Spatial and Temporal Coordination of *oskar* mRNA Localization and Translation During *Drosophila* Oogenesis

by

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ABSTRACT

In the fruit fly, *Drosophila melanogaster*, accumulation of *osk* mRNA at the posterior pole of the oocyte and local translation initiate assembly of the pole plasm, which is required for germ cell formation and posterior patterning of the embryo. I have used fluorescence *in situ* hybridization (FISH) in combination with immunofluorescence and laser scanning confocal microscopy to examine the spatial and temporal control of *osk* transcript localization and translation. *Drosophila* oocytes develop within cysts of 16 interconnected cells. One cell in each cyst differentiates to form the oocyte while the remaining cells form nurse cells that produce RNAs and proteins that are transported to the oocyte. *osk* mRNA is produced by the nurse cells and accumulates in the oocyte throughout oogenesis, but is only specifically localized to the posterior pole and translated during mid to late oogenesis. My studies help define distinct steps in the *osk* mRNA localization process. An early step in posterior localization is removal of *osk* mRNA from most of the cortex, leading to accumulation in the oocyte interior. This process requires microtubules, the microtubule motor protein Kinesin I, the actin binding protein Tropomyosin, and the RNA binding protein Staufen. Transcript then moves from the oocyte interior to the posterior pole through a microtubule independent process. The genes *cappuccino*, *chickadee*, *spire*, *armitage*, *maelstrom*, *par-1* and *gurken* are all required for this next step in *osk* mRNA localization. The final capturing or tethering *osk* mRNA at the cortex requires an intact actin filament system, but additional components of this anchoring system remain to be identified. I also find that *osk* mRNA first begins to accumulate at the posterior pole during oogenesis stage 8, but protein is not detectable until stage 9. In addition, *grk* and *par-1* mutations that block *osk* mRNA localization to the posterior pole and lead to transcript accumulation in the interior do not prevent translation; again, Osk protein production is only observed during stage 9 and later. These observations indicate that posterior localization is neither

sufficient nor necessary to trigger *osk* mRNA translation, which appears to be under tight temporal control.

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LIST OF ABBREVIATIONS

armi	*armitage*
bcd	*bicoid*
Bcd	Bicoid protein
Bic-D	*Bicaudal-D*
Bic-D	Bicaudal-D protein
BLE1	bicoid localization element
bp	base-pair
BREs	Bruno response elements
capu	*cappuccino*
chic	*chickadee*
cy5	Cyanine 5
DEPC	diethyl pyrocarbonate
DER	*Drosophila* epidermal growth factor receptor
EGF	epidermal growth factor
exu	*exuperantia*
Exu	Exuperantia protein
grk	*gurken*
Grk	Gurken protein
egl	*egalitarian*
FISH	fluorescence *in situ* hybridization
hb	*hunchback*
kD	kilodalton
khc	Kinesin I heavy chain
mael	*maelstrom*
MTOC	microtubule organizing center
nos	*nanos*
nt	nucleotide
osk	*oskar*
Osk	Oskar protein
PBS	Phosphate buffered saline
spn-E	*spindle-E*
spir	*spire*

stau	*staufen*
sww	*swallow*
TmII	*Tropomyosin*
TGF-β	Transforming growth factor-β
UTR	untranslated region
vls	*valois*

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INTRODUCTION

The localization of mRNAs to discrete regions of the cell is one general mechanism for the efficient targeting of proteins to their site of function (Bashirullah et al., 1998; Gottlieb, 1990; Lipshitz and Smibert, 2000; Micklem, 1995). In developing oocytes and embryos as well as in numerous cell types in a diversity of organisms, mRNA localization plays important roles in a variety of processes including axis specification, cell differentiation and polarization.

Drosophila melanogaster serves as an excellent model system in which to analyze mRNA localization. During *Drosophila* oogenesis, the proper localization of mRNAs and the proteins they encode figures prominently in oocyte differentiation and the establishment of the embryonic axes (Lasko, 1999).

Drosophila oogenesis

Each *Drosophila* ovary consists of approximately ten to twenty ovarioles, each composed of a string of progressively developing egg chambers (Figure 1a). Oogenesis begins with the asymmetric division of a germline stem cell in a specialized structure called the germarium located at the anterior of the ovariole (King, 1970; Spradling, 1993). In region 1 of the *Drosophila* germarium, the first division of the stem cell produces a daughter stem cell and a cystoblast (Figure 1b). Subsequently, the cystoblast undergoes four mitotic divisions. As a result of incomplete cytokinesis at each division, cytoplasmic bridges called ring canals interconnect the sixteen resulting germline cells, the cystocytes. During these divisions, a branching vesicular organelle called the fusome spans the cystocytes via the ring canals (de Cuevas and Spradling, 1998; McKearin, 1997; Storto and King, 1989). The fusome anchors one pole of each mitotic spindle, establishing an invariable pattern to the cystocyte divisions (Carpenter, 1975; King, 1970). The resulting sixteen-cell cyst contains eight cells with one ring canal, four cells with two, two cells with three, and two cells with four. The sixteen cell cyst enters region 2a of the germarium, where the two cells with four ring canals, the pro-oocytes, enter prophase I and

accumulate centrioles that migrate from the nurse cells through the ring canals (Carpenter, 1975; Grieder et al., 2000; King, 1970; Mahowald and Strassheim, 1970). In region 2b, only one pro-oocyte is selected to differentiate into an oocyte. The oocyte nucleus remains arrested in meiotic prophase and is transcriptionally dormant whereas the remaining pro-oocyte exits meiosis and begins to endoreplicate its DNA, along with the fourteen cystocytes, to become a transcriptionally active polyploid nurse cell (King, 1970).

Oocyte determination

The mechanism of oocyte determination is not known; however, the oocyte is defined by the differential accumulation of specific factors such as *oskar* (*osk*) and *Bicaudal-D* (*Bic-D*) RNAs and Bicaudal-D (Bic-D), Egalitarian (Egl), Oo18 RNA-binding (Orb) and Cup proteins (Ephrussi et al., 1991; Keyes and Spradling, 1997; Kim-Ha et al., 1991; Lantz et al., 1994; Mach and Lehmann, 1997; Suter and Steward, 1991; Wharton and Struhl, 1989). *Bic-D* and *egl* appear to be two major components in these oocyte-determining processes. RNAs and proteins that define the oocyte fail to localize in a single cystocyte in *Bic-D* and *egl* mutants (Huynh and St Johnston, 2000; Mach and Lehmann, 1997; Mohler and Wieschaus, 1986; Ran et al., 1994; Schupbach and Wieschaus, 1991; Suter and Steward, 1991; Theurkauf et al., 1993). Subsequently, *Bic-D* and *egl* mutants produce cysts lacking an oocyte and the sixteen cystocytes differentiate into polyploid nurse cells (Mohler and Wieschaus, 1986; Schupbach and Wieschaus, 1991).

The transport of mRNAs to the oocyte is dependent on the polarized microtubule network established early in oogenesis (Theurkauf, 1994b; Theurkauf et al., 1993). Treatment with microtubule depolymerizing drugs disrupts the accumulation of specific factors in the pro-oocyte, resulting in the failure of the determination of an oocyte (Koch and Spitzer, 1983; Theurkauf et al., 1993). Immunocytological examination reveals that mutations in *Bic-D* and *egl* similarly affect oocyte determination as a result of defects in microtubule organization (Theurkauf et al., 1993). In *Bic-D* mutants, the initial establishment and later maintenance of the microtubule

organizing center (MTOC) in the germarial cyst is disrupted (Oh and Steward, 2001). *egl* mutants, by comparison, initially establish a polarized microtubule network in region 2a; however, in region 2b this network degenerates, and within region 3 an organized focus is no longer observed (Theurkauf et al., 1993). These observations provide evidence that a polarized microtubule network is necessary for the transport of factors to the future oocyte and its eventual determination.

Microtubule organization during *Drosophila* oogenesis

The oocyte cytoskeleton reorganizes several times during the course of oocyte development (Knowles and Cooley, 1994; Theurkauf, 1994b; Theurkauf et al., 1992). Early in oogenesis, in region 2b in the germarium, a clearly defined anterior microtubule organizing center (MTOC) is first observed in the oocyte and is maintained through stage 1 (Figure 2a, arrowhead in a′) (Theurkauf et al., 1993). During stages 2-6 of oogenesis, microtubules extend from a MTOC positioned at the posterior pole of the oocyte and pass through the ring canals adjoining the oocyte and the neighboring nurse cells (Figure 2b, arrowhead in b′) (Theurkauf et al., 1993). Additionally, microtubules are observed in the nurse cells. These nurse cell microtubules do not appear to originate from the oocyte and do not nucleate from well-defined MTOCs. During stage 7 and early 8, the posterior MTOC degenerates as a result of a *grk*-dependent signaling event and the majority of microtubules reorganize at the anterior of the oocyte (Theurkauf et al., 1992). Microtubules are oriented in such a way that the minus-ends, the nucleation site of microtubules, are proximal to the oocyte cortex. The plus-ends of microtubules, the growing ends, are oriented towards the center of the oocyte. There is an evident gradient of microtubules along the cortex of the oocyte, with the highest abundance towards the anterior (arrowheads in Figure 2c′) and with a decline in density towards the posterior pole (arrow in Figure 2c, c′). This anterior to posterior gradient in microtubule density is maintained throughout stages 8-10a. During stage 10b, the next major microtubule reorganization event takes place. Cytoplasm from the nurse cells dumps

into the oocyte concomitant with subcortical bundling of the microtubules and cytoplasmic streaming within the oocyte (Gutzeit, 1986; Theurkauf et al., 1992) (Figure 2d, arrowhead in d′).

Axis specification during *Drosophila* oogenesis

Axis specification in the *Drosophila melanogaster* embryo and formation of the germline are established during oogenesis by the asymmetric positioning of morphogenetic factors, mRNAs and proteins, in the oocyte (Figure 3) (Lasko, 1999; St Johnston and Nusslein-Volhard, 1992). Through much of oogenesis, the oocyte is transcriptionally inactive, and the majority of the mRNAs and proteins are synthesized in the nurse cells and transported to the oocyte through the ring canals (Mahajan-Miklos and Cooley, 1994). Genetic analyses have identified RNAs and proteins that are essential for specification of the dorsal-ventral and anterior-posterior axes. Several extensively researched examples include the maternal RNAs *oskar* (*osk*), *bicoid* (*bcd*), *gurken* (*grk*) and *nanos* (*nos*), and Vasa (Vas) and Staufen (Stau) proteins.

gurken

grk RNA is transcribed in the nurse cells early during oogenesis. During the later stages of oogenesis, *grk* RNA is also transcribed by the oocyte nucleus, in contrast to the majority of maternal factors (Neuman-Silberberg and Schupbach, 1993). *grk* mRNA is involved in defining both the anterior-posterior and dorsal-ventral axes (Gonzalez-Reyes et al., 1995; Neuman-Silberberg and Schupbach, 1993). The establishment of the anterior-posterior axis is dependent on the signaling between the oocyte and the posterior follicle cells. Early in oogenesis, *grk* mRNA is localized at the posterior pole of the oocyte where it is translated. Gurken (Grk) protein, a member of the transforming growth factor α (TGFα) family, signals to the posterior follicle cells, via the *Drosophila* epidermal growth factor (EGF) receptor homologue *torpedo/DER*, to adopt a posterior rather than anterior fate (Gonzalez-Reyes et al., 1995; Neuman-Silberberg and Schupbach, 1993; Ray and Schupbach, 1996; Roth et al., 1995). As early as stage 7 of oogenesis, upon induction of the posterior follicle cells, an unknown signal

back to the oocyte induces a major rearrangement of the oocyte microtubule cytoskeleton, establishing the anterior-posterior microtubule gradient network (Gonzalez-Reyes et al., 1995; Roth et al., 1995). This reorganization of microtubules leads to the repositioning of the oocyte nucleus from the posterior pole to the anterodorsal margin of the oocyte (Neuman-Silberberg and Schupbach, 1993; Spradling, 1993). Similar to anterior-posterior axis specification, *grk* mRNA localized to the anterodorsal region is locally translated and activates the *Drosophila* EGF receptor in the adjacent follicle cells, specifying the fate of these cells as dorsal anterior (Neuman-Silberberg and Schupbach, 1993; Price et al., 1989; Schejter and Shilo, 1989).

bicoid

bcd mRNA, the primary anterior defining morphogen, is synthesized in the nurse cells and transported through the ring canals to localize at the anterior cortex adjoining the nurse cells (Berleth et al., 1988; Driever and Nusslein-Volhard, 1988b; St Johnston et al., 1989). The transport of *bcd* mRNA into the oocyte and anchoring to the anterior cortex is dependent upon *exuperantia* (*exu*), *swallow* (*sww*), *staufen* (*stau*) and an intact microtubule network (Macdonald et al., 1991; Marcey et al., 1991; Pokrywka and Stephenson, 1991; Pokrywka and Stephenson, 1995; St Johnston et al., 1989). Additionally, the *bcd* 3′ untranslated region (UTR) has been identified as an essential *cis*-acting element for the proper localization of *bcd* RNA (Macdonald et al., 1993; Macdonald and Struhl, 1988).

Localization of *bcd* mRNA to the anterior of the oocyte is comprised of three distinct steps. Initially, *bcd* RNA associates with Exu protein in a microtubule dependent process and this Exu-*bcd* complex is transported by microtubules to the oocyte. Exu is not necessary for transport within the nurse cells, but is critical for anterior localization upon entering the oocyte (Cha et al., 2001; Macdonald et al., 1991; Marcey et al., 1991). A 53 base-pair element identified within the *bcd* 3′ UTR, the bicoid localization element (BLE1), is required for the Exu-dependent anterior localization (Macdonald et al., 1993; Macdonald and Struhl, 1988). Once localized to the

anterior, Swallow is responsible for the maintenance of cortical anchoring of *bcd* mRNA during later oogenesis (Stephenson et al., 1988). Lastly, Stau protein has been shown to associate with the *bcd* 3′ UTR, including the BLE1, to prevent diffusion of the RNA once it is released into the egg cytoplasm during egg activation (Ferrandon et al., 1994).

Following fertilization, *bcd* mRNA is translated to produce a gradient of Bicoid (Bcd) protein extending over the anterior half of the embryo (Berleth et al., 1988; Driever and Nusslein-Volhard, 1988a; Driever and Nusslein-Volhard, 1988b; St Johnston et al., 1989). Bcd protein directs the expression of zygotically expressed segmentation genes that establish anterior patterning in the developing embryo (St Johnston and Nusslein-Volhard, 1992).

OSKAR

oskar is required for pole plasm assembly and abdominal patterning

oskar serves a dual role during *Drosophila* development. Localization of *osk* RNA at the posterior pole of the oocyte is essential for the assembly of the pole plasm, which is required for both germ cell formation and posterior patterning (Ephrussi et al., 1991; Kim-Ha et al., 1991; Lehmann and Ephrussi, 1994). Several genes are required for pole plasm formation: *osk* (Lehmann and Nusslein-Volhard, 1986), *stau*, *vasa* (*vas*), *valois* (*vls*) (Schupbach and Wieschaus, 1986), *tudor* (*tud*) (Boswell and Mahowald, 1985), *cappuccino* (*capu*) and *spire* (*spir*) (Manseau and Schupbach, 1989). Embryos derived from females which are homozygous mutant for these genes lack polar granules, fail to form pole cells, and show defects in abdomen formation due to failure of *nanos* (*nos*) mRNA localization and translation (Gavis and Lehmann, 1994; Wang and Lehmann, 1991).

Pole plasm assembly and posterior polarity are established by an ordered series of events whereby one gene product is responsible for localizing the next. The localization of Stau protein to the posterior pole is dependent upon the functions of *capu* and *spir* (St Johnston et al., 1991). Stau protein is in turn required for posterior localization of *osk* mRNA (Ephrussi et al., 1991;

Kim-Ha et al., 1991; St Johnston et al., 1991). These initial steps are prerequisite for the posterior localization of Vas (Hay et al., 1990; Lasko and Ashburner, 1990) as well as Tudor and Valois proteins, whose functions are not precisely known. Oskar is also required for the posterior localization of *nos*, which is required for determining abdominal cell fate (Lehmann and Nusslein-Volhard, 1991; Wang and Lehmann, 1991). Vas protein, already localized to the posterior of the oocyte, is required for the translation of *nos* (Gavis and Lehmann, 1994). With the aid of Pumilio, Nos protein binds to *hunchback* (*hb*) RNA (Murata and Wharton, 1995) resulting in the translational repression of *hb* mRNA in the posterior of the embryo, thus establishing abdominal cell fate (Barker et al., 1992; Tautz and Pfeifle, 1989).

***oskar* mRNA localization**

osk mRNA, transcribed in the nurse cells, is transported to and enriched within the oocyte as early as germarial stage 2b (Ephrussi et al., 1991; Kim-Ha et al., 1991). The transport of *osk* RNA into the oocyte and eventual localization to the posterior pole requires the microtubule cytoskeleton (Clark et al., 1994; Pokrywka and Stephenson, 1994). Through stage 6, a microtubule organizing center is situated at the posterior pole of the oocyte and microtubules nucleated from this site extend through the ring canals into the nurse cells (Theurkauf et al., 1993). *osk* RNA is localized to the posterior cortical region presumably by minus-end directed movement along microtubules. During stage 7-8, a *grk*-dependent signaling event results in a major rearrangement of the oocyte microtubule network, such that the posterior MTOC disassembles and microtubules begin to nucleate from the anterior of the oocyte (Gonzalez-Reyes et al., 1995; Roth et al., 1995). By stage 8 of oogenesis, *osk* RNA is observed at the anterior marginal cortex with slight accumulation at the posterior pole (Ephrussi et al., 1991; Kim-Ha et al., 1991). From stage 8 onwards, the exclusion of *osk* RNA from the cortex is accomplished by the plus-end directed microtubule motor Kinesin in conjunction with an intact microtubule network (Cha et al., 2002). *osk* RNA is exclusively localized to the posterior pole by stage 9, when it is first translated (Ephrussi et al., 1991; Kim-Ha et al., 1991).

cis-acting elements required for _osk_ mRNA localization

The *osk* 3′ UTR contains sequence elements required for *osk* mRNA localization. Construction of a transgenic mutant with a 728bp deletion within the 1043bp osk 3′ UTR failed to localize osk mRNA (Kim-Ha et al., 1993). Further analysis of mutants lacking regions in the 3′ UTR identified sequence elements responsible for distinct steps in the *osk* RNA localization process. The region between nt 532-791 of the osk 3′ UTR is necessary for the accumulation of *osk* mRNA in the oocyte, whereas a distinct region between nt 1-242 is required for posterior localization (Kim-Ha et al., 1993).

trans-acting elements required for _osk_ mRNA localization

osk mRNA localization also requires *trans*-acting factors. Throughout oogenesis Stau, a putative RNA binding protein, colocalizes with *osk* mRNA, and *stau* mutations block *osk* RNA localization to the posterior pole (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991). It has been proposed that *osk* RNA and Stau protein form a complex that is transported to the posterior pole of the oocyte. The microtubule network is apparently intact, yet *osk* RNA fails to localize to the posterior pole in *stau* mutant oocytes, suggesting that Stau may mediate microtubule-dependent transfer of *osk* RNA to the posterior pole (Ephrussi et al., 1991; Kim-Ha et al., 1991). Following localization of Stau protein and *osk* RNA to the posterior pole of the oocyte, *osk* mRNA is translated. In the absence of Osk protein, *osk* RNA is liberated from the posterior pole at stage 10 of oogenesis (Ephrussi et al., 1991; Kim-Ha et al., 1991; Rongo et al., 1995). Osk protein thus appears to anchor *osk* mRNA at the posterior pole (Breitwieser et al., 1996; Markussen et al., 1995).

Translational regulation of _oskar_ mRNA

The localization of RNAs restricts production of proteins to sites where they are required. Generally, RNAs are translationally silent until they reach their prescribed destination within the cell. Translational repression during transport is imperative, as premature or ectopic translation

of RNAs leads to developmental defects (Driever et al., 1990; Ephrussi and Lehmann, 1992; Gavis and Lehmann, 1992; Kim-Ha et al., 1995; Smith et al., 1992).

Translational regulation of *osk* mRNA depends on *cis*-acting sequence elements in both the 3′ and 5′ UTRs and *trans*-acting factors that interact with these sequence elements. The 3′ UTR contains elements regulating repression of translation (Kim-Ha et al., 1995; Kim-Ha et al., 1993). Bruno, encoded by the *arrest* gene, colocalizes with *osk* RNA at the posterior pole and represses premature translation of *osk* transcript (Kim-Ha et al., 1995; Webster et al., 1997). Bruno recognizes a 7-9nt repeated sequence, the Bruno response elements (BRE), in the 3′ UTR of *osk* mRNA (Kim-Ha et al., 1995; Rongo et al., 1995; Webster et al., 1997). Mutations in the BREs result in premature translation of unlocalized *osk* mRNA (Kim-Ha et al., 1995). This result indicates that under wild-type conditions, unlocalized *osk* mRNA is translationally repressed. A 50kD protein (p50), also interacts with the BRE in the 3′ UTR and appears to be required for translational repression of *osk* mRNA (Gunkel et al., 1998). A third protein, Bicaudal-C, an RNA binding protein, is implicated in *oskar* translational repression, although it has not been demonstrated to bind to *osk* mRNA (Saffman et al., 1998). In *Bic-C* mutants, *osk* mRNA is dispersed in the oocyte and is ectopically translated (Saffman et al., 1998).

The 3′ UTR alone is not sufficient for translational activation of transcript localized at the posterior pole; heterologous transcripts containing the full-length *osk* 3′ UTR are localized, but not translated (Rongo et al., 1995). Additional sequences within the *osk* mRNA thus appear to be necessary for translation. An element between two alternative start codons within the 5′ UTR of *osk* is required to terminate BRE-mediated repression (Gunkel et al., 1998). Translational derepression is additionally linked to the binding of p50, and a 68kD protein, p68, to the 5′ UTR (Gunkel et al., 1998). Several additional proteins have also been implicated in *osk* translation although their precise roles are yet to be determined. Included are Oskar itself (Markussen et al., 1997; Markussen et al., 1995); Stau (Kim-Ha et al., 1995; St Johnston et al., 1991; St Johnston

and Nusslein-Volhard, 1992); the DEAD-box RNA helicase Vasa, which interacts with Osk and Bruno (Markussen et al., 1995; Webster et al., 1997); and Aubergine, a protein required for efficient *osk* mRNA translation (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995; Wilson et al., 1996). The localization of *osk* mRNA to the posterior pole and translational repression/derepression ensures the restriction of Osk protein and pole plasm determinants to the posterior of the oocyte and proper establishment of the anterior-posterior axis.

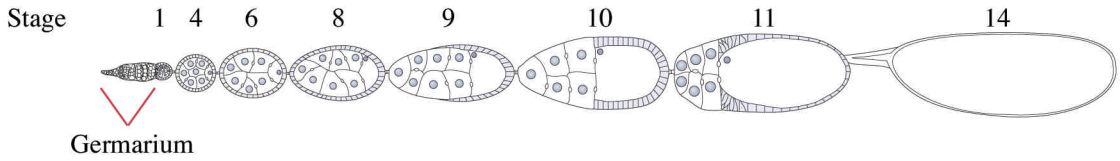
Figure 1. Formation and development of *Drosophila* egg chambers.

(a) Schematic representation of a *Drosophila* ovariole. Each ovariole consists of a string of developing egg chambers increasing in age from anterior (left) to the posterior (right). Egg chambers formed in the germarium exit to the vitellarium where they continue to grow and mature. Beginning with stage 8, the oocyte volume increases as cytoplasm is transported from the nurse cells and yolk is taken up by the oocyte. During stage 10b-11, the nurse cells rapidly dump their cytoplasm into the oocyte. Oocyte maturation is completed during stages 12-14 with the advent of complete incorporation of nurse cell cytoplasm and deposition of the chorion to the oocyte exterior.

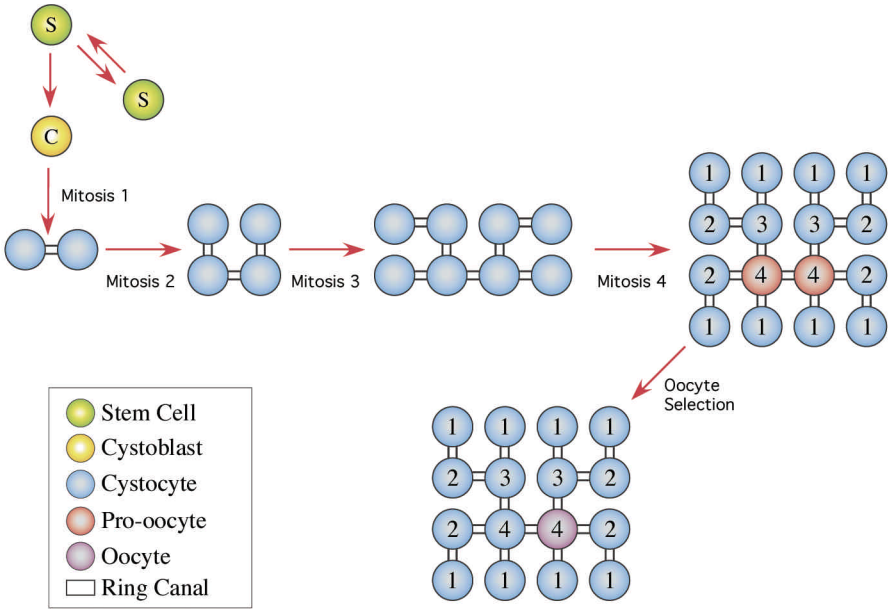
(b) Cyst formation and oocyte determination. The first division of the stem cell produces a daughter stem cell and a cystoblast. The cystoblast undergoes four mitotic divisions. As a result of incomplete cytokinesis at each division, cytoplasmic bridges called ring canals interconnect the sixteen resulting germline cells, the cystocytes. The resulting sixteen-cell cyst contains eight cells with one ring canal, four cells with two, two cells with three and two cells with four. One of the two cells with four ring canals, the pro-oocytes, invariably becomes the oocyte and the remaining pro-oocyte and remaining cystocytes are fated to become polyploid nurse cells.

(c) Germarial regions and oocyte positioning. Region 1 contains the germ line stem cells and mitotically dividing cystocytes. Region 2a, the two cells with four ring canals, pro-oocytes (red), enter meiosis. In region 2b, one of the pro-oocytes is selected to become the oocyte (purple) while the other reverts to a nurse cell fate. Somatic follicle cells encapsulate the sixteen-cell cyst. In region 3/stage 1, the cyst is spherical in shape with the oocyte positioned at the posterior and the fifteen nurse cells anterior to it.

a Ovariole



b Germline Cyst Formation



c Germarium - Oocyte Positioning

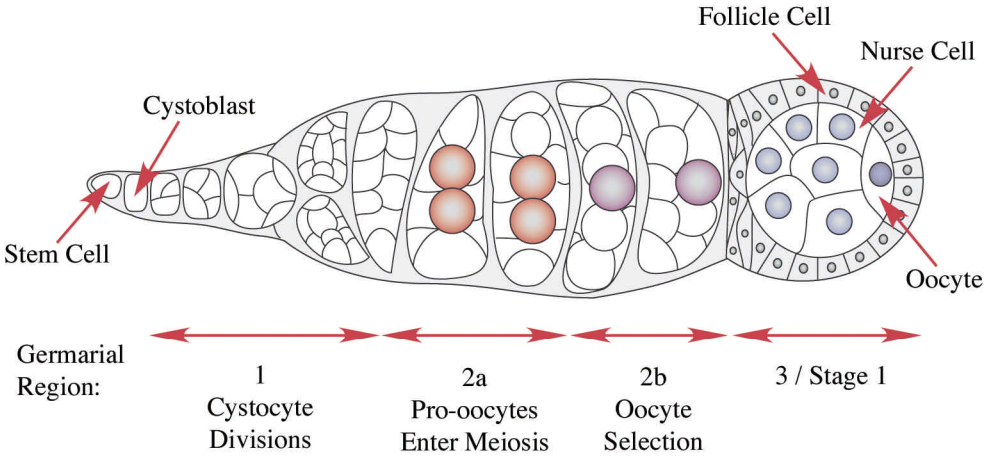


Figure 2. Microtubule organization during *Drosophila* oogenesis

Isolated egg chambers from wild-type females were fixed and microtubule structure was visualized with FITC conjugated anti-α-Tubulin (clone DM1A, Sigma). (a and a′) Germarium and stage 1 egg chamber. Anterior microtubule organizing center (MTOC) (arrowhead) observed in stage 1 oocyte (asterisk). (b and b′) Stage 4 egg chamber. MTOC observed at the posterior of the oocyte (arrowhead). During stage 7, the posterior MTOC degenerates and microtubules reorganize at the anterior of the oocyte. (c and c′) Stage 9 egg chamber. A gradient of microtubules is seen along the lateral cortical surface. The highest abundance of microtubules is observed along the anterior (arrowheads in c′) and decreases towards the posterior (arrow in c′). This organization is maintained until stage 10a. (d and d′) Stage 10b. Microtubules become bundled at the subcortical region (arrowhead in d′) accompanying ooplasmic streaming and the influx of nurse cell cytoplasm. These representations are consistent with previous observations (Theurkauf et al., 1992).

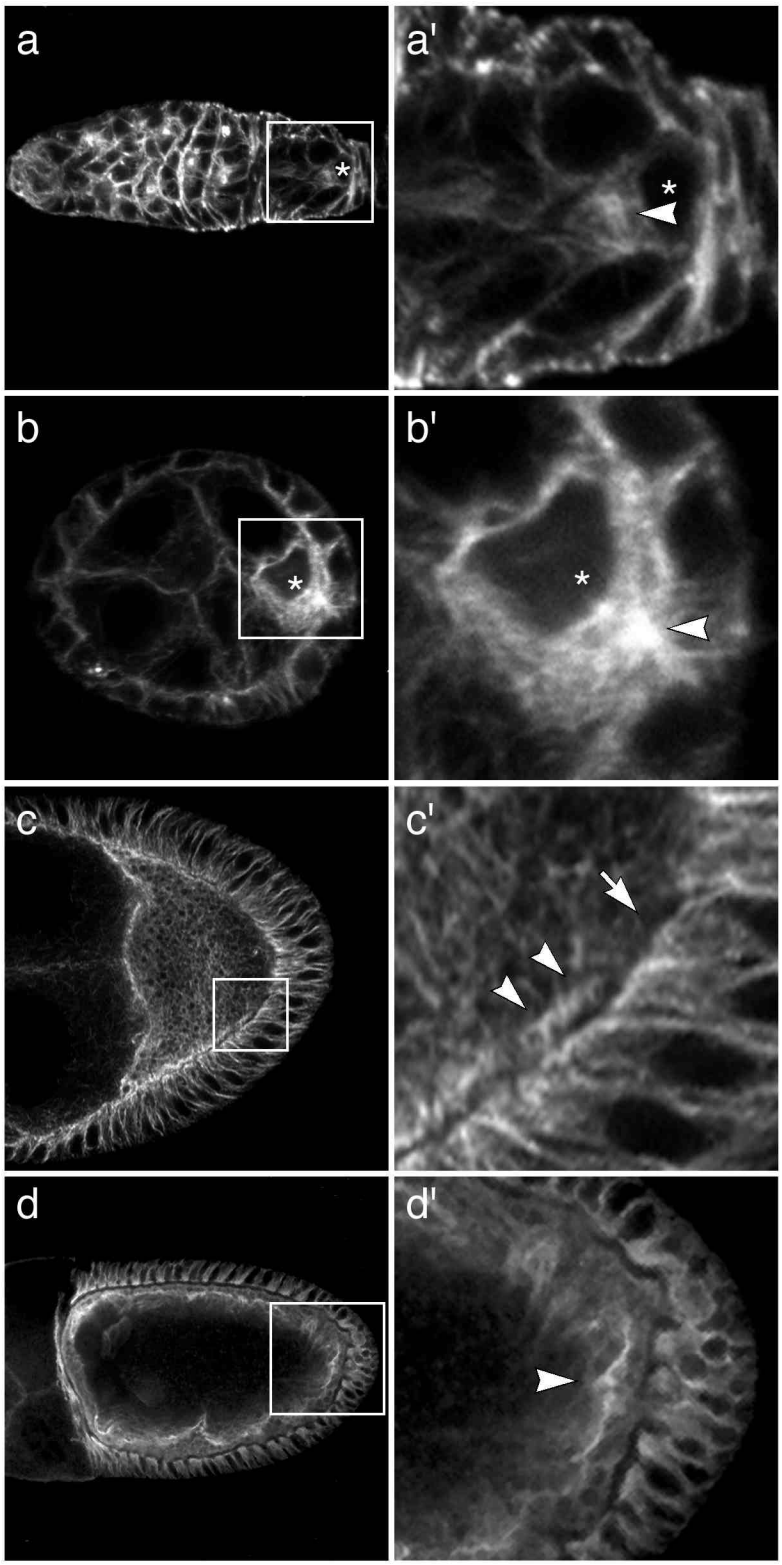
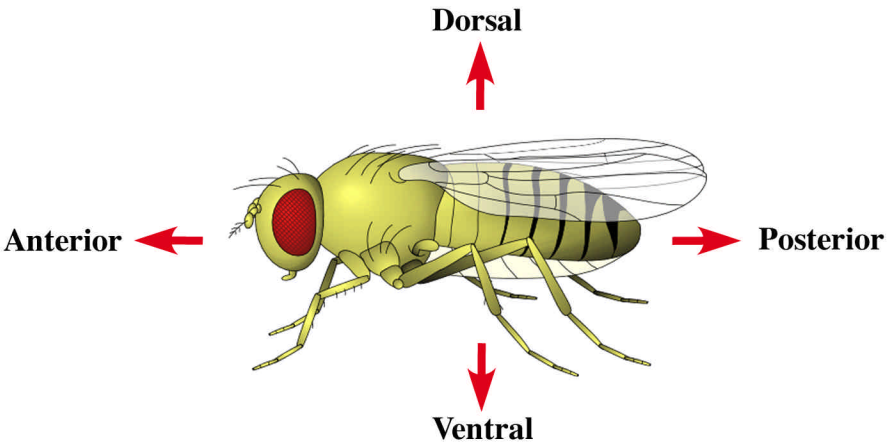
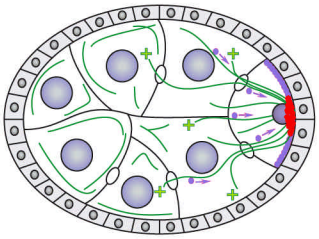

a
a'
b
b'
c
c'
d
d'

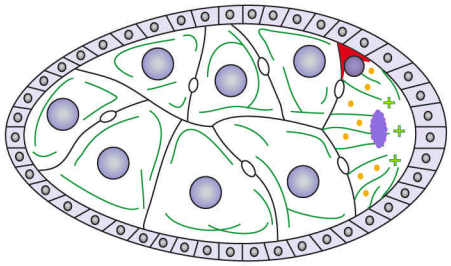
Figure 3. Schematic representation of microtubule organization and mRNA localization during *Drosophila* oogenesis

The anterior-posterior and dorsal-ventral axes of the *Drosophila* oocyte and subsequent embryo are established by the asymmetric distribution of morphogenetic factors during oogenesis. During stages 2-6 of oogenesis, the microtubules (green) are focused at the posterior pole of the oocyte forming a microtubule organizing center (MTOC). During this period, mRNAs and proteins begin to accumulate in the developing oocyte. *oskar* (*osk*) mRNA (purple), the primary posterior defining factor, accumulates at the posterior pole by minus-end microtubule directed transport. During stage 7-8, microtubules undergo dramatic reorganization as a result of a *grk*-dependent signaling event. The posterior MTOC appears to be inactivated and the microtubules nucleate from the anterior and lateral cortical surfaces and establish an anterior to posterior gradient. The minus-ends of the microtubules lie at the cortex while the plus-ends are oriented towards the oocyte cytoplasm. Accompanying microtubule reorganization, specific mRNAs localize. *gurken* (*grk*) mRNA (red), which defines future dorsal-ventral polarity, accumulates between the oocyte nucleus and the area proximal to the dorsal-anterior cortex. *bicoid* (*bcd*) mRNA (orange), the primary anterior-defining morphogen, begins to accumulate in the oocyte during these early stages. *osk* mRNA, which had accumulated at the posterior cortex, pools in the center of the oocyte. By stage 9, these essential mRNAs have localized to their final destinations. *osk* mRNA is localized to the posterior pole, *bcd* mRNA along the anterior cortex of the oocyte adjacent to the nurse cells and *grk* mRNA at the antero-dorsal margin flanking the oocyte nucleus. The next major microtubule reorganizing event occurs at stage 10b when cytoplasm from the adjoining nurse cells begins to dump into the oocyte. The microtubules bundle along the cortex concomitant with streaming of the oocyte cytoplasm, which evenly mixes the nurse cell cytoplasm with the contents of the oocyte. The localized RNAs remain anchored to their respective locations. At stage 11, the remaining nurse cell cytoplasm is dumped into the oocyte.

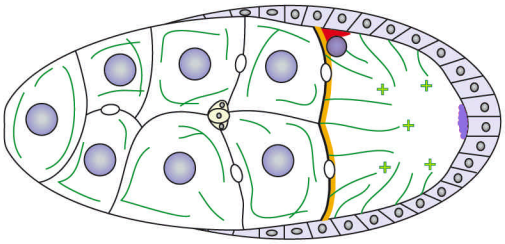




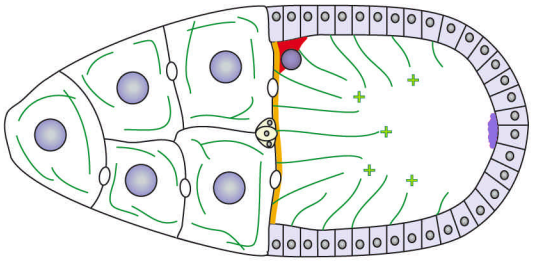
Stage 6



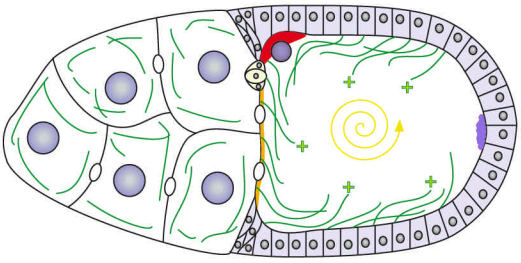
Stage 8



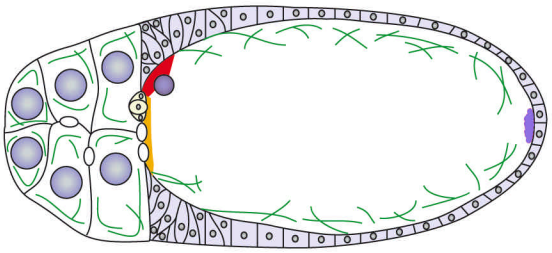
Stage 9



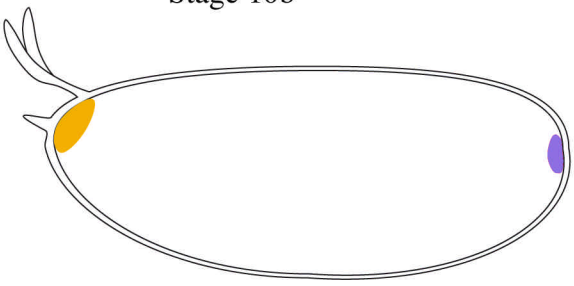
Stage 10a



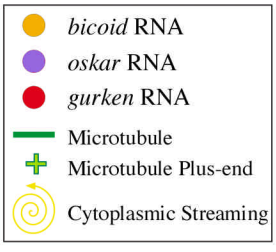
Stage 10b



Stage 11



Stage 14



METHODS

Drosophila stocks and germline clones

The following allelic combinations were used during the course of this study:

Genotype	Reference
$capu^{1}/capu^{1}$	(Manseau and Schupbach, 1989)
$chic^{1320}/chic^{1320}$	(Verheyen and Cooley, 1994)
$spir^{1}/spir^{1}$	(Manseau and Schupbach, 1989)
$armi^{72.1}/armi^{72.1}$	H. Cook and W. Theurkauf, unpublished
grk^{HK}/grk^{2B6}	(Neuman-Silberberg and Schupbach, 1993; Schupbach, 1987)
$stau^{D3}/stau^{D3}$	(Lehmann and Nusslein-Volhard, 1991)
$mael^{r20}/Df(3L)79E\text{-}F$	(Clegg et al., 1997)
$TmII^{gs1}/TmII^{gs1}$	(Erdelyi et al., 1995)
Kinesin I heavy chain germline clone	(Brendza et al., 2000; Chou et al., 1993)
osk^{54}/osk^{54}	(Lehmann and Nusslein-Volhard, 1991)
$spn\text{-}E^{1}/spn\text{-}E^{1}$	(Gillespie and Berg, 1995)
$par\text{-}1^{6323}/par\text{-}1^{w3}$	(Shulman et al., 2000)

Kinesin heavy chain (*khc*) null germline clones were generated by crossing *w;p{w*$^{+}$*,FRT}42B c* khc^{27}/CyO females to *yw p{hs-FLP};p{w*$^{+}$*,FRT}42B, p{*Ovo^{D1}*}55D/CyO* males (Brendza, 2000; Chou, T.B., 1993). Mitotic recombination in the germline stem cells of second and third instar larvae with the genotype *yw p{hs-FLP}/w;p{w*$^{+}$*,FRT}42B, p{*Ovo^{D1}*}55D/ p{w*$^{+}$*,FRT}42B c* khc^{27} was induced via heat-shock treatments. Oregon R served as the wild-type strain in all experiments.

In vitro transcription and fluorescent labeling of RNA

Full-length (2.9 kb) *osk* antisense RNA probes were synthesized *in vitro* by run-off transcription from the plasmid pBluescriptKS-osk (generously provided by R. Lehmann) restriction digested with HincII. Cyanine 5 (cy5)-conjugated UTP ribonucleotides were incorporated during the transcription reaction. Typically 1 µg DNA was digested in a volume of 30 µl with 10 Units Hinc II, incubated at 37 °C for 1 hour, phenol:chloroform:isoamyl alcohol

(25:24:1) extracted and ethanol precipitated using 1/10 volume 3M NH_4OAc. Transcription reaction components were combined on ice and included: 1 µg DNA, 1 mM ATP, 1 mM CTP, 1 mM GTP, 0.65 mM UTP, 0.55 mM cyanine 5-UTP (PerkinElmer Life Sciences, Inc.), 1× T7 transcription buffer (5×, Promega), 40 Units T7 RNA polymerase (20 U/µl, Promega) in a final volume of 18 µl. The reagents were mixed and centrifuged briefly prior to incubation at 37 °C for 2 hours. Following incubation, the transcription reaction was immediately terminated by the addition, on ice, of EDTA pH 8.0 to a final concentration of 20 µM. Conjugated RNAs were used immediately following dilution to 80 µl with dH_2O (DEPC treated) without further purification measures. A typical transcription reaction yields 1 µg/µl labeled RNA.

Isolation and fixation of egg chambers

Egg chambers were obtained by hand dissecting ovaries from yeast-paste fed females 3 to 5 days after eclosion and fixed as previously described (Theurkauf, 1994a). Briefly, egg chambers were isolated in 1× Robb's medium (55 mM potassium acetate, 40 mM sodium acetate, 100 mM sucrose, 10 mM glucose, 12 mM $MgCl_2$, 1.0 mM $CaCl_2$, 100 mM HEPES pH 7.4). Egg chambers were not permitted to remain in Robb's medium for more than 10 minutes. Following dissection, intact ovaries were transferred to 1:1 2× fixation buffer (2× fixation buffer: 200 mM cacodylic acid (dimethylarsinic acid, Sigma), 200 mM sucrose, 80 mM potassium acetate, 20 mM sodium acetate, 20 mM EGTA, pH 7.2):16% formaldehyde. Egg chambers were fixed for 10 minutes and allowed to settle. Following fixation, egg chambers were rinsed three times in PBST (1× Phosphate buffered saline (PBS), 0.05% Triton X-100). Intact ovaries were teased apart into individual ovarioles in PBST. Egg chambers were transferred to 1% Triton X-100 in PBS, for extraction, and incubated for 2 hours at room temperature with gentle rocking. After extraction, egg chambers were rinsed once in PBST and two times in PBT (1× PBS, 0.1% Tween[å]-20). Fixed egg chambers were processed immediately via *in situ* hybridization.

Cytoskeletal inhibitor treatment

Flies were fed yeast paste 3 days prior to harvesting egg chambers. The microtubule deploymerization agent colcemid (Sigma) was added to the yeast paste, at a concentration of 50 µg/ml, 20 hours prior to egg chamber harvesting. For disruption of F-actin, egg chambers were isolated in Robb's medium and incubated in the same medium, containing 20 µg/ml cytochalasin D (Sigma), for 10 minutes at room temperature. Egg chambers were immediately fixed following cytochalasin D treatment.

RNA and Protein Localization Analysis – dual fluorescence *in situ* hybridization (FISH) and immunofluorescence

Whole-mount *in situ* hybridization was performed as previously described (Tautz and Pfeifle, 1989) with modifications. Briefly, fixed egg chambers were washed two times with PBT (PBS, 0.1% Tween^{å}-20), rinsed in 1:1 PBT: Hyb-B Buffer (50% formamide, 5× SSC), rinsed in Hyb-B Buffer and prehybridized in Hyb-A Buffer (50% formamide, 5× SSC, 0.2 mg/ml DNA from salmon testes, 0.1 mg/ml yeast tRNA, and 0.05 mg/ml heparin) for 1 hour at 63 °C. RNA probes (1 µg/sample) were denatured at 80 °C for 10 minutes, placed on ice, centrifuged briefly, resuspended in 30 µl Hyb-A Buffer and added to the egg chambers. Egg chambers were incubated for 16 hours at 63 °C. Following hybridization, room temperature Hyb-B Buffer was added and egg chambers were permitted to settle for 15 minutes at 63 °C. Hyb-B Buffer was exchanged two additional times and finally diluted with PBT at room temperature. Egg chambers were rinsed three times with PBT. Prior to primary antibody incubation, egg chambers were blocked in 1% BSA in PBST for 1 hour at room temperature with gentle agitation. Egg chambers were incubated at 4 °C with gentle agitation for 16 hours with polyclonal rabbit anti-Osk (Ephrussi et al., 1991) diluted 1:75 in 1% BSA in PBST. Egg chambers were incubated overnight at 4 °C with gentle agitation. Following primary antibody incubation, egg chambers were rinsed in six changes of PBST. The secondary antibody, goat anti-rabbit IgG AlexaFluor 488-conjugated (Molecular Probes) was diluted 1:200 in PBST and egg chambers were incubated overnight at 4 °C with gentle agitation. Following secondary antibody incubation, egg chambers

were rinsed in six changes of PBST and mounted in PBS-glycerol mounting medium (9:1, glycerol:10× PBS).

Images were acquired with an inverted Leica DMIRB/E laser scanning confocal microscope. Leica TCS NT image acquisition software was used. All images were acquired under identical imaging conditions for a given experimental protocol. Single confocal optical sections are represented in the figures.

RESULTS

Characterization of *osk* mRNA and Osk protein localization during *Drosophila* oogenesis

Fluorescence *in situ* hybridization (FISH) in combination with immunofluorescence and subsequent analysis by laser scanning confocal microscopy provides a high-resolution method to detect both *osk* mRNA and Osk protein during *Drosophila* oogenesis. The simultaneous examination of RNA and protein allows a spatial and temporal correlation between transcript localization and protein expression.

Characterization of *osk* mRNA distribution in wild-type oocytes has defined distinct steps in the localization of *osk* mRNA to the posterior pole. Initially, as early as germarial stage 2b, *osk* mRNA becomes enriched in the oocyte (Ephrussi et al., 1991; Kim-Ha et al., 1991). During stages 2 through 6, *osk* mRNA is clearly localized to the posterior cortex of the oocyte (arrowheads in Figure 4a). During these early stages, microtubules appear to nucleate from a MTOC at the oocyte posterior (Theurkauf et al., 1993). The posterior accumulation of *osk* transcript is likely to be the result of minus-end directed movement from the nurse cells to the oocyte, given that mutants affecting dynein, a minus-end directed motor, show defects in oocyte determination and the transfer of *osk*, *bcd*, and other RNAs from the nurse cells to the oocyte (McGrail and Hays, 1997). Posterior cortical localization is maintained until stage 7, when the next major microtubule reorganization event occurs. As a result of *grk* signaling between the oocyte and the posterior follicle cells, the posterior microtubule organizing center disassembles and microtubules are organized along the anterior and lateral cortices of the oocyte, producing an anterior-to-posterior density gradient (Gonzalez-Reyes et al., 1995; Roth et al., 1995; Theurkauf et al., 1992). At this time, Kinesin I, a plus-end directed microtubule motor, removes *osk* mRNA from the lateral cortex, resulting in the accumulation of *osk* mRNA in the center of the oocyte (Figure 4d and g) (Cha et al., 2002). In approximately 47% of stage 8 oocytes, *osk* mRNA is

detected solely at the anterior/central region, while 53% show *osk* mRNA both at the posterior pole and in the central cytoplasmic pool (Figure 5).

While *osk* mRNA is present in the oocyte throughout oogenesis, *osk* mRNA first localizes to the posterior pole during stage 8 (Figure 4g), and Osk protein is only detected at approximately stage 9 (Figure 4k), after essentially all detectable *osk* transcript is at the posterior pole of the oocyte (Figure 4j) (Gunkel et al., 1998; Kim-Ha et al., 1995; Rongo et al., 1995). These observations suggest that cortical localization is not sufficient to prompt *osk* mRNA translation. Instead, it appears that translational activation is triggered by temporal signals that may function in conjunction with spatial cues.

Kinesin I, Tropomyosin, and Staufen are required for removal of *osk* mRNA from the oocyte cortex.

Kinesin I heavy chain (*khc*), a plus-end-directed motor (Vale et al., 1985), is required for *osk* mRNA localization to the posterior pole of the oocyte (Brendza et al., 2000). To further elucidate the role of Kinesin in *osk* mRNA localization, FISH analysis was used to examine *osk* mRNA distribution in *khc* mutant oocytes that were generated as germ line clones (Brendza et al., 2000). During early oogenesis, *osk* mRNA localizes to the posterior cortex in *khc* mutant oocytes (Figure 6d); however, the association of *osk* mRNA with the cortex appears to be not as tight as seen in wild-type (Figure 6a). While *osk* mRNA normally pools in the center and begins to translocate to the posterior pole in stage 8 oocytes (Figure 6b), in *khc* mutants, *osk* RNA is found along the oocyte cortex with no accumulation in the interior (Figure 6e). This broad cortical distribution is maintained through later stages of oogenesis (Figure 6f). Similar uniform cortical localization is observed following microtubule disruption. These observations indicate that Kinesin is required to move *osk* mRNA from the cortex to the oocyte interior (Cha et al., 2002).

Mutants in *Tropomyosin* (*TmII*) exhibit defects in the localization and/or maintenance of pole plasm components at the posterior of the oocyte (Erdelyi et al., 1995). Through early oogenesis, the localization of *osk* mRNA to the posterior cortex in *TmII* mutant oocytes (Figure

6g) appears wild-type. However, during stage 8, *osk* transcript accumulates along the anterior margin proximal to the nurse cells, the lateral cortex, and at the posterior pole (Figure 6h). The internal cytoplasmic accumulation of *osk* RNA is never observed. Uniform distribution of *osk* mRNA along the oocyte cortex is seen through stage 10 (Figure 6i), and *osk* mRNA is never restricted to a tight region at the posterior pole of the oocyte.

Stau, a presumptive RNA binding protein (Lasko, 1999) colocalizes with and is necessary for the posterior localization of *osk* mRNA to the posterior of oocyte (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991). In *stau* mutants, accumulation of *osk* mRNA to the posterior cortex during early oogenesis appears normal (Figure 6j). During stage 8, however, *osk* transcript is seen along the anterior margins and posterior pole, but the internal pool is never observed (Figure 6k). The localization of *osk* mRNA along the anterior and lateral cortices as well as at the posterior of the oocyte is maintained through the later stages of oocyte development (Figure 6l), and exclusive accumulation of *osk* mRNA to the posterior is never observed. Together, these results suggest a role for Staufen, Tropomyosin and Kinesin I in the efficient removal of *osk* mRNA from the oocyte cortex.

capu, _chic_ and _spir_ induce premature ooplasmic streaming and block posterior localization of _osk_ mRNA

capu, chic and spir mutants all disrupt microtubule organization, trigger premature microtubule-based ooplasmic streaming, and block *osk* mRNA localization (Emmons et al., 1995; Manseau and Schupbach, 1989; Theurkauf, 1994b). Through oogenesis stage 6, *osk* mRNA accumulates normally at the posterior cortex of the oocyte in *capu*, *chic* and *spir* mutants (Figure 7d, g, and j). During midoogenesis, *osk* mRNA accumulates in the center of the oocyte (Figure 7e, h, and k), as in wild-type (Figure 7b). *capu*, *chic* and *spir* mutations all lead to premature ooplasmic streaming during stages 8-9 (Emmons et al., 1995; Manseau and Schupbach, 1989; Theurkauf, 1994b), and these dramatic cytoplasmic movements may hinder the final step in *osk* mRNA localization. During stage 10, *osk* mRNA is usually localized to the posterior pole in wild-type (Figure 7c).

By contrast, in *capu*, *chic* and *spir* mutants, *osk* mRNA remains dispersed in the cytoplasm, displaced somewhat towards the anterior pole (Figure 7f, i and l). *capu*, *chic* and *spir* are therefore required for the efficient movement of *osk* mRNA from the interior to the posterior pole of the oocyte.

par-1 and _grk_ are required for microtubule asymmetry and posterior localization of _osk_ mRNA

Mutations in *par-1* and *grk*, as in *capu*, *spir* and *chic*, block *osk* mRNA localization to the posterior pole (Shulman et al., 2000; Tomancak et al., 2000). However, unlike *capu*, *spir*, and *chic*, *par-1* and *grk* do not lead to premature ooplasmic streaming. In *par-1* and *grk* mutants, the accumulation of *osk* mRNA at the posterior cortex of the oocyte through stage 6 appears wild-type (Figure 8d and g). Stage 8 *par-1* and *grk* mutant egg chambers accumulate *osk* mRNA in a central cytoplasmic region in the oocyte (Figure 8e and h), comparable to the *osk* mRNA localization pattern in wild-type (Figure 8b). During the later stages of oogenesis when *osk* mRNA normally localizes at the posterior pole (Figure 8c), *osk* mRNA is mislocalized to an ectopic site in the center of the oocyte in *par-1* and *grk* mutants (Figure 8f and i).

The ectopic localization of *osk* mRNA in *par-1* and *grk* mutants correlates with observed defects in microtubule organization. In wild-type, stage 8 and 9 oocytes, a cortical microtubule gradient is established with the highest concentration at the anterior and a decline in density towards the posterior (Theurkauf et al., 1992). *osk* mRNA localizes to the microtubule-poor posterior pole, presumably because Kinesin cannot efficiently remove *osk* mRNA from this region (Cha et al., 2002). In *par-1* and *grk* mutants, however, cortical microtubules persist at the posterior pole (Cox et al., 2001; Gonzalez-Reyes et al., 1995; Huynh et al., 2001; Shulman et al., 2000; Tomancak et al., 2000). Therefore, in *grk* and *par-1* mutant oocytes, Kinesin-dependent movement on cortical microtubules prevents *osk* mRNA localization to the oocyte posterior, trapping *osk* transcript in the oocyte interior.

armi, _mael_ and _spn-E_ are required for microtubule asymmetry and posterior localization of _osk_ mRNA

armi, *mael* and *spn-E* have demonstrated defects in microtubule organization, *osk* mRNA localization, and translational regulation of *osk* mRNA (H. Cook and W. Theurkauf, unpublished observations)(Clegg et al., 1997; Gillespie and Berg, 1995). *osk* RNA FISH analysis of oocytes isolated from *armi*, *mael* and *spn-E* mutant females revealed a defect in *osk* mRNA localization that manifests itself early in oogenesis. During the early stages of oogenesis, *osk* transcript accumulates at the posterior cortical region in wild-type oocytes (Figure 9a); however, in *armi*, *mael* and *spn-E* mutant oocytes *osk* mRNA is dispersed in the oocyte cytoplasm (Figure 9d, g, and j). During stage 8, *osk* transcript accumulates in the oocyte center in wild-type (Figure 9b). Similarly, although apparently more diffuse, *osk* mRNA accumulates in a central cytoplasmic region in stage 8 mutant oocytes (Figure 9e, h, and k). Late in oogenesis, during stage 9-10, *osk* mRNA has tightly localized to the posterior pole in wild-type oocytes (Figure 9c). However, in *armi* and *mael* mutant oocytes, *osk* mRNA is seen accumulating at an ectopic site in the cytoplasm towards the anterior of the oocyte (Figure 9f and i). Some egg chambers derived from *armi* and *mael* mutants show an accumulation of *osk* RNA at the posterior, but the predominant accumulation remains in the central cytoplasmic region. Mutants in *spn-E* produce very few late stage egg chambers and in those examples, *osk* RNA is distributed at the posterior of the oocyte but is not tightly localized (Figure 9l). The inefficient localization of *osk* mRNA to the posterior pole observed in mutant oocytes suggests a direct or indirect role for *armi*, *mael* and *spn-E* in the effective localization of *osk* mRNA to the oocyte posterior.

***armi*, *mael* and *spn-E* affect temporal control of *osk* translation**

Localized *osk* mRNA translation at the posterior of the oocyte is essential for the proper posterior patterning of the embryo, and defects in the spatial and temporal control of Osk protein production result in severe developmental defects (Driever et al., 1990; Ephrussi and Lehmann, 1992; Gavis and Lehmann, 1992; Kim-Ha et al., 1995; Smith et al., 1992). FISH, in conjunction with immunofluorescence, was used to simultaneously examine the localization of *osk* mRNA

and Osk protein. In wild-type, *osk* mRNA is expressed through most of oogenesis, but Osk protein is only observed during stage 9 (inset in Figure 10b), when *osk* mRNA is strictly localized at the posterior pole (inset in Figure 10a). As described earlier, in egg chambers isolated from *armi*, *mael* and *spn-E*^{1} mutant females, *osk* mRNA accumulates in the oocyte but does not localize to the posterior pole during early oogenesis (Figure 10d, g and j), In addition, we find that Osk protein is prematurely expressed in these mutants (Figure 10e, h and k) (H. Cook and W. Theurkauf, unpublished observations). *armi*, *mael* and *spn-E* are therefore required for translational silencing of *osk* mRNA during early oogenesis.

***par-1* and *grk* are required for spatial control of *osk* translation**

By contrast, mutations in *par-1* and *grk* alter the spatial control of *osk* mRNA translation. In wild type, *osk* mRNA is only translated after localization to the posterior pole is complete, during stage 9. *osk* mRNA translation is never observed when *osk* transcript is pooled in the center of the oocyte (inset in Figure 11a and b) during stage 8. In *par-1* and *grk* mutants, however, *osk* mRNA fails to localize at the posterior pole and accumulates in the center of the oocyte during stages 9 and 10 (Figure 11g and j), and this ectopically localized transcript is translated (Figure 11h and k). Similarly, *armi* mutants show ectopic expression of Osk protein in the center of stage 9 and 10 oocytes (Figure 11d). *par-1* and *grk*, are therefore required for proper spatial control of *osk* mRNA translation, while *armi* is required for both spatial and temporal control of Osk expression.

Microtubule Depolymerization – the role of cytoskeletal factors in short-range *osk* mRNA capturing/maintenance

osk mRNA localization within the oocyte appears to require direct transport to the desired location and capturing or anchoring at the localization site. *osk* RNA injected in close proximity to the posterior cortex localizes following microtubule disruption (Glotzer et al., 1997). This suggests the existence of a short-range, microtubule-independent mechanism for RNA capturing (Glotzer et al., 1997).

Uniform cortical distribution of *osk* RNA is observed in oocytes isolated from flies that had been fed the microtubule depolymerizing agent colcemid (Figure 12d) (Cha et al., 2002). Furthermore, egg chambers isolated from wild-type flies, and subsequently treated with cytochalasin D, which disrupts actin polymerization, show displacement of *osk* RNA and Osk protein from the entire oocyte cortex (arrowhead in inset, Figure 12i) (Cha et al., 2002). These observations indicate that a microtubule-independent process, which requires actin, anchors *osk* mRNA at the cortex. The exact mechanism of *osk* mRNA localization to the cortex remains to be determined. To this end, mutant flies showing *osk* mRNA localization defects were fed the microtubule depolymerizing drug colcemid, and the isolated egg chambers were analyzed for *osk* RNA localization.

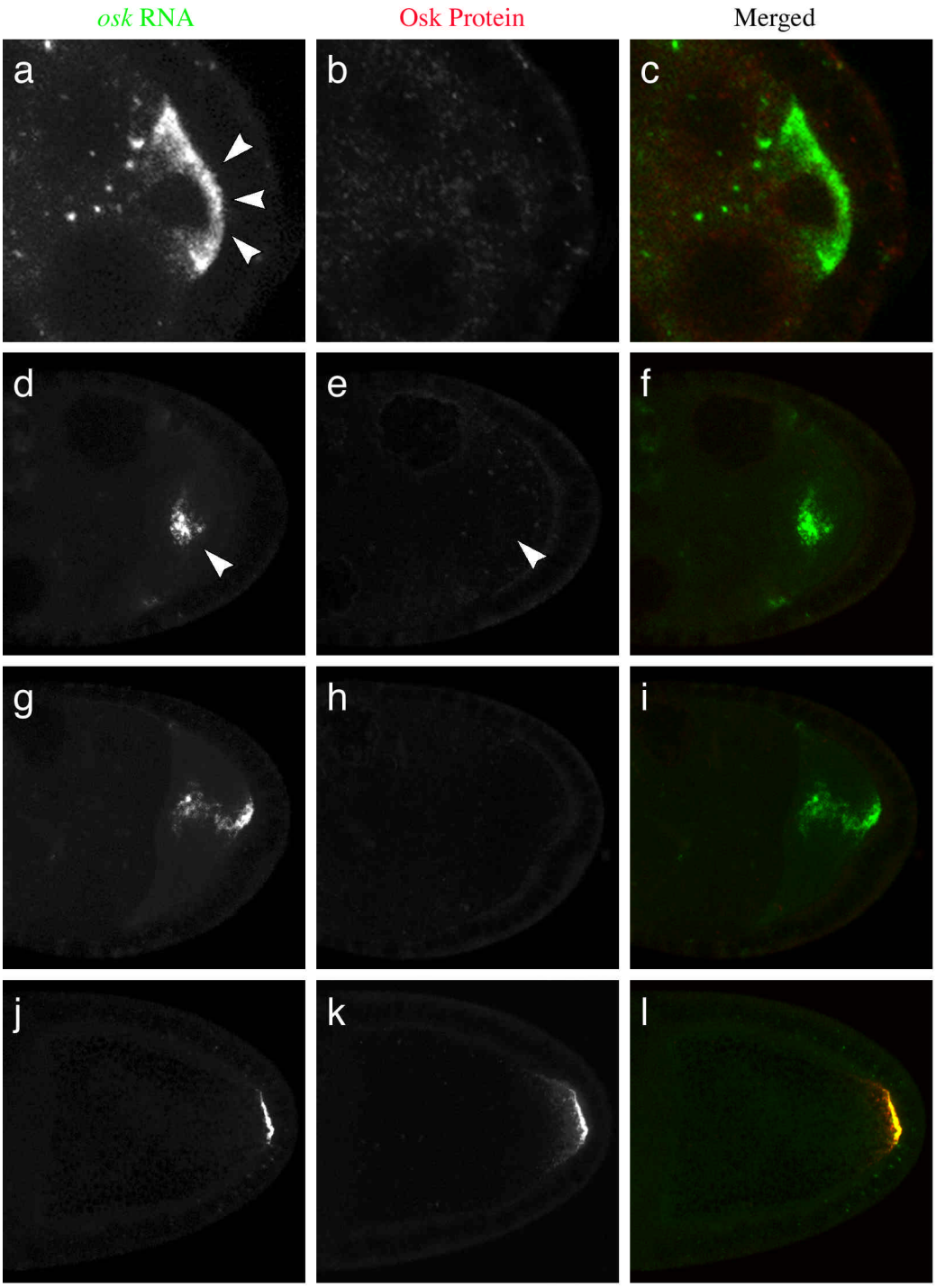
In egg chambers from wild-type flies treated with colcemid for 20 hours, *osk* RNA shows a broad cortical distribution (arrowheads in Figure 13b) in contrast to the discrete localization of *osk* RNA at the posterior pole observed in untreated egg chambers (Figure 13a). To determine the specificity of the cortical *osk* RNA distribution following microtubule depolymerization, FISH analysis was performed to determine the distribution of other RNAs, *bcd* and *cyclin B*, following colcemid treatment. *bcd* mRNA, which normally localizes to the anterior margin of the oocyte adjacent to the nurse cells (Figure 13c), becomes dispersed in the oocyte cytoplasm on microtubule depolymerization (Figure 13d). *cyclin B* mRNA is normally localized along the anterior and lateral cortices (arrowheads in Figure 13e) and enriched at the posterior pole (arrow in Figure 13e). Following microtubule depolymerization, *cyclin B* transcript exhibits diffuse distribution at the anterior margins (arrowheads in Figure 13f) and is no longer detectable at the posterior pole (arrow in Figure 13f). Therefore, cortical distribution of *osk* mRNA following microtubule depolymerization is specific to *osk* RNA.

All of the mutants screened thus far (Figure 14d, f, h, j, l, n, p, r and t) have the capacity of localizing *osk* mRNA to the cortex in the absence of microtubules. This analysis has not revealed a candidate directly required in the capturing/anchoring of *osk* transcript to the oocyte

cortex. The possibility exists that actin alone is responsible for the capturing and tethering of osk RNA once it reaches its destination. Alternatively, a gene yet unidentified or not yet examined may act in association with the actin cytoskeleton to anchor *osk* RNA at the cortex.

Figure 4. Dual detection of *osk* mRNA and Osk protein in wild-type oocytes.

The simultaneous detection of *osk* RNA and Osk protein permits the spatial correlation of *osk* transcript localization and Osk protein production. (a-c) Stage 5 oocyte. *osk* RNA is localized to the posterior cortical region of the oocyte (arrowheads in a). (b) During early oogenesis, Osk protein is not detectable. (d-f) Stage 8 oocyte. *osk* RNA accumulates transiently in the central cytoplasm (arrowhead in d) without any detectable level of Osk protein (arrowhead in e). (g-i) Stage 8 oocyte. *osk* RNA is localized continuously between the central pool and the posterior pole. Even after the localization of transcript to the posterior, no protein is detected. (j-l) Stage 9 oocyte. *osk* RNA is strictly localized to the posterior pole and Osk protein is observed. (l) Colocalization of *osk* RNA and Osk protein at the posterior of the oocyte. (c, f, i and l) Composites of the *osk* RNA and Osk protein images. *osk* RNA is represented in green, Osk protein in red and colocalization of *osk* RNA and Osk protein represented in yellow.


osk RNA
Osk Protein
Merged
a
b
c
d
e
f
g
h
i
j
k
l

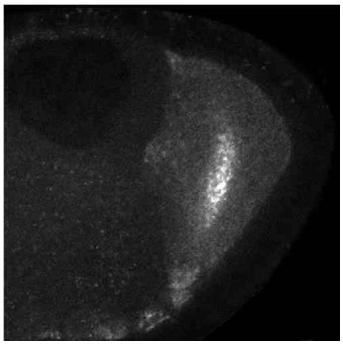
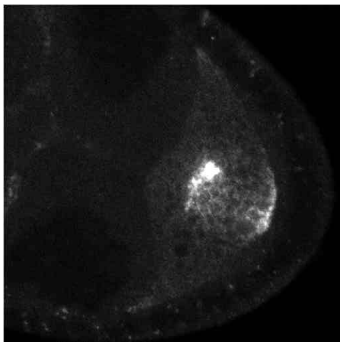
	 Central	 Central-Posterior
Individuals / Percentage (n=136)	63 / 46.6%	73 / 53.6%

Figure 5. Quantification of the transient transport intermediate in stage 8 wild-type oocytes.

osk fluorescence *in situ* hybridization was performed and individual stage 8 egg chambers were scored for *osk* RNA localization patterns. Two predominant patterns were observed, a centralized pool or an intermediate central-posterior distribution of *osk* RNA.

Figure 6. *osk* RNA localization in *khc, TmII* and *stau* mutant oocytes.

Cy5-conjugated *osk* antisense probes were used to detect *osk* transcript *in situ* in oocytes isolated from wild-type, Kinesin I Heavy Chain (*khc*) germline clone flies, $TmII^{gs1}/TmII^{gs1}$ and $stau^{D3}/stau^{D3}$ mutant females. (a-c) *osk* RNA localization in wild-type oocytes. (a) *osk* RNA is localized to the posterior cortical region in early wild-type oocytes. (b) During stage 8, *osk* transcript is transiently localized to the central region and posterior pole. (c) In stage 10 wild-type oocytes, *osk* RNA is exclusively localized to the posterior pole. (d) In early *khc* mutant oocytes, *osk* mRNA accumulates at the posterior cortex, however, distribution of *osk* transcript in the oocyte cytoplasm is observed as well. (g and j) In *TmII* and *stau* mutant stage 5-6 oocytes, *osk* RNA is localized to the posterior cortical region, similar to wild-type. (e) During midoogensis in *khc* mutants, *osk* RNA is distributed along the cortex of the oocyte. (h) During stage 8 in *TmII* mutant oocytes, *osk* RNA localizes to the anterior cortical region proximal to the nurse cells and weakly along the cortex. (k) Stage 8 *stau* mutant oocytes show anterior cortical and posterior localization of *osk* RNA. In the three mutants examined here no central accumulation of *osk* RNA, as observed in wild-type, was detected. (f and i) During stage 10, *osk* RNA is detected along the cortex in *khc* and *TmII* mutant oocytes. (l) The anterior-cortical and posterior localization of *osk* RNA observed during stage 8 is maintained during the later stage of oogenesis in *stau* mutant oocytes.

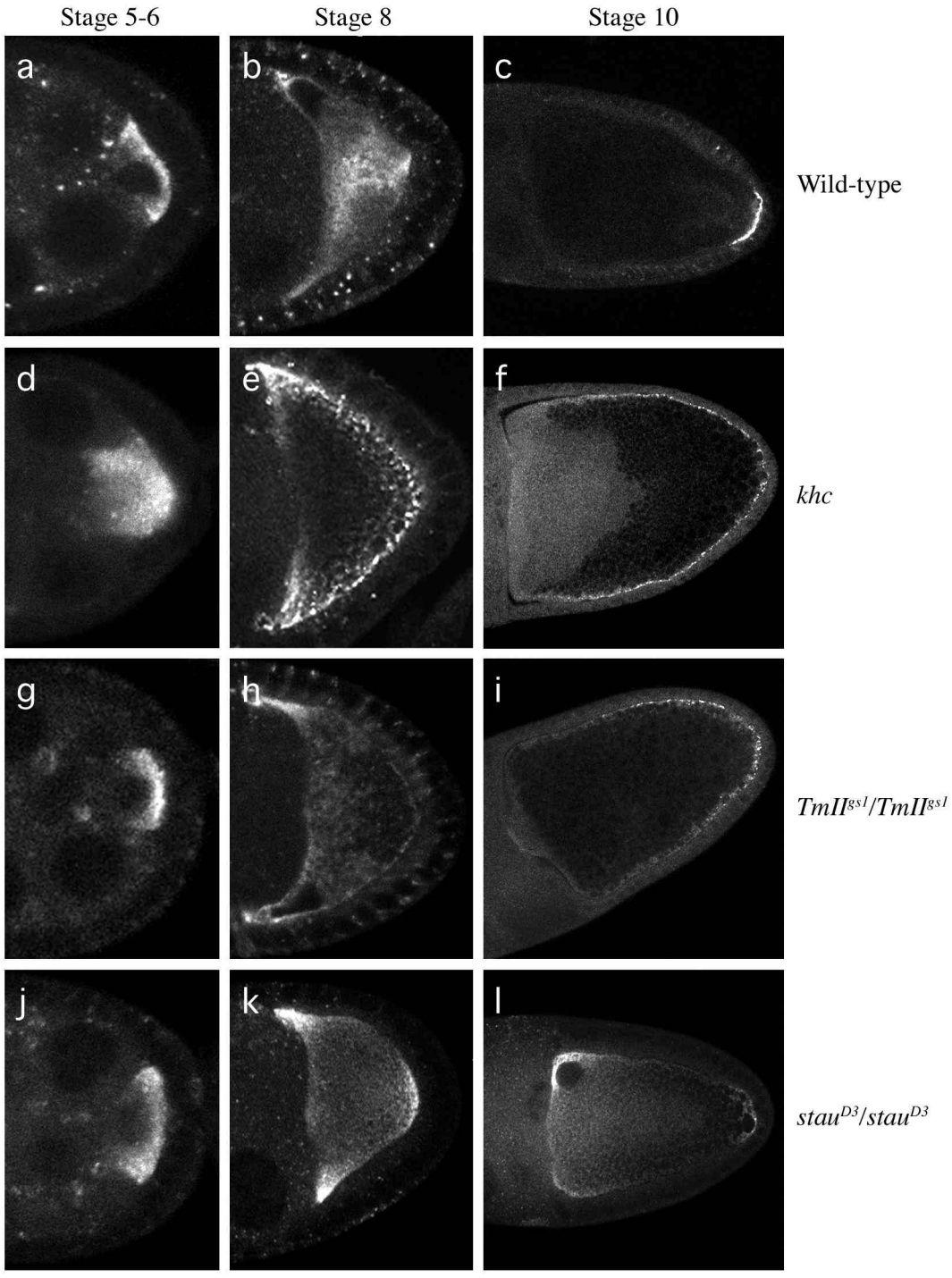

Stage 5-6
Stage 8
Stage 10
a
b
c
Wild-type
d
e
f
khc
g
h
i
TmII^gs1/TmII^gs1
j
k
l
stau^D3/stau^D3

Figure 7. *osk* RNA localization in *capu*, *chic* and *spir* mutant oocytes.

Cy5-labeled *osk* antisense probes were used to detect *osk* transcript *in situ* in oocytes isolated from wild-type, $capu^{1}/capu^{1}$, $chic^{1320}/chic^{1320}$ and $spir^{1}/spir^{1}$. Single confocal sections are shown. (a-c) *osk* RNA localization in wild-type oocytes. (a) *osk* RNA is localized to the posterior cortical region in early wild-type oocytes. (b) During stage 8, *osk* transcript is transiently localized to the central cytoplasmic region and posterior pole of wild-type oocytes. (c) In stage 10 oocytes, *osk* RNA is strictly localized to the posterior pole. (d, g, and j) During early oogenesis, up to stage 6, *capu*, *chic*, and *spir* mutant oocytes show posterior cortical localization. (e, h, and k) The localization of *osk* RNA in mutant oocytes during stage 8 appears, to some extent, wild-type. However, *osk* RNA is more diffuse than wild-type and little transcript accumulates at the posterior pole. (f, i, and l) *osk* mRNA localization to the posterior pole is not observed in *capu*, *chic* and *spir* mutant oocytes. Instead, a diffuse accumulation towards the anterior/central region is observed.

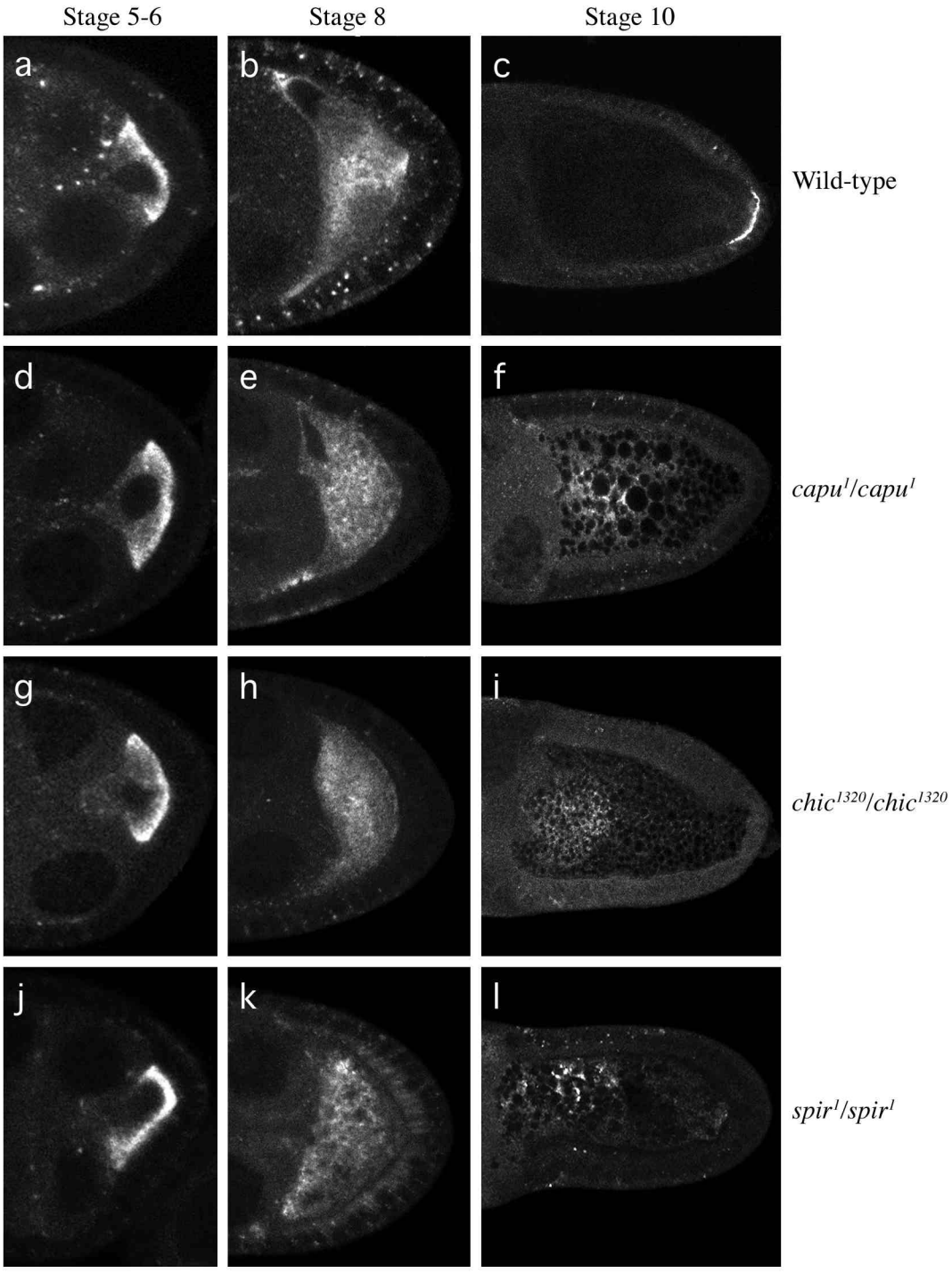

Stage 5-6
Stage 8
Stage 10
a
b
c
Wild-type
d
e
f
capu1/capu1
g
h
i
chic1320/chic1320
j
k
l
spir1/spir1

Figure 8. *osk* RNA localization in *par-1* and *grk* mutant oocytes.

Cy5-conjugated *osk* antisense probes were used to detect *osk* transcript *in situ* in oocytes isolated from wild-type, and $par\text{-}1^{w3}/par\text{-}1^{6323}$ and grk^{HK}/grk^{2B6} mutants. (a-c) *osk* RNA localization in wild-type oocytes. (a) During early oogenesis in wild-type, *osk* RNA accumulates at the posterior region of the oocyte. (b) During stage 8, *osk* transcript is observed in a transient central pool with accumulation at the posterior of the oocyte. (c) In stage 10 wild-type oocytes, *osk* transcript shows strict localization to the posterior cortex. (d and g) *par-1* and *grk* mutant oocytes exhibit a normal posterior localization pattern of *osk* RNA during early oogenesis. (e and h) During stage 8, similar to wild-type, the intermediate pool of transcript is readily detectable in the *par-1* and *grk* mutant oocytes. (f and i) During stage 10, when the transcript is observed at the posterior of the oocyte in wild-type, *osk* RNA remains accumulated in the center of *par-1* and *grk* mutant oocytes.

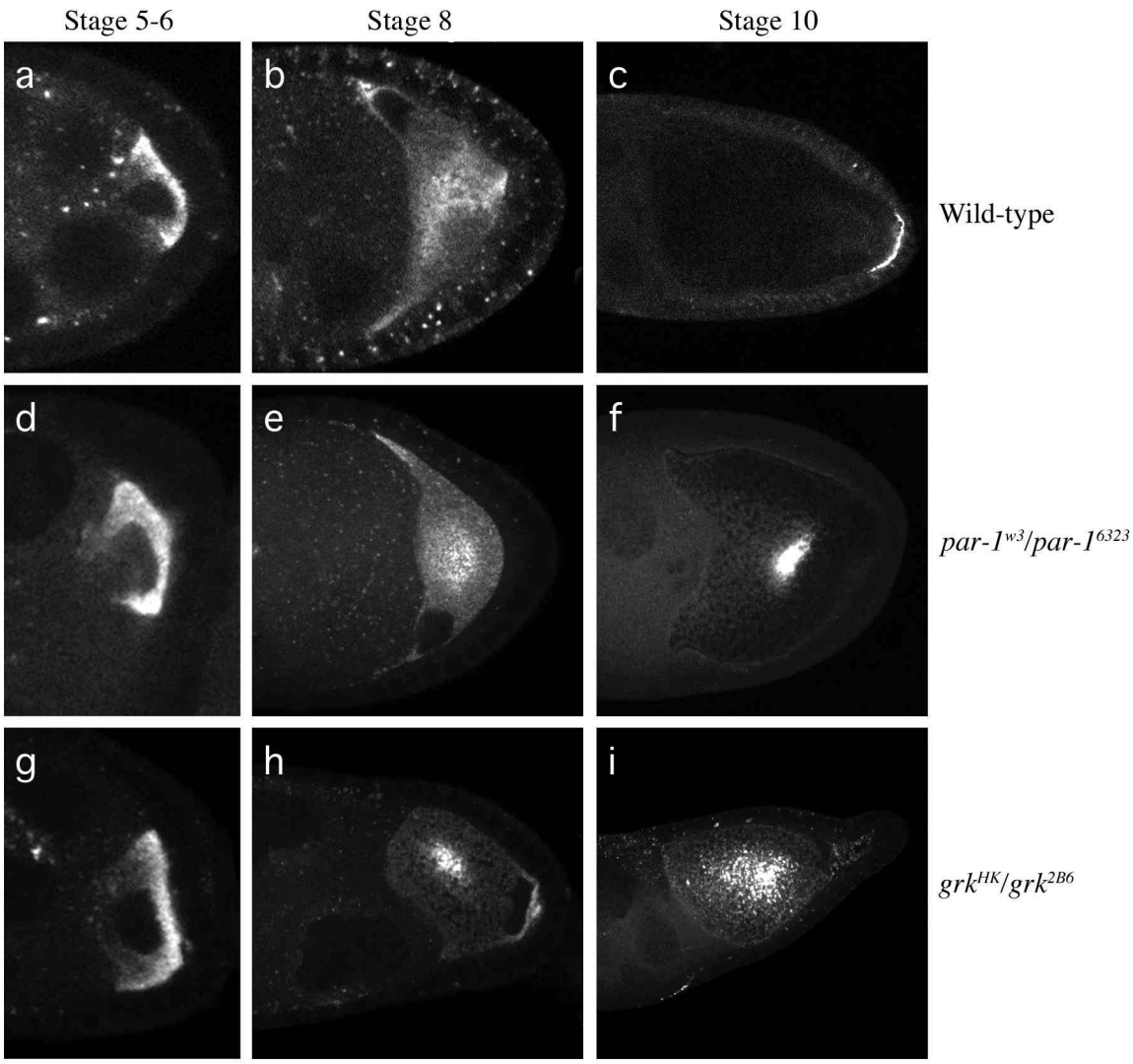

Stage 5-6
Stage 8
Stage 10
a
b
c
Wild-type
d
e
f
par-1w3/par-16323
g
h
i
grkHK/grk2B6

Figure 9. *osk* RNA localization in *armi, mael* and *spn-E* mutant oocytes.

Cy5-conjugated *osk* antisense probes were used to detect *osk* transcript *in situ* in oocytes isolated from wild-type, $armi^{72.1}/armi^{72.1}$, $mael^{r20}/Df$ and $spn\text{-}E^{1}/spn\text{-}E^{1}$ mutant flies. (a-c) *osk* RNA localization in wild-type oocytes. (a) *osk* RNA is localized to the posterior cortical region in early wild-type oocytes. (b) During stage 8, *osk* transcript is transiently localized to the central region and posterior pole of wild-type oocytes. (c) In stage 10 wild-type oocytes, *osk* RNA is strictly localized to the posterior pole. (d, g, and j) In contrast to the tight posterior cortical localization of *osk* RNA observed in wild-type, *osk* RNA is distributed throughout the cytoplasm in stage 5-6 *armi, mael* and *spn-E* mutant oocytes. (e, h, and k) *osk* RNA appears to accumulate in the central cytoplasm in mutant oocytes during stage 8, similar to wild-type. (f and i) During stage 9-10 in *armi* and *mael* mutant oocytes, *osk* transcript accumulates predominantly in the central cytoplasm. Occasionally, *osk* RNA is observed at the posterior pole in *mael* mutants. (l) Later stage egg chambers are rarely observed in *spn-E* mutants; however, when they are seen, *osk* RNA diffusely accumulates at the posterior pole.

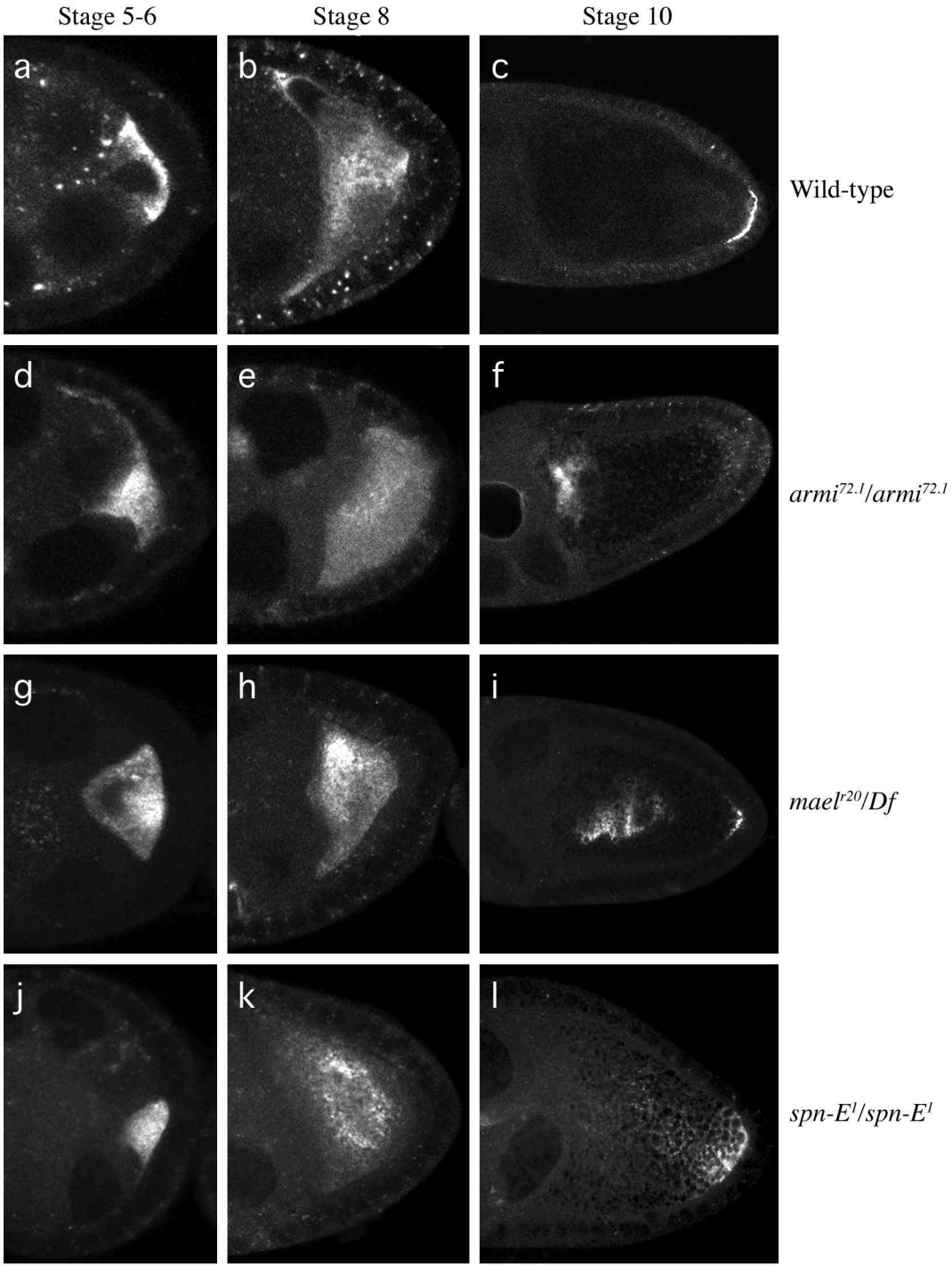

Stage 5-6
Stage 8
Stage 10
a
b
c
Wild-type
d
e
f
armi72.1/armi72.1
g
h
i
mael r20/Df
j
k
l
spn-E1/spn-E1

Figure 10. Premature Osk protein expression in *armi*, *mael* and *spn-E* mutant oocytes.
Egg chambers from wild-type, $armi^{72.1}/armi^{72.1}$, $mael^{r20}/Df$ and $spn\text{-}E^{1}/spn\text{-}E^{1}$ mutant flies were isolated and processed for FISH with *osk* antisense RNA probes followed by Anti-Osk immunolabeling. (a) During stage 5-6, *osk* RNA is detected at the posterior cortical surface in wild-type oocytes. (b) No Osk protein is detected in wild-type during the early stages of oogenesis. (inset a) At stage 9, once *osk* RNA localizes exclusively to the oocyte posterior, Osk protein is observed (inset b). (d, g, and j) *osk* transcript is distributed throughout the oocyte in stage 5-6 *armi*, *mael* and *spn-E* mutant oocytes. (e, h, and k) Osk protein is detected in the early oocytes from *armi*, *mael* and *spn-E* mutants. (c, f, i and l) Composites of the *osk* RNA and Osk protein images. *osk* RNA is represented in green, Osk protein in red and colocalization of *osk* RNA and Osk protein represented in yellow. (f, i and l) Moderate colocalization of *osk* transcript and Osk protein is observed in *armi*, *mael* and *spn-E* oocytes.

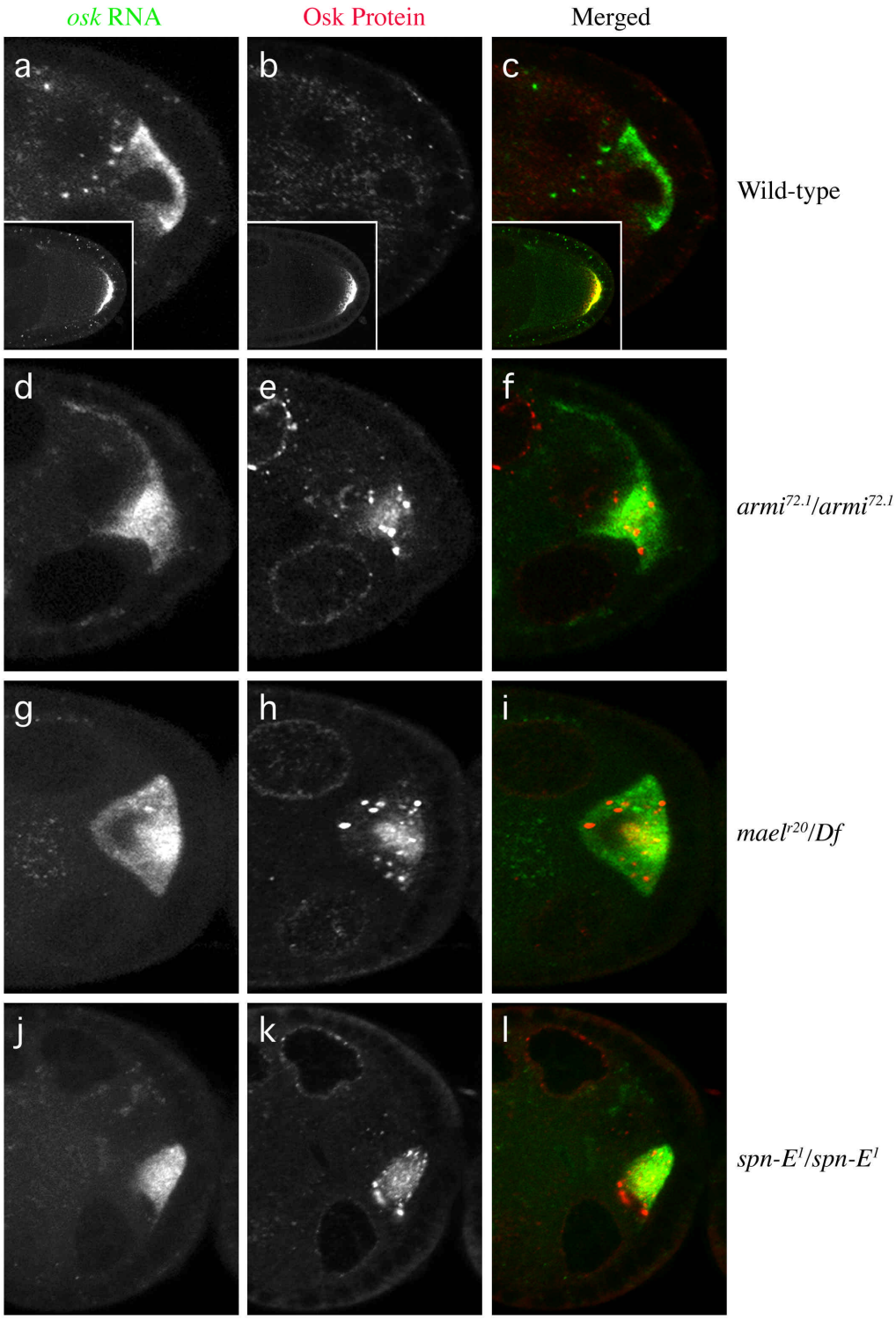

osk RNA
Osk Protein
Merged
a
b
c
Wild-type
d
e
f
armi$^{72.1}$/*armi*$^{72.1}$
g
h
i
mael^{r20}/*Df*
j
k
l
spn-E^{1}/*spn-E*^{1}

Figure 11. Ectopic expression of Osk protein in *armi*, *par-1* and *grk* mutant oocytes.
osk RNA and Osk protein are simultaneously detected by dual FISH and immunofluorescence. Wild-type stage 8 and 10 egg chambers are shown in panels a, b, and c. (inset a) *osk* RNA is centrally localized in stage 8 wild-type oocytes. (inset b) Stage 8 oocytes have no detectable Osk protein. (a) By contrast, in stage 10 wild-type oocytes, *osk* RNA is strictly localized to the posterior pole where it is translated to Osk protein (b). (c) Overlaying the *osk* RNA image and the Osk protein image shows essentially complete colocalization of *osk* transcript with Osk protein. (d, g and j) *osk* RNA is ectopically localized in the central cytoplasmic region in stage 10 $par\text{-}1^{w3}/par\text{-}1^{6323}$, grk^{HK}/grk^{2B6} and $armi^{72.1}/armi^{72.1}$ mutant oocytes. (e, h, and k) Ectopic expression of Osk protein is observed in *par-1*, *grk armi* mutant oocytes. (c, f, i and l) Composites of the *osk* RNA and Osk protein images. *osk* RNA is represented in green, Osk protein in red and colocalization of *osk* RNA and Osk protein represented in yellow. (f, i, and l) Colocalization of *osk* RNA and Osk protein is observed at the ectopic site in *par-1*, *grk* and *armi* oocytes.

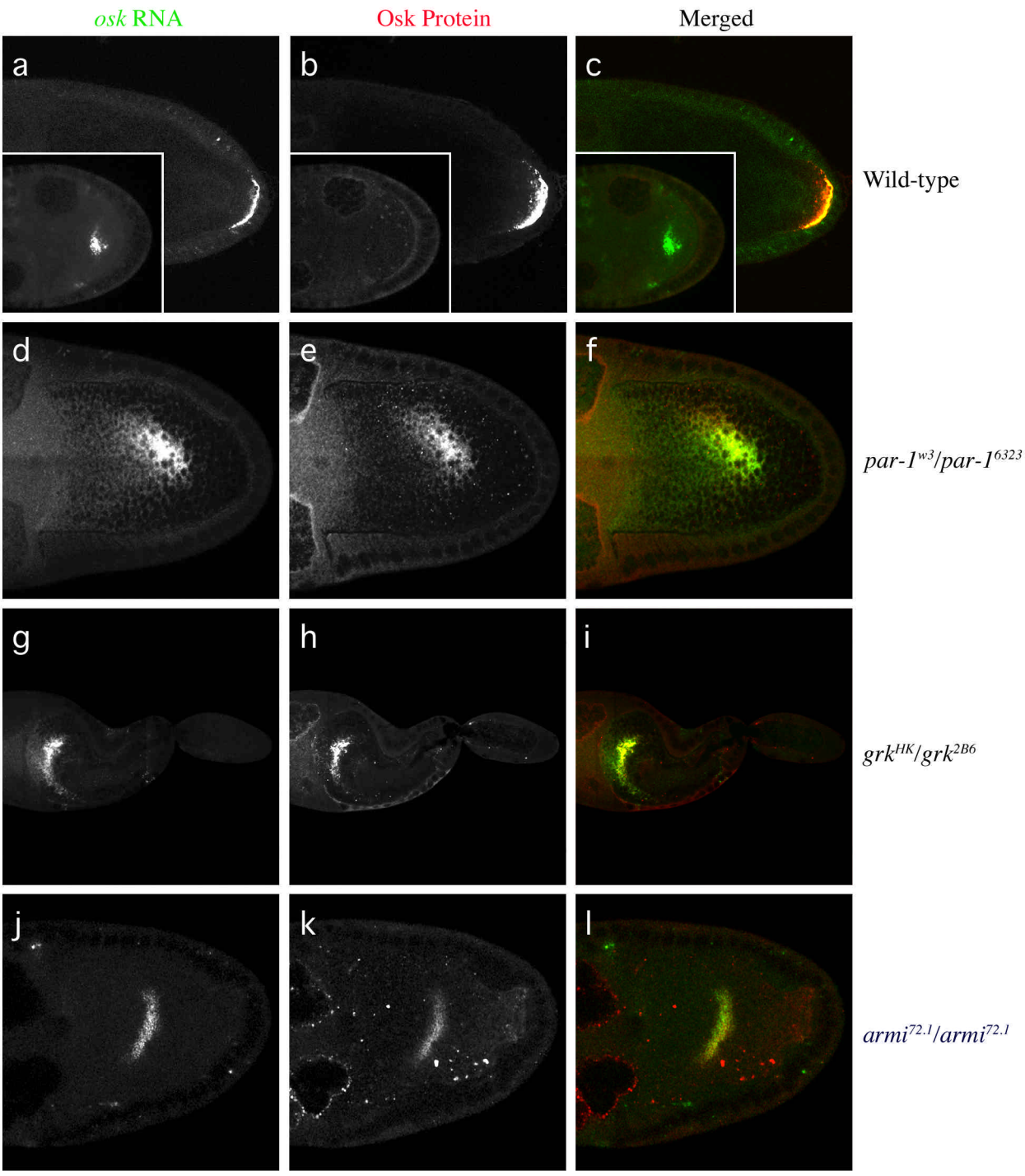

osk RNA
Osk Protein
Merged
a
b
c
Wild-type
d
e
f
par-1^{w3}/*par-1*^{6323}
g
h
i
grk^{HK}/*grk*^{2B6}
j
k
l
armi$^{72.1}$/*armi*$^{72.1}$

Figure 12. Cytoskeletal inhibitor treatment of wild-type egg chambers

Egg chambers were isolated from wild-type flies that had been fed the microtubule depolymerizing drug colcemid or egg chambers were isolated and subsequently treated with cytochalasin D, which disrupts actin polymerization. Following fixation, samples were processed for *osk* antisense FISH and Anti-Osk immunofluorescence. (a-c) Untreated stage 10 wild-type oocytes. (c) *osk* RNA and Osk protein are exclusively colocalized to the posterior pole of the oocyte. (d-f) Colcemid-treated egg chambers exhibit uniform distribution and colocalization of *osk* RNA and Osk protein along the entire cortex proximal to the overlying follicle cell layer. (g-i) Following cytochalasin-D treatment, *osk* RNA and Osk protein are found to be dissociated from the cortex (arrowhead in i, inset), yet remain colocalized. (c, f and i) Composites of the *osk* RNA and Osk protein images. *osk* RNA is represented in green, Osk protein in red and colocalization of *osk* RNA and Osk protein represented in yellow.

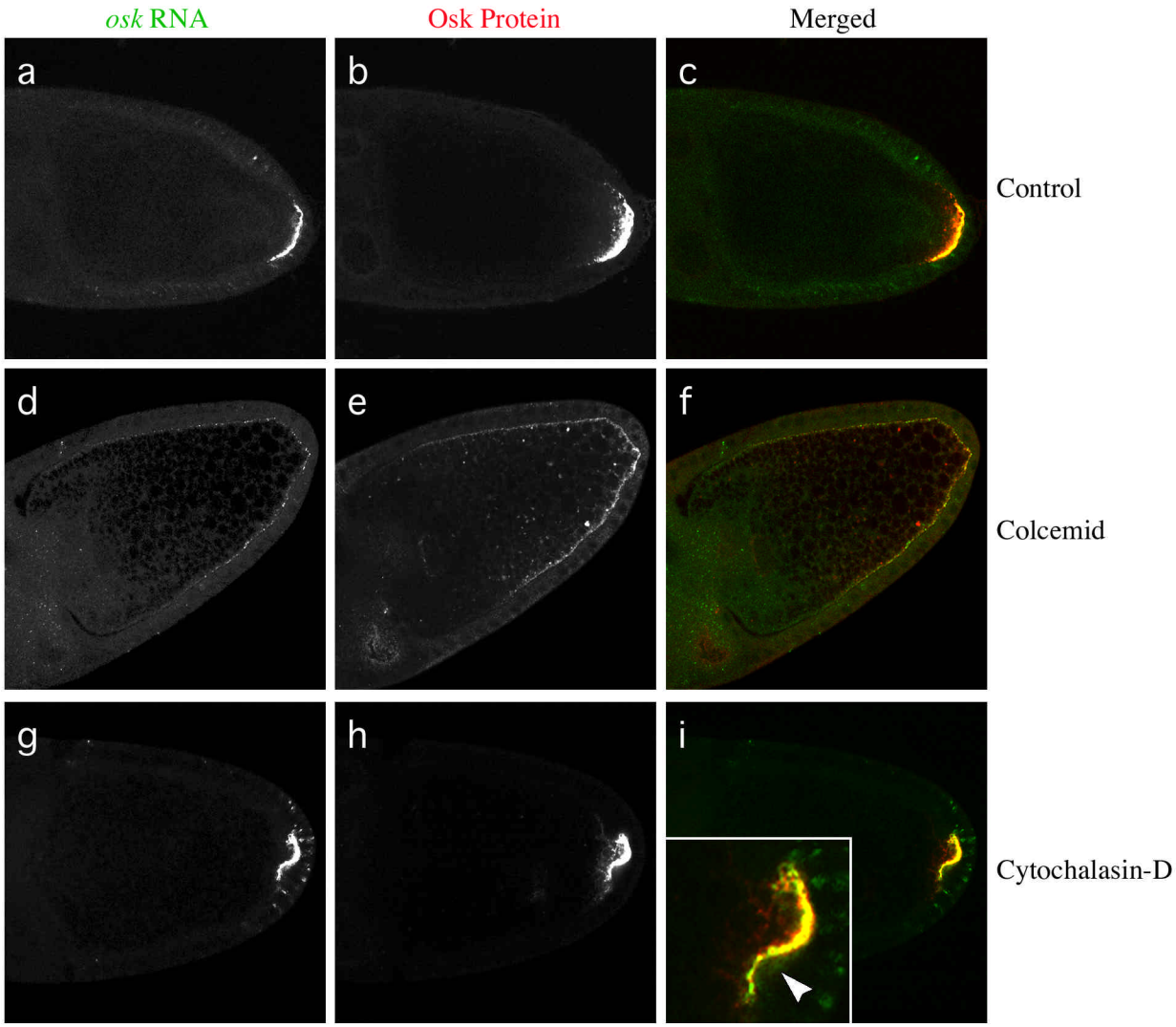

osk RNA
Osk Protein
Merged
a
b
c
Control
d
e
f
Colcemid
g
h
i
Cytochalasin-D

Figure 13. Localization of *osk, bcd, cyclin B* RNA in control and colcemid treated wild-type oocytes

(a) In stage 10 wild-type oocytes, *osk* RNA is exclusively localized to the posterior pole of the oocyte. (c) *bcd* RNA is localized to the anterior cortex and (e) *cyclin B* RNA localizes to the anterior lateral cortex (arrowheads in e) and accumulates at the posterior pole as well (arrow in e). After microtubule depolymerization, (b) *osk* RNA redistributes over a broad region of the oocyte cortex (arrowheads), whereas (d) *bcd* RNA is released into the oocyte cytoplasm and is no longer localized. (f) *cyclin B* is released from the posterior cortex (arrow) on microtubule depolymerization, but remains diffuse at the anterior margins of the oocyte (arrowheads in f).

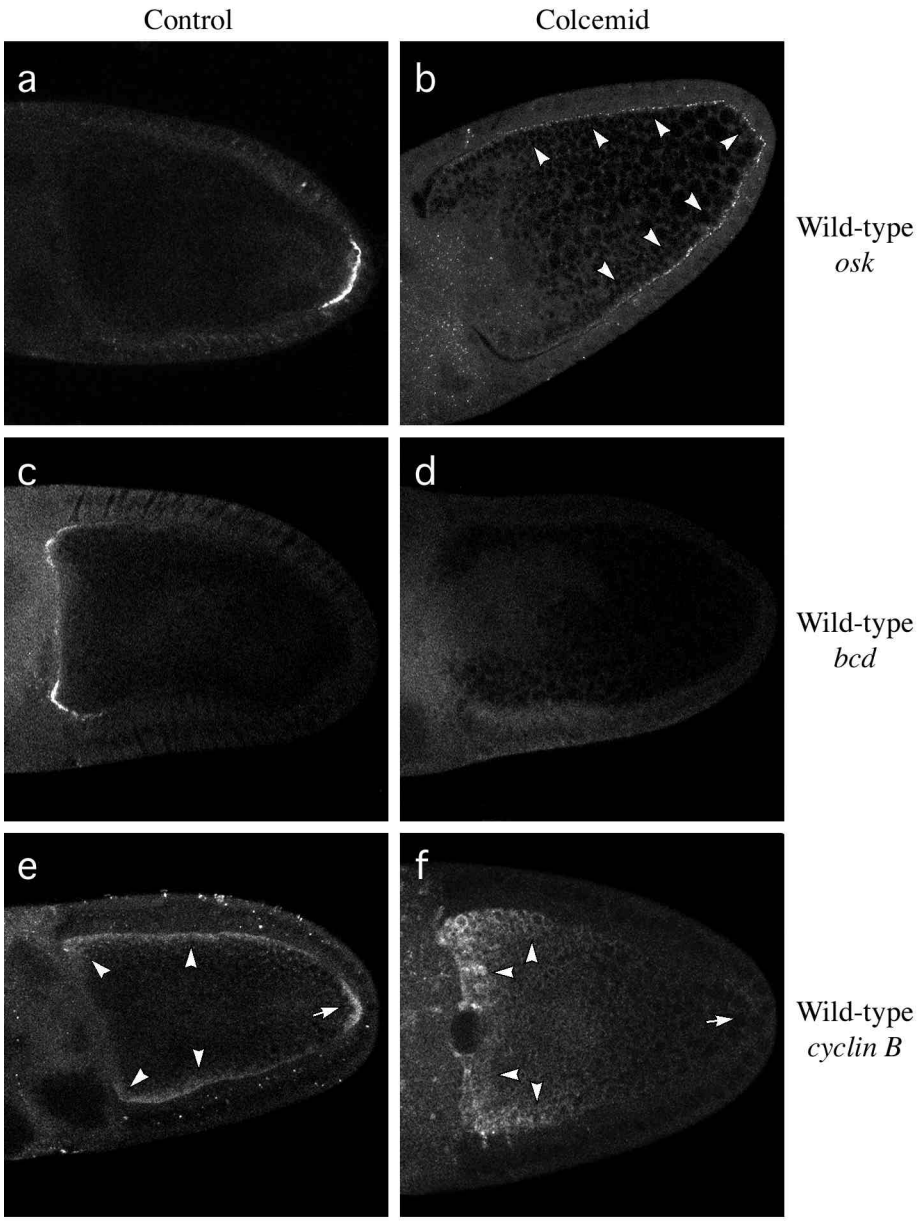
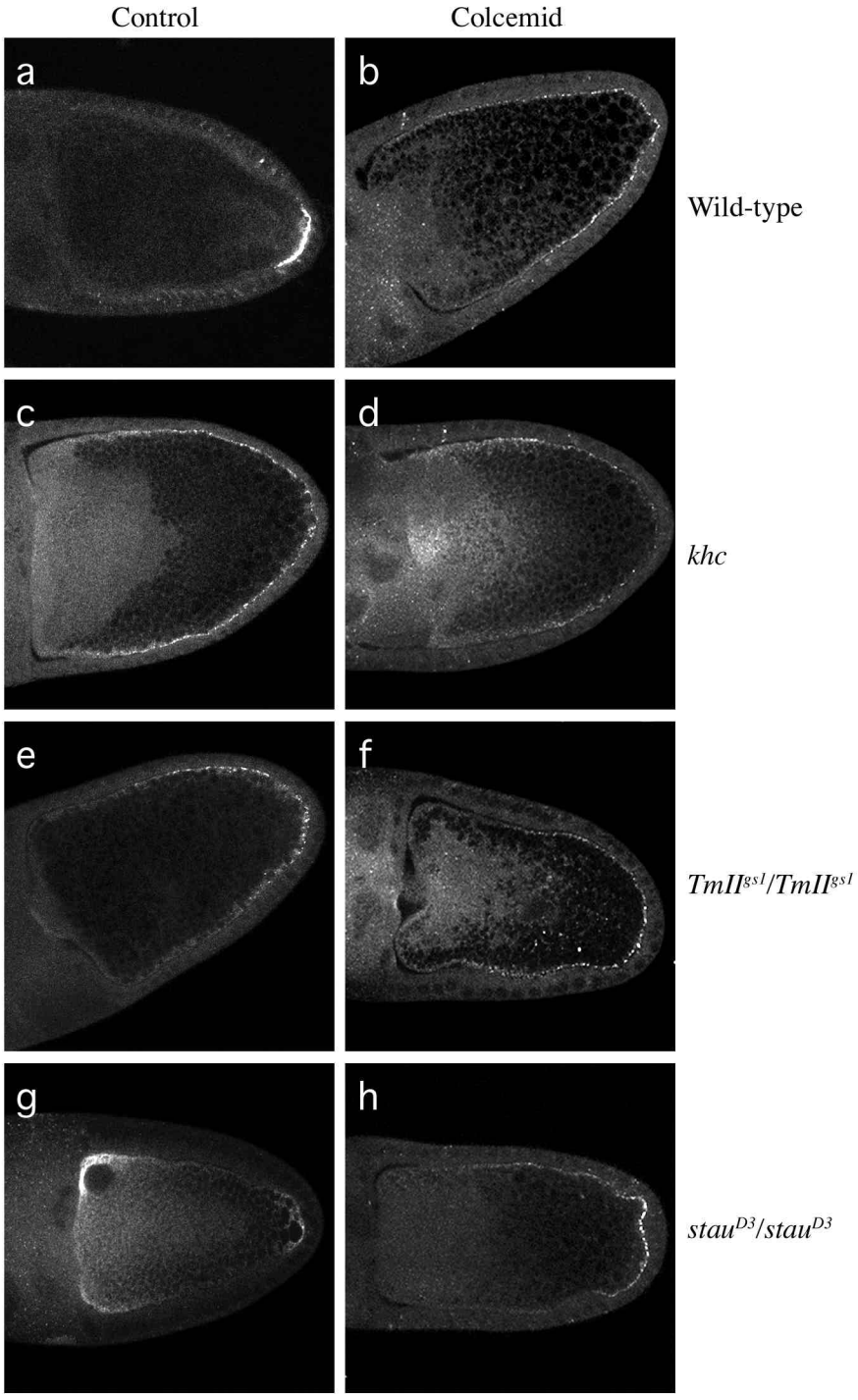
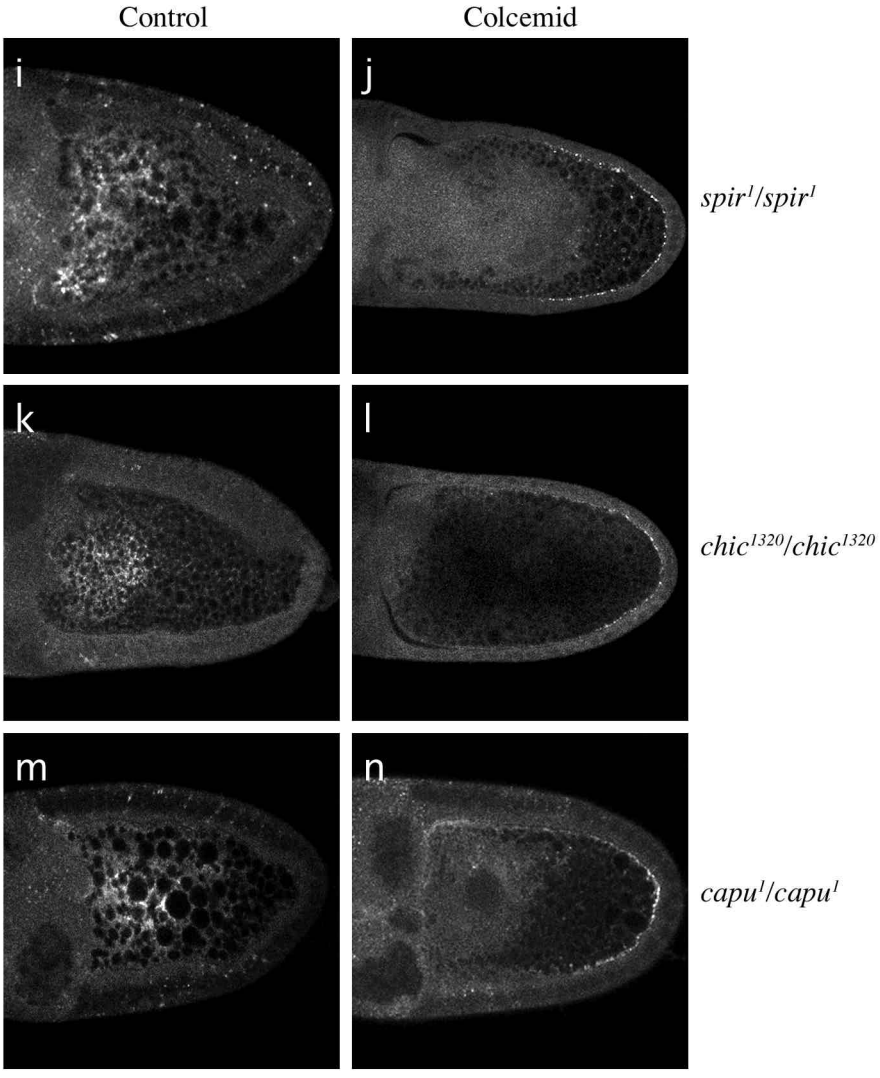
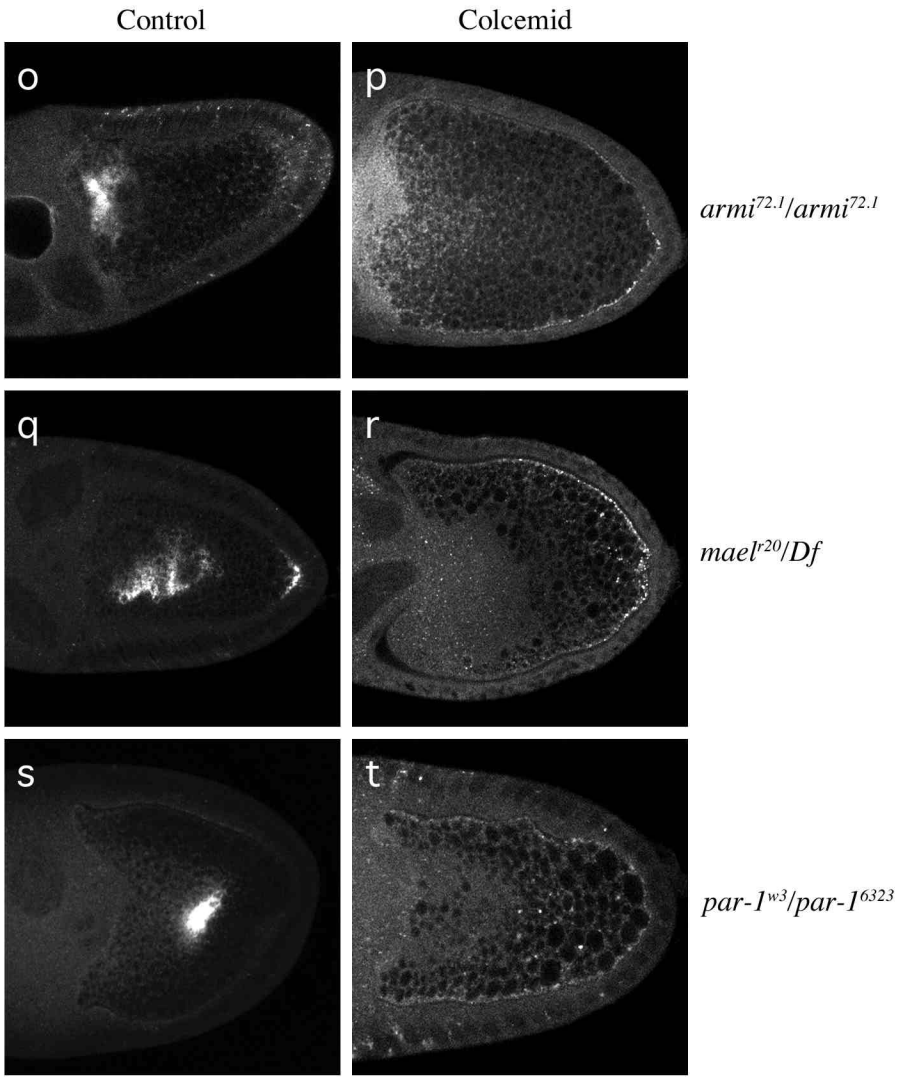

Control
Colcemid
a
b
c
d
e
f
Wild-type
osk
Wild-type
bcd
Wild-type
cyclin B

Figure 14. Microtubule deployerization

Egg chambers were isolated from wild-type and mutant flies that had been fed colcemid, which depolymerizes microtubules. Samples were examined by *osk* FISH. (a, c, e, g, i, k, m, o, q and s) Untreated stage 10 oocytes. (b, d, f, h, j, l, n, p, r and t) Colcemid treated oocytes. All mutants screened showed uniform cortical localization of *osk* RNA in the absence of microtubules.


Control
Colcemid
a
b
Wild-type
c
d
khc
e
f
TmII^gs1/TmII^gs1
g
h
stau^D3/stau^D3


Control
Colcemid
i
j
k
l
m
n
spir1/spir1
chic1320/chic1320
capu1/capu1


Control
Colcemid
o
p
q
r
s
t
armi72.1/armi72.1
mael r20/Df
par-1w3/par-16323

DISCUSSION

In *Drosophila*, the embryonic axes are specified during oogenesis in a process that requires the precise asymmetric localization of RNAs and proteins (Riechmann and Ephrussi, 2001; van Eeden and St Johnston, 1999). Proper localization of *osk* RNA and local translation at the posterior pole of the oocyte are essential first steps in the assembly of the pole plasm, which is required for germ cell formation and posterior polarity (Ephrussi et al., 1991; Ephrussi and Lehmann, 1992; Kim-Ha et al., 1991; Lehmann and Ephrussi, 1994). I have used a modified whole-mount *in situ* hybridization protocol employing fluorescently labeled *osk* RNA antisense probes in conjunction with anti-Osk immunofluorescence and subsequent analysis by confocal microscopy to examine the temporal and spatial distribution of transcript and protein during *Drosophila* oogenesis.

Multi-step *osk* mRNA localization pathway

My studies help define distinct steps in the *osk* mRNA localization process (Figure 15). Initially, *osk* mRNA, transcribed in the nurse cells, becomes enriched in the future oocyte (Ephrussi et al., 1991; Kim-Ha et al., 1991). This initial accumulation of *osk* mRNA in the oocyte is dependent upon *Bic-D* and *egl* (Mohler and Wieschaus, 1985; Mohler and Wieschaus, 1986). During stages 2 through 6, *osk* mRNA localizes to the posterior cortex of the oocyte. Optical sectioning of the oocyte reveals distinct localization to the cortical region neighboring the overlying somatic follicle cells. During these early stages, microtubules appear to nucleate from a MTOC at the oocyte posterior (Theurkauf et al., 1992). The observed posterior accumulation of *osk* transcript is likely to be the result of minus-end directed movement along microtubules from the nurse cells to the oocyte (McGrail and Hays, 1997). During stages 7 and 8, as a result of a *grk*-dependent signaling event, the posterior MTOC disassembles and microtubules reorganize at the anterior and lateral cortices and establish an anterior-to-posterior gradient of microtubules (Gonzalez-Reyes et al., 1995; Theurkauf et al., 1992). A prominent central cytoplasmic

accumulation of *osk* RNA is observed during stage 8. The accumulation of *osk* RNA to the center of the oocyte is dependent on Kinesin I, a plus-end-directed motor, and the microtubule cytoskeleton, which function together to remove *osk* mRNA from the microtubule-rich cortex, thus preventing ectopic pole plasm assembly (Cha et al., 2002). Lastly, during stage 9 of oogenesis, *osk* mRNA translocates from the interior to the posterior pole of the oocyte where microtubule density is low. While lack of microtubules at the posterior cortex may not wholly account for posterior localization of osk mRNA, studies have also suggested the involvement of the actin cytoskeleton in a short-range microtubule-independent process (Erdelyi et al., 1995; Glotzer et al., 1997). Microtubule-independent translocation of *osk* mRNA from the cytoplasm to the posterior pole with subsequent capturing/anchoring by actin is supported by the observations that *osk* mRNA associates with the cortex in the absence of microtubules, and actin depolymerization disrupts cortical localization of *osk* mRNA (Cha et al., 2002). Localization of *osk* mRNA to the posterior pole results in localized translation and thus establishes the proper positioning for pole plasm assembly and axis specification.

Genetic dissection of the steps involved *osk* mRNA localization

Mutants that perturb *osk* mRNA localization were examined in an attempt to dissect the steps involved in localizing *osk* RNA to the posterior pole of the oocyte (Table 1). The initial step of removing *osk* transcript from the cortex requires Kinesin I and an intact microtubule network (Cha et al., 2002). Likewise, Tropomyosin and Staufen appear to be required for efficient movement of *osk* mRNA from the cortex to the center of the oocyte. Stau, an RNA binding protein, has been directly implicated in the localization of *bcd* RNA to the anterior of the oocyte (Ferrandon et al., 1994), and may have a direct role in the localization of *osk* mRNA. In Stau null mutant oocytes, *osk* RNA remains at the cortex, despite an apparently intact microtubule network (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991). Moreover, Stau protein colocalizes with *osk* RNA in the *khc* mutant (Brendza et al., 2000). *osk* mRNA, Kinesin and Staufen, which together, could form a complex and participate in excluding *osk* mRNA from

the cortex in a microtubule-dependent manner. Inhibitor and cytological studies have implicated the actin cytoskeleton in anchoring *osk* mRNA at the oocyte cortex. Mutants in Tropomyosin block *osk* mRNA movement from the cortex to the interior. Tropomyosin, an actin binding protein, stabilizes filamentous actin (Broschat et al., 1989) and my results suggest that it may antagonize binding of *osk* mRNA to the cortex, allowing for the cortical removal of *osk* mRNA during midoogenesis.

The localization of *osk* mRNA is microtubule dependent and several mutants that disrupt this process do so by directly or indirectly altering the organization of the oocyte microtubule network. In wild-type oocytes, as a result of a *grk*-dependent signaling event, the microtubules, once focused at the posterior of the oocyte, disassemble and reorganize at the anterior and establish an anterior to posterior gradient during stage 7-8 of oogenesis (Theurkauf et al., 1992). This organization is maintained until the next microtubule reorganization at stage 10b when cytoplasmic streaming takes place concurrently with the bundling of microtubules along the cortex of the oocyte (Gutzeit, 1986; Theurkauf et al., 1992).

Previous live analysis in wild-type oocytes showed that from stages 8 through 10a, only non-coordinated cytoplasmic movement is observed towards the anterior of the oocyte, while more posterior regions demonstrate hardly any cytoplasmic movement (Theurkauf, 1994c). During stages 10b through stage 12, however, the entire oocyte cytoplasm undergoes rapid, coordinated, and unidirectional streaming (Theurkauf, 1994c). Concomitant with ooplasmic streaming, microtubules bundle along the subcortical region of the oocyte (Gutzeit, 1986; Theurkauf et al., 1992). In *capu*, *spir*, *chic*, and *mael* mutant oocytes, however, rapid unidirectional ooplasmic streaming and bundling of microtubules along the oocyte cortex occur prematurely, as early as stage 8 (Clegg et al., 1997; Emmons et al., 1995; Manseau and Schupbach, 1989; Theurkauf, 1994c). These mutants do not localize *osk* mRNA to the posterior pole and *osk* transcript remains distributed towards the anterior cortex of the oocyte. Premature

streaming, or the changes in microtubule organization that accompany streaming, thus appear to prevent translocation of *osk* mRNA from the interior cytoplasm to the posterior pole.

Mutations in *armi*, *spn-E*, *grk* and *par-1* also disrupt microtubule organization. During early oogenesis in *armi* mutants, the posterior MTOC fails to form, whereas later in oogenesis, a higher density of microtubules is observed at the posterior of the oocyte (H. Cook and W. Theurkauf, unpublished). In *spn-E*, *par-1* and *grk* mutant oocytes, microtubules are similarly distributed along the oocyte cortex (Cox et al., 2001; Gillespie and Berg, 1995; Gonzalez-Reyes et al., 1995; Huynh et al., 2001; Shulman et al., 2000). All of these mutations lead to central ooplasmic accumulation of *osk* mRNA, with little accumulation at the posterior pole of the oocyte.

Previous studies have shown that the establishment of the anterior-posterior axis and suppression of microtubules at the posterior pole is contingent upon *grk*-dependent signaling from the oocyte to the posterior follicle cells (Gonzalez-Reyes et al., 1995; Neuman-Silberberg and Schupbach, 1993). Grk signaling induces the posterior cells to adopt a posterior rather than anterior fate (Gonzalez-Reyes et al., 1995; Neuman-Silberberg and Schupbach, 1993; Ray and Schupbach, 1996; Roth et al., 1995). Upon induction, an unknown signal back to the oocyte induces major rearrangement of the oocyte microtubule cytoskeleton and initiates microtubule depolymerization at the posterior cortex (Gonzalez-Reyes et al., 1995; Roth et al., 1995). The exact nature of this signal and its target are not known. Par-1 is expressed in the somatic follicle cells and accumulates at the posterior pole of the oocyte during stages 8 and 9 (Cox et al., 2001; Shulman et al., 2000; Tomancak et al., 2000). *par-1* mutants maintain posterior cortical microtubules and obstruct *osk* mRNA localization (Shulman et al., 2000; Tomancak et al., 2000). The mammalian Par-1 Kinase homologue phosphorylates microtubule-associated proteins, destabilizing the microtubules (Bohm et al., 1997; Drewes et al., 1997). *grk*-signaling may therefore regulate Par-1 association with the posterior pole, resulting in local depolymerization of microtubules, which allows *osk* mRNA movement to the cortex. Continued microtubule

nucleation at the posterior cortex in mutant oocytes thus produces Kinesin-dependent movement of *osk* mRNA away from the cortex, maintaining cytoplasmic accumulation of *osk* mRNA.

Actin involvement in *osk* mRNA capturing/anchoring

The actin cytoskeleton has been implicated in the microtubule-independent short-range capturing/anchoring of *osk* mRNA to the cortex (Erdelyi et al., 1995; Glotzer et al., 1997). A role for actin in cortical localization is supported by the observation that disruption of the actin cytoskeleton with cytochalasin-D prevents cortical localization of *osk* mRNA (Cha et al., 2002). To determine if any known posterior patterning mutants disrupt cortical anchoring, *osk* RNA localization was examined following microtubule deploymerization. Surprisingly, all of the mutants screened thus far (Table 1) showed normal cortical localization of *osk* RNA in the absence of microtubules. Actin alone may be responsible for cortical capturing and tethering *osk* mRNA, or an unidentified gene may act in association with the actin cytoskeleton to anchor *osk* mRNA at the cortex.

Translational regulation

In wild-type egg chambers, *osk* mRNA translation is repressed until stage 9, when *osk* mRNA is exclusively localized to the oocyte posterior. During stage 8, however, a significant fraction of oocytes show some *osk* mRNA localization to the posterior cortex, but no posterior accumulation of Osk protein. This observation indicates that cortical localization alone is not sufficient to initialize translation and suggests that *osk* mRNA translation is also under tight temporal control. One group of mutants, *armi*, *mael* and *spn-E* (H. Cook and W. Theurkauf, unpublished observations) disrupts temporal control of *osk* mRNA translation. In these mutants, Osk protein is produced throughout oogenesis and is independent of cortical localization. In *grk* and *par-1* mutants, by contrast, Osk protein is not produced until stage 9, but protein production occurs in the absence of cortical localization. These mutants could affect *osk* translation by restricting components of the translational machinery to the cortex.

My studies help define distinct steps in the *osk* mRNA localization process. An early step in posterior localization is removal of *osk* mRNA from most of the cortex, leading to accumulation in the oocyte interior. This process requires microtubules, the microtubule motor protein Kinesin I, the actin binding protein Tropomyosin, and the RNA binding protein Staufen. Transcript then moves from the oocyte interior to the posterior pole through a microtubule independent process. The genes *cappuccino*, *chickadee*, *spire*, *armitage*, *maelstrom*, *par-1* and *gurken* are all required for this next step in *osk* mRNA localization. The final capturing or tethering *osk* mRNA at the cortex requires an intact actin filament system, but additional components of this anchoring system remain to be identified. I also find that *osk* mRNA first begins to accumulate at the posterior pole during oogenesis stage 8, but protein is not detectable until stage 9. In addition, *grk* and *par-1* mutations that block *osk* mRNA localization to the posterior pole and lead to transcript accumulation in the interior do not prevent translation; again, Osk protein production is only observed during stage 9 and later. These observations indicate that posterior localization is neither sufficient nor necessary to trigger *osk* mRNA translation, which appears to be under tight temporal control.

Figure 15. A model for the multi-step process of *osk* mRNA localization

(a) During the early stages of oogenesis, a MTOC is established at the oocyte posterior. Microtubules (green) nucleated from this site extend through the ring canals into the neighboring nurse cells. *osk* mRNA (purple), synthesized in the nurse cells, is transported to the oocyte by microtubules and tightly localizes to the posterior cortex adjacent to the overlying follicle cell layer. (b) During stage 8, following microtubule reorganization, a gradient of microtubules is formed. The highest density of microtubules is observed towards the anterior and declining in density towards the posterior. *osk* transcript pools in the central cytoplasmic region of the oocyte as a function of cortical removal involving Kinesin I heavy chain, *Tropomyosin II* and *staufen*. (c) The microtubule-poor posterior pole region supports *osk* RNA binding to the cortical actin cytoskeleton. *osk* mRNA localization to the posterior pole is dependent on the functions of *cappuccino*, *spire*, *chickadee*, *maelstrom*, *armitage*, *par-1* and *spindle-E*.

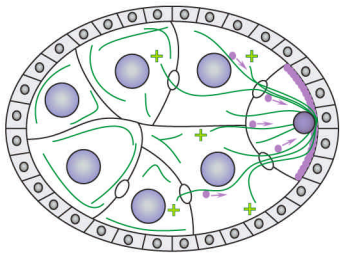
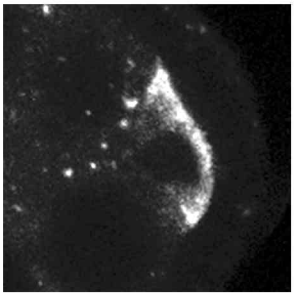
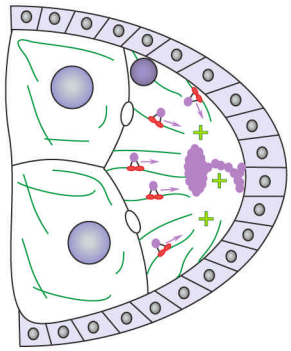
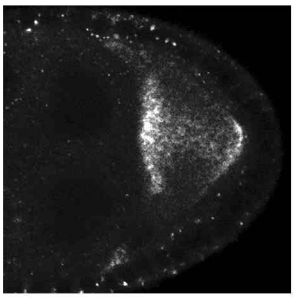
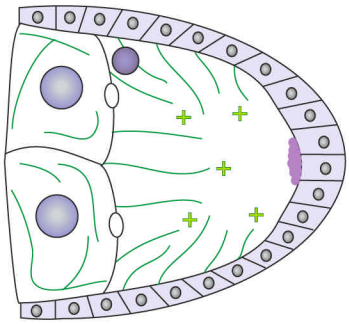
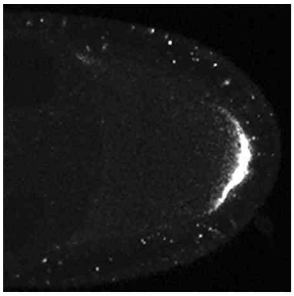
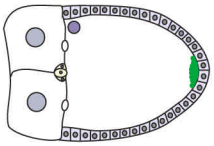
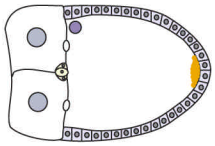
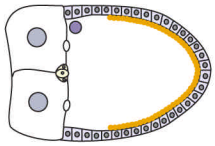
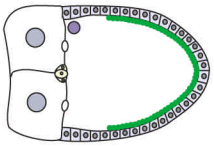
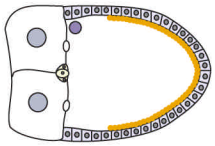
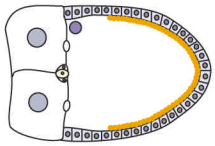
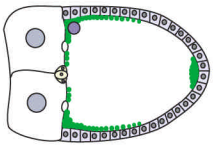
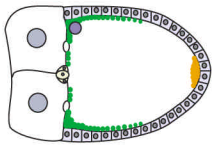
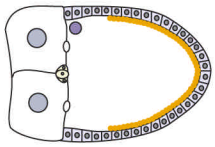
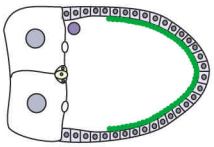
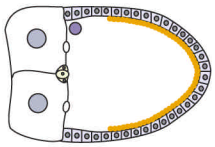
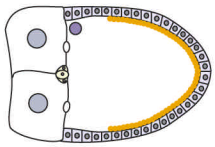
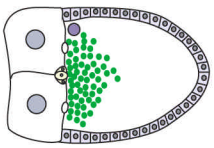
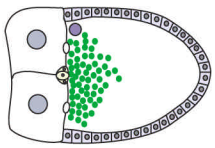
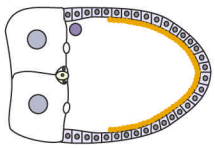
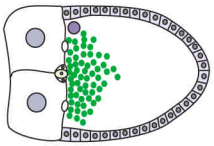
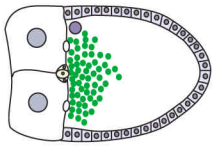
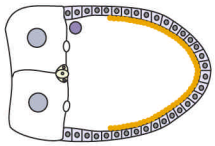
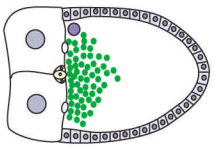
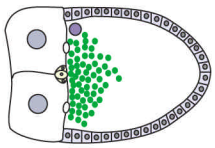
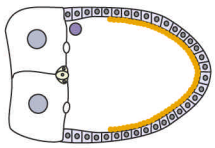
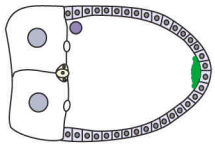
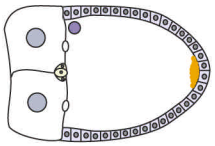
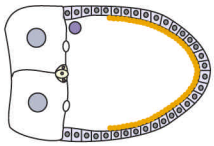
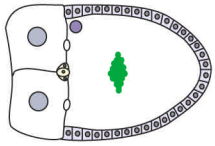
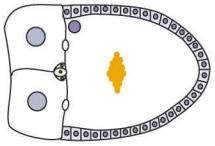
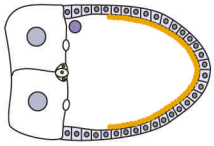
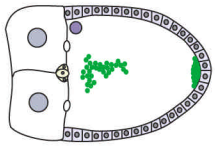
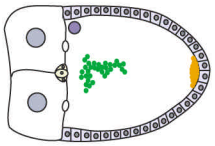
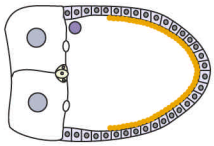
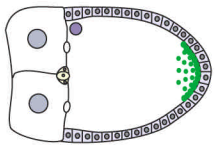
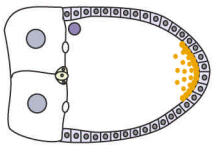
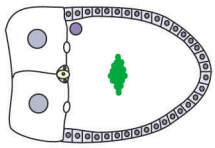
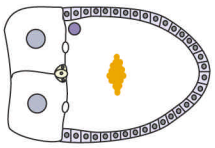
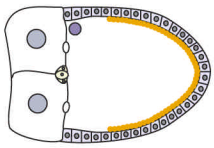
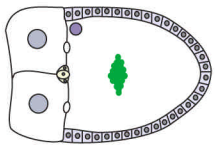
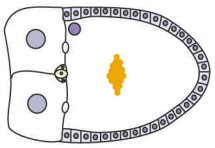
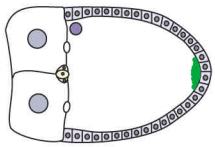
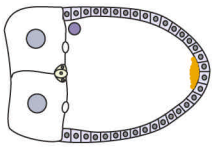
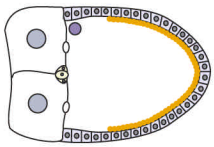
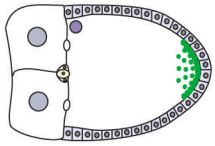
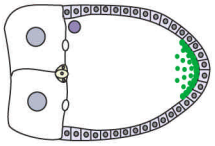
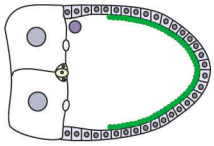
a Stage 2-6 		Transport to the oocyte and cortical binding: Microtubules Actin
b Stage 7-8 		Cortical removal of *osk* mRNA: *Kinesin I heavy chain* *Tropomyosin II* *staufen*
c Stage 9-10a 		Posterior localization of *osk* mRNA: *cappuccino* *spire* *chickadee* & Translational control: *maelstrom* *armitage* *spindle-E* *par-1* *gurken*

Table 1. Experimental Results Summary - *osk* FISH and Osk protein immunofluorescence analysis data.

The table summarizes the results from *osk* FISH and Osk protein immunofluorescence analysis in untreated egg chambers and egg chambers collected from flies fed the microtubule depolymerizing drug colcemid. Results are representative for stage 10 egg chambers. *osk* RNA is denoted in green and *osk* RNA and Osk protein colocalization in orange. Osk protein expression in wild-type oocytes is observed at stage 9 when transcript is completely localized to the posterior pole of the oocyte. Several mutants have been identified where premature translation is observed. In these mutants, Osk protein is seen as early as germarial stage 1. Several mutants have been identified with ectopic Osk expression. With the exception of the protein null osk^{54}/Df, upon microtubule depolymerization, wild-type and all the mutants analyzed demonstrate *osk* RNA localization and subsequent expression at the oocyte cortex.

	osk RNA ●	*osk* RNA + Osk Protein (● colocalization)	Osk Protein Expression	Localization Following Colcemid Treatment
Wild-type			~stage 9+	
TmII^{gs1}/*TmII*^{gs1}			~stage 9+	
stau^{D3}/*stau*^{D3}			~stage 9+	
khc glc			~stage 9+	
capu^{1}/*capu*^{1}			No Detectable Protein	
chic^{1320}/*chic*^{1320}			No Detectable Protein	
spir^{1}/*spir*^{1}			No Detectable Protein	

	osk RNA ●	*osk* RNA + Osk Protein (● colocalization)	Osk Protein Expression	Localization Following Colcemid Treatment
Wild-type			~stage 9+	
armi$^{72.1}$/*armi*$^{72.1}$			Premature Translation	
mael^{r20}/*Df*			Premature Translation	
spn-E^{1}/*spn-E*^{1}			Premature Translation	NA
par-1^{6323}/*par-1*^{w3}			~stage 9+	
grk^{HK}/*grk*^{2B6}			~stage 9+	NA
vas^{1}/*vas*^{rj36}			~stage 9+	
osk^{54}/*Df*			No Detectable Protein	

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