mRNA maintenance, based on the tight localization of this isoform at the oocyte posterior (Markussen et al., 1995). Consistent with this, it has been reported that *osk* mRNA is maintained at the posterior of the early embryo only in the presence of Long Osk (Rongo et al., 1997). In contrast, Short Osk function appears restricted to pole plasm assembly (Markussen et al., 1995; Breitwieser et al., 1996).

We show that Long Osk anchors both *osk* mRNA and Short Osk at the posterior cortex of the oocyte, ensuring their stable maintenance during late oogenesis when cytoplasmic streaming occurs. Through this anchoring, Long Osk plays a dual role in *Drosophila* development, guaranteeing both spatial restriction and integrity of the pole plasm throughout oogenesis, ensuring proper patterning and efficient germline formation. This analysis unravels the crucial association of transport and anchoring mechanisms when localization of developmental determinants must be durably maintained.

MATERIALS AND METHODS

Fly stocks, transgenes and transformation

The wild-type reference stock was Oregon R. The *osk* RNA null allele, osk^{A87}, is described elsewhere (M. Erdélyi, D. St Johnston and A. E., unpublished). $Df(3R)p^{XT103}$, which uncovers *osk*, has been previously described (Lehmann and Nüsslein-Volhard, 1986). The recipient stock for P element transformation was w^{1118}. Transformation was performed according to standard procedure (Rubin and Spradling, 1982). Transgene *oskM139L* was previously described (Markussen et al., 1995). The substitution (ATG to CGC) at the first translation initiation codon of the *osk* open reading-frame in the transgene *oskM1R* was introduced by oligonucleotide-directed mutagenesis using PCR. All constructs were cloned into the pCaSpeR4 vector (Pirrotta, 1988) for germline transformation.

Construction of the *osk-K10* 3′UTR transgene was as previously described (Riechmann et al., 2002). *oskM1R-K10* 3′UTR was generated by subcloning a *Bam*HI-*Xba*I *osk* cDNA fragment containing the substitution ATG→CGC at the first translation initiation codon, into pUASP2 (Rorth, 1998) linearized by the same enzymes. Construction of the transgene $m1^{414}lacwt$ has been described previously (Gunkel et al., 1998).

Immunological and colorimetric detection in situ

Whole-mount antibody staining of ovaries and embryos was performed according to Tomancak et al. (Tomancak et al., 2000) and RNA in situ hybridization of embryos was as described by Mata et al. (Mata et al., 2000). Preparation of ovarian extracts (Markussen et al., 1997) and western blotting analysis have been previously described (Markussen et al., 1995). The polyclonal rabbit anti-Osk antibody used in this work was generated against an *Eco*RV-*Sph*I fragment of the Blue-osk cDNA (Ephrussi et al., 1991) and was a gift from Andreas Jenny. Membranes were stripped and re-probed with an α-tubulin monoclonal antibody DM 1A (1:2000, Sigma). Rat anti-Vasa polyclonal serum, used to visualize pole cells, was previously described (Tomancak et al., 1998). β-Galactosidase activity was detected in situ in ovaries as described in elsewhere (Clark et al., 1994). Stained ovaries were mounted in 80% glycerol.

FISH and FISH/immunofluorescence double-labeling of ovaries

An *osk* RNA probe was synthesized by run-off transcription from the plasmid oskTAAXba linearized with *Xba*I (Ephrussi and Lehmann, 1992) and labeled with digoxigenin-11-UTP ribonucleotides (Roche). For in situ hybridization, ovaries were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) for 30 minutes and rinsed three times 10 minutes in PBT (PBS + 0.5% Tween 20). Subsequent steps were as previously described for RNA in situ hybridization of embryos (Wilkie et al., 1999). The *osk* digoxigenin-labeled probe was visualized by TSA detection using FITC-tyramide (NEN-life Sciences, UK). After overnight equilibration in N-propylgallate, ovaries were mounted on slides and images were captured with a Leica laser scanning confocal microscope.

FISH/antibody double-labeling was performed according to Hughes and Krause (Hughes and Krause, 1999) with the following modifications. Ovaries were fixed in 4% paraformaldehyde/PBS for 30 minutes and rinsed three times for 10 minutes in PBT. Ovaries were successively pre-hybridized 10 minutes in HYB/PBT (1:1) (see

Hughes and Krause, 1999) and 1 hour in HYB at 64°C. Hybridization was carried out at 64°C. After the post-hybridization washes, the ovaries were directly incubated with anti-digoxigenin (1:200) and rabbit anti-Osk antibodies (1:1000) in PBT during 12-15 hours at room temperature. Ovaries were washed three times for 10 minutes in PBT and incubated with a Rhodamine-coupled anti-rabbit secondary antibody (1:300, Jackson ImmunoResearch Laboratories) during 2 hours before proceeding with the TSA-based detection of *osk* RNA.

Rescue and fertility analysis

For rescue analysis, eggs were collected on apple juice plates and hatch rates scored by counting hatched and unhatched eggs, after aging for 24 hours at 25°C. Fertility of hatchers was tested by mating the female offspring with wild-type males.

RESULTS

Long Osk maintains *osk* mRNA at the posterior of the oocyte during late oogenesis

To directly investigate the process of *osk* RNA maintenance during oogenesis, we expressed the two Osk isoforms separately in *osk* mRNA null females (M. Erdélyi, D. St Johnston and A. E., unpublished) and observed *osk* mRNA distribution in ovaries. We expressed each isoform independently from the *oskM1R* and *oskM139L* transgenes, in which the first or the second start codon was mutated, respectively (see Materials and Methods) (Markussen et al., 1995). In situ hybridization reveals that both transgenic *osk* mRNAs accumulate in the oocyte during the early stages of oogenesis and concentrate at the posterior pole during mid-oogenesis (Fig. 1A), as is the case for endogenous *osk* mRNA in wild-type egg chambers (Kim-Ha et al., 1991; Ephrussi et al., 1991). During stage 10, however, a striking difference in the distribution of the two *osk* mRNAs is observed. In the case of transgenic lines expressing only Long Osk, *osk* mRNA remains tightly localized at the posterior pole of late stage oocytes and in the early embryo (lower panels), as it does when both isoforms are expressed from a wild-type rescuing transgene (upper panels). In contrast, in oocytes expressing only Short Osk, the bulk of *osk* mRNA detaches from the posterior cortex of the oocyte and disperses massively (middle panels). The penetrance of delocalization is high: 95% of late stage 10 oocytes (n=118) show significant RNA detachment, resulting in only trace amounts of *osk* mRNA detected at the posterior pole of freshly laid eggs (see arrow). Notably, this pattern of delocalization is distinct from that observed in the *osk* mutants osk^{84} and osk^{346}, which are predicted to produce only truncated Osk peptides (Kim-Ha et al., 1991). Indeed, whereas osk^{84} mRNA spreads diffusely during stage 10 and remains visible only in a restricted area close to the posterior pole (Fig. 1B, top panel), *oskM1R* mRNA disperses in tight aggregates that are readily detected in the anterior half of the oocyte (Fig. 1B, bottom panel). Western blotting analysis of ovary extracts reveals that similar quantities of Short and Long Osk are produced from the two transgenes *oskM1R* and *oskM139L* (Fig. 1C). This indicates that a qualitative, rather than a quantitative, difference accounts for the differential ability of Short and Long Osk to maintain *osk* mRNA at the posterior pole of the oocyte after mid-oogenesis. Thus, we conclude that the two Osk isoforms differ in their intrinsic competence to maintain *osk* mRNA and that only Long Osk fulfils this function.

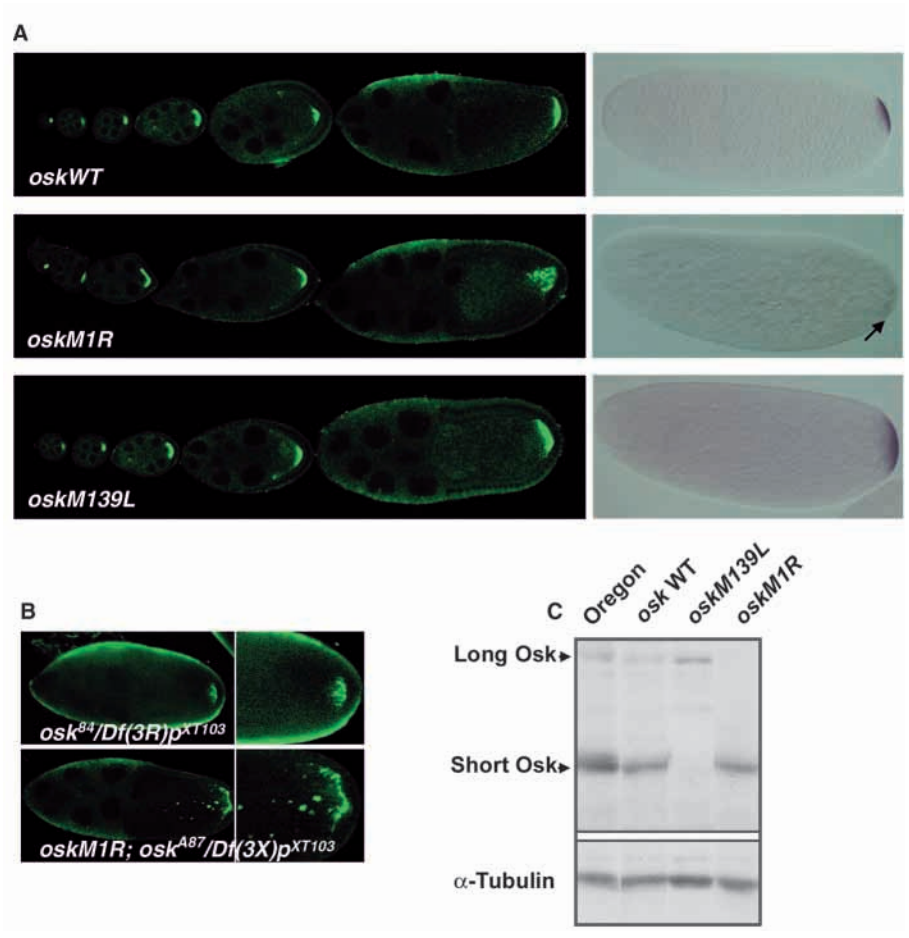


Fig. 1. *osk* mRNA maintenance at the posterior pole of the oocyte requires Long Osk. (A) *osk* mRNA in situ hybridization to whole-mount ovaries (left panels) and freshly laid eggs (right panels; anterior is towards the left and dorsal towards the top) of *osk* RNA-null females $osk^{A87}/Df(3R)p\text{-}XT103$, expressing either a wild-type *osk* transgene (upper panels), *oskM1R* encoding Short Osk (middle panels) or *oskM139L* encoding Long Osk (lower panels). The arrow indicates residual *osk* mRNA in embryos produced by females expressing Short Osk alone. (B) Comparison of the delocalization pattern of *osk* mRNA in stage 10 oocytes of the *osk* nonsense mutant $osk^{84}/Df(3R)p\text{-}XT10$ (upper panel) and of the RNA-null mutant $osk^{A87}/Df(3R)p\text{-}XT103$ expressing *oskM1R*. (C) Western blot analysis of Osk isoforms produced in ovaries shown in A, and of α-Tubulin (an internal loading control). Oregon R is the wild-type reference strain.

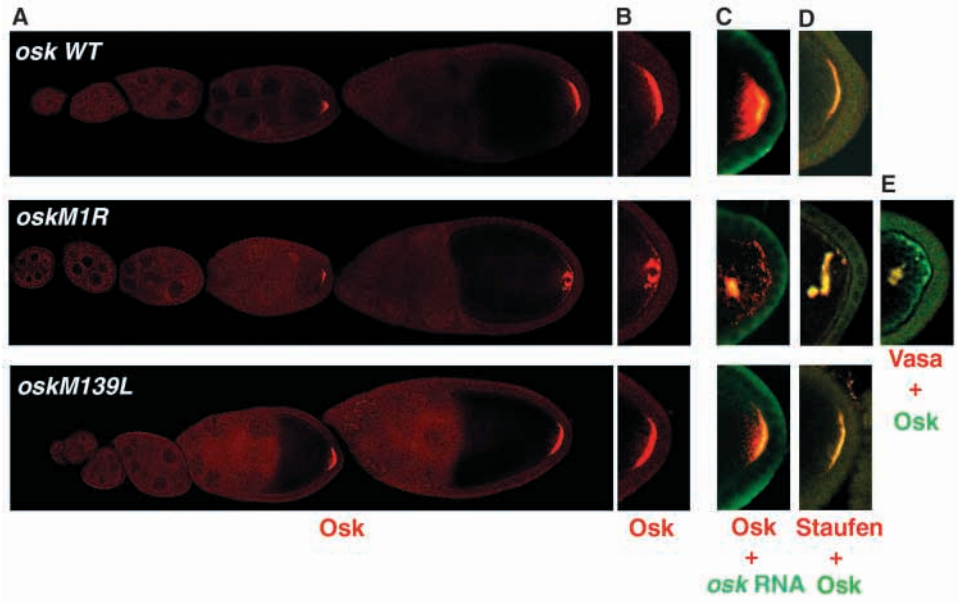


Fig. 2. Long Osk, but not Short Osk, can anchor at the posterior pole of the oocyte. (A-E) Confocal images of Osk isoforms detected by immunostaining of *osk* RNA-null ovaries osk^{A87}*/Df(3R)p-XT103,* expressing either a wild-type *osk* transgene (upper panels), *oskM1R* encoding Short Osk (middle panels) or *oskM139L* encoding Long Osk (lower panels). The rabbit anti-Osk polyclonal serum used in these experiments detects both Osk isoforms. During late stage 10, Long Osk remains tightly anchored at the posterior pole in a crescent-shaped pattern (bottom panels), as observed when both isoforms are expressed (top panels). At the same stage, Short Osk shows either a 'dotty' localization close to the posterior cortex (3%) or is strongly detached in aggregated particles that disperse in the bulk ooplasm (97%) (middle panels). (B) Magnification of the posterior tip of the oldest egg chamber shown in A. (C-E) Co-localization of pole plasm components at the posterior tip of stage 10 egg chambers. (C) Merged confocal images of *osk* mRNA (green) and Osk protein (red) simultaneously detected by in situ hybridization and antibody staining. Merged confocal images of immunohistochemically detected Staufen (red) and Osk (green) (D), and of Oskar (green) and Vasa (red) (E).

Long Osk, but not Short Osk, anchors at the posterior pole of the oocyte

Possible explanations of the failure of Short Osk to maintain *osk* mRNA at the oocyte posterior might be that Short Osk lacks elements crucial for its association with an anchoring target at the cortex and/or lacks the binding activity necessary for RNA sequestration. To investigate these possibilities, we first analyzed the distribution of the two Osk isoforms when expressed separately during oogenesis, in the same *osk* RNA null mutant background (Fig. 2). Both isoforms are detected during mid-oogenesis at the posterior pole of the oocyte where *osk* mRNA is localized (Rongo et al., 1995; Markussen et al., 1995). However, during late stage 10 of oogenesis, Short Osk disperses in aggregates from its initial cortical location in 97% of egg chambers (n=84), in contrast to Long Osk, which persists tightly anchored at the oocyte cortex (Fig. 2A, middle and lower panels). Higher magnification of the posterior pole at this stage shows that only small amounts of Short Osk are maintained at the oocyte cortex, in comparison with Long Osk (Fig. 2B). Hence, Long Osk, but not Short Osk, has the capacity to anchor to the oocyte cortex, where *osk* mRNA is localized and translated. To assess whether Short Osk has the capacity to associate with its own mRNA, we then examined the released Short Osk and *osk* mRNA aggregates in *oskM1R* oocytes, by simultaneous detection of RNA and protein. The merged confocal image reveals that Short Osk and *osk* mRNA co-localize in the released particles (Fig. 2C, middle panel). This demonstrates that Short Osk, although unable to anchor at the cortex, is closely associated with its own mRNA as is Long Osk (Fig. 2C, lower panel).

We next investigated localization of other pole plasm components in egg chambers expressing a single Osk isoform. We examined the distribution of Staufen, an RNA-binding protein associated with *osk* mRNA throughout oogenesis, and of Vasa, a DEAD-box RNA helicase recruited by Short Osk and required for subsequent recruitment of the other pole plasm components (St Johnston et al., 1991; Lasko and Ashburner, 1990; Hay et al., 1990; Breitwieser et al., 1996). In oocytes expressing only Short Osk, Staufen and Vasa are detected in the same Short Osk/*osk* RNA detaching granules (Fig. 2D, middle panel, Fig. 2E), showing that Short Osk detachment causes pole plasm delocalization. By contrast, in ovaries expressing only Long Osk, Staufen remains associated with Long Osk at the posterior pole of the oocyte (Fig. 2D, lower panel), whereas Vasa is not recruited (data not shown) (Breitwieser et al., 1996).

Co-expression of the two Osk isoforms mimics wild-type maintenance and accumulation of Short Osk during oogenesis

To determine whether expression of Long Osk would suffice to rescue the *osk* mRNA and Short Osk anchoring defects observed in *oskM1R* transgenic ovaries, we generated flies expressing both Short and Long Osk from the *oskM1R* and *oskM139L* transgenes. Remarkably, co-expression of Long Osk entirely suppresses the granular detachment of both *oskM1R* mRNA and its protein product, Short Osk (Fig. 3B,D). In situ hybridization reveals a pattern of *osk* mRNA maintenance indistinguishable from that observed in stage 10 oocytes expressing the two Osk isoforms from a wild-type *osk* transgene (compare Fig. 3B with Fig. 1A). Similarly, the characteristic crescent-shaped distribution of Osk isoforms observed in wild-type oocytes is detected at the posterior pole of oocytes produced by the doubly transgenic females (Fig. 3D). This result demonstrates that the nucleotide substitutions in *oskM1R* mRNA, replacing the first translation initiation codon, do not interfere with RNA cortical anchoring. It also rules out that ongoing translation from M1 is required per se for RNA maintenance. Rather, we conclude that expression of Long Osk from the *oskM139L* transgene is responsible for

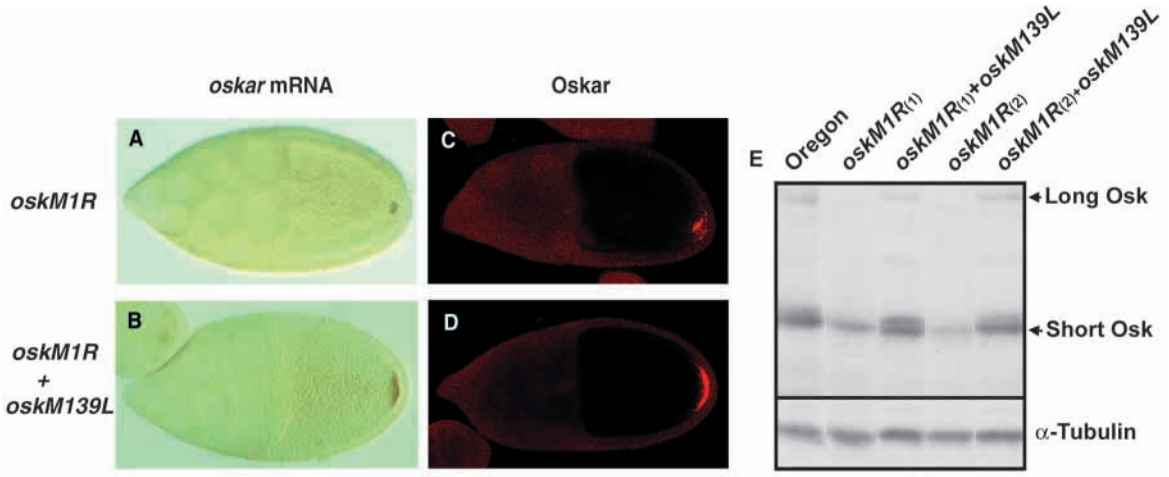


Fig. 3. Long Osk anchors both *osk* RNA and Short Osk at the posterior pole of the oocyte. (A,B) *osk* mRNA and (C,D) Osk isoforms in ovaries of *osk* RNA-null females osk^{A87}*/Df(3R)p-XT103*, expressing only *oskM1R* (A,C) or both *oskM1R* and *oskM139L* (B,D). Co-expression of Short Osk and Long Osk (B,D) suppresses the granular detachment of both *osk* mRNA and Short Osk observed when Short Osk is expressed alone (A,C). Note that mRNA detection in A,B is colorimetric, rather than fluorescence based, as shown in Fig. 1A. (E) Long Osk expression promotes Short Osk accumulation during oogenesis. Quantification of Short Osk in ovary extracts from two independent lines (noted 1 and 2) expressing *oskM1R* alone or together with *oskM139L*. A significantly greater amount of Short Osk, both phosphorylated and unphosphorylated, accumulates in the presence of Long Osk.

anchoring, in trans, of both *oskM1R* mRNA and Short Osk after mid-oogenesis.

To determine if cortical anchoring influences Short Osk accumulation during oogenesis, we compared the amount of Short Osk produced in transgenic females expressing *oskM1R* either alone or together with *oskM139L*. Western blotting of ovary extracts reveals that significantly higher levels of Short Osk are produced in females also expressing Long Osk (Fig. 3E). Indeed, wild-type levels of Short Osk are detected when the two transgenes are co-expressed, demonstrating that Long Osk promotes Short Osk accumulation during oogenesis. Long Osk presumably controls Short Osk levels by maintaining *osk* mRNA at the posterior pole of the oocyte, where it is translated; it is also possible that Long Osk acts directly to stabilize Short Osk.

Short Osk anchoring during oogenesis is required for wild-type patterning and fertility

Given that a localized source of Osk activity is required for correct anteroposterior patterning and fertility, it seemed likely that loss of Short Osk anchoring would affect these processes. However, conflicting results have been reported concerning the extent to which Short Osk alone can support patterning and germline formation in the embryo (Markussen et al., 1995; Rongo et al., 1997). We therefore took advantage of the *oskM1R* transgene to evaluate carefully the consequence of expressing Short Osk alone on these processes.

We first analyzed the hatching rate of embryos produced by two lines expressing *oskM1R*. Both of the lines exhibit a moderate reduction in hatch rate (71% and 78%) compared with the control line expressing a wild-type *osk* transgene (88%) (Fig. 4A). In contrast, the progeny of *oskM139L* females, which only express Long Osk, do not hatch, as previously shown (Markussen et al., 1995). However, females bearing both *oskM1R* and *oskM139L* produce embryos whose hatch rate is very similar to that of lines expressing a wild-type *osk* transgene, showing that Long Osk is required to achieve wild-type hatch rates. We further looked for cuticle defects in unhatched embryos produced by *oskM1R* females. Approximately half of the abnormal cuticles exhibit strictly posterior group defects (Fig. 4A,B, left panel). Strikingly, the remaining half shows either only anterior group defects or both anterior and posterior group defects, ranging from subtle reduction to complete deletion of head structures (Fig. 4A,B, middle and right panels). In some cases, anterior duplication of the filtzkorper is also observed (data not shown), revealing posteriorization of the anterior in these embryos. Such anterior defects can be caused by translation of unlocalized *osk* mRNA, which is recognizable by the diffuse distribution of Osk throughout oocyte before stage 7 (Kim-Ha et al., 1995). Indeed, immunostaining for Short Osk in early *oskM1R* oocytes, using our anti-Osk serum at unusually high concentrations, reveals a very faint, but reproducible, diffuse signal above the nonspecific background (data not shown). However, although ovaries co-expressing both *oskM1R* and *oskM139L* still show premature Osk signal (data not shown), both anterior and posterior patterning defects are suppressed in the progeny (Fig. 4A,C). We therefore conclude that the patterning defects of *oskM1R* embryos are primarily the result of Short Osk delocalization from the posterior pole of the oocyte, which is rescued by co-expression of Long Osk (Fig. 3). Thus, both posterior and anterior defects can be explained by the delocalization of pole plasm and its abdomen-inducing activity from the posterior of the oocyte.

To determine whether germline formation is also impaired in the absence of Short Osk anchoring, we first examined the pole cells of embryos produced by *oskM1R* females. Embryos from the two different *oskM1R* lines show a dramatic reduction in the number of pole cells formed at the posterior pole, compared with embryos of the *osk* wild-type transgene control (Fig. 5A,C). In contrast, embryos from females co-expressing *oskM1R* and *oskM139L* develop pole cells similar in number

A

Transgenes	% hatching	% of patterning defects in abnormal cuticles of unhatchers: posterior	anterior	posterior and anterior
osk WT	88*	0	0	0
oskM1R + oskM139L	84	0	0	0
	91	0	0	0
oskM1R	71	50	30	20
	78	50	25	25
oskM139L	0	100	-	-

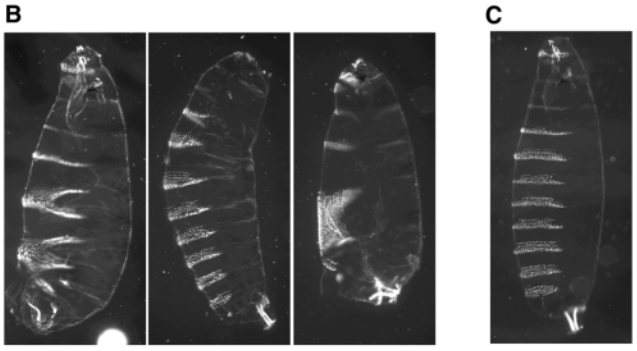


Fig. 4. Proper patterning requires the combinatorial activity of Long Osk and Short Osk. (A) Hatching rate of embryos and cuticle analysis of unhatchers with patterning defects produced by *osk* RNA-null females *osk^{A87}/Df(3R)p-XT103*, expressing the indicated transgenes. Number of eggs scored to calculate hatch rates are *osk* wild type (*n*=90); *oskM1R* [*n*=125 (line 1) and 49 (line 2)]; *oskM1R+oskM139L* [*n*=561 (line 1) and 147 (line 2)]; *oskM139L* (*n*>300). [(1) and (2) refer to the lines analyzed by western blotting in Fig. 3E.] *The remaining 12% of unhatchers develop cuticles without obvious patterning defects. (B) Posterior defects (left panel), anterior defects (middle panel), and both anterior and posterior defects (right panel) observed in progeny of females expressing only *oskM1R*. None of these defects is detected when *oskM1R* and o*skM139L* are co-expressed (C). Anterior is towards the top and ventral is towards the left.

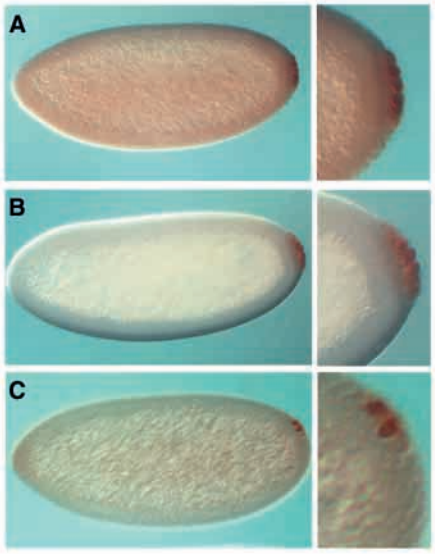
to the wild-type *osk* control (Fig. 5B). We directly evaluated the fertility of adult female progeny, by scoring their ovaries (Fig. 5D). Notably, only half of the progeny of *oskM1R* females develop wild-type ovaries. The rest are sterile, developing either rudimentary ovaries consisting only of the terminal filaments or, in rare cases, tiny ovaries (‘atrophic’). More importantly, wild-type fertility is restored in both lines in which the *oskM1R* transgene is co-expressed with *oskM139L*. This demonstrates that Long Osk is required to achieve wild-type fertility, despite its inability to assemble the pole plasm (Markussen et al., 1995; Breitwieser et al., 1996). Given our previous results showing that Long Osk anchors Short Osk and *osk* mRNA at the posterior pole of the oocyte, we conclude that the anchoring activity of Long Osk is required to generate the high local source of Short Osk necessary for wild-type germline formation.

Long Osk is necessary for Osk anchoring around the oocyte cortex

It has been shown that when *osk* mRNA is translated throughout the oocyte, as can be achieved by replacing the *osk* 3′UTR with that of *K10 (osk-K10)*, an ectopic accumulation of Osk around the entire oocyte cortex is observed (Fig. 6A) (Riechman et al., 2002). Although both Osk isoforms are recognized by the antibody in this experiment, our results concerning Osk anchoring at the posterior pole would predict that the cortical signal is dependent on the ectopic expression of Long Osk. To test this possibility, we ectopically expressed Short Osk alone, by fusing the *oskM1R*-coding region to the *K10* 3′UTR *(oskM1R-K10)*, and analyzed its distribution in a wild-type background. Strikingly, Short Osk is no longer detected around the oocyte cortex (Fig. 6B). Western blot analysis of protein extracts from *osk-K10* and *oskM1R-K10 ovaries* shows that similar amounts of Short Osk are produced from the two transgenes (Fig. 6C), indicating that, even when unlocalized, Short Osk is stable. Thus, we conclude that Long Osk is required for Osk anchoring to all regions of the oocyte cortex.

The N-terminal extension of Long Osk is necessary but not sufficient for posterior anchoring

Because Long Osk contains an N-terminal extension not present in Short Osk, we tested whether this difference is responsible for the unique property of Long Osk to anchor at the oocyte cortex. A previous study has shown that a fusion between the N-terminal extension of Long Osk and β-



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Transgene	Ovary in progeny (%): Wild type	atrophic	rudimentary
osk WT	100	-	-
oskM1R + oskM139L	(1) 100	-	-
	(2) 100	-	-
oskM1R	(1) 54	6	40
	(2) 49	0	51

Fig. 5. Long and Short Osk are required for efficient pole cell formation. (A-C) Vasa immunostaining reveals pole cells in embryos produced by *osk^{A87}/Df(3R)p-XT103* females expressing a wild-type *osk* transgene (A), the two transgenes *oskM1R* and *oskM139L* (B) or only *oskM1R* (C). Compared with embryos produced by females expressing both Osk isoforms (A,B), a significant reduction in pole cell number is observed in embryos produced by females expressing only Short Osk (C). (D) Fertility of the adult female progeny was evaluated by ovary dissection. Note that the two independent lines expressing *oskM1R* or both *oskM1R* and *oskM139L* were subjected to Osk western blotting analysis [denoted as (1) and (2) in Fig. 3E]. The number of ovaries dissected in each case was *oskWT* (*n*=41), *oskM1R* [*n*=54 (line 1) and 51 (line 2)], *oskM1R* and *oskM139L* [*n*=52 (1) and 61 (2)].

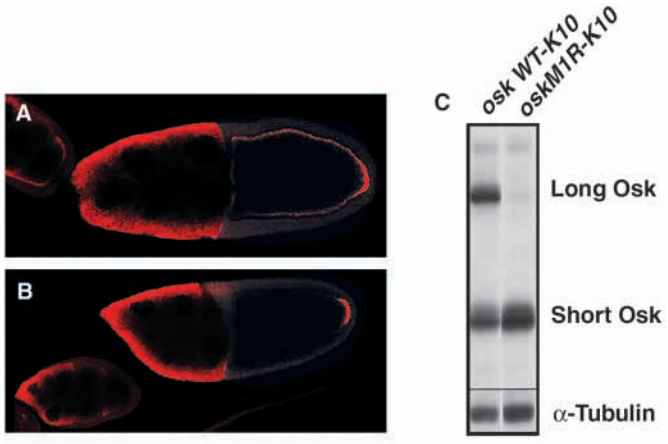

Fig. 6. Ectopic anchoring of Osk to the entire oocyte cortex requires Long Osk. Ectopic overexpression of the two Osk isoforms (A) and Short Osk alone (B) from the *UAS-osk-K10 3′UTR* and *UAS-oskM1R-K10 3′UTR* transgenes, respectively, in wild-type ovaries. When both isoforms are expressed (A), Osk is readily detected along the entire cortex of oocytes of stages 7/8 to 10. When only Short Osk is expressed (B), no cortical staining is observed other than at the posterior pole, where endogenous Osk (Long and Short) is expressed and localized. In both cases, the brightness of the immunostaining in the subcortical region of the nurse cells reveals the massive overproduction of Osk isoforms. (C) Analysis of overexpressed Osk isoforms produced from the transgenic ovaries by western blotting.

galactosidase (Osk-β-galactosidase fusion) accumulates at the posterior tip of the oocyte in wild-type egg chambers (Gunkel et al., 1998). This accumulation relies on the Long Osk region of the fusion, as wild-type β-galactosidase is not enriched at the posterior pole, even when translated from a posterior-localized transgenic mRNA (N. Gunkel and A. E., unpublished). To examine if endogenous Osk plays a role in the anchoring of the Osk-β-galactosidase fusion protein in wild-type egg chambers, we expressed this fusion in a background in which full-length Osk is not produced ($osk^{84}/Df(3R)p^{XT103}$). The fusion protein is tightly localized at the posterior of $osk^{84}/Df(3R)p^{XT103}$ oocytes at stage 10 (Fig. 7C). However, after stage 10, Osk-β-galactosidase diffuses away from the posterior pole and is no longer detected (Fig. 7D). In contrast, the fusion protein remains localized at the posterior pole of wild-type oocytes during late oogenesis (Fig. 7A,B), as previously reported (Gunkel et al., 1998). Thus, we conclude that the N-terminal extension of Long Osk is required but is not sufficient for posterior anchoring during the late stages of oogenesis.

DISCUSSION

After mid-oogenesis, at the posterior tip of the *Drosophila* oocyte, a region morphologically distinct from the remaining ooplasm forms under the control of Osk. This spatially restricted region, the pole plasm, contains the information required for specification of the abdomen and of the germ cells of the future embryo. In this study, we have shown that Long Osk anchors both *osk* mRNA and Short Osk at the posterior cortex of the oocyte. Unanchored Short Osk, the pole plasm-inducing isoform, nucleates a functional but untrapped pole plasm whose components, such as Staufen or Vasa, disperse in the bulk ooplasm at the onset of oocyte streaming. The loss of pole plasm components from the posterior pole of the oocyte results in a significant impairment of fertility in the progeny. In addition, the erratic dispersion of the posterior-inducing activity of Osk throughout the oocyte ooplasm causes anteroposterior patterning defects during embryogenesis.

Both Short and Long Osk associate with *osk* mRNA but only Long Osk can maintain RNA localization during late oogenesis

At mid-oogenesis, the transport of *osk* mRNA to the posterior pole of the oocyte requires the plus-end motor Kinesin I, a polarized microtubule network and an intact actin cytoskeleton (for a review, see Riechmann and Ephrussi, 2001) (Brendza et al., 2000; Theurkauf et al., 1992; Manseau et al., 1996; Erdélyi et al., 1995; Baum et al., 2000). The polarized and sustained transport of *osk* mRNA can also account for its maintenance at the posterior pole of the oocyte from stage 7 to 10. In contrast, at stage 10, an active process of mRNA maintenance must exist, as the polarized microtubule network is disassembled and a subcortical array of microtubules forms and promotes vigorous cytoplasmic streaming. Indeed, several lines of evidence indicate that, as of stage 10, Osk protein maintains *osk* mRNA localization. First, *osk* mRNA delocalizes during stage 10 in the three *osk* nonsense mutants osk^{54}, osk^{84} and osk^{346}, predicted to produce truncated Osk peptides of 179, 253 and 323 amino acids, respectively (Kim-Ha et al., 1991). However, our failure to detect these peptides by western analysis (N. V. and A. E., unpublished) suggests that they are

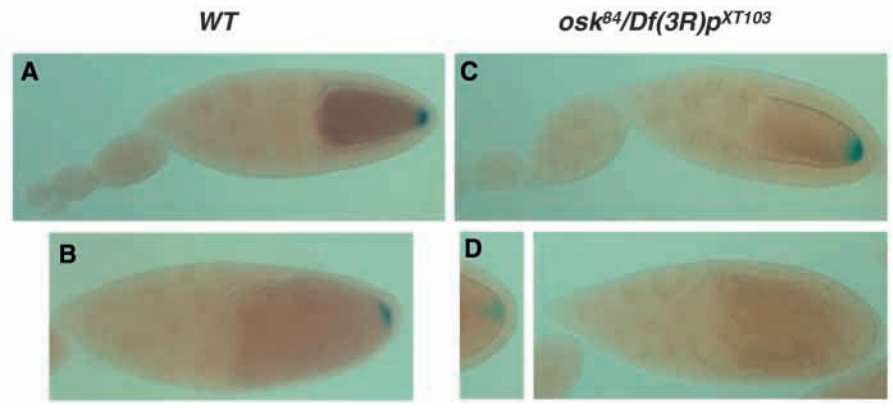

Fig. 7. The N-terminal extension of Long Osk is not sufficient for posterior anchoring. β-Galactosidase staining of wild-type (A,B) and $osk^{84}/Df(3R)p^{XT103}$ ovaries (C,D) expressing the $m1^{414}lacwt$ transgene, which encodes a translational fusion of the first 139 amino acids of Long Osk to β-galactosidase (Gunkel et al., 1998). The chimeric protein is detected at the posterior pole of wild-type oocytes during stage 10 onwards (A,B), whereas in the absence of endogenous Osk activity, the fusion delocalizes during stage 10 (C,D).

unstable and the nonsense *osk* alleles are protein null. Second, the maintenance of a transgenic *nos-osk3′UTR* mRNA at the posterior pole of stage 10B oocytes requires Osk protein (Rongo et al., 1995). Finally, posterior accumulation of fluorescent *osk* mRNA, injected into living oocytes at stage 10-11, occurs by a trapping mechanism dependent on endogenous Osk protein (Glotzer et al., 1997). Our analysis shows that, although it supports expression of Short Osk, *oskM1R* transgenic mRNA detaches from the posterior pole during late stage 10. In contrast, we show that Long Osk, which is dispensable for pole plasm formation (Markussen et al., 1995), is competent and required to persistently confine *osk* mRNA at the posterior pole of the oocyte during late oogenesis until early embryogenesis. Thus, *osk* mRNA maintenance is an active process mediated by Long Osk. Maintenance of the three nonsense *osk* mRNAs is rescued in heterozygous females (Kim-Ha et al., 1991). Our present work reveals that this rescue in trans can only be attributed to the Long Osk isoform encoded by the wild-type *osk* gene. Consistent with this, we show that Long Osk can also maintain localization of the transgenic *oskM1R* mRNA, which encodes only Short Osk.

In the absence of Long Osk, Short Osk also detaches from the posterior cortex of stage 10 oocytes, in concert with *oskM1R* mRNA. Both Short Osk and *osk* mRNA delocalize in dense aggregates, suggesting that they might be associated. Consistent with this, we have shown that they co-localize in the same released aggregates. It is noteworthy that the delocalizing pattern of *oskM1R* mRNA is significantly different from that of osk^{84} mRNA, which diffuses without forming aggregates. Because osk^{84} mRNA encodes an unstable Osk peptide, we conclude that aggregate formation is dependent on Short Osk. The pole plasm protein Vasa, which is a component of the polar granules, the germline granules of *Drosophila*, is also detected in these aggregates. This suggests that the aggregates contain nascent but untethered polar granules, whose assembly might be initiated by Short Osk-mediated clustering of *osk* mRNA. The ability of Short Osk to package macro-molecular complexes is supported by our observation that it can oligomerize, in a yeast two-hybrid assay (A. Jenny and A. E., unpublished). Given the underexpression of Long Osk relative to Short Osk in wild-type ovaries, multimerization of Short Osk could also explain the apparent non-stoichiometric competence of Long Osk to anchor Short Osk at the oocyte cortex.

Because both Long and Short Osk can sequester *osk* mRNA, it is likely that the same region in the two isoforms mediates RNA association. However, no RNA-binding activity has been reported for Osk, which does not exhibit any predicted RNA-binding motif in its coding sequence. Thus, the association of the two Osk isoforms with *osk* mRNA most probably involves adaptor(s). One such candidate could be Staufen, a double-strand RNA-binding protein suspected to bind *osk* mRNA directly (Micklem et al., 2000). Staufen is required for posterior maintenance of *osk* mRNA, as revealed by its delocalization in the temperature-sensitive mutant $stau^{C8}$ (Rongo et al., 1995). We have shown that, in ovaries expressing each Osk isoform individually, Staufen either co-localizes with the Long Osk/*osk* mRNA complex at the posterior pole of the oocyte or co-segregates with the Short Osk/*osk* mRNA complex in the released aggregates, as expected of an adaptor factor. Previous analysis has shown that Staufen binds to Short Osk in a yeast two-hybrid assay, but binds quite poorly to Long Osk (Breitwieser et al., 1996), which does not reflect the robust ability of Long Osk to maintain *osk* mRNA localization that our study reveals. Thus, whether the association of the two Osk isoforms with *osk* mRNA relies on a direct interaction with Staufen or with another adaptor factor remains to be elucidated.

Are pole plasm assembly and anchoring activities of Osk mutually exclusive?

The observation that Long Osk but not Short Osk can anchor at the cortex suggests that the N-terminal extension of Long Osk mediates anchoring. Surprisingly, the extension is not sufficient for this function, as revealed by its failure to maintain an Osk-β-galactosidase fusion in Osk protein-null oocytes at the onset of oocyte streaming. This suggests that at least two separate docking modules, one in the N-terminal extension of Long Osk and a second in the region shared by the two Osk isoforms, cooperate to form a robust anchoring domain. Two observations support this hypothesis. First, the Osk-β-galactosidase fusion remains localized during stages 8 to 10 of oogenesis in Osk protein-null oocytes, whereas native β-galactosidase translated from a posterior localized RNA fails to accumulate at this location. Second, even in the absence of Long Osk, a residual amount of Short Osk remains localized at the posterior pole of the oocyte during late oogenesis and supports substantial posterior patterning and partial fertility of the progeny. Thus, two docking modules appear to be involved in Long Osk anchoring, but neither alone is sufficient for this process.

An important result of this work is that Long Osk anchors Short Osk, the pole plasm-inducing isoform, at the posterior pole of oocyte. Strikingly, although it contains the entire Short Osk sequence, Long Osk can not recruit pole plasm components (Markussen et al., 1995; Breitwieser et al., 1996). It has previously been proposed that the N-terminal extension of Long Osk exerts an inhibitory effect on downstream protein-interaction domains (Breitwieser et al., 1996). We propose that this inhibition is caused by folding of the robust anchoring domain of Long Osk, masking the pole plasm-recruiting activity of this isoform. By contrast, the absence of the N-terminal anchoring module would allow Short Osk to nucleate pole plasm assembly. Hence, anchoring and pole plasm nucleation might be structurally mutually exclusive activities.

Osk anchoring maintains pole plasm integrity and restriction at the posterior pole of the oocyte

Our results show that, in the absence of Long Osk-anchoring activity, which causes a massive dispersion of Short Osk from the posterior pole of the oocyte, effective abdominal patterning in the embryo can nonetheless be achieved. Consistent with this, strong impairment of *osk* mRNA localization and translation in mutants in Tropomyosin II, an actin-binding protein, and Barentsz, a putative component of the transport machinery, has little consequence on abdominal development (Erdélyi et al., 1995; van Eeden et al., 2001). This demonstrates that, with regard to abdomen formation, a substantial excess of Short Osk is present at the posterior pole of the wild-type oocyte. However, our results indicate that dispersion of untethered Short Osk from the posterior of *oskM1R* oocytes can hinder anterior development. We cannot exclude a contribution of the weak premature translation of *oskM1R* to

these anterior patterning defects. However, the complete suppression of these defects by co-expression of *oskM139L*, demonstrates that the anchoring activity of Long Osk can restrict the pole plasm-inducing activity of Short Osk to the posterior. Anchoring of the bulk of Short Osk to the posterior pole would lead to titration of limiting pole plasm components from any residual ectopically localized Osk.

In contrast, Short Osk delocalization causes a significant reduction of germ cell precursors and fertility of the progeny. Our results provide yet another demonstration of the correlation between Osk protein dose and the number of pole cells formed. Indeed, whereas overexpression of Osk at the posterior pole increases pole cell number (Ephrussi and Lehmann, 1992; Smith et al., 1992), its underexpression, caused by defects in RNA localization or translation, impedes pole cell formation (Erdélyi et al., 1995; Lie and Macdonald, 1999; Chang et al., 1999; van Eeden et al., 2001). In our analysis, restoration of Short Osk anchoring by co-expression of Long Osk enhances Short Osk accumulation and restores germ-plasm integrity. This demonstrates that Long Osk guarantees accumulation of high levels of Short Osk in the subcortical region of the egg that is subsequently incorporated into pole cells. It has been shown that when the *D. virilis* Osk homolog is expressed in *Drosophila melanogaster* oocytes, it efficiently rescues the posterior patterning defects of *osk* mutants, but does not support pole cell formation in the embryos (Webster et al., 1994). Transgenic *D. virilis* Osk fails to maintain *osk* mRNA localization in *D. melanogaster*, which led Webster et al. to hypothesize that *virilis* Osk is not competent to anchor at the posterior pole of the *D. melanogaster* oocyte. This conclusion is entirely consistent with our demonstration that Osk-mediated anchoring of the pole plasm is a critical step during *Drosophila* germline formation.

Given that both efficient germ cell formation and, to a lesser extent, proper patterning rely on Long Osk-mediated anchoring of the pole plasm, an important issue in the future will be the characterization of the mechanism by which Long Osk is tethered to the cortex.

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