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***Curriculum* Human Genetics**

The translational repressor Cup is required for germ plasm assembly and germ cell development in *Drosophila*

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Abbreviations

Osk	**Oskar**
osk	***oskar***
L-Osk	**Long Oskar**
S-Osk	**Short Oskar**
Vas	**Vasa**
Tud	**Tudor**
Stau	**Staufen**
bcd	***bicoid***
nos	***nanos***
Nos	**Nanos**
Pum	**Pumilio**
EJC	**exon-exon junction complex**
eIF4E	**eukaryotic translation initiation factor 4E**
eIF4G	**eukaryotic translation initiation factor 4G**
Pgc	**primordial germ cells**
m^7GpppN	**7-methyl-guanosine**
UTR	**untranslated region**
mRNP	**messenger ribonucleoprotein**

BRE	**Bruno responsive elements**
grk	***gurken***
MTOC	**microtubule organising centre**

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Abstract

RNA localization is a cellular mechanism used to localize proteins to sub-cellular domains and to control protein synthesis regionally. In oocytes, RNA localization has profound implications for development, generating asymmetric protein distributions that promote morphological and functional cell polarization and establish regional fates in the future embryo. One such fate is that of the germ cell lineage.

In *Drosophila*, germ cell formation depends on maternal inherited factors localized in the posterior pole region of oocytes and early embryos, named germ plasm. Oskar (Osk), a key determinant of germ plasm assembly, is both necessary and sufficient for germ-line formation and posterior patterning The localization of *oskar* (*osk*) mRNA starts during oogenesis and it is mediated by trans-acting factors several of which have been shown to have roles in post-transcriptional regulation of RNA, such as splicing, translational control and degradation. However, most of the molecular mechanisms underlying *osk* mRNA localization are still unclear (Zhou Y. and King ML, 2004).

During the course of my study, I have demonstrated that Cup, a translational regulator of specific mRNAs, localizes to both nuage, a germ-line perinuclear organelle assembled during early oogenesis, and germ plasm. Moreover, Cup protein interacts with Osk and Vasa (Vas) to assure anchoring, stabilization and/or maintenance of germ plasm particles to the posterior pole of oocytes and early embryos. According to these results, homozygous *cup* mutant embryos display a reduced number of germ cells, respect to the heterozygous *cup* mutants. The latest embryos exhibit, in turn, less germ cells than wild type. Finally, *cup* and *osk* interact genetically, since reducing *cup* copy number further decreases the total number of germ cells observed in heterozygous *osk* mutant embryos. In this thesis, I shed light on a novel role of Cup during germ plasm assembly and germ cell development in *Drosophila*.

Introduction

1. mRNA localization

The process of mRNA localization and regulated translation has classically been considered to be a mechanism used by a handful of transcripts to spatially and temporally restrict gene expression to discrete sites within highly polarized, asymmetric cells. To date, the best-studied examples of mRNA localization all involve transcripts whose protein products play specialized roles within well-defined sub-cellular compartments (**Figure 1**).

In the fruit fly *Drosophila*, the localization of mRNAs, such as *bicoid* (*bcd*), *osk* and *nanos*, to anterior and posterior poles of the oocyte helps to establish morphogen gradients that underlie the proper spatial patterning of the developing embryo (**Figure 1A**) (Johnstone and Lasko, 2001). Similar processes occur in oocytes of the frog Xenopus, where the mRNA of a T-box transcription factor VegT asymmetrically localizes to the vegetal pole and induces the endodermal and mesodermal cell fates in the future embryo (**Figure 1B**) (Kinget al.,2005; Zhang et al., 1998). In fibroblasts, *Pkp4* mRNA localizes to the lamellipodia, where its translation is required for cytoskeletal-mediated motility (**Figure 1C**). During brain development, local translation of mRNAs in axonal growth cones allows neurons to respond to local environmental cues as the distal axonal processes navigate toward their synaptic partners (Lin and Holt, 2007). In particular, in the axonal growth cone of the frog Xenopus, the mRNA encoding β-actin protein is transported and *in situ* translated (**Figure 1D**).

Recently, numerous mRNAs have been found to be asymmetrically localized in cells: for example, a high-throughput *in situ* hybridization analysis of more than 3000 transcripts in *Drosophila* embryos showed that more than 70% of the transcripts are distributed in a spatially distinct manner and most of them are localized into sub-cellular

compartments (**Figure 2**) (Lecuyer et al., 2007). Moreover, the identification of mRNAs from purified dendritic and/or synaptic compartments of neurons (Miyashiro et al., 1994) have generated lists of localized mRNAs numbering in the hundreds .This suggests that mRNA localization is a more common biological phenomenon than previously thought.

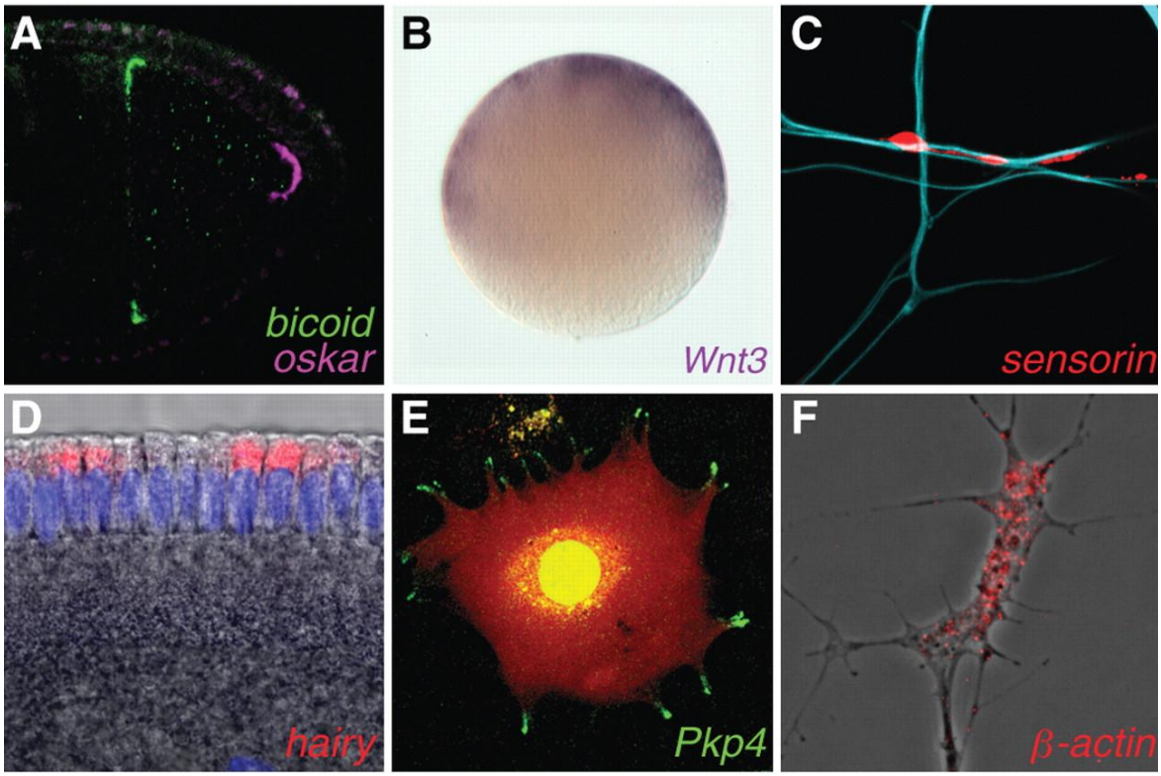

Figure1. Examples of asymmetrically localized mRNAs. (A) Differential localization of mRNA determinants within the *Drosophila* oocyte. (B) Animal localization of a transcript encoding a signaling molecule required for axis development in the egg of Xenopus. (C) mRNA enrichment in synapses of an Aplysia sensory neuron in response to contact with a target motor neuron (blue). (D) Apical localization of an mRNA in the *Drosophila* embryo, which facilitates entry of its transcription factor product into the nuclei (purple). (E) mRNA localization in pseudopodial protrusions of a cultured mammalian fibroblast (red signal indicates the cell volume). (F) mRNA enrichment within a Xenopus axonal

growth cone. mRNAs were visualized by means of in situ hybridization except in (E), in which the MS2–green fluorescent protein (GFP) system was used (Modified from Holt et al., 2009).

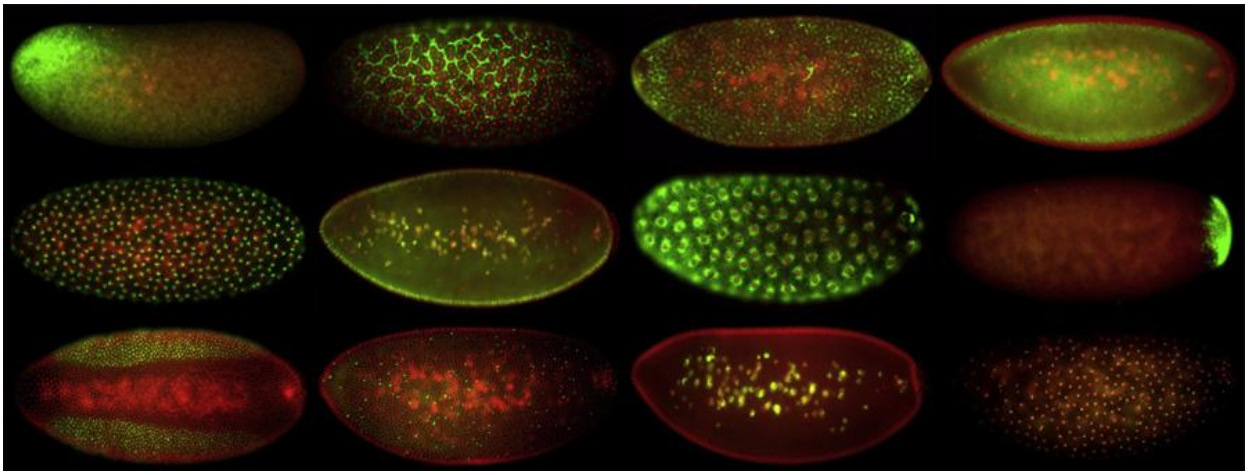

Figure 2. Many mRNAs Show Specific Patterns of Subcellular Localization. High-resolution fluorescent in situ analysis of 25% of mRNAs encoded by the *Drosophila* genome revealed that 71% of these display striking patterns of subcellular localization in early embryos. Some of these patterns are illustrated in this montage of photomicrographs, in which nuclei are in red and mRNAs in green. The anterior pole of the embryo is to the left, and the posterior pole to the right (Adapted from Martin and Ephrussi, 2009)

1.1. General mechanisms of mRNA localization

Eukaryotes have evolved different strategies to localize mRNAs into different sub-cellular compartments. Studies in diverse systems such as oocytes, embryos, and somatic cells

have demonstrated the existence of several potential mechanisms by which RNAs can be localized. These include the active directional transport of RNA on cytoskeletal elements, general degradation and localized RNA stability, random cytoplasmic diffusion and trapping, and vectorial transport from the nucleus to a specific target. A combination of mechanisms may be used to localize different RNAs; however, the most convincing evidence exists for the mechanisms of active directional transport on cytoskeletal elements and degradation combined with localized stability.

The targeting of mRNAs to specific sub-cellular sites by active and directed transport of the transcript to a subcellular site involves multiple steps.

First, the cellular ‘‘address’’ of transcripts is encoded by cis-acting elements in the RNA. As detailed below, these cis-acting elements, called ‘‘localization elements’’ or ‘‘zipcodes,’’ are most frequently found in the 3’ untranslated region (UTR), although in some cases they are present in the 5’UTR or in the coding sequence. Localization elements are recognized by specific RNA-binding proteins that often function both in transcript localization and translational regulation.

Several studies indicate that the processing of pre-mRNAs in the nucleus is required for the recruitment of RNA-binding proteins and factors, which are then involved in mRNA cytoplasmic localization (Giorgi and Moore, 2007). A striking example of the role of nuclear processing in transcript localization within the cytoplasm involves the exon-exon junction complex (EJC), a set of proteins that bind to mRNAs during splicing. In *Drosophila*, the core proteins of the EJC, eIF4AIII, Barentsz, Mago Nashi, and Tsunagi, (fly orthologs of MLN51, Magoh, and Y14), are deposited upstream of exon-exon junctions on mRNAs concomitant with splicing and are thought to remain bound to the mRNA in the cytosol until they are removed during the first round of translation. Several of these proteins were first identified as genetic mutants affecting *osk* localization in

Drosophila oocytes, and subsequently all four members of the EJC core were shown to be essential for this process. Consistent with the requirement for splicing in EJC deposition, the correct localization of *osk* mRNA to the oocyte posterior pole was shown to depend on the presence of the first intron (Hachet and Ephrussi, 2004), in addition to the 3'UTR.

Second, the mRNP is exported from the nucleus and matures in the cytosol, where additional proteins are recruited to modify its composition.

Third, the complex of RNAs and RNA-binding proteins, called ribonucleoproteins (RNPs), in many cases forms part of a larger structure called an RNA transport granule, which is transported by motor proteins along cytoskeletal elements to its final destination in the cell, where additional mechanisms anchor the RNA in place to prevent its diffusion.

Finally, there are mechanisms to maintain the RNA in a translationally repressed state during delivery and to regulate its translation at the right place and right time after delivery (Martin and Ephrussi, 2009).

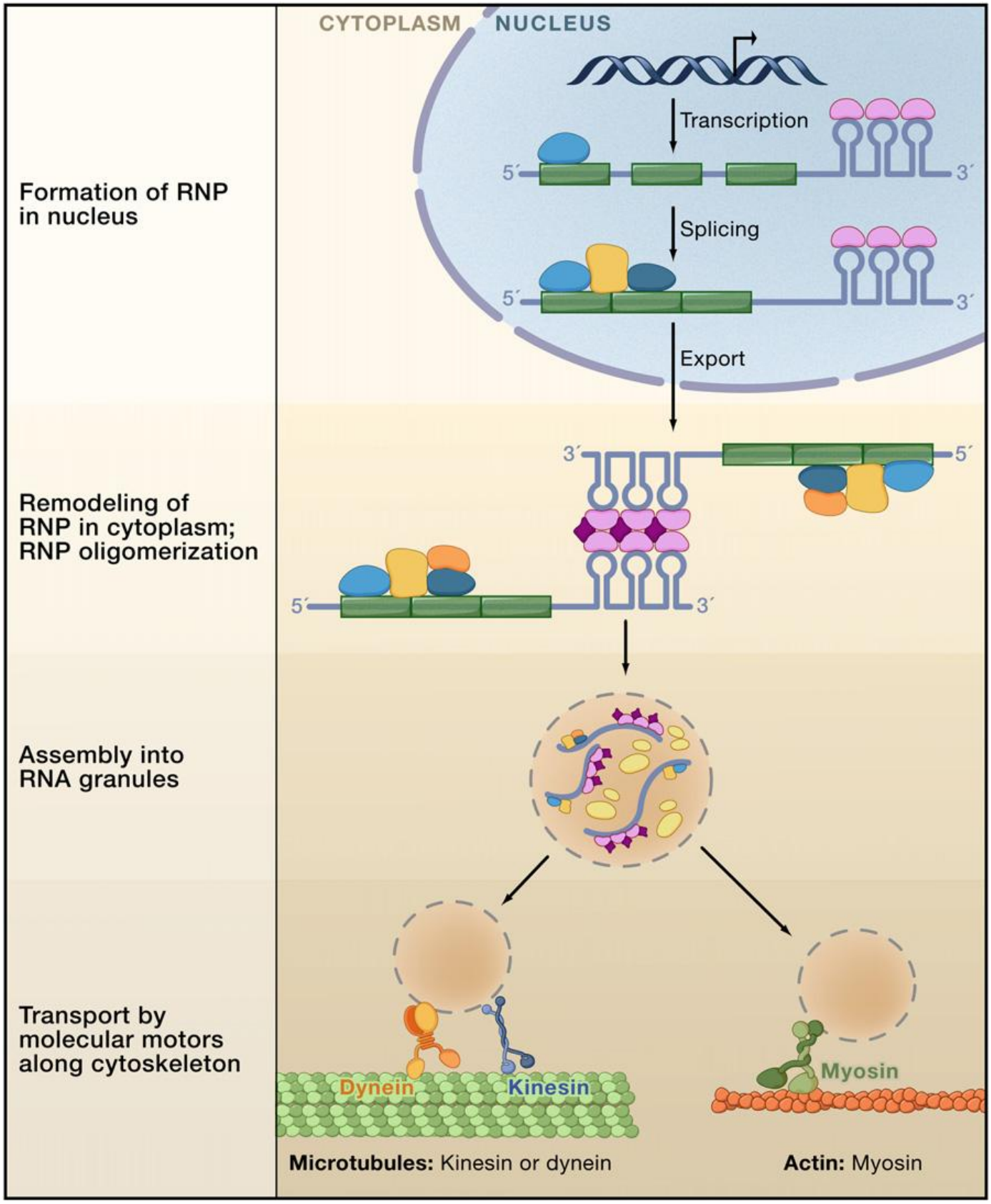
CYTOPLASM
NUCLEUS
Transcription
5′
3′
Splicing
5′
3′
Export
Formation of RNP in nucleus
3′
5′
5′
3′
Remodeling of RNP in cytoplasm; RNP oligomerization
Assembly into RNA granules
Dynein
Kinesin
Myosin
Transport by molecular motors along cytoskeleton
Microtubules: Kinesin or dynein
Actin: Myosin

Figure 3. mRNA Localization Is a Multistep Process. The pre-mRNA (exons in green; introns, 5' and 3'UTRs in grey) has cis-acting localization elements in its primary sequence. These are usually in the 3'UTR and often form stem-loop structures. RNA-binding proteins (blue and purple) bind the pre-mRNA. During splicing, additional RNA-binding proteins (golden and dark blue) are added to form a ribonucleoprotein (RNP) complex. Following export into the cytoplasm, the RNP is remodeled as additional proteins (orange, dark purple) are added. In some cases, the RNP can form oligomers with other RNPs through protein-protein interactions. In the cytoplasm, RNPs are assembled into RNA granules that are likely a heterogeneous population of structures containing diverse RNAs, ribosomal subunits (yellow), as well as many factors involved in translational regulation. Recent studies suggest a dynamic relationship between RNA transport granules, P-bodies, and stress granules. The RNA granules associate with motor proteins and are transported by cytoskeletal elements to their final destination (Adapted from Martin and Ephrussi, 2009).

1.2 Importance of mRNA localization

What are the advantages of regulating gene expression by mRNA localization? The most obvious is that it allows gene expression to be spatially restricted within the cytoplasm. A second advantage is that this spatially restricted gene expression can be achieved with high temporal resolution: local stimuli can regulate translation on-site instead of requiring a signal to be delivered to the nucleus to initiate transcription, followed by mRNA export, cytoplasmic translation, and subsequent targeting of the protein to the site of stimulation. A third advantage is one of economy: localized mRNAs can be translated multiple times to generate many copies of a protein, which is much more efficient than translating mRNAs elsewhere in the cell, then transporting each protein individually to a distinct site. A fourth advantage is that the local translation of proteins can protect the rest of the cell from proteins that might be toxic or deleterious in other cellular compartments (Martin and Ephrussi, 2009).

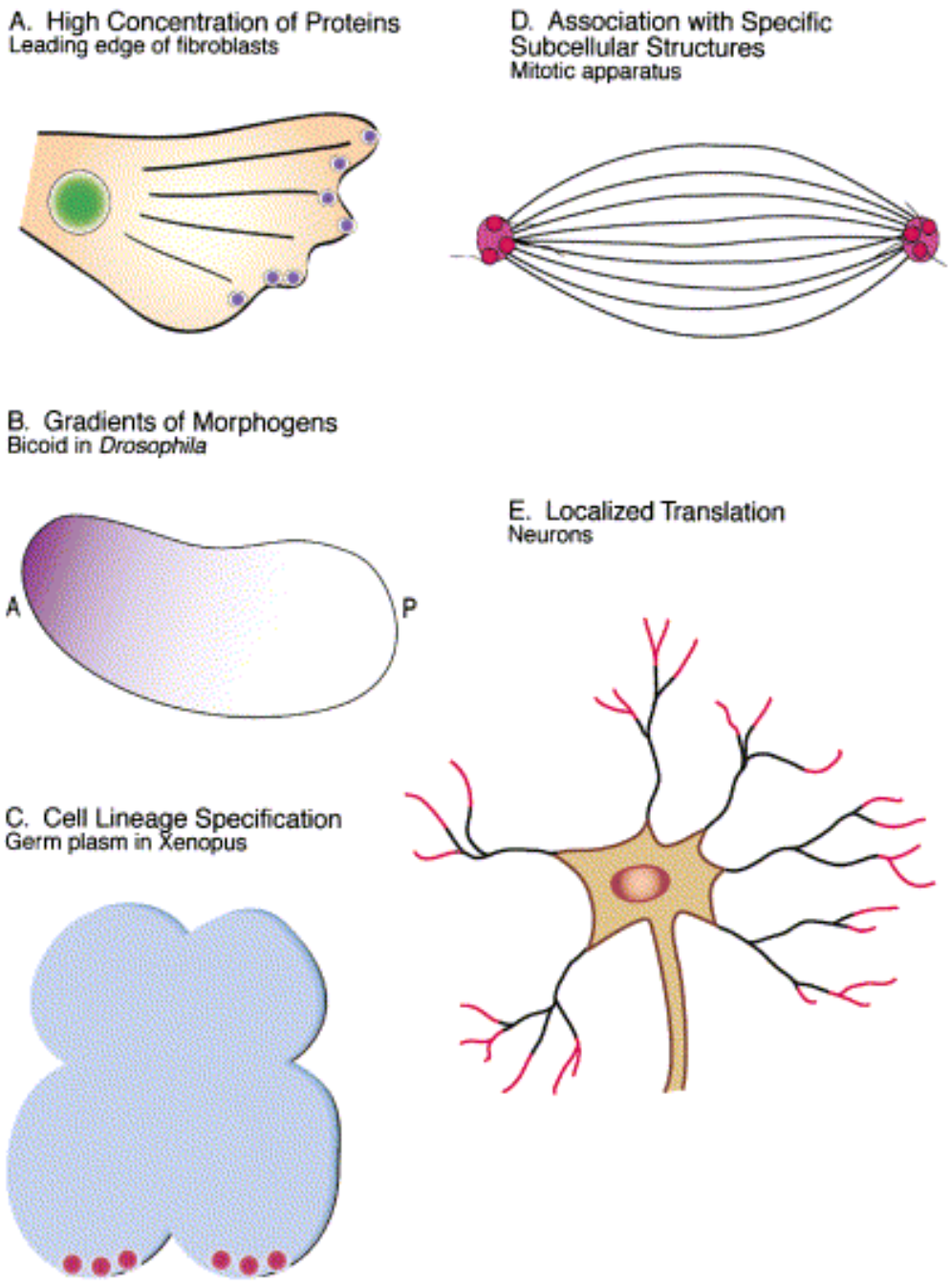

Figure 4. Examples of the Roles of Localized RNAs in Different Systems (A) The production of high levels of protein in specific regions of cells as represented by the accumulation of localized β-actin mRNA and protein at the leading edge of a fibroblast.(B) The production of gradients of morphogens in oocytes and embryos as in the case of *bicoid* mRNA localization in *Drosophila* producing a gradient of Bicoid

protein.(C) Cell lineage specification. Localized RNAs are used in a variety of systems in which they are partitioned unequally into daughter cells or into blastomeres of embryos to determine a cell lineage. The case depicted is the localized RNAs involved in specifying the germ cell lineage in amphibians. (D) Association of RNA with different organelles or cell structures. This example is of the localization of *cyclin B* mRNA at the poles of the mitotic spindle.(E) Some RNAs are localized to a specific region of a cell or embryo to allow for localized translation such as RNAs at the synapses of neurons (red) (Modified from Martin and Ephrussi, 2009).

1.3 mRNA Localization and translational regulation

In metazoa, translational control is a critical process to accomplish spatio-temporal restriction of protein production. The localization of RNAs restricts production of proteins to sites where they are required and the translational repression during transport is imperative, as premature or ectopic translation of RNAs leads to developmental defects. During late oogenesis and early embryogenesis of most multicellular organisms, ranging from worms to humans, transcription is silent, thus only maternally inherited mRNAs determine *de novo* synthesis of proteins. During oocyte development these maternal mRNAs, which are transcribed during early oogenesis and stored in the cytoplasm, are localized asymmetrically and this localization is often coupled to their translational regulation.

In eukaryotes, translation initiation is facilitated by the 5' cap structure, m^7GpppN, which is present on all mRNAs transcribed in the nucleus. The eukaryotic translation initiation factor 4E (eIF4E), the rate-limiting component for cap-dependent translation initiation, physically interacts with the cap structure.

Successively, eIF4E associates with the eukaryotic translation initiation factor 4G (eIF4G), a scaffold protein that binds several initiation factors (PABP, eIF4A, eIF4B, eIF1, eIF1A, eIF2, eIF3, among others) and the ribosomal subunit, thus forming a complex that, after mRNA circularization, begins translation. Binding of the PABP protein was shown to further stabilize the cap interaction. PABP binding to eIF4G is enhanced by contacts with the poly (A) tail and RNA maximal cap-binding activity likely depends upon the formation of a full eIF4E–eIF4G–PABP–poly (A) complex.

A key point of cap-dependent translational control is represented by events favouring the dissociation of the eIF4E–eIF4G interaction.

Among these, the eIF4E binding proteins (4E-BPs) play the role of an important repressor of translation, preventing the association of eIF4E with eIF4G. 4E-BPs share with eIF4G the eIF4E-binding motif YXXXXLΦ (where X is any amino acid and Φ is a hydrophobic residue) that interacts with the convex dorsal surface of eIF4E.

In addition to the 4E-BPs several other eIF4E binding proteins have been identified in a number of different species, which can compete for the assembly of a translationally active eIF4E–eIF4G complex and hence act as regulators of translation. These are usually specialized proteins that play specific roles during developmental processes. The best characterized examples are the X. laevis Maskin, the *Drosophila* Bicoid (BCD) and Cup proteins, which share with eIF4G and the 4EBPs the eIF4E-binding motif.

Maskin protein acts in an mRNA-specific manner and interacts with the cytoplasmic polyadenylation binding protein (CPEB) that in turn recognizes a uridine-rich sequence (CPE) present in the 3’ untranslated region (3’ UTR) of selected mRNAs. The repression itself is brought about by a direct Maskin-eIF4E interaction (**Figure 5**).

The third mechanism is represented by the BCD protein, which can both directly interact with a specific mRNA (*caudal* mRNA) and disrupt the eIF4E–eIF4G complex

(**Figure 5**) Moreover, an additional eIF4E interacting protein, Cup, was identified in *Drosophila* and shown to act via a mechanism similar to that of Maskin. It has been suggested that Cup plays crucial roles in translational control and in the localization of eIF4E during *Drosophila* oogenesis and ovary development (Piccioni et al., 2005).

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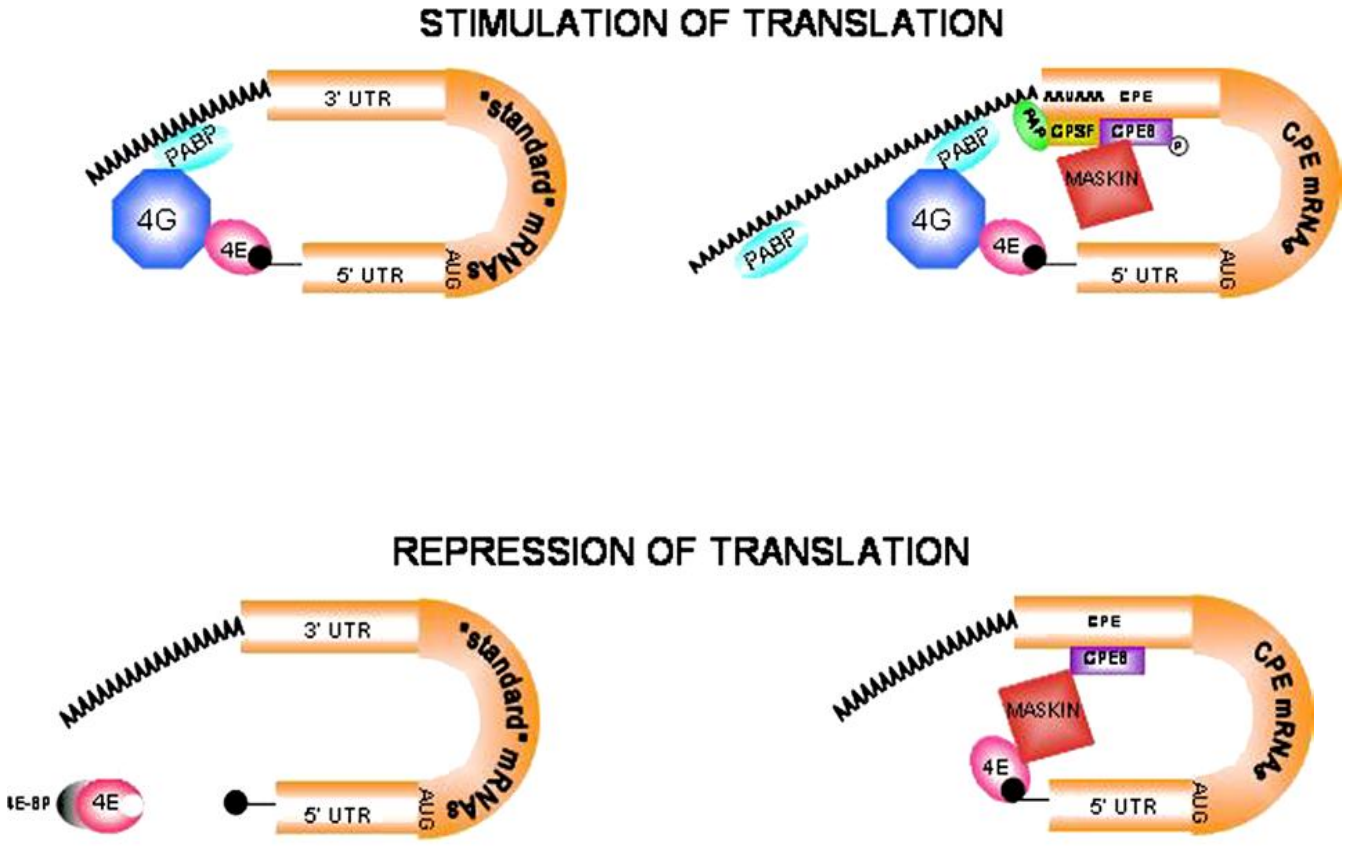


Figure 5. Modes of translational regulation during oogenesis and early development. (**A**)–(**B**). Stimulation of eukaryotic mRNA translation. (**A**) 'Standard' mRNAs. The poly (A) binding protein (PABP) binds the scaffolding protein eIF4G (4G) that in turn interacts with eIF4E (4E), thus promoting mRNA circularization and translation. (**B**) CPE-containing mRNAs are activated by cytoplasmic polyadenylation. The cytoplasmic polyadenylation element binding protein (CPEB) binds specific U-rich sequences named cytoplasmic polyadenylation elements (CPEs). Phosphorylated CPEB increases its affinity for the cleavage and polyadenylation specificity factor (CPSF). CPSF in turn interacts with the canonical nuclear polyadenylation signal AAUAAA and recruits poly(A) polymerase (PAP) to the 3' end of the mRNA. The consequent poly (A) tail elongation stimulates translation of dormant mRNAs. (**C**)–(**F**) Repression of eukaryotic mRNA translation. (**C**) 'Standard' mRNAs. eIF4E-binding proteins (4E-BPs) are inhibitory proteins that regulate the availability of eIF4E for interaction with eIF4G. 4E-BPs sequester eIF4E molecules that are either free or cap-bound. Hyperphosphorylation of 4E-BPs prevents interaction with eIF4E, thus allowing interaction with eIF4G and translation to start (see panel **A**). (**D**) CPEB mediates both activation and repression of translation. The repressive role of CPEB involves the interaction with Maskin. Maskin associates with the translation initiation factor eIF4E and excludes eIF4G from interacting with eIF4E, thus blocking initiation of translation of CPE-containing mRNAs. The Maskin-eIF4E complex is disrupted by cytoplasmic polyadenylation triggered by CPEB, allowing eIF4G to bind eIF4E and activate translation. Translation repression is relieved when PABP binds to the poly(A) tail and helps eIF4G displace Maskin and bind eIF4E (see panel B). (**E**) Bicoid (BCD) is a specific repressor of *caudal* (*cad*) mRNA translation. The anterior determinant BCD protein acts not only as a transcriptional activator of segmentation genes, but also interacts directly with both the 3'UTR of *caudal* mRNA and eIF4E to disrupt the eIF4E-eIF4G complex, thus preventing translation initiation of ubiquitously distributed *cad* mRNA. (**F**) Translational inhibition of *oskar* (*osk*) and other mRNAs. Cup

is a translational regulator that acts, by interacting simultaneously with eIF4E and RNA-bound repressor molecules, in translational inhibition of specific mRNAs. Accordingly, two repressors, Bruno and Smaug, suppress translation of *osk* and *nanos* (*nos*) mRNAs respectively by binding to their 3'UTRs prior to their posterior localization in the egg (*osk*) and embryo (*nos*). (Adapted from Piccioni et al., 2005).

2. Germ cell development

One of the most important cell fate decisions made in early development is the choice between becoming either a somatic or a germ cell. Only the germ cell lineage passes genetic information onto the next generation and has a genetic program of differentiation that preserves totipotency. In most sexually reproducing organisms, the gametes are derived from a precursor stem cell population, the primordial germ cells (PGCs). In many organisms, these cells originate extra-gonadally early in development and migrate through somatic tissues to reach the developing gonads. How cells are selected to become germ cells is an important question that has intrigued biologists for over a century. In species as diverse as *Drosophila*, C. elegans, zebrafish and Xenopus, the formation of germ cells is linked with the presence of a specialized cytoplasm called the germ plasm. The germ plasm is a morphologically distinct structure, assembled during oogenesis, which harbors the germ cell determinants. It contains clusters of mitochondria and unique electron-dense organelles called germinal granules. These granules and the germ plasm in general are composed of specific RNAs and proteins that genetic studies in *Drosophila* and embryological experiments in Xenopus have shown to be important to germ cell formation. RNAs recruited to the germ plasm will often not be translated until much later during embryogenesis when PGCs differentiate. RNAs are targeted to germ plasm through distinct cellular mechanisms that also operate in somatic cells. Therefore, the

germ line is a useful model system for studies on how cells in general sort classes of RNAs and target them to specific intra-cellular locations (Santos and Lehmann, 2004).

In this thesis, I will show in detail how RNA localization process is so crucial for germ cell specification in *Drosophila*.

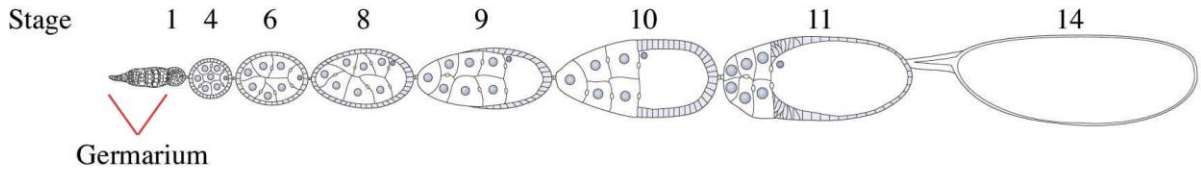
2.1 *Drosophila* oogenesis

The oocyte of *Drosophila melanogaster* has been a key model system for the analysis of mRNA localization, because of its large size and the availability of numerous genetic tools in this organism. An adult *D. melanogaster* female has one pair of ovaries, each of which comprises16 to 20 ovarioles. Each ovariole contains a series of developing egg-chambers at different stages of maturation (**Figure 6A,B**). The ovariole can be divided into an anterior germarium and a posterior vitellarium (**Figure 6C**). The germarium has been divided into four regions (1, 2a, 2b, 3) (**Figure 6C**). It contains the germline and somatic stem cells (GSC and SSC, respectively) other support cells, and the youngest developing cysts, that derive from the differentiation of the germline stem cells. The vitellarium contains egg chambers from stage 1 to stage 14, classified according to their size and morphology (King, 1970).

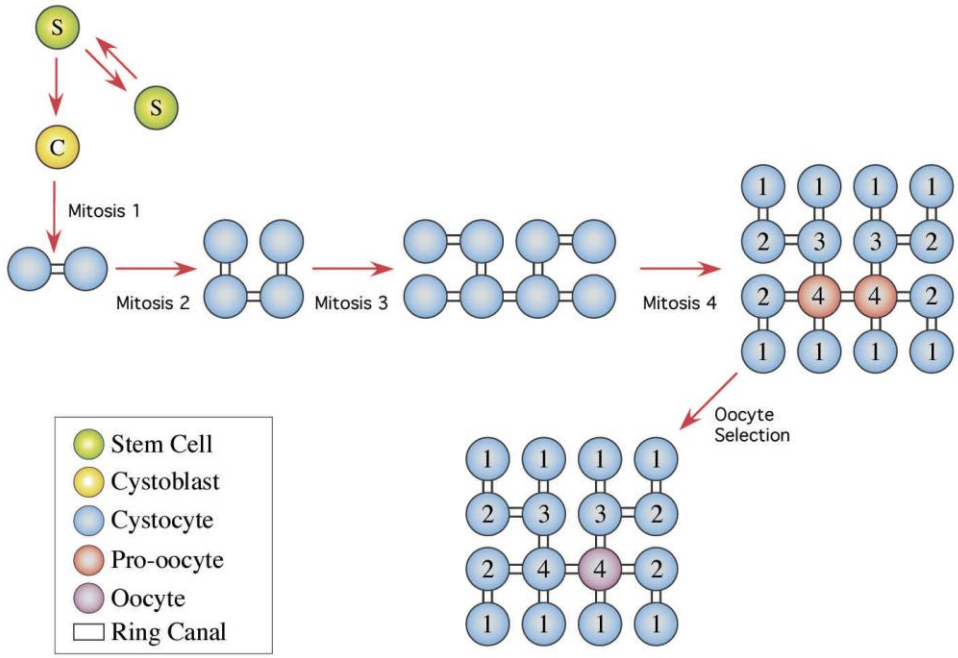
At the tip of the germarium, germ stem cells divide asymmetrically to generate a new stem cell and a cystoblast. The cystoblast then undergoes four rounds of mitotic cell divisions with incomplete cytokinesis, resulting in a syncytium of 16 cells called a germline cyst (Huynh and St Johnston, 2004) .Among these 16 cells, which are interconnected via the actin -rich ring canals, one of the two cells with four ring canals is selected to develop into an oocyte, while the other 15 cells differentiate into nurse cells.

The germline cyst and the surrounding somatic follicular epithelial cells, which are derived from somatic stem cells in the germarium, give rise to a stage 1 egg -chamber (S1) that will mature gradually in the vitellarium. According to their morphology and size, egg-chamber development has been divided into 14 stages, S1 to S14 (King, 1970) (**Figure 6A**).

a Ovariole



b Germline Cyst Formation



c Germarium - Oocyte Positioning

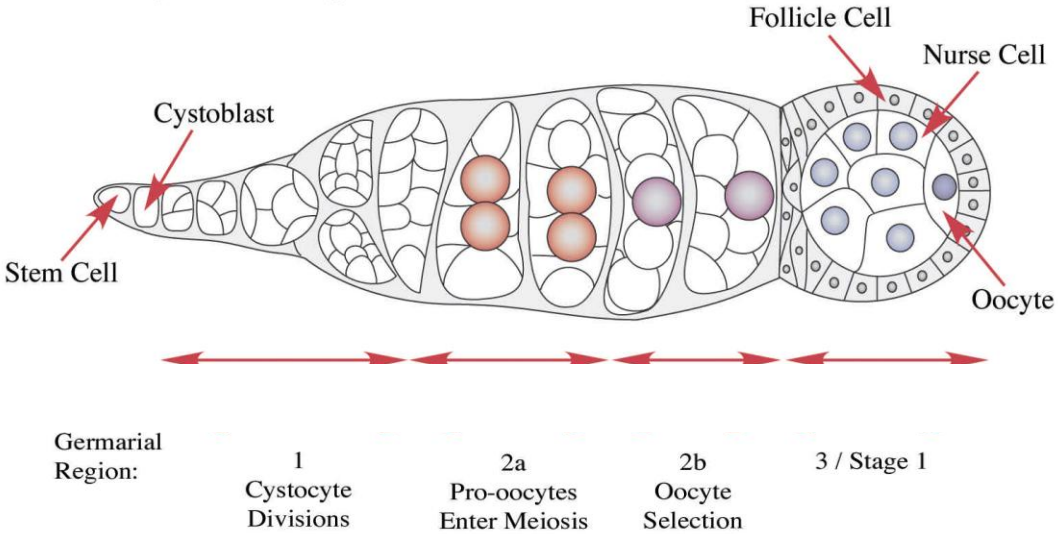


Figure 6. Formation and development of *Drosophila* egg chambers.
(a) Schematic representation of a *Drosophila* ovariole. Each ovariole consists of a string of developing egg chambers increasing in age from anterior (left) to the posterior (right). Egg chambers formed in the germarium exit to the vitellarium 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.

2.2 Specification of primordial germ cells in *Drosophila*

Germ cell specification begins during oogenesis, when maternally germ-line determinants, produced by the nurse cells, are transported and then localized in the oocyte. During early oogenesis, nuage components encircle nurse cell nuclei and appear to be involved in many processes including assembly, transport, and stabilization of germ-line RNP particles. During late oogenesis, a specialized cytoplasm, termed germ plasm, is constituted to the posterior of the oocyte. This germ plasm contains polar granules, organelles resembling

the nuage ultra-structure and composition, which are associated with germ-line specific RNP determinants, like Osk, Vas, and Tudor proteins (Frohnhofer et al., 1986; Saffman and Lasko, 1999) (**Figure 7**).

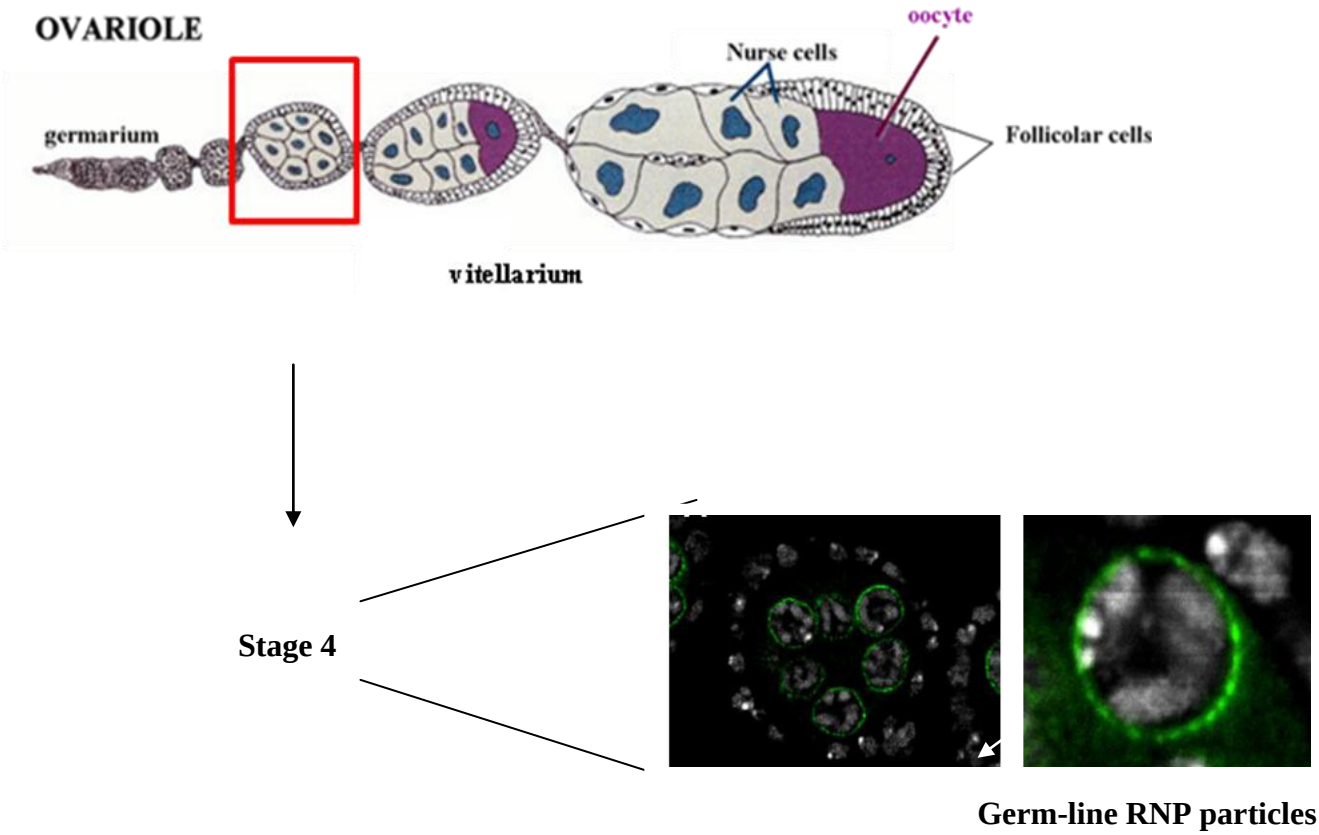


Figure 7. Nuage is assembled at stage 4 of oogenesis. *Drosophila* egg chamber, where stage 4 is highlighted by red square. Immunostaining experiment using anti-Tudor antibody as a nuage marker. As is shown, Tudor RNP particles encircle around nurse cell nuclei.

Assembly of the germ plasm is achieved by sequential and genetically defined pathways, where localization-dependent translational control processes contribute to confine protein synthesis in space and time. The posterior determinant Osk directs formation of the germ-cell lineage and abdominal embryonic structures (Ephrussi et al., 1992). To achieve this aim, the first key event is the accumulation of *osk* mRNA at the posterior of the oocyte. *osk* transcript is produced by nurse cells and enclosed within dynamic RNP particles containing specific localization and translation factors, as well exon-exon junction complex (EJC) components Mago nashi (Mago), Y14, eIF4AIII, Barentsz (Btz) and HRP48 (Hachet and Ephrussi, 2001; Huynh et al., 2004; Newmark and Boswell, 1994; van Eeden et al., 2001; Yano et al., 2004; Trucco et al., 2009).

Then, the RNA binding protein Staufen (Stau) and several microtubule motor proteins are recruited to *osk* RNPs, thus assuring their transport to the posterior cytoplasm of the oocyte (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995; Besse and Ephrussi, 2008). In addition, the translational regulator Cup coordinates both *osk* mRNA localization and its translation through a 5'-3' interaction mediated by the eIF4E-Cup-Bruno complex. Cup antagonizes the binding of eIF4E to the eukaryotic translation initiation factor 4G (eIF4G) thus repressing the translation of unlocalized *osk* mRNA (Wilhelm et al., 2003; Nakamura et al., 2004; Nelson et al., 2004).

Following localization, *osk* mRNA can be translate and it produces Long and Short Osk protein isoforms, which have different functions in the oocyte. Long Osk anchors both *osk* mRNA and Short Osk, as well as Stau, at the posterior cortex of the oocyte, whereas Short Osk recruits all the downstream germ plasm components, including Vasa (Vas), Tudor (Tud), and Nanos (Nos) (Ephrussi et al., 1991; Kim-Ha et al., 1991; Vanzo and Ephrussi, 2002). (**Figure 8**).

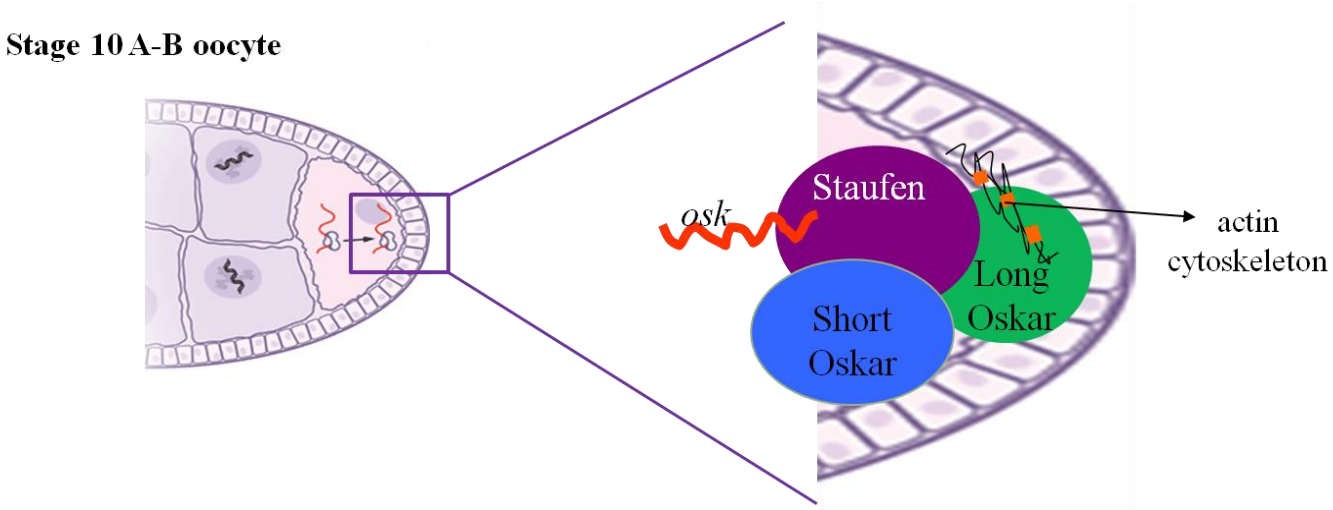


Figure 8. Scheme of germ plasm assembly in *Drosophila*

Both dosage and spatial restriction of Osk protein are tightly coupled to the number of germ cells formed: in *osk* mutant embryos fewer germ cells are present, since reduced germ plasm components are recruited (Ephrussi and Lehmann, 1992) and in addition ectopic functional germ cells are produced when Osk protein is ectopically expressed at the anterior pole of the embryo (Ephrussi et al., 1991; Kim-Ha et al., 1991; Ephrussi and Lehmann, 1992) (**Figure 9**).

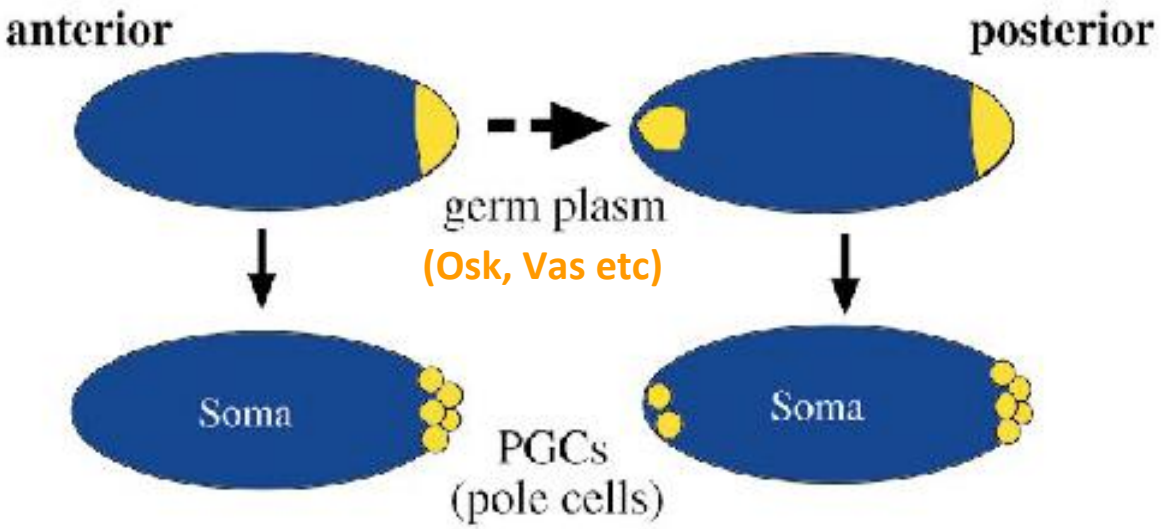


Figure 9. Germ cell determinants in the Drosophila embryo. Germ plasm (yellow) is sufficient to specify the germ cells. Anterior is to the left and posterior to the right. Germ plasm is maternally deposited and localized to the posterior. Injection of this specialized cytoplasm into the anterior of the embryo (top right), results in germ cells forming at this ectopic site (bottom right, Illmensee and Mahowald, 1974) (Adapted from Starz-Gaiano and Lehmann, 2000).

2.3 *osk* 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). 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. 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. *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).

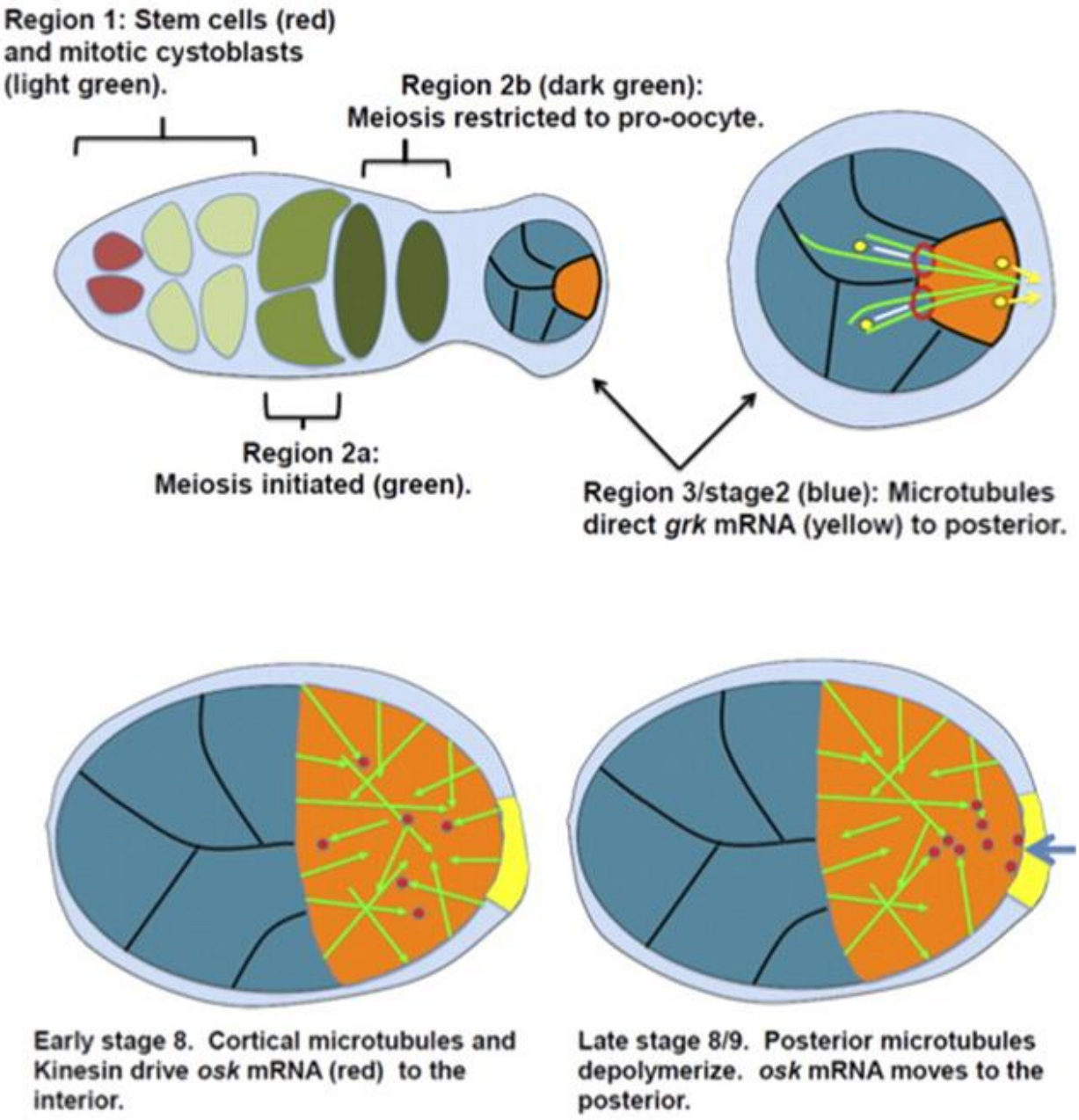


Figure 10. Microtubule polarity and *osk* mRNA localization

2.4 *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).

2.5 Translational regulation of *osk* mRNA

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. 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). In addition, the translational regulator Cup coordinates both *osk* mRNA localization and its translation through a 5'-3' interaction mediated by the eIF4E-Cup-Bruno complex. Cup antagonizes the binding of eIF4E to the eukaryotic translation initiation factor 4G (eIF4G) thus repressing the translation of unlocalized *osk* mRNA (Wilhelm et al., 2003; Nakamura et al., 2004; Nelson et al., 2004).

These results indicate 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 *osk* 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 Osk 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 Vas, 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).

2.6 Other genes involved in germ cell development in *Drosophila*

Several genes act downstream or together with Osk to make germ plasm. Vas, an ATP-dependent RNA helicase, has a role in *osk* translation, localizes with *osk* to the polar granules, and has been shown to mark germ cells in many organisms, including humans.

Tudor (Tud) protein localizes to the polar granules and the mitochondria. It has been suggested that Tud is involved in exporting mitochondrial ribosomal RNAs to the cytoplasm and that mitochondrial factors are necessary in the cytoplasm for germ cell formation (Bardsley et al., 1993; Kashikawa et al., 1999). A few germ plasm components have been identified that affect germ cell formation and determination without affecting embryonic patterning or the morphological specialization of the germ plasm. These genes include the mitochondrial 16S large rRNA (mtlr RNA) and the nuclear pore component germ cell-less (*gcl*). The polar granule component (*pgc*) gene encodes a non-translated RNA and is also localized to the posterior. This gene is not required for germ cell formation but plays a role in the later aspects of germ cell development

The *nanos* gene is required early at the posterior pole for proper abdomen development but not for germ cell formation. *nanos* (*nos*) RNA is synthesized during oogenesis and localized to the posterior pole. Initial localization and translation of nanos are necessary for proper abdomen development. Once the germ cells form, *nos* RNA and protein are sequestered into these cells.

Pumilio protein is present throughout the early embryo including the germ cells. *nos* and *pum* mutant germ cells (i.e. germ cells formed in embryos derived from homozygous *nos* or *pum* mutant females) undergo additional divisions, fail to migrate to the mesoderm, and adhere to each other in a clump prior to reaching the somatic gonad. During later stages of development, most mutant germ cells die and fail to contribute to the germ line. In addition, these mutant cells precociously express a number of genes that are normally expressed only later or not at all in the germ line, implicating Nanos and Pumilio in transcriptional repression (Saffman and Lasko, 2004).

2.7 Germ cell migration in *Drosophila*

During blastoderm formation, the nuclei localized within the germ plasm are the first to cellularize: about 10 nuclei become surrounded by membranes at this time (approximately 1.5 h after egg laying) and these cells undergo up to two divisions to give rise to about 40 pole cells, specifically localized at the posterior pole.

Upon formation, germ cells adhere to the future posterior midgut. During gastrulation, as the germband extends, they are carried along the dorsal side of the embryo in close association with the posterior midgut primordium. As the primordium invaginates, the germ cells are carried to the inside of the embryo. Next, they actively migrate across the epithelium of the posterior midgut primordium and then dorsally along its basal side. Finally, germ cells migrate away from the midgut toward the adjacent mesoderm where they associate with somatic gonadal precursor cells. During germ band retraction, germ cells and the associated somatic gonadal precursor cells migrate anteriorly until the gonadal cells round up to coalesce into the embryonic gonad (Santos and Lehmann, 2004) (**Figure 11**).

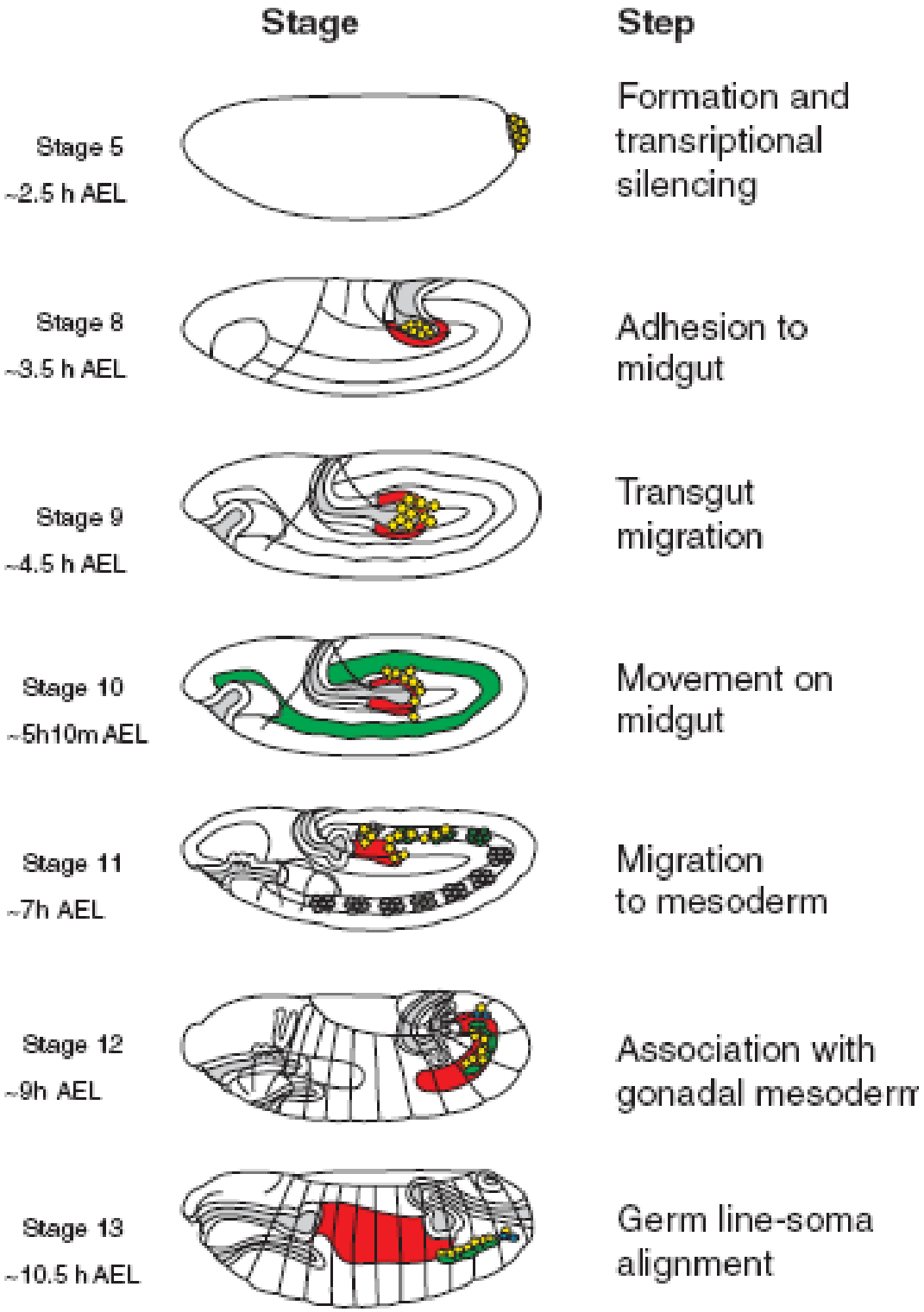

Stage
Step
Stage 5
~2.5 h AEL
Formation and transriptional silencing
Stage 8
~3.5 h AEL
Adhesion to midgut
Stage 9
~4.5 h AEL
Transgut migration
Stage 10
~5h10m AEL
Movement on midgut
Stage 11
~7h AEL
Migration to mesoderm
Stage 12
~9h AEL
Association with gonadal mesoderm
Stage 13
~10.5 h AEL
Germ line-soma alignment

Figure 11. Stages of PGC migration in Drosophila and genes involved. Schematic drawings of embryos after fertilization, with the anterior to the left and dorsal on top. Yellow, germ cells; red, midgut; green, all mesoderm at stage 10, somatic gonadal precursor clusters from stage 11 onward; blue, male specific somatic gonadal precursors, drawn as in male gonad morphogenesis (Adapted from Santos and Lehmann, 2004).

2.8 Germ Cell Specification in Other Model Organisms

As in *Drosophila***,** in *Xenopus* germ cell specification also requires a specialized germ plasm. At stage 1 of embryogenesis, germ plasm associates with the plasma membrane and is found in large patches or aggregates at the vegetal pole of an embryo. Aggregation of germ plasm was shown to depend on microtubules and the activity of kinesin-like protein 1 (Xklp-1). Contraction waves and cytoplasmic ingression triggered by fertilization facilitate formation of germ plasm islands and its gradual internalisation. During blastula stages germ plasm remains at its peri-membrane location and is inherited by one of the daughter cells via asymmetric division of blastomeres. By the onset of gastrulation, germ plasm bearing cells reach the critical germ plasm/ total cell volume ratio and are specified as primordial germ cells (PGCs). At this stage of development germ plasm is relocated to the peri-nuclear region of PGCs and associates with the yolk-free cytoplasm (nuage). By the end of gastrulation new-born PGCs are positioned to the posterior endoderm, undergo the first wave of mitotic division, and stay passively within the endoderm. At stage 25-26 PGCs start to move laterally and then dorsally. At stage 31-33 the yolk-free cytoplasm disappears and the germ plasm seems to disperse between yolk-granules around the nucleus. Next, actively migrating germ cells undergo two waves of mitotic divisions and end up in the dorsal endodermal crest by stage 40. After the formation of the dorsal mesentery (stage 43-44), they move along it to the dorsal body

wall, then laterally to colonize the region of the future gonads, which are formed by stage 50 (Houston and King, 2000) (**Figure 12**).

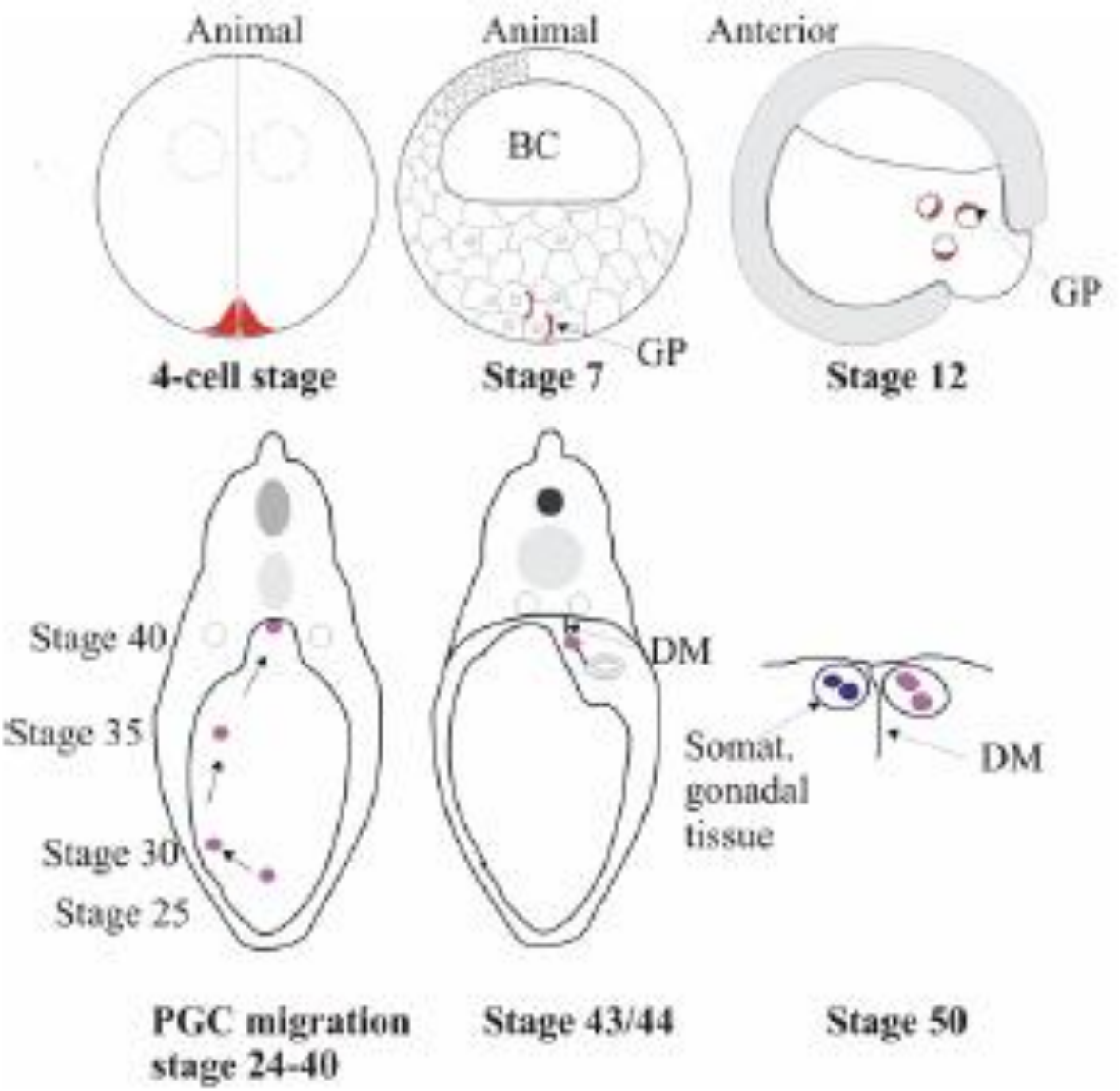


Figure 12. Germ plasm/ PGC development in Xenopus. During early cleavage stages, accompanied by cytoplasmic ingression and contraction waves, germ plasm (red) accumulates in granular germ patches and islands at the tip of the vegetal pole of an embryo. At blastula stages the germ plasm is associated with plasma membranes and inherited by one of daughter cells via asymmetrical cell division. By gastrula stage, germ plasm translocates to the peri-nuclear region of specified germ cells, which are positioned

to the posterior endoderm by gastrulation movements and undergo the first wave of mitotic division. By stage 25 PGCs start active migration laterally and then dorsally to reach the dorsal crest. Upon formation of the dorsal mesentery at stage 43-44, PGCs migrate to the dorsal body wall, from where they move laterally, associate with somatic gonadal precursor cells and form the gonads by stage 50. Abbreviations: GP- germ plasm, BC- blastocoel, DM- dorsal mesentery. (Modified from Houston and King, 2000).

Zebrafish germ cells form in four clusters at the embryo's vegetal margin, which are distributed randomly with regard to the future dorsal-ventral axis. During gastrulation, the four clusters move dorsally and align with the anterior and lateral trunk mesoderm. Two clusters on each side of the embryo join and migrate together posteriorly where they align with the gonad. Live observations suggest that germ cells move individually and that periods of directional movement are interrupted by pausing to sense guidance cues (Reichman-Fried et al., 2004) (**Figure 13**).

In the mouse, germ cells move from their site of induction toward the primitive streak and enter the posterior endoderm, where they spread along the endoderm. At stage 9, they reorient along the dorsal side of the hindgut and exit the gut toward the dorsal body wall from where they migrate into the genital ridge. Live observation of mouse germ cell migration in tissue slices revealed that germ cell migration is completed by the time the dorsal mesentery forms. These studies also show that germ cells seem to move individually and do not necessarily rely on contacts between germ cells, as was previously suggested by experiments in tissue culture (Santos and Lehmann, 2004) (**Figure 13**).

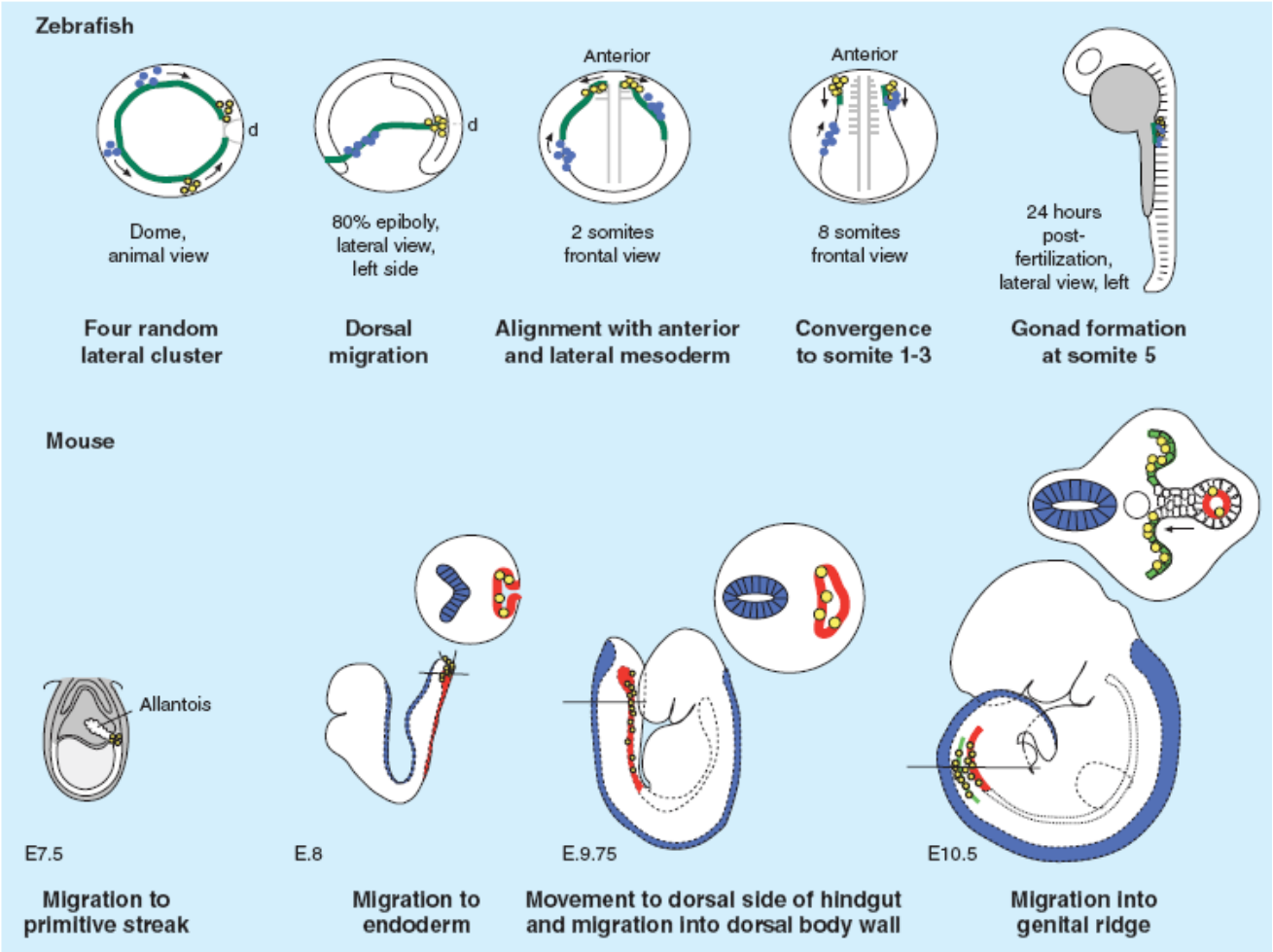


Figure 13. Germ cell migration in zebrafish and mouse. Top: zebrafish germ cell migration. Germ cells (yellow and blue) form in four clusters and are attracted toward the dorsal side of the embryo (d), but do not cross the dorsal midline. High levels of the chemokine SDF-1 (green) are found along the migratory route. The SDF-1 receptor CXCR4 is expressed in the germ cells and mediates guidance to the future gonad. Bottom: mouse germ cell migration (lateral views with enlarged cross section next to each stage, line indicates level of section). E7.5: Germ cells (yellow) moving toward the primitive streak; grey, extra-embryonic ectoderm. At stage E8, germ cells migrate along endoderm (red); insert shows germ cells in endoderm; blue, neural fold. At E9.75, germ cells move on the hindgut (red) toward its dorsal side into the lateral body wall. The neural tube (blue) is closed. At E10.5, most germ cells have left the hindgut and are in the genital ridge (red and green) (Modified from Santos and Lehmann, 2004).

3 The *Drosophila cup* gene

The *Drosophila cup* gene is crucial for diverse aspects of female germ-line development. The gene codes for a 1132-amino acid long protein, maternally deposited in the egg, that is required for normal oogenesis to assure cyst formation, germ-line chromosome structure, and oocyte development and growth. In situ hybridization and immunohystochemical analysis revealed that the *cup* mRNA and protein are confined to the cytoplasm of all germ-line cells, and are undetectable within nurse cell or oocyte nuclei, as well as somatic follicle cells. Ovaries derived from *cup* mutant females exhibit aberrant nurse cell nuclear morphology and immature egg chambers, whose development arrests between stages 5 and 14.

In recent years, Cup has been shown to interact with diverse proteins and mRNAs to accomplish a variety of functions during oocyte maturation and first stages of development (Piccioni et al., 2005)

3.1Manteinance and survival of female germ-line stem cells.

Cup directly interacts with Nos to promote normal development and survival of the female germ-line stem cells. Nos, a translational regulator directing posterior patterning in the *Drosophila* embryo, possesses evolutionary conserved functions in the ovary. nos^{RC} mutant female flies show severe egg-laying defects, because stem cells in the adult germ-line are not maintained due to a progressive degeneration of their plasma membranes. Cup is a functional partner of Nos specifically during germ-line stem cell development, since a reduction in the level of Cup substantially suppresses the oogenesis defects of hemizygous nos^{RC} mutant females. Cup and Nos colocalize to the cytoplasm of the germ-line stem

cells, cystoblasts, and cysts within the germarium, consistent with the genetic interaction described above. These data support the idea that Cup inhibits/lowers Nos activity, perhaps titrating it away from its regulatory targets. It appears that the amount of Nos *in vivo* is critical, and it is Cup that regulates this amount. Whether or not this mechanism of *nos* regulation by *cup* is conserved during male germ-line development still remains to be ascertained. In addition, cup genetically interacts with *ovarian tumor* (*otu*) to attain normal egg chamber formation and growth, thus suggesting that they cooperate in a common specialized pathway. Indeed otu is involved, similarly to *cup,* in cyst development and growth (Piccioni et al, 2005).

3.2 Cup controls the translation and localization of specific mRNAs

During *Drosophila* oogenesis and early embryogenesis, Cup plays crucial roles in the control of translation and localization of select mRNAs. It was moreover shown to be required for the proper localization and accumulation of the eukaryotic translation initiation factor 4E (eIF4E) at the posterior end of the oocyte. Cup antagonizes the binding of eIF4E to eIF4G, a crucial step in translation initiation, by competing for the same eIF4E-binding motif and is thus supposed to repress translation even though it has never been demonstrated that dissociation of Cup from eIF4E allows translation to proceed. However, Cup is found associated to specific mRNAs, which are transported during oogenesis and whose translation is blocked during transport, such as the *osk*, *gurken* (*grk*) and *cyclinA* mRNAs. Cup is recruited to the *osk* mRNA in the oocyte and to the *nanos* (*nos*) mRNA in the embryo by the Bruno and Smaug proteins, respectively, which

specifically contact these mRNAs. Thus, Cup likely functions to coordinate localization and translation of a number of mRNAs during *Drosophila* oogenesis, whose transcripts encode products distributed asymmetrically within the oocyte (Piccioni et al., 2005).

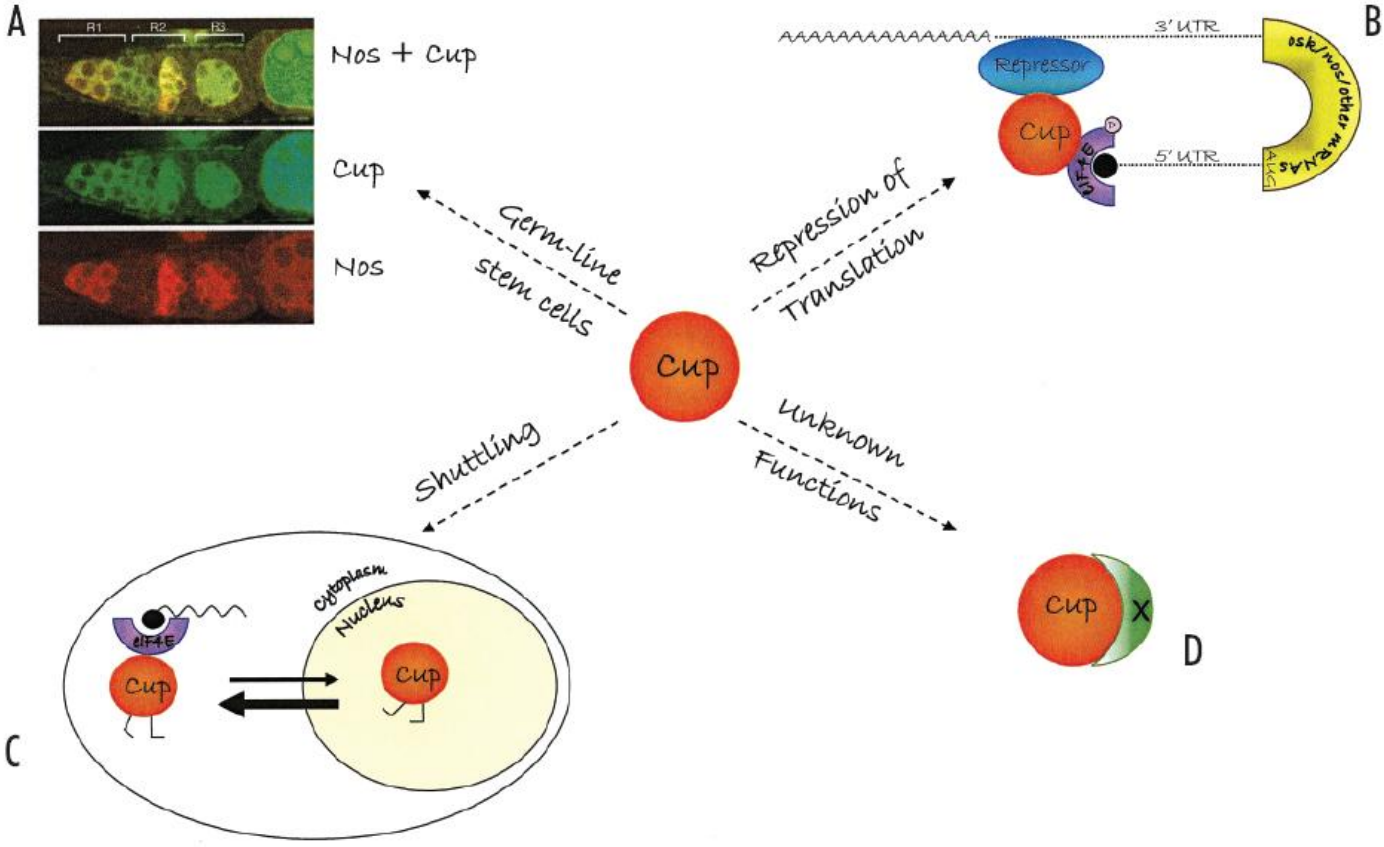


Figure 14. Cup is a multi-functional protein acting during Drosophila ovary development and early embryogenesis. Cup orchestrates the activities of different mRNAs and proteins to achieve a broad range of biological functions. (A) Cup interacts with Nos to promote maintenance and survival of the female germ-line stem cells. Cup and Nos colocalize during early germ cell development in the germarium, the terminal region of the ovary that contains stem cells and most immature egg chambers. Cup (in

green) is localized in the cytoplasm of all germ cells, whereas Nos (in red) distribution is not uniform. Cup and Nos colocalize to the cytoplasm of the stem cells, cystoblasts, and cysts within the germarium, in agreement with their genetic and biochemical interaction. The identification of the Nos-Cup interaction was the first step towards the understanding of how nos and cup function during germ-line development. Regions 1, 2 and 3 of the germarium are indicated. (B) Cup-mediated translational repression and localization of select mRNAs. Cup, like Maskin, bridges eIF4E and 3'-UTR-bound factors to keep mRNAs repressed. Cup is able to antagonize the binding of eIF4E to eIF4G, a crucial step in translation initiation, thus inhibiting translation. Cup likely functions to coordinate translation and localization of a number of mRNAs distributed asymmetrically within the oocyte. During transport, Cup possibly inhibits translation by antagonizing the interaction between eIF4G and eIF4E. Subsequently, upon localization of the transcript, the interaction between eIF4E and Cup could be weakened, e.g., due to the action of a local trigger mechanism(s) such as phosphorylation, thus allowing eIF4G to interact with eIF4E and translation to start. (C) Cup is a nucleo-cytoplasmic shuttling protein. In Schneider cells (S2), Cup, which is mainly cytoplasmic at equilibrium (indicated by arrows size), shuttles between the nucleus and the cytoplasm. This result suggests that Cup likely exerts its functions in the cytoplasm forming a complex with eIF4E to control translation of specific mRNAs. In ovaries, Cup has not yet been shown to shuttle into and out the nurse cell or oocyte nuclei. However, Cup has been found to interact with Nup154, an evolutionary conserved protein of the nuclear pore complex (NPC). (D) Cup may exert still unknown functions. The identification of additional interactors of Cup may shed light on novel, still unexplored functions. For example, Cup, similar to its putative human orthologue eIF4E-Transporter, could be localized within P bodies and therefore be involved in mRNA degradation.

3.3 Cup mediated translational control and ovary development

Interestingly, the localization and the overall amount of the eIF4E protein, which in *Drosophila* normally accumulates at the posterior end of the oocyte, are altered in a *cup* mutant background. This result reveals that Cup acts more generally to control translation within the oocyte, likely affecting the translation of numerous different mRNAs. Accordingly, the mutation of *cup* has a considerable effect on oocyte maturation and ovary development. Moreover, a reduction of the eIF4E gene dosage causes a significant aggravation of the *cup* mutant ovary phenotype, with both the development and the

growth rate of the egg chambers affected. Based on these findings and on the results of other studies a model was proposed for the function of Cup within the ovary. According to this model, Cup functions to mediate both the localization and translational repression during transport of a number (or possibly the majority) of mRNAs confined at the posterior end of the oocyte. eIF4E and the associated mRNAs would be transported via the interaction between Cup and a localization factor such as Btz. During transport, Cup would inhibit translation by antagonizing the interaction between eIF4G and eIF4E. Subsequently, upon localization of the transcript, the interaction between eIF4E and Cup would be weakened, e.g., due to the action of a local trigger mechanism(s) such as phosphorylation, thus allowing eIF4G to interact with eIF4E and translation to start (Zappavigna et al.,2004).

3.4 Cup orthologues

A search of the protein database for a mouse homologue of the *Drosophila* Cup protein identified the product of the *Clast4* gene. A subsequent search for Clast4 orthologues identified gene products

from other vertebrates, ranging from human to *Xenopus laevis,* which share variable but high degree of identity with the full-length mouse Clast4 protein. In *Homo sapiens*, the Clast4 homologue corresponds to eIF4E-Transporter (4E-T), which is approximately 90% identical at the amino acid level. Within mammalian cells, 4E-T protein mediates the nuclear import of eIF4E and, similarly to Cup, is a nucleo-cytoplasmic shuttling protein. Interestingly, Cup shares a high degree of homology with a short segment of the 4E-T protein (amino acids 565–630 that span a portion of the Nos-binding domain), but the nuclear localization signal (NLS) and nuclear export signals (NESs) found within 4E-T do

not have an obvious counterpart within Cup. *Clast4* mRNA and protein are highly expressed within the cytoplasm of growing oocytes. The Clast4 protein is stable during this developmental window and post-translationally modified by phosphorylation upon oocyte meiotic maturation. Additionally, Clast4 directly interacts with eIF4E by means of a shortened and functional eIF4E-binding motif (YXXXXL).Taken together these data suggest that Clast4, similar to *Drosophila* Cup, may act at the translational level during murine female germline development. Moreover, 4E-T binds eIF4E in vivo within cytoplasmic foci enriched in factors required for mRNA degradation (known as mRNA processing bodies or P bodies; thus suggesting that these proteins and their orthologues might also promote 5' to 3' mRNA degradation (Piccioni et al., 2005).

Aim of thesis

In this thesis, I show that Cup physically interacts with Osk and co-localizes with Osk protein and other polar granule factors to the nuage and germ plasm during oogenesis. Furthermore both *cup* gene products are expressed during early and late embryogenesis, where they are specifically detected within germ cells. In addition, heterozygous *cup* mutant ovaries have reduced levels of Stau, Osk, and Vas proteins at the posterior pole of the oocyte, thus indicating that Cup contributes to promote germ plasm assembly through accumulation, stabilization and/or maintenance of these maternal germ-line determinants. Furthermore, as consequence of this Cup function, the germ plasm appears affected in early heterozygous *cup* mutant embryos: although *osk* mRNA/Stau particles reach the posterior pole as in wild type embryos, a significant amount of these complexes is not properly anchored to the cortex and appears mislocalized. Consistent with the translation activation of only correctly localized *osk* mRNA, reduced levels of Osk protein and other downstream germ plasm components, (i.e. Vas protein), are detected at the posterior pole and, consequently, reduced germ cell number is found in heterozygous *cup* mutant embryos. Finally, I show that *cup* and *osk* interact genetically, since reducing *cup* gene copy number further decreases the number of germ cells number detected in heterozygous *osk* mutant embryos. Taken together, my data demonstrate that Cup, through the binding with Osk and other germ plasm components, is required to assure correct germ cell formation and maintenance during *Drosophila* development.

Results

1. Cup is a component of the nuage and of the germ plasm

During *Drosophila* oogenesis, the nuage is localized around the nurse cell nuclei and contains developmental key components, such as Vas and Tud proteins (Saffman and Lasko, 1999). Previously, it was reported that the translational regulator Cup is expressed in all germ-line cells throughout oogenesis and, around stage 4, accumulates as large aggregates into the perinuclear cytoplasm of the nurse cells, similar to the distribution of known nuage components (Keyes and Spradling, 1997; Saffman and Lasko, 1999). In addition, Snee et al. (2007) identified Bruno as a new component of nuage. Bruno binds Cup and specific 3'UTR sequences (named Bruno responsive elements, BRE) within *osk* mRNA, thus supporting the idea that translational regulation events are coupled with the formation and/or reorganization of RNA-protein complexes.

To determine if Cup is a novel nuage component, I performed double immunostaining experiments on developing egg chambers using Tud as marker. In wild-type stage 4 egg chambers, Tud and Cup signals are concentrated in the region surrounding the nurse cell nuclei (**Figure 15**), in agreement with previous reports (Keyes and Spradling, 1997; Grimaldi et al., 2007; Bardsley et al., 1993). As shown in the overlay (**Figure 15C, F**), Tudor and Cup co-localize in the form of perinuclear clusters in the nuage. Nevertheless, in homozygous *cup* (data not shown) and *tudor* (Snee et al., 2004) mutant ovaries, the organization of perinuclear nuage appears unaltered. In addition, Cup also accumulates in cytoplasmic particles (**Figure 15B,E**), which seem to arise from the nuage and move from the nurse cell cytoplasm to the posterior pole of the oocyte to be assembled in polar granules (Styhler et al., 2002; Snee at al., 2004).

Emerging evidences suggest that Cup is also a germ plasm component: First, Cup accumulates to the posterior pole of stage 10 oocytes, where it co-localizes with Osk and

Stau proteins (Wilhelm et al., 2003; Piccioni et al., 2009); Second, Thomson et al. (2008) performed immuno-precipitation experiments to identify novel polar granule components associated with Vas and Tud and isolated several P body related proteins, including the Cup protein; Third, Thomson et al. (2008) hypothesize a direct association between polar granules and ER exit sites, where Cup has been shown to aggregate on ER discrete foci (Wilhelm et al., 2005).

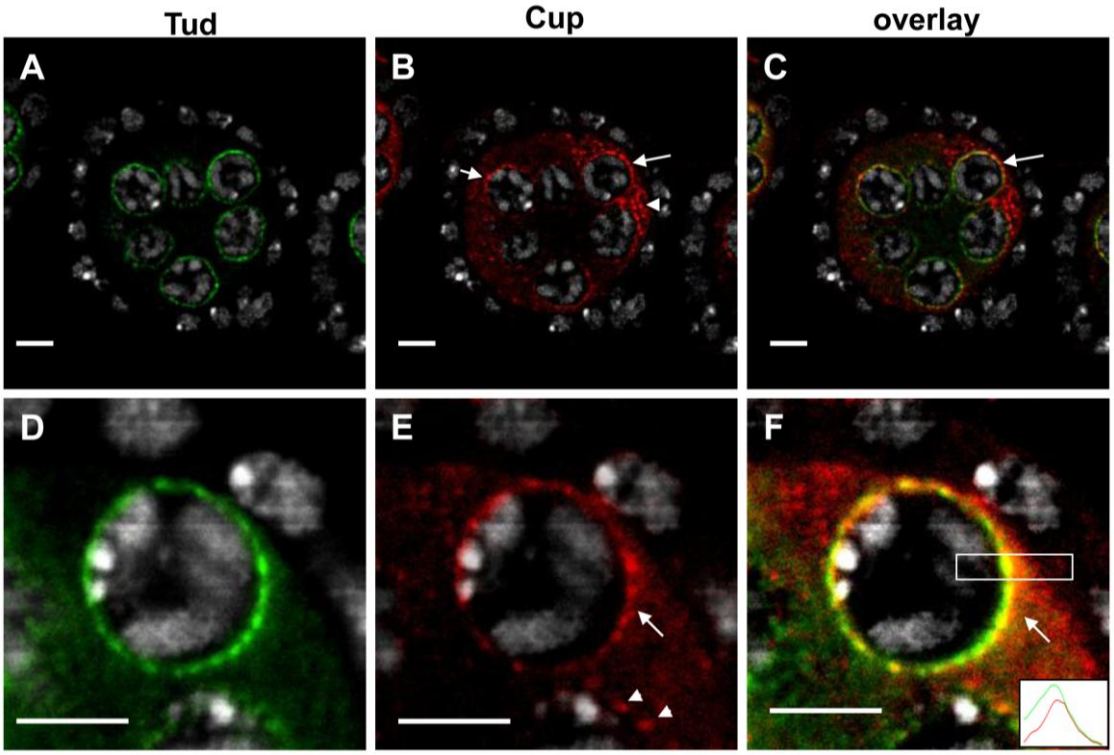


Figure 15. Cup is a component of nuage. (**A-C**) Wild-type ovaries were immunostained with rabbit anti-Tud antibody (shown in green, A) and rat anti-Cup antibody (shown in red, B) and the nuclei are highlighted by DAPI staining (shown in grey). (**D-F**) Closer view of nurse cell nuclei shown in (A-C), respectively. At Stage 4, Cup forms aggregates around the nurse cell nuclei, (B and E, arrows) and co-localizes with the nuage marker Tud in the perinuclear region (C and F, arrows). In addition, cytoplasmic particles enriched of Cup protein are detected in the proximity of the nurse-cell nuclei (B and F, arrowheads). The inset in F shows an intensity profile across the perinuclear region of the

stage 4 egg chamber (region of the white square). The fluorescence intensities of both profiles peak at a similar position at the nuclear foci and demonstrate the overlapping localization. The scale bars are 5 µm in all the panels.

To confirm that Cup is a novel germ plasm component, I performed immunostaining experiments on wild-type ovaries, comparing the distribution of Cup with that of main germ plasm molecules such as Stau, Osk, and Vas. During early oogenesis, Cup and Vas localize within the cytoplasm of developing oocytes, when *osk* mRNA is present but not yet translated. In stage 10 oocytes, Vas protein reaches the posterior pole where *osk* mRNA begins to be locally translated (Lasko and Ashburner, 1990; Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al, 1995). As shown in **Figure 16,** Cup co-localizes with Stau, Osk, and Vas proteins at the posterior pole of stage 10 oocytes. These results shed light on a yet unexplored role of Cup during nuage and germ plasm formation.

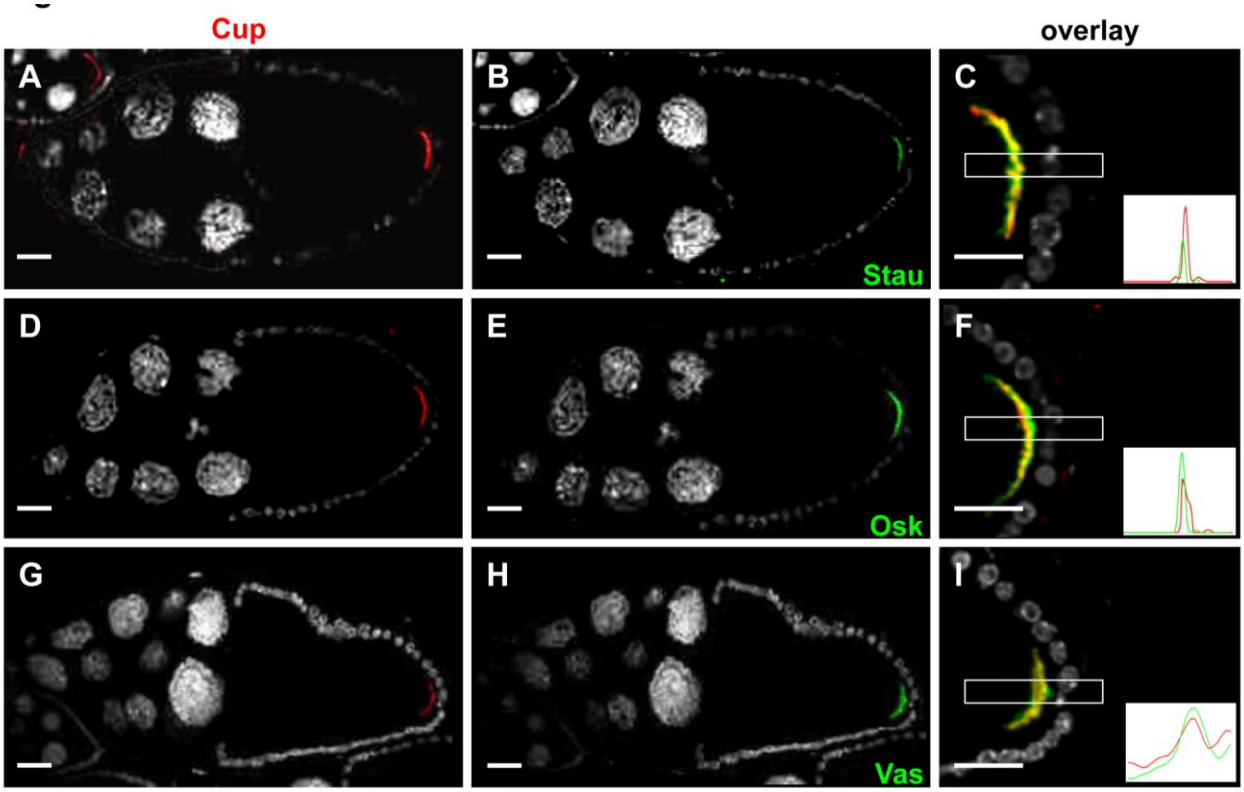


Figure 16. Cup localizes to the germ plasm. Confocal images of wild-type stage 10 egg chambers to visualize Cup (shown in red; A, D and G) and Stau, Osk and Vas (shown in green; B, E and H, respectively). The scale bars are 20 µm.
Enlarged view of stage 10 oocytes shows the co-localization of Cup with Stau (C), Osk (F) and Vas proteins (I); the inset in (C, F and I) shows the intensity profile across the posterior pole oocyte (region of white square) and confirms the overlapping localization. The scale bars are 10 µm.

2. Cup associates with Osk and Vas

In order to test if Cup physically interacts with germ plasm components other than Stau (Piccioni et al., 2009), wild-type ovary extracts were immuno-precipitated with anti-Osk and anti-Vas antibodies and the precipitates analyzed by Western blot using an anti-Cup antibody. As shown in **Figure 17A,B**, a clearly detectable amount of Cup consistently and specifically co-precipitates with either Osk and Vas. In addition, two different Cup fragments interact directly with Osk in yeast (**Figure 17C**). This latest result suggests that Cup-Osk association does not require any RNA intermediate and that Cup is part of a multi-protein complex, including Stau, Osk and Vas, involved in germ plasm assembly.

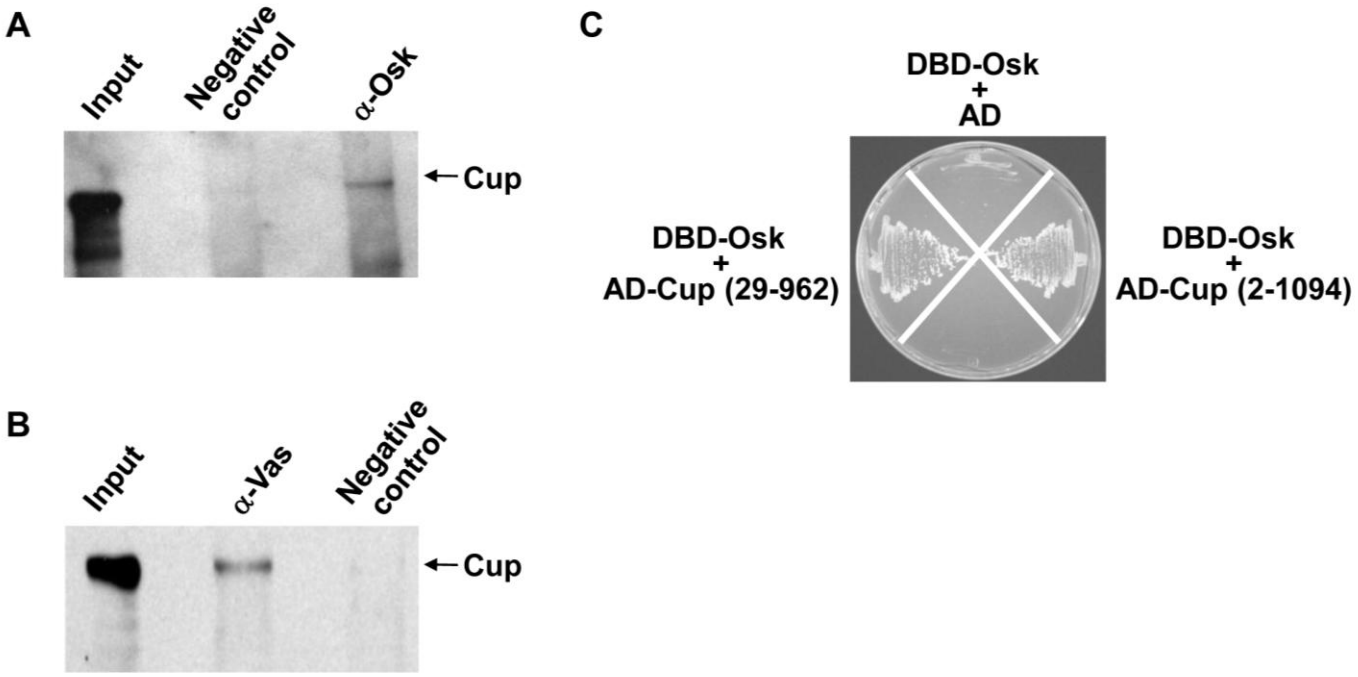
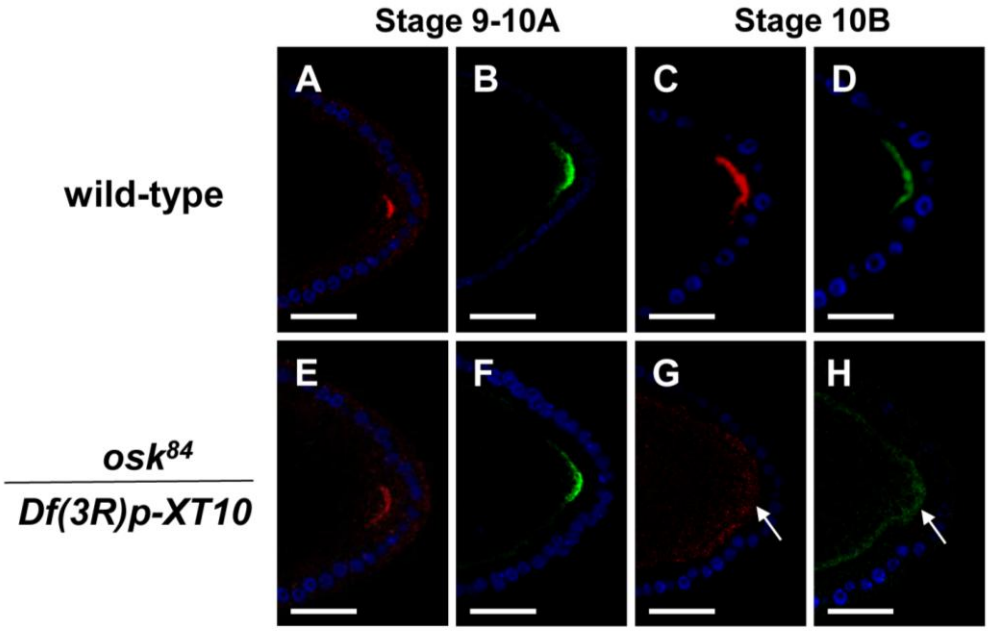


Figure 17. Cup associated with Osk and Vas. (**A-B**) Co-immunoprecipitation assay. Ovary extracts were immunoprecipitated by rabbit anti-Osk and rabbit anti-Vas antibodies, and the precipitates analyzed by Western blot using rat anti-Cup antibody. Cup consistently and specifically co-precipitated with Osk (A) and Vas (B). Rabbit pre-bleed serum was used as a negative control. (**C**) Yeast two-hybrid assay. Doubly transformed yeast expressing a GAL4 DNA-binding domain (DBD)-Osk fusion (amino acids 122-650) and either the GAL4 transcriptional activation domain (AD) alone or AD-Cup fusion proteins, as indicated. Protein-protein interactions stimulate transcription of the HIS3 reporter, allowing growth on selective media lacking His and containing 3 mM 3-aminotriazole.

During the organization of the germ plasm, germ-line components are hierarchical localized and anchored to the posterior pole of the mature oocyte (Thomson and Lasko, 2005). To examine the involvement of Cup in this process, I analyzed Cup localization at the posterior cortex of oocyte in *osk* and *vas* alleles. In both wild-type and *osk*^{84}*/Df(3R)p-XT10* stage 9-10A oocytes, similar to Stau protein localization (St Johnston et al., 1991; Kim-Ha et al., 1991; Vanzo et al., 2002; **Figure 18A,E**), Cup accumulates at the posterior cortex (Fig. 4B,F). In contrast to wild-type controls, Cup (**Figure 18D,H**) and Stau (St Johnston et al., 1991; **Figure 18C,G**), detaches from the posterior pole of *osk*^{84}*/Df(3R)p-XT10* stage 10B oocytes, thus resulting in a significant reduction, about 50%, of posteriorly localized proteins (quantification of fluorescent signals is shown in **Figure 18I**).



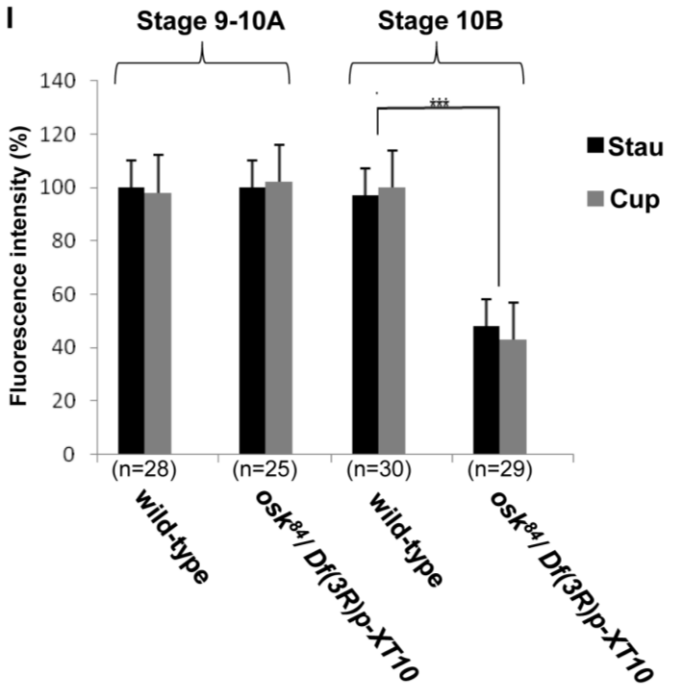


Figure 18. Cup posterior localization is affected in *osk* mutant oocytes. (**A-H**) Wild-type and *osk* non sense mutant oocytes (*osk*^{84}*/Df(3R)p-XT10*) stained with anti-Stau (shown in red; A, E, C, G) or anti-Cup (shown in green; B, F, D, H) antibodies. During stage 9 -10 A, Stau and Cup are detected at the posterior pole of both wild-type and *osk*^{84}*/Df(3R)p-XT10* oocytes (A-B and E-F, respectively). During late stage 10 (10B), Stau and Cup remain tightly anchored forming a crescent-shaped pattern at the posterior pole of wild-type oocytes (C,D). At same stage in *osk*^{84}*/Df(3R)p-XT10* ovaries, Cup, as well as Stau (H,G, respectively), detached away from the cortex and a significantly reduced amount (at least 50%) is detected to the posterior pole of the oocyte (H, arrow). The nuclei (shown in blue) are highlighted by DAPI staining. Each panel shows single stained oocyte. The scale bars are 10 µm in all the panels. (**I**) Quantification of the effect shown in upper panel: comparison of fluorescent signal intensities showed that the reduction of Stau and Cup is specific and significant at P<0.001 (***); student's *t* test.

As far as *vas* alleles are concerned, homozygous *vas*^{1} females undergo normal oogenesis and lay a wild-type number of eggs that develop into embryos lacking abdominal segments (Lasko and Ashburner, 1991). In the null *vas*^{PH165} allele, approximately 70% of homozygous egg chambers appear normal until stage 6 and degenerate afterwards; 20% continue beyond this stage, most arrested at stage 10; finally, a small number of oocytes are able to complete oogenesis, thus reaching stage 14 (Styhler et al., 1998). In early stages of *vas*^{1} and *vas*^{PH165} egg chambers, Cup protein is normally distributed (data not shown), while in the majority of stage 10 egg chambers, Cup, even if correctly localized at the posterior pole, is less abundant (**Figure 19B,C**; quantification of fluorescent signals is shown in **Figure 19F**). Moreover, about 20% (N=69) of stage 10 *vas*^{1} egg chambers display a punctuate aspect of Cup at the posterior pole (**Figure 19D**). This interspersed Cup distribution is also observed at the posterior pole of few stage 10 *vas*^{PH165} oocytes (**Figure 19E**), where *osk* mRNA signal is greatly reduced and diffused (Styhler et al 1998).

These results demonstrate that Osk directs accumulation and anchoring of Cup, as well as the other germ plasm determinants, at the posterior pole of late stage 10 oocytes. Importantly, these analyses also show that any destabilization of germ plasm assembly, as occurs in *vas* mutants, alters Cup posterior localization. Taken together, the data shown is this thesis indicate that Cup is a novel component of the germ plasm.

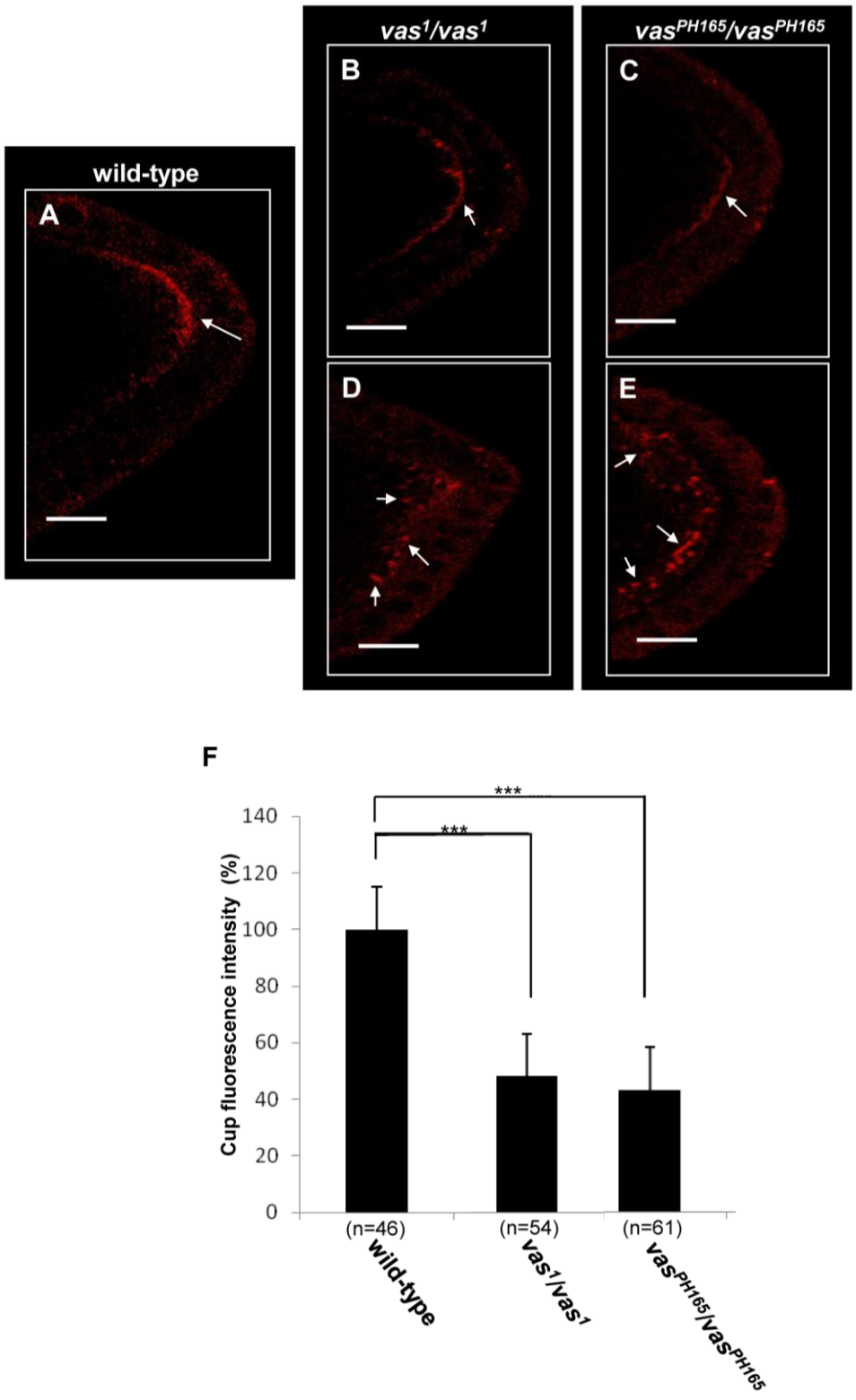


Figure 19. Disruption of Cup localization in *vas* mutant oocytes. (**A**-**E**) Wild-type and homozygous *vas* mutant oocytes (*vas*^{1} and *vas*^{PH165}) were stained with anti-Cup antibody.

At stage 10, 80% of homozygous *vas* mutant ovaries showed a posterior enrichment of Cup protein, correctly localized to the cortex of the oocyte but at a reduced level compared with wild-type (B, C and A, respectively). In the remaining 20% of homozygous *vas* mutant oocytes, Cup signals are detected within numerous sites of punctuate fluorescence around the posterior cortex thus indicating that Cup is not stable anchored to the posterior pole (D,E). Scale bars are 10 μm in all the panels. (**F**) Quantification of the effect shown in (A-E): comparison of fluorescent signal intensities showed that the reduction of Cup is specific and significant at P<0.001 (***). Student's *t* test.

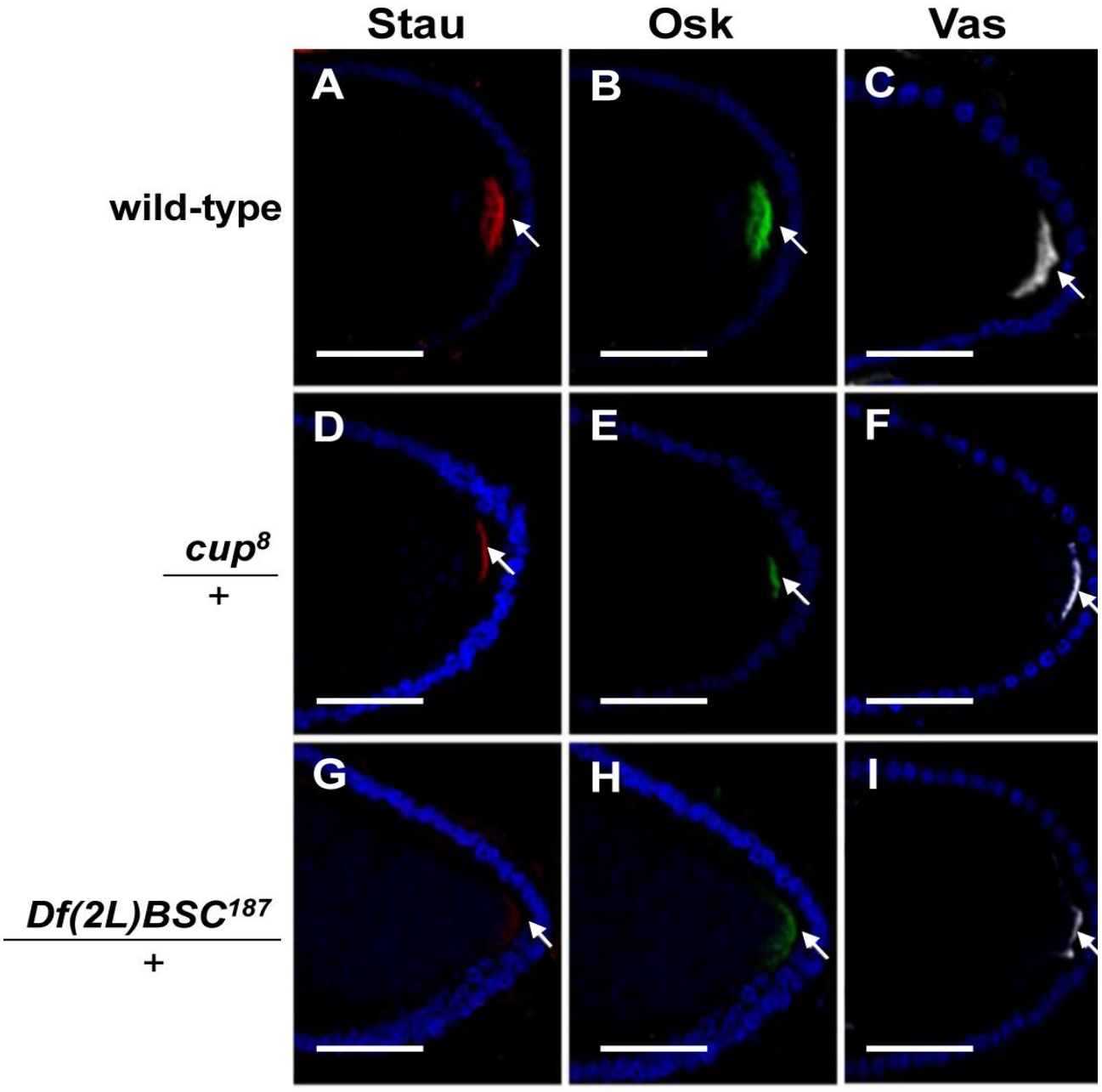
3. Cup anchors the germ plasm at the posterior pole of stage 10 oocytes

Homozygous *cup* mutants are sterile and their ovaries display a wide range of defects; in contrast, heterozygous *cup* mutants are fertile with wild-type egg chamber development (Keyes and Spradling, 1997; Piccioni et al., 2009).

To understand the role of Cup protein during germ plasm assembly, I carefully analyzed the localization of several germ plasm determinants in ovaries with reduced *cup* gene dosage. During mid-oogenesis (stage 8) of cup^{8} /+ and *Df(2L)BSC187/+* egg chambers, Osk protein is normally localized, coincident with Stau accumulation at the posterior pole (data not shown).

Later in oogenesis, when the first steps of germ plasm assembly take place and Osk anchoring becomes crucial (Ephrussi et al., 1991; Vanzo et al., 2007), I detect a significant reduction of localized Stau, Osk and Vas proteins at the posterior cortex of heterozygous *cup* stage 10B oocytes, in comparison to wild-types (**Figure 20**). Western blot analyses on total protein extracts derived from *cup* mutant ovaries (cup^{8} /+ and *Df(2L)BSC187/+*) , reveal levels of Stau, Osk and Vas comparable to wild-types (data not

shown). This observation indicates that the detected diminution in posterior localization of these germ plasm determinants does not depend upon protein degradation but rather from the failure of the germ plasm to remain localized at the posterior pole.



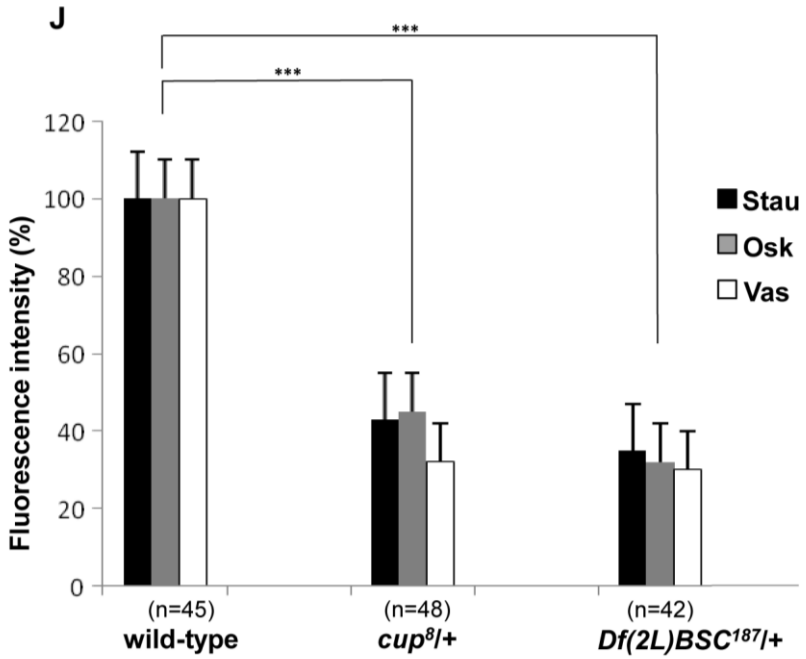


Figure 20. Cup promotes the accumulation of the germ plasm determinants at the posterior pole of the oocyte. (**A-I**) Stage 10 wild-type and heterozygous *cup* mutant egg chambers (cup^{8}/+ and $Df(2L)BSC^{187}$/+) stained with anti-Stau (shown in red; A, D, and G), anti-Osk (shown in green; B, E and H) or anti-Vas (shown in grey; C, F and I) antibodies. Compared with wild-type oocytes, Stau, Osk and Vas protein levels are reduced at the posterior cortex of the heterozygous *cup* mutant oocytes. For comparison, wild-type stage 10 oocytes are shown in the top panels. The nuclei (shown in blue) were highlighted by DAPI staining. Each panel shows single stained stage 10 oocyte. Scale bars are 10 µm in all the panels. (**J**) Quantification of fluorescent signals indicates that the decrease of Stau, Osk and Vas is significant at $P<0.001$ (***); Student's *t* test

Moreover, Cup does not co-localize with F-actin at the posterior pole of stage 10 oocytes (**Figure 21**) suggesting that Cup mediates the anchoring of germ plasm

determinants at posterior pole of stage 10 oocytes through direct interaction with Osk, whose long isoform (L-Osk) is specifically associated with F-actin projections (Vanzo et al., 2007).

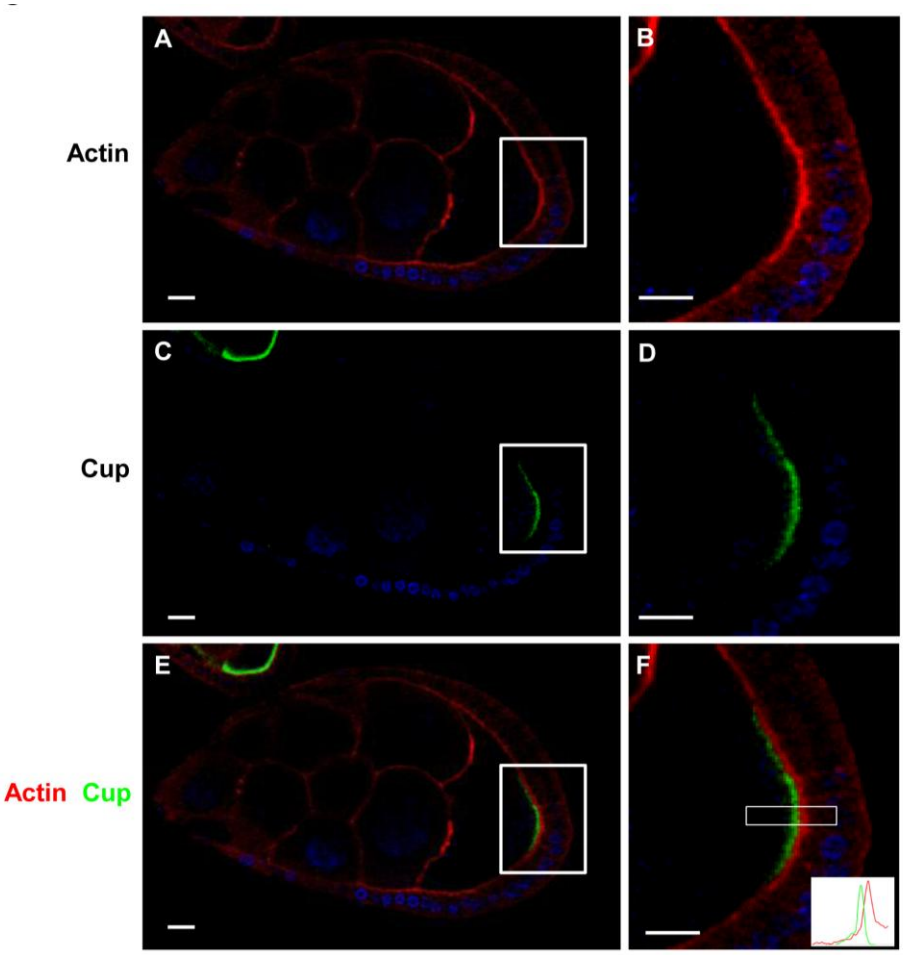


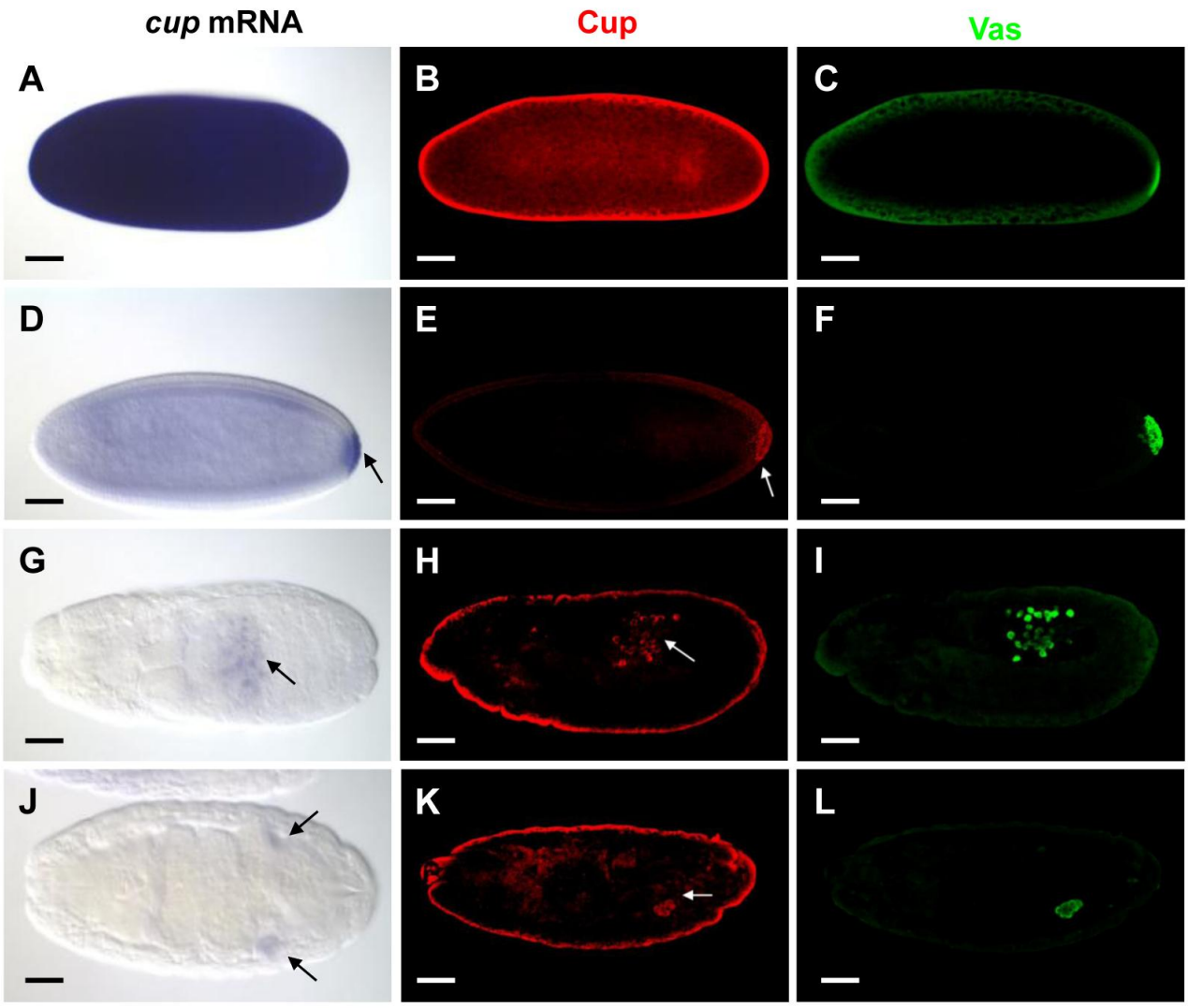
Figure 21. Cup does not colocalize with actin. (**A, C, E**) Wild-type stage 10 oocytes stained with Texas Red-X phalloidin (A) and anti-Cup antibody (C). Merged image is shown in (E). The scale bars are 20 µm. (**B**, **D**, **F**) Magnification of the posterior tip of the oocytes shown in A, C and E respectively. The inset in the panel F represents the intensity profile across the posterior pole of the embryo (region of white square), where the

different subcellular distribution of actin (texas red phalloidin) and Cup protein are shown. The scale bars are 10 µm.

In conclusion, Cup appears to be required specifically for the stable accumulation of germ plasm determinants at the posterior cortex of stage 10 egg chambers.

4. *cup* gene products are expressed in germ cells

Given the new role of Cup in the germ plasm assembly, I asked whether or not Cup may exert an important yet unknown function during germ cell determination. To this aim, I analyzed *cup* gene product distribution during *Drosophila* embryogenesis by *in situ* hybridization and immunostaining experiments on wild-type embryos at different stages of development. In stage 2, *cup* mRNA and protein are uniformly distributed in the whole embryos (**Figure 22A,B**). During blastoderm formation (stage 4), *cup* mRNA and protein are concentrated at the posterior pole to become incorporated within newly formed germ cells (**Figure 22D,E**). Subsequently, *cup* gene products accumulate specifically in the pole cells at stage 10, when they migrate through the posterior midgut primordium, and during stage14, when the germ cells reach their final destination (**Figure 22G,H,J,K**).


cup mRNA
Cup
Vas
A
B
C
D
E
F
G
H
I
J
K
L

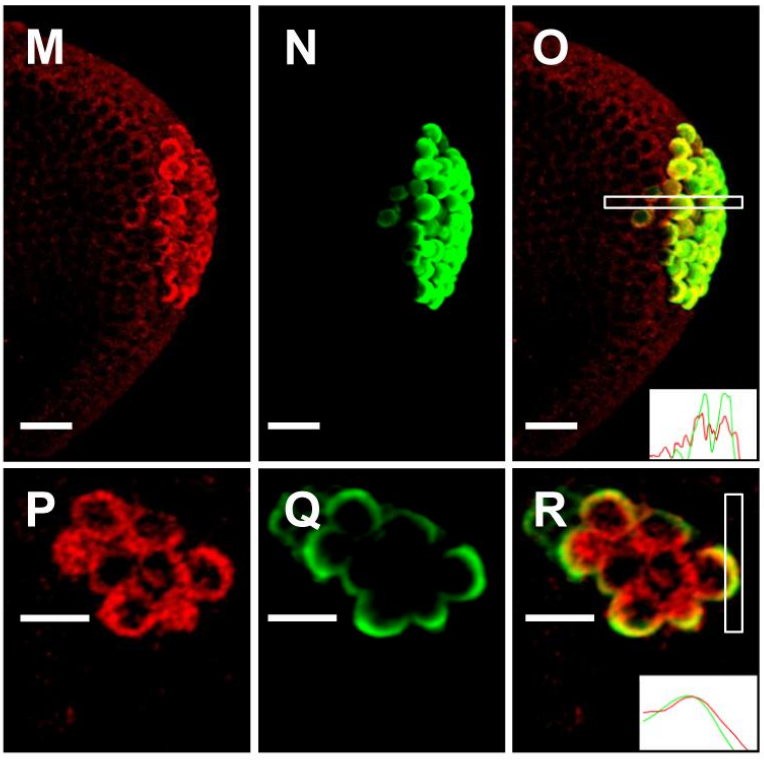
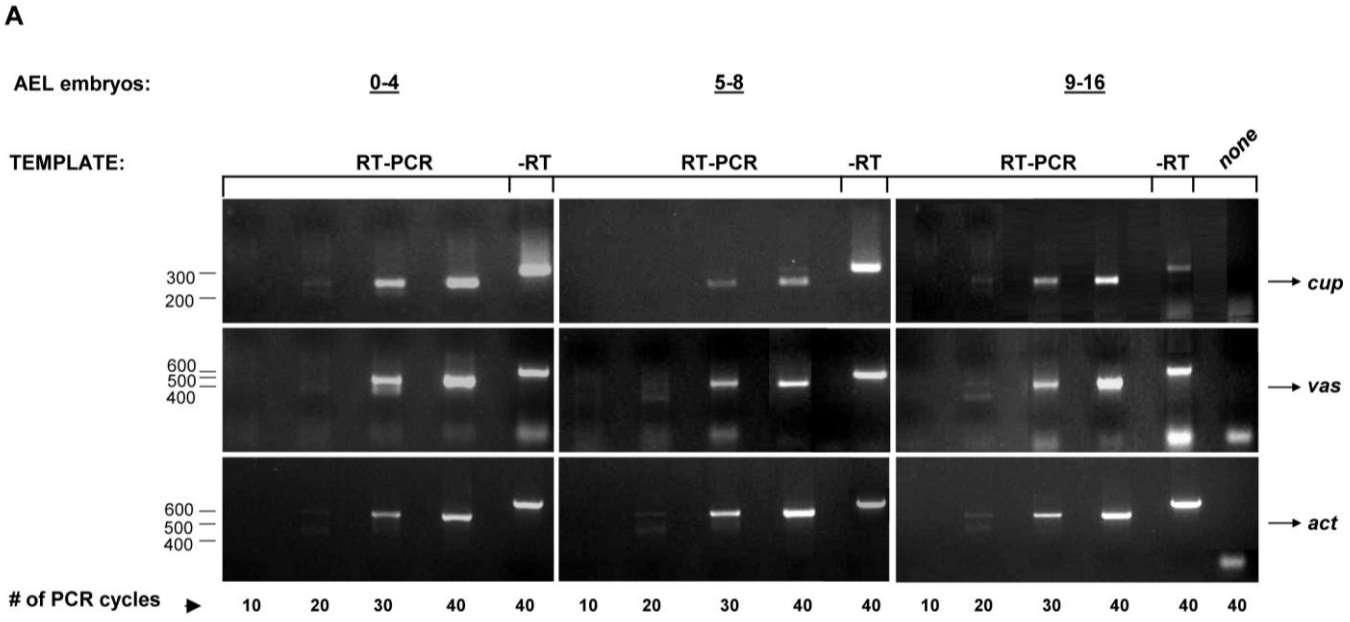

M
N
O
P
Q
R

Figure 22. *cup* gene products localize in germ cells during embryogenesis. (**A-L**) At stage 2, *cup* mRNA and protein are expressed in whole embryos (A,B); at cellularization of the embryo (stage 4), *cup* gene products accumulate in germ cells (D,E), which are marked with anti-Vas antibody (C, F, I, and L). Specific *cup* mRNA and protein signals localize within germ cells in migration (stage10, G,H), and also during stage 14, when the germ cells reach their final destination (panels J,K). The scale bars are 50 µm. (**M-R**) Magnification of Vas positive cells within stage 4 and 14 embryos stained with anti-Cup antibody. The scale bars are 20 µm in (M-O) and 10 µm in (P-R). The inset in the panels O and R show the intensity profile across the posterior pole of the embryo and inside a single germ cell (region of white square) and confirms the overlapping localization.

cup gene product expression during whole embryogenesis was confirmed by semi-quantitative RT-PCR and Western blotting analyses (**Figure 23**). Interestingly, Cup has the same sub-cellular localization displayed by Vas, a well known germ cell marker, within developing germ cells (**Figure 22M-R**).



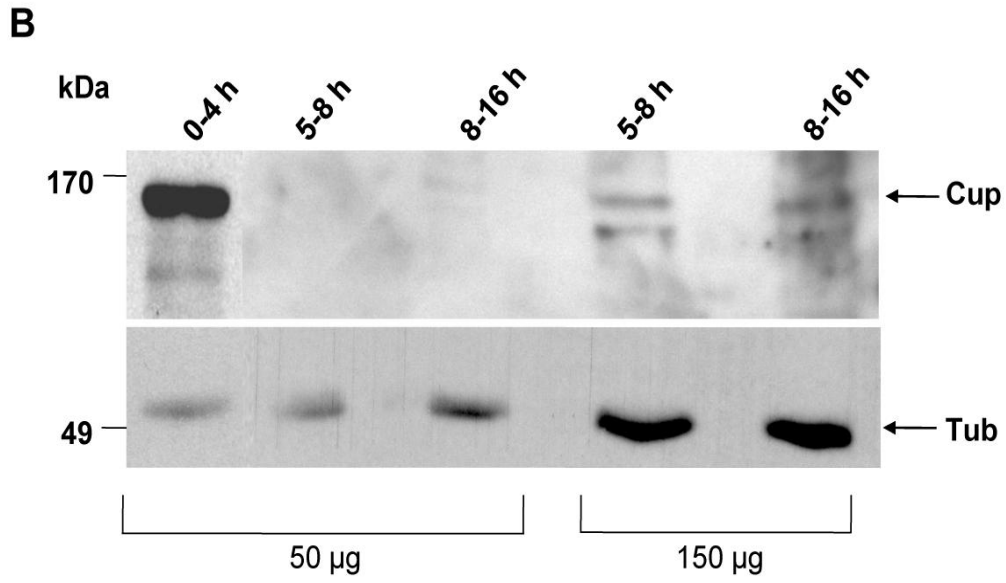


Figure 23. *cup* gene products are expressed in germ cells during embryogenesis. (A) The agarose gel displays RT-PCR products amplified with *cup* (top), *vas* (middle) and *actin42A*- specific primers (bottom). Total RNAs were isolated from wild-type embryos (0-4, 5-8, 9-14 hrs after egg laying (AEL), respectively) and used as templates in RT-PCR reactions. Aliquots were removed after the indicated number of cycles. **(B)** Total protein extracts (50 ng and 150 ng) derived from wild type staged embryos (0-4, 5-8, 9-14 hrs AEL) were analyzed by Western blot using anti-Cup antibody. Note that the amount of Cup protein present during 5-8 and 9-14 hrs AEL embryos is very low compared to the containment of Cup within 0-4 hrs embryos.

To further demonstrate that Cup tightly associates with Osk during germ plasm assembly, I examined whether or not Cup is recruited by Osk to the ectopic site in embryos carrying the *osk-bcd* 3'UTR transgene. It is known that misexpression of Osk

protein at the anterior pole of embryos induces ectopic recruitment of all germ-line factors, thus leading to the formation of completely functional germ cells at the anterior (Ephrussi and Lehmann, 1992). As shown in **Figure 24**, anterior germ cells of *osk-bcd* 3'UTR embryos show expression of Cup protein, simultaneously with Osk. However, both proteins maintain also their posterior localization. Taken together, these data show for the first time that *cup* gene products are expressed throughout embryogenesis, and localize specifically in germ cells, demonstrating a strict functional correlation between Cup and Osk during germ cell assembly.

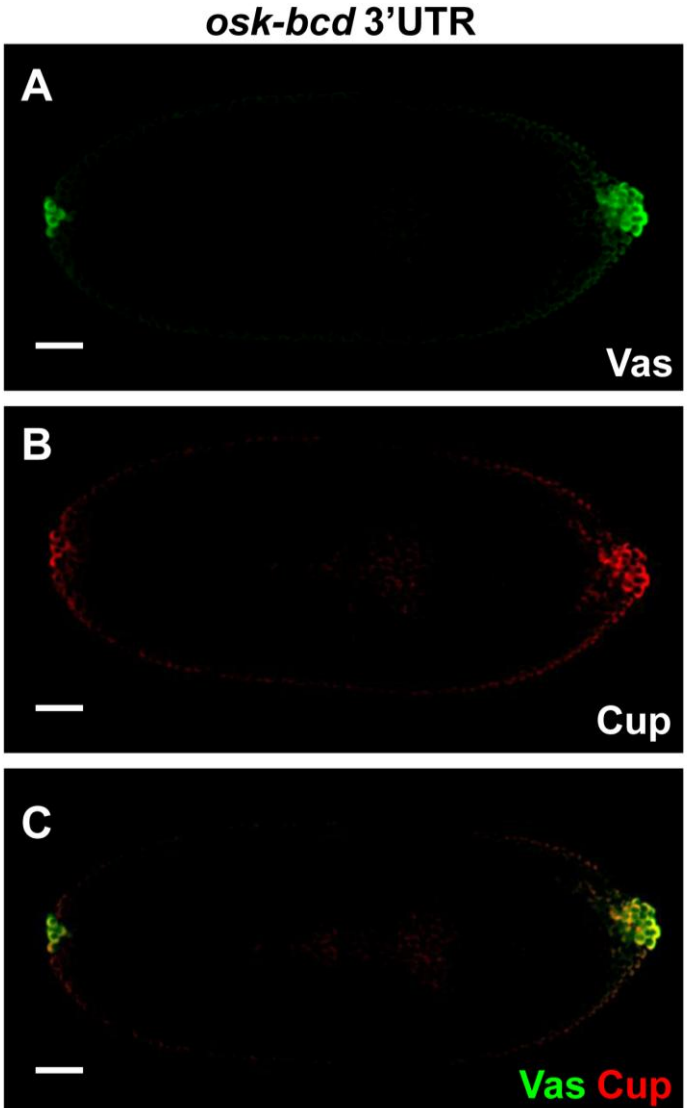
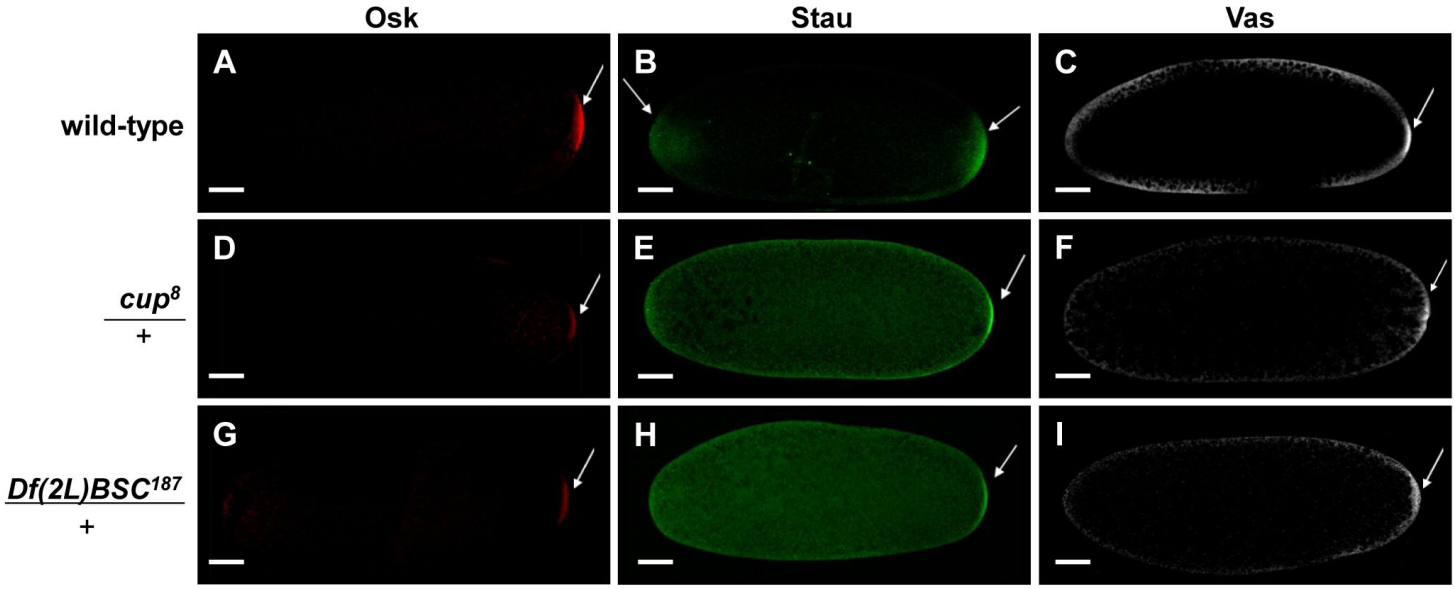


Figure 24. Cup localizes within anterior germ cells of *osk-bcd* mutant embryos. Germ cell staining of cellular blastoderm-stage embryos laid by wild-type females expressing the *osk -bcd* 3'UTR. Germ cells are stained with anti-Vas antibody. Cup localizes in ectopic, anterior germ cells. Note that Cup specific signal is also concentrated into the posterior germ cells (B). Merged image is shown in (C). The scale bars are 50 µm.

5. Reduction of *cup* gene dosage alters posterior localization of Osk, Stau and Vas in embryos

In order to analyze the functional relation between Cup and key germ plasm proteins, such as Osk, Stau, and Vas during early embryogenesis, I performed immunostaining experiments on heterozygous *cup* mutant embryos (0-2 hours AEL). Consistent with the results obtained during egg chamber development (**Figure 25**), heterozygous *cup* mutant embryos (cup^{8}/+ and *Df(2L)BSC187/+*), screened as described in Materials and Methods, also showed a reduced amount of localized Osk, Stau, and Vas at the posterior pole (**Figure 25A-I**; quantification of fluorescence signals is shown in the **Figure 25J**).


Osk
Stau
Vas
wild-type
A
B
C
cup8
+
D
E
F
Df(2L)BSC187
+
G
H
I

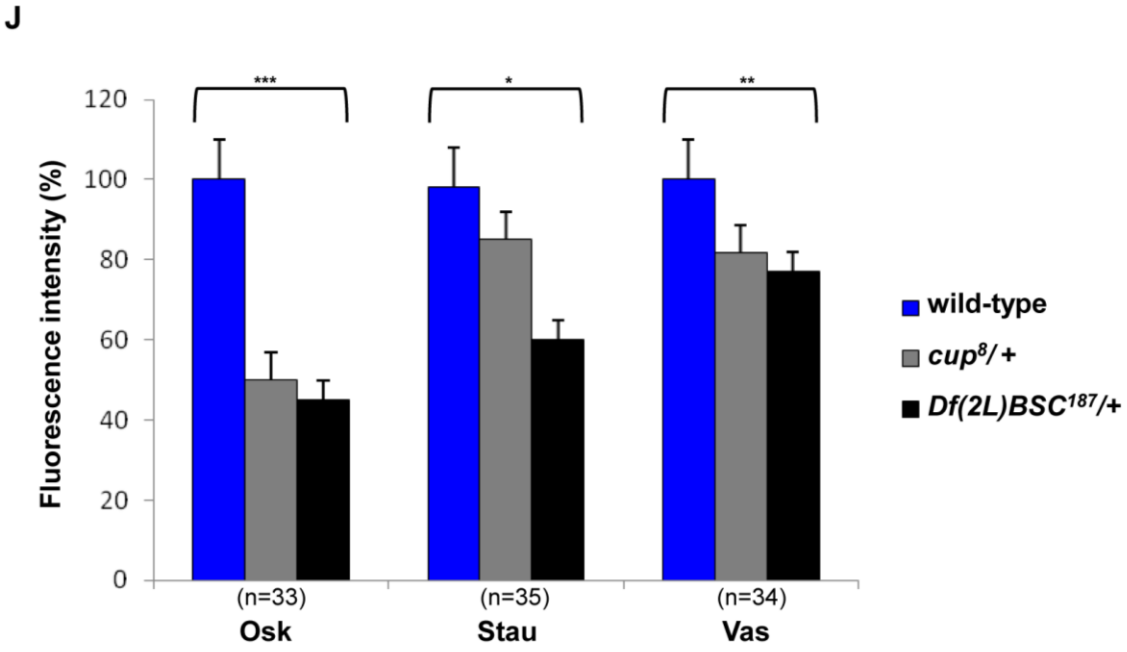

J
Fluorescence intensity (%)
120
100
80
60
40
20
0
***
*
**
(n=33)
(n=35)
(n=34)
Osk
Stau
Vas
wild-type
cup8/+
Df(2L)BSC187/+

Figure 25. Osk, Stau and Vas levels are reduced in heterozygous *cup* mutant embryos. (**A-I**) Wild-type and heterozygous *cup* mutant preblastoderm- embryos (*cup*^{8}/+ and *Df(2L)BSC*^{187}/+) stained with anti-Osk (shown in red; A, D and G), anti-Stau (shown in green; B, E and H) or anti-Vas (shown in grey, C, F and I) antibodies. Compared with wild-type embryos (A-C), Osk, Stau and Vas are reduced at the posterior of the heterozygous *cup* mutant embryos (D-I). Note that Stau fluorescence signal is also distributed throughout the whole cytoplasm of both heterozygous *cup* mutant embryos (E and H). The scale bars are 50 µm in all the panels. (**J**) Quantification of effect shown in (A-I). Comparison of fluorescent signal intensities showed that the reduction of Osk, Stau and Vas is specific and significant at P<0.001 (***), at P<0.05 (*) and <0.01 (**), respectively; Student's *t* test.

Moreover, Stau is diffusely distributed throughout the whole cytoplasm of these embryos. Accordingly, Western blot analysis on total embryo extracts (0-2 hours) showed a significant reduction of Osk and Vas protein levels in heterozygous *cup* mutants when compared to wild-type controls (**Figure 26A,C,D**). On the contrary, Stau levels appeared unchanged (**Figure 26B,D**), thus indicating that, similarly to what occurs in *cappuccino* and *spire* mutants (St Johnston et al., 1991), Stau protein is not properly localized but not degraded. In addition, heterozygous *osk* mutant embryos (0-2 hours) obtained from two different alleles (*osk*^{54} and *Df(3R)p-XT10*) showed a consistent reduction of the total amount of Cup protein (**Figure 26E,F**), strengthening the scenario, where Cup plays a crucial effect *in vivo* on Osk protein accumulation and vice-versa, given that they are interacting molecules of the same germ plasm complex.

Taken together, these results demonstrate that Cup is necessary to guarantee adequate levels of Osk, Stau, and Vas at the posterior pole of embryos, which are essential for functional germ cell assembly.

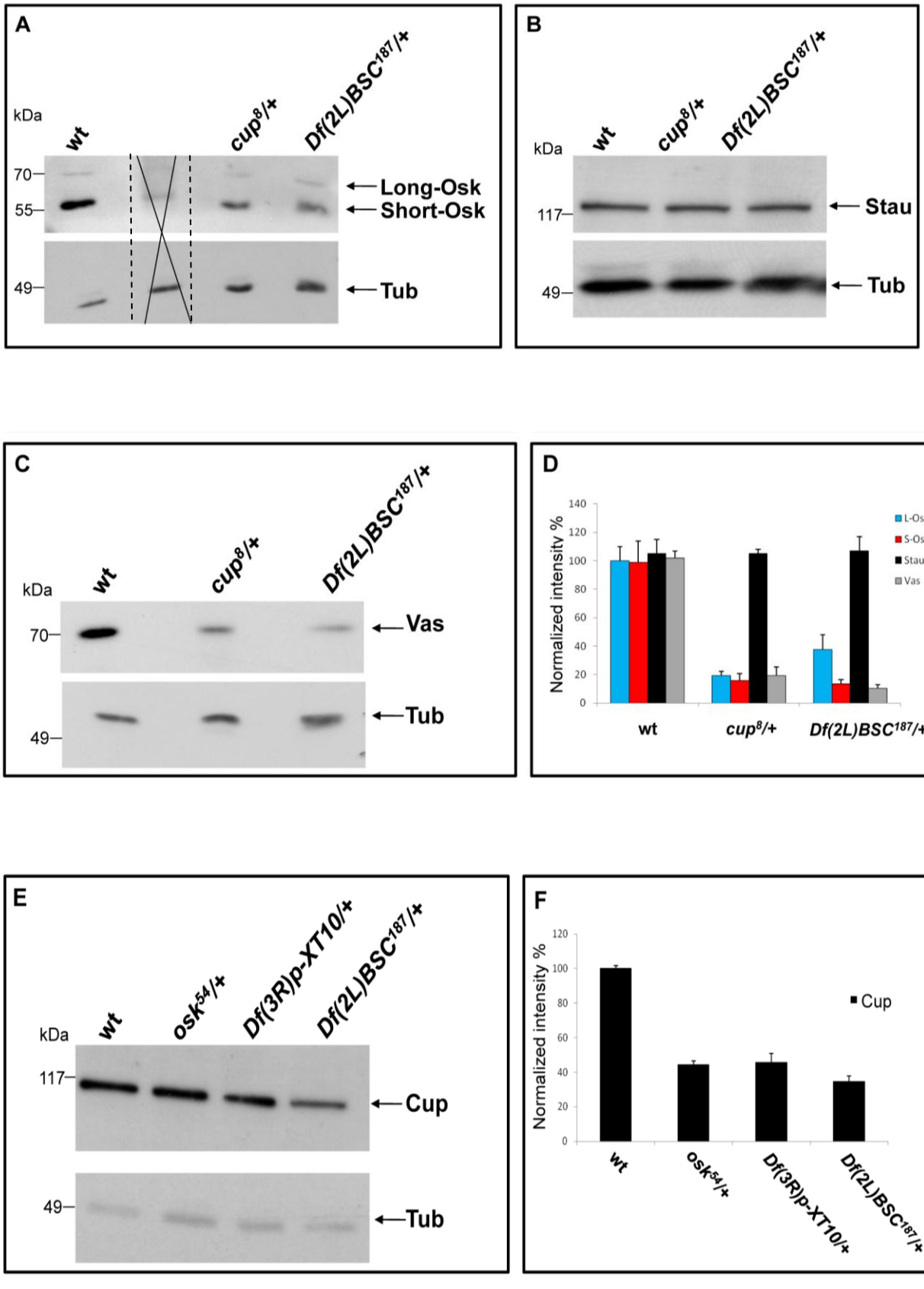
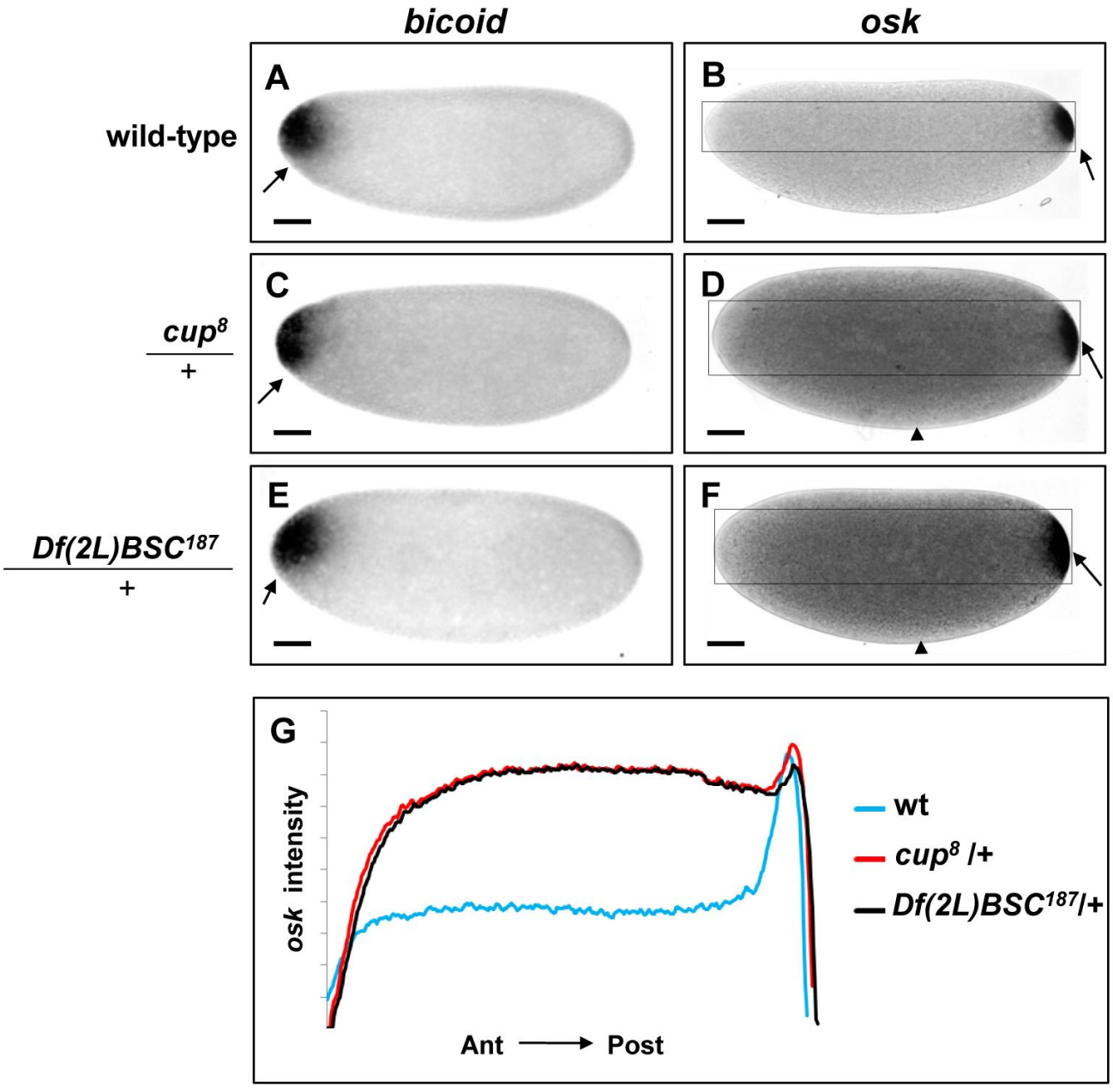

A
kDa
wt
cup8/+
Df(2L)BSC187/+
70
55
49
Long-Osk
Short-Osk
Tub
B
kDa
wt
cup8/+
Df(2L)BSC187/+
117
49
Stau
Tub
C
kDa
wt
cup8/+
Df(2L)BSC187/+
70
49
Vas
Tub
D
Normalized intensity %
140
120
100
80
60
40
20
0
L-Osk
S-Osk
Stau
Vas
wt
cup8/+
Df(2L)BSC187/+
E
kDa
wt
osk54/+
Df(3R)p-XT10/+
Df(2L)BSC187/+
117
49
Cup
Tub
F
Normalized intensity %
120
100
80
60
40
20
0
Cup
wt
osk54/+
Df(3R)p-XT10/+
Df(2L)BSC187/+

Figure 26. Western blot analyses. (**A-C**) Western blot detection of Osk (A, full membrane where the lanes to consider are indicated), Stau (B) and Vas (C) protein in extracts of wild-type and heterozygous *cup* mutant (*cup*^{8}/+ and *Df(2L)BSC*^{187}/+) preblastoderm embryos. α-Tubulin was used as loading control (lower panels). (**D**) Densitometry analysis of each band visualized by Western blot, using Image J software. The quantification showed that the levels of Long and Short Osk isoforms as well as Vas (normalized to Tubulin) were considerably reduced in the heterozygous *cup* mutant compared to those of wild-type embryos (arrows, A and C respectively; quantification is shown in D). On the contrary, the total level of Stau protein were unchanged (arrow, B; quantification is shown in D), confirming that the fluorescence signal observed in whole cytoplasm of the heterozygous *cup* mutant embryos represented probably the amount of Stau protein not correctly localized and not degraded (**Figure 25E,H**). (**E**) Western blot detection of Cup protein in extracts of wild-type and heterozygous *osk* mutant (*osk*^{54}/TM3 and *Df(3R)p-XT103*/TM3) preblastoderm embryos. I also used protein extract obtained from heterozygous *Df(2L)BSC*^{187} preblastoderm embryos, carrying one copy of *cup* gene as internal control. α-Tubulin was used as loading control (lower panel). (**F**) Densitometry analysis of each band visualized by Western blot. The quantification showed that Cup levels (normalized to Tubulin) were appreciably reduced in the heterozygous *osk* mutant compared to those of wild-type embryos (arrows, E; quantification is shown in F).

6. *osk* mRNA expression and localization are affected in heterozygous *cup* mutant embryos.

To understand whether or not the reduction of Osk protein described above is due to alteration of *osk* mRNA, I performed *in situ* hybridization experiments on 0-2 hours embryos. In both wild-type and heterozygous *cup* mutant embryos, I detected, in addition to an *osk* mRNA signal at the posterior pole (**Figure 27B,D,F**), a gradient of the mRNA extending from the posterior to the whole cytoplasm of the embryos (**Figure 27D,F**). Accordingly, quantitative RT-PCR experiments (**Figure 27G**, panel on the right) revealed

a significant increase of total levels of *osk* mRNA in heterozygous *cup* mutant embryos (0-2 hours) when compared to those in wild-types. By contrast, the total amount of *bcd* mRNA (**Figure 27G**) and its localization pattern remained unchanged (**Figure 27A,C,E**), demonstrating that the reduction of Cup activity specifically affects *osk* mRNA expression and localization.



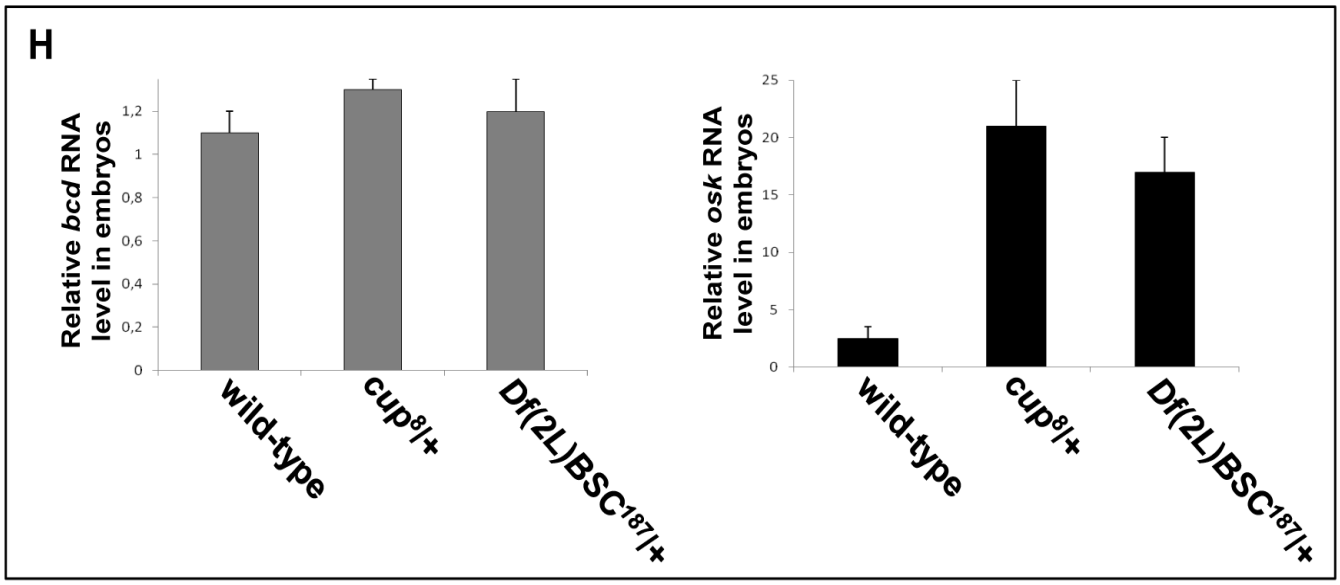
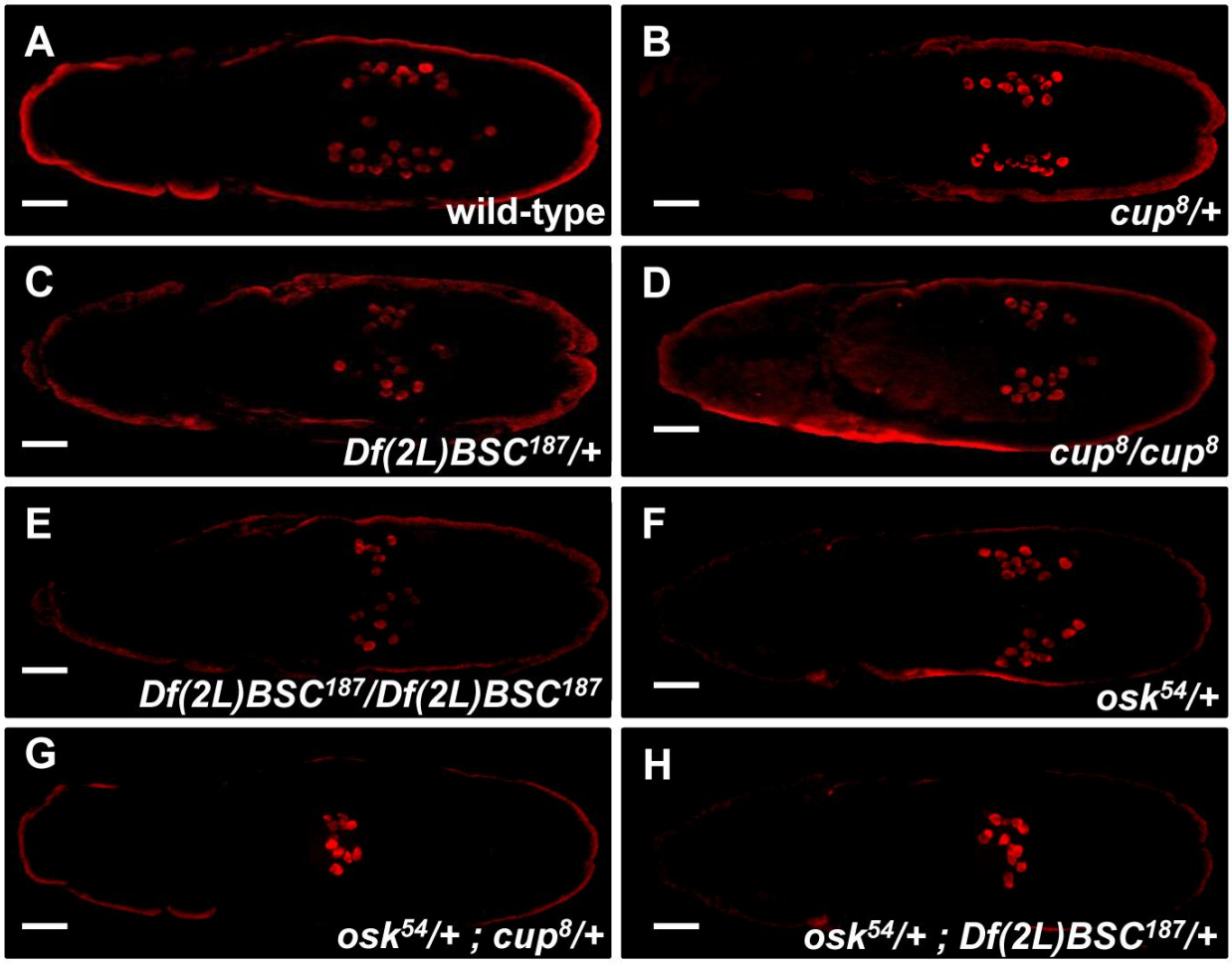


Figure 27. *osk* mRNA levels are affected in heterozygous *cup* mutant embryos. (**A-F**) *In situ* hybridization experiments on wild-type and heterozygous *cup* mutant preblastoderm embryos (*cup*^{8}/+ and *Df(2L)BSC*^{187}/+). *osk* mRNA is localized at the posterior pole of heterozygous *cup* mutant (D and F), as in wild-type embryos (B). Note that *osk* mRNA signal is also distributed throughout the whole cytoplasm of heterozygous *cup* mutant compared with wild-type embryos (D, F and B, respectively). On the contrary, *bicoid* (*bcd*) mRNA is correctly localized at the anterior pole of heterozygous *cup* mutant, as in wild type embryos. The scale bars are 50 µm in all the panels. (**G**)**. Intensity profile of *osk* mRNA intensity.** Note that *osk* signal is detected from the anterior to posterior pole of wild-type and *cup* mutant embryos. **(H) *osk* mRNA levels are increased in heterozygous cup mutant embryos.** RT-PCR quantification of *osk* mRNA normalized to *rp49* mRNA shows a strong increase of total *osk* mRNA levels in heterozygous *cup* mutant compared with the level in wild-type preblastoderm- embryo (right panel). On the contrary, total *bcd* mRNA levels were unchanged in both wild-type and *cup* mutant preblastoderm embryos (left panel).

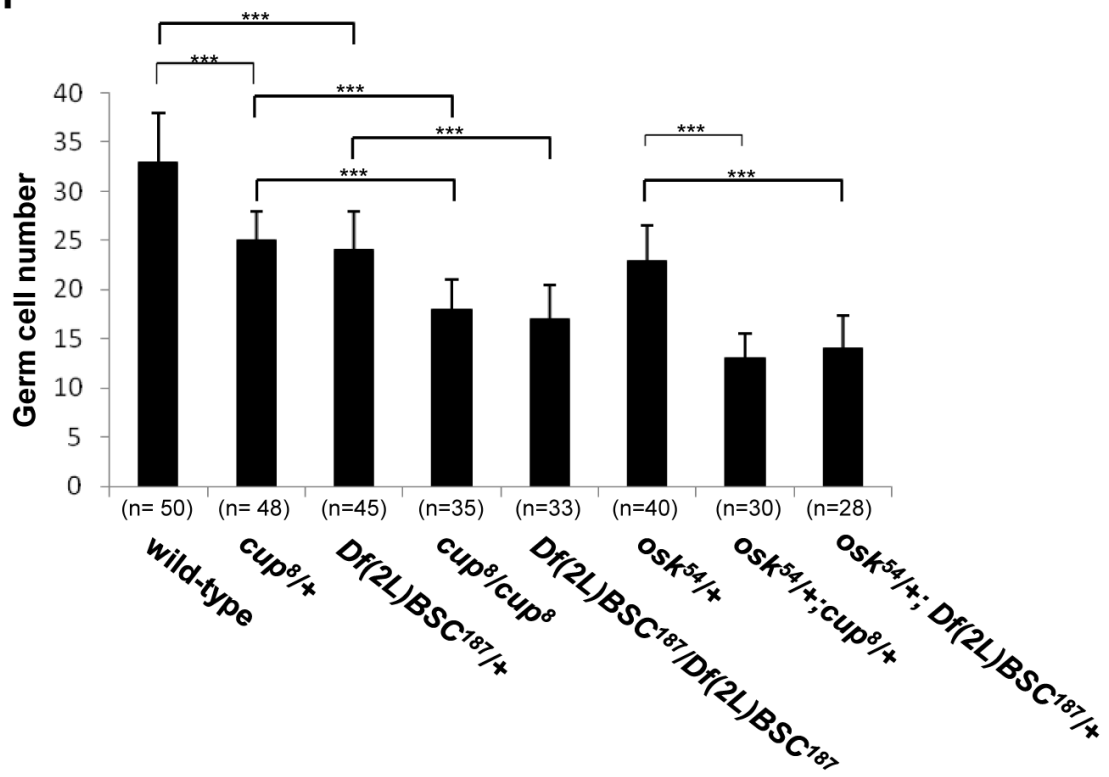
7. *cup* is required for germ cell formation and interacts genetically with *osk*

In *Drosophila* embryos, germ cell number depends on proper germ plasm accumulation during oogenesis. To investigate if Cup plays a pivotal role during germ cell development, embryos derived from heterozygous *cup* mutant females (*cup*^{8} and *Df(2L)BSC187*) were collected and the germ cells visualized by staining with anti-Vas antibody. Heterozygous *cup* mutant embryos have a reduced average number of 25 cells, instead of the canonical 33 cells present in wild type embryos (**Figure 28A-C**). This number is further decreased in homozygous *cup* mutant embryos (**Figure 28D,E**). These results appear to be a direct consequence of maternal germ plasm determinant reduction observed at the posterior pole of heterozygous *cup* mutant ovaries and early embryos.

To demonstrate if *cup* and *osk* genetically interact, I further analyzed double heterozygous *cup-osk* mutant embryos: whereas *osk*^{54}/+ and *cup*/+ heterozygous mutant embryos display an average of 23/25 germ cells (Ephrussi et al., 1991; Lehmann and Nusslein-Volhard, 1986; **Figure 28B,C,F**), double heterozygous *cup* and *osk*^{54} mutant embryos have only an average of 12 germ cells (**Figure 28G,H**). Quantification of germ cell average number is shown in **Figure 28I**.


A
wild-type
B
cup8/+
C
Df(2L)BSC187/+
D
cup8/cup8
E
Df(2L)BSC187/Df(2L)BSC187
F
osk54/+
G
osk54/+ ; cup8/+
H
osk54/+ ; Df(2L)BSC187/+

I


Germ cell number
0
5
10
15
20
25
30
35
40
***
***
***
***
***
***
***
(n= 50)
(n= 48)
(n=45)
(n=35)
(n=33)
(n=40)
(n=30)
(n=28)
wild-type
cup8/+
Df(2L)BSC187/+
cup8/cup8
Df(2L)BSC187/Df(2L)BSC187
osk54/+
osk54/+;cup8/+
osk54/+; Df(2L)BSC187/+

J

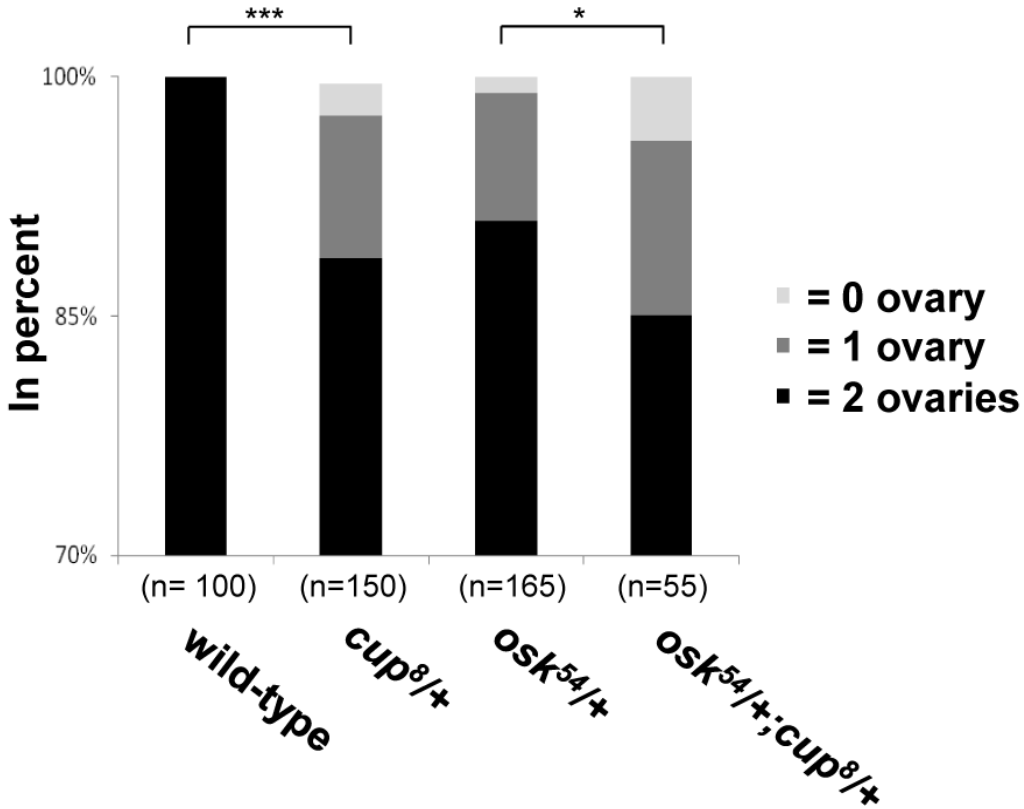

***
*
100%
85%
70%
In percent
(n= 100)
(n=150)
(n=165)
(n=55)
wild-type
cup8/+
osk54/+
osk54/+;cup8/+
= 0 ovary
= 1 ovary
= 2 ovaries

Figure 28. *cup* assures germ cell development and interacts genetically with *osk*. (**A-I**) Germ cells of stage 11 embryos of indicated genotypes were stained with anti-Vas antibody (A-H) and counted (I). The number of germ cells in heterozygous *cup* mutant embryos (cup^{8}/+ and $Df(2L)BSC^{187}$/+; 24 cells on average; B and C) was reduced when compared with the number observed in wild-type embryos (33 cells on average; A). Homozygous *cup* mutant embryos (cup^{8}/ cup^{8} and $Df(2L)BSC^{187}$/ $Df(2L)BSC^{187}$) showed a further reduction of the germ cell number (18 cells on average; D and E). The number of germ cells in osk^{54} /+; cup^{8}/+ and osk^{54} /+; $Df(2L)BSC^{187}$/+ (12 cells on average; G and H) was half that of the osk^{54} /+ embryos (23 cells on average; F). P<0,001(***); Student's *t* test. (**J**) The grandchildless phenotype (absence of ovaries) was quantified by dissecting female offspring of the indicated genotypes. cup^{8}/+ females display a strong grandchildless phenotype compared with wild-type females (*** statistically significant at P<0.001; $\chi 2$). osk^{54} /+; cup^{8}/+ females showed a weak but significant grandchildless phenotype when compared with osk^{54} /+ females (* statistically significant P<0.05; $\chi 2$)

Finally, females derived from heterozygous cup^{8} mutant alleles, similarly to osk^{54}, show a penetrant grandchildless phenotype, a condition when mutant females produce sterile progeny. 9% of *cup* heterozygous mutant female offsprings display only a single ovary, and 2% have a complete absence of ovaries. By contrast, progeny of double heterozygous *cup* and *osk* females show a low but significant penetrance of the grandchildness phenotype (**Figure 28J**).

Taken together, these experiments demonstrate the role *in vivo* of *cup-osk* interaction during embryogenesis and suggest that *cup* is necessary to maintain proper germ cell number during late embryo development.

Discussion

In this thesis, I've discuss a novel role of Cup during oogenesis and embryonic development. Cup is expressed in all germ line cells during egg chamber development and localizes to both nuage and germ plasm. Moreover, Cup physically interacts with Osk and Vas to guarantee the anchoring and stabilization of germ plasm at the posterior cortex of oocytes. As a consequence, embryos laid by heterozygous *cup* mutant mothers display a reduced number of germ cells. Finally, I demonstrated that *cup* and *osk* genetically interact, since reducing *cup* gene dosage in heterozygous *osk* mutant embryos further decreases the number of germ cells.

1. Cup localizes within nuage

In *Drosophila* ovary, three classes of ribonucleic nuclear proteins (RNPs) have been distinguished on the basis of their localization on morphological structures, such as nuage, sponge bodies, and polar granules.

Nuage particles are Tudor, Vas, and Gustavus positive (Bardsley et al., 1993; Hay et al., 1988a; Hay et al., 1988b; Styhler et al., 2002), which are accumulated as large aggregates around the outer membrane of nurse cell nuclei (Saffman and Lasko, 1999). Sponge bodies are Exuperantia, Me31B, and Gustavus positive and are composed of electron-dense matrix of ER-like vesicles localized in the nurse cell and, even if to a lesser degree, in the oocyte cytoplasm (Wilsch-Brauninger et al. 1997; Nakamura et al 2001; Styhler et al., 2002). Polar granules are Osk, Vas, Stau, and Tudor positive and assembled in RNP particles, including *osk* mRNA, mitochondrial ribosomal RNA, and polysomes. These granules are specifically localized at the posterior end of *Drosophila* oocytes and early embryos and are associated with determinants of the germ-line and posterior

structures (Breitwieser et al., 1996; Hay et al., 1988b; Bardsley et al., 1993; St Johnston et al., 1991; Kobayashi et al., 1993).

Previous studies demonstrate that Cup is a component of sponge bodies (Snee and Macdonald, 2009) and polar granules (Thomson et al., 2008). The distribution pattern I observed indicates that Cup is also a component of the nuage where it co-localizes with Tud (**Figure 15**).

This evidence is also supported by several studies demonstrating that nuage, sponge bodies and polar granules contain shared components. For example, nuage cytoplasmic foci appear to be enriched of mRNA degradation proteins, such as dDCP1 and dDCP2 (Lin et al., 2008; Lim at al., 2009), as well Me31B (Coller et al., 2001; Lim at al., 2009), which has been found to colocalize with eIF4E and Cup (Wilhelm et al., 2003; Nakamura et al., 2004). Both dDCP1 and Me31B, together with the polysomal apparatus, are also components of the *osk* mRNP complex in the oocyte (Nakamura et al., 2001; Braat et al., 2004) and are also components of the polar granules.

Several studies demonstrated that nuage mRNP particles are first assembled into the nucleus of nurse cells and are involved in different cellular processes, such as splicing, nucleo-cytoplasmic mRNA export and regulation of mRNA translation. In this model, the nuage particles might also contain shuttle proteins and RNAs that move between the nucleus and cytoplasm (Findley et al., 2003; Kloc et al., 2005). Accordingly, Hachet and Ephrussi (2004) demonstrated that the splicing of *osk* mRNA in the nucleus is coupled to its cytoplasmic localization: In particular, the nuclear shuttling proteins Y14/Tsunagi and Mago nashi, which are core components of the exon–exon junction complex (EJC), are required for splicing and in turn for *osk* mRNA cytoplasmic localization. It has been demonstrated that Cup is a nucleo-cytoplasmic protein (Zappavigna et al., 2004) and

interacts genetically with Nup154 (Grimaldi et al, 2007), an evolutionary conserved nuclear pore complex protein (NPC), thus further suggesting a participation of Cup in nucleo-cytoplasmic shuttling activities involving proteins and/or mRNAs.

This finding, together with recent identification of Bruno as a new element of nuage (Snee et al., 2007), supports the idea that translational regulation events are coupled with formation and/or reorganization of RNP complexes in nuage particles.

From all these results it appears likely that nuage particles and sponge bodies are in fact assembled by heterogeneous but related RNPs that differ in their molecular composition and may be dynamic in their structures (Decker and Parker, 2006; Lin et al., 2006).

2. Cup plays a role in germ plasm assembly

Germ plasm assembly is a stepwise process occurring during oogenesis. Accumulation of *osk* mRNA at the posterior of egg chambers is necessary for correct germ plasm assembly, which requires a polarized microtubule network, the plus-end motor kinesin I. and the activity of several genes (*capu, spire, par-1. mago nashi, barentz, stau, tsunagi, rab11 and vls*; St. Johnston, 1991; Wellington et al., 1999; Mohr et al., 2001;Hachet and Ephrussi, 2001; van Eeden et al., 2001; Dollar et al., 2001; Anne et al, 2005). Localization of *osk* mRNA is strictly linked to the control of its translation, as unlocalized *osk* mRNA is silent. Upon localization at the posterior pole, the relieve of *osk* translational repression involves several factors, including Orb, Stau, and Aubergine (Chang et al., 1999; Micklem et al., 2000; Harris and Macdonald, 2001). Localized Osk protein, in turn, triggers a cascade of events that results in the recruitment of all factors

necessary for establishment of functional germline structures, such as Vas, Tud, Stau proteins and *nanos*, *germ less* mRNAs (Mahowald, 2001). Posterior anchoring of Osk requires the functions of Vas (Breitwieser et al., 1996) and Tudor (Thomson and Lasko, 2004), as well as Osk itself (Ephrussi et al., 1991; Kim-Ha et al., 1991; Markussen et al., 1995) to direct proper germ plasm assembly. Misexpression of Osk at the anterior pole of oocytes causes ectopic pole plasm formation (Ephrussi and Lehmann, 1992), indicating that Osk is the key organizer of pole plasm assembly. Moreover, it has been demonstrated that endocytic pathways acting downstream of Osk regulates F-actin dynamics, which in turn are necessary to anchor pole plasm components to the oocyte cortex (Tanaka and Nakamura, 2008).

As far as Cup is concerned, it has been demonstrated that Cup is engaged in translational repression of unlocalized mRNAs, such as *osk* (Wihlem et al., 2003; Nakamura et al., 2004), *gurken* (*grk*) (Clouse et al., 2008) and *cyclinA* (Sagimura and Lilly 2006) during early oogenesis.

The results shown in this thesis establish that Cup is also a novel germ plasm component. First, Cup co-localizes with Osk (Wilheim et al 2003, Nakamura 2004), Stau (Piccioni et al., 2009), and Vas (this work; **Figure 16**) at the posterior pole of stage 10B oocytes. Second, biochemical evidences indicate that Cup directly interacts with Osk, Stau (Piccioni et al. 2009), and Vas (**Figure 17**), and that *osk* mRNA and the actin cytoskeleton structures (**Figure 21**) are dispensable for Osk binding. These results are in agreement with those reported by Breitwieser et al. (1996), where Vas localization occurs not through its association with localized RNAs, but rather through Osk protein interaction, which in turn represents an essential step in polar granule assembly.

As a consequence of these interactions, Cup protein is mislocalized in *osk* and *vas* mutant stage 10 oocytes (**Figure 18,19**), demonstrating that Osk and Vas are essential to achieve a correct localization of Cup at the posterior cortex of stage 10 oocytes. This study suggests that the presence of Cup, Osk, Stau and Vas are needed for a correct germ plasm assembly. Moreover, several immuno-precipitation experiments using anti-Tud and anti-Vas antibodies identified numerous P-body related proteins, including Cup, as novel polar granule components (Thomson et al., 2008).

All of this results suggest that Cup plays at least two different roles at stage 10 of oogenesis: Cup represses translation of unlocalized *osk* mRNA (Nakamura et al., 2004) and is also necessary to anchor and/or maintain Stau, Osk, and Vas at the posterior cortex (**Figure 20**). This novel function of Cup is supported by the findings that, when *cup* gene dosage is reduced, Stau, Osk, and Vas are partially anchored and/or maintained at the posterior pole, even if these proteins are not degraded. Consequently, pole plasm assembly is disturbed and *cup* mutant females lay embryos with a reduced number of germ cells. Since the role of Cup, a known multi-functional protein during the different stages of egg chamber development, cannot be easily studied in homozygous *cup* ovaries, it is not surprising that the involvement of Cup in pole plasm assembly remained undiscovered until now (**Figure 29**).

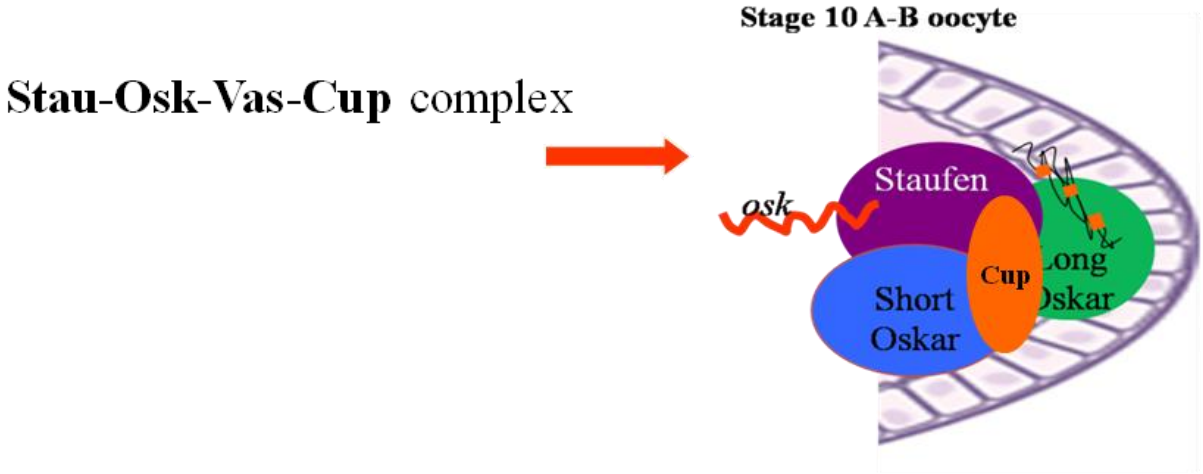


Figure 29. Cup anchors the germ plasm at posterior pole stage 10 oocyte.

3. Cup plays a role during germ cell formation.

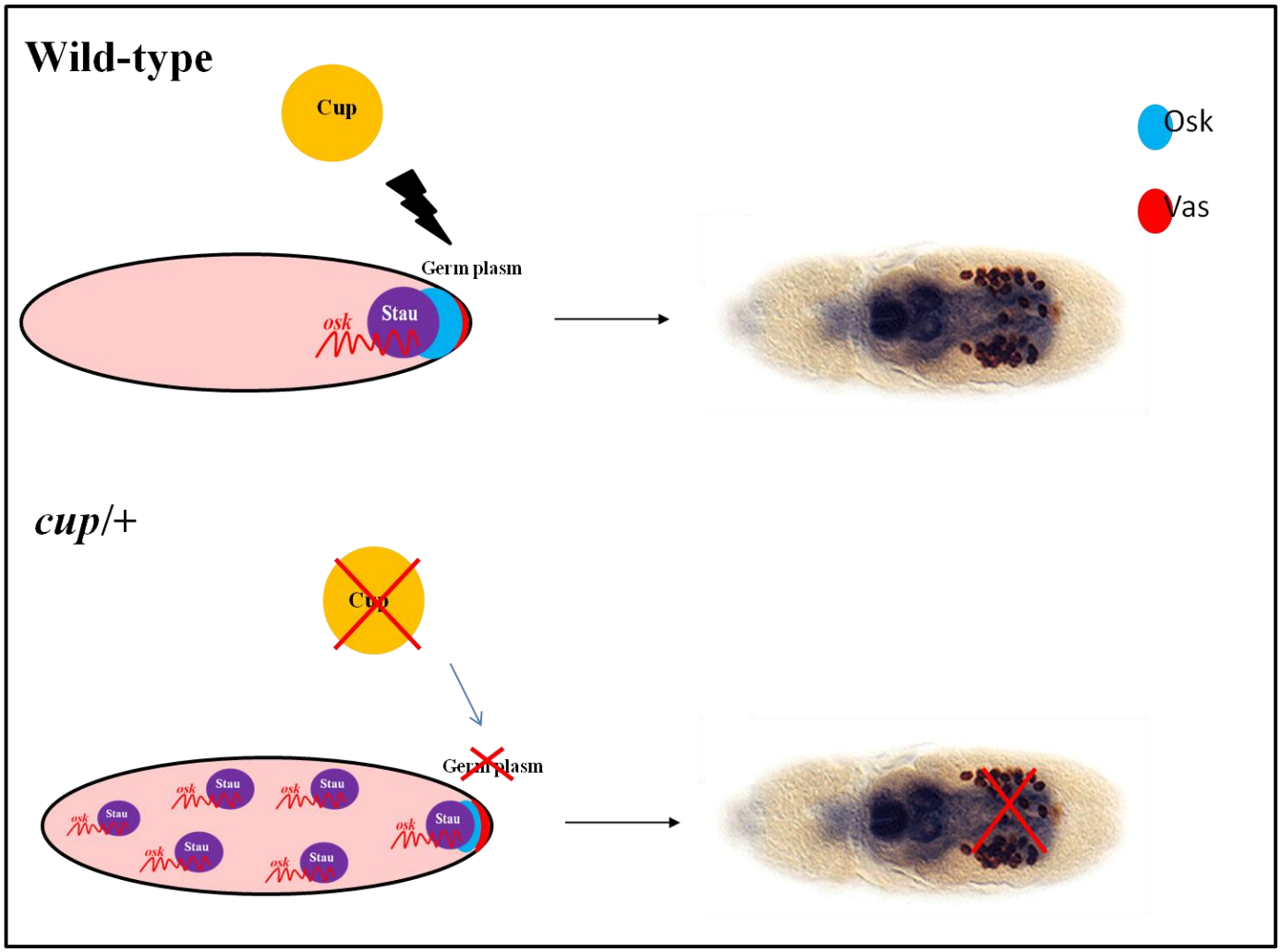
During embryogenesis, Cup exerts similar functions. In particular, Osk, Stau, and Vas proteins and *osk* mRNA are not properly maintained and/or anchored at the posterior pole of embryos laid by heterozygous *cup* mutant mothers (**Figure 30A**).

In addition, reduced levels of Osk and Vas are detected in total embryo extracts. For these reasons, I cannot exclude a direct/indirect involvement of Cup in the activation of *osk* mRNA translation. In this scenario, Cup could act more like an adapter protein involved in the translational regulation of *osk* mRNA, probably through interactions with specific binding proteins, as Bruno for the repression and Stau, Vas and Osk itself for the activation, respectively (**Figure 30B**). In agreement with this hypothesis, it has been demonstrated that Stau mediates its function in *osk* translation through protein-protein

interactions (Micklem et al., 2000) and Vas, as well as Osk, is required for efficient activation of *osk* translation (Webster 1997; Gunkel, 1998).

In addition, Gunkel et al. (1998) demonstrated that *osk* translation requires a functional interaction between its 5′ and 3′ ends, and in this scenario Cup could act like a bridge to raise *osk* mRNA circularization and then its translation

A



B

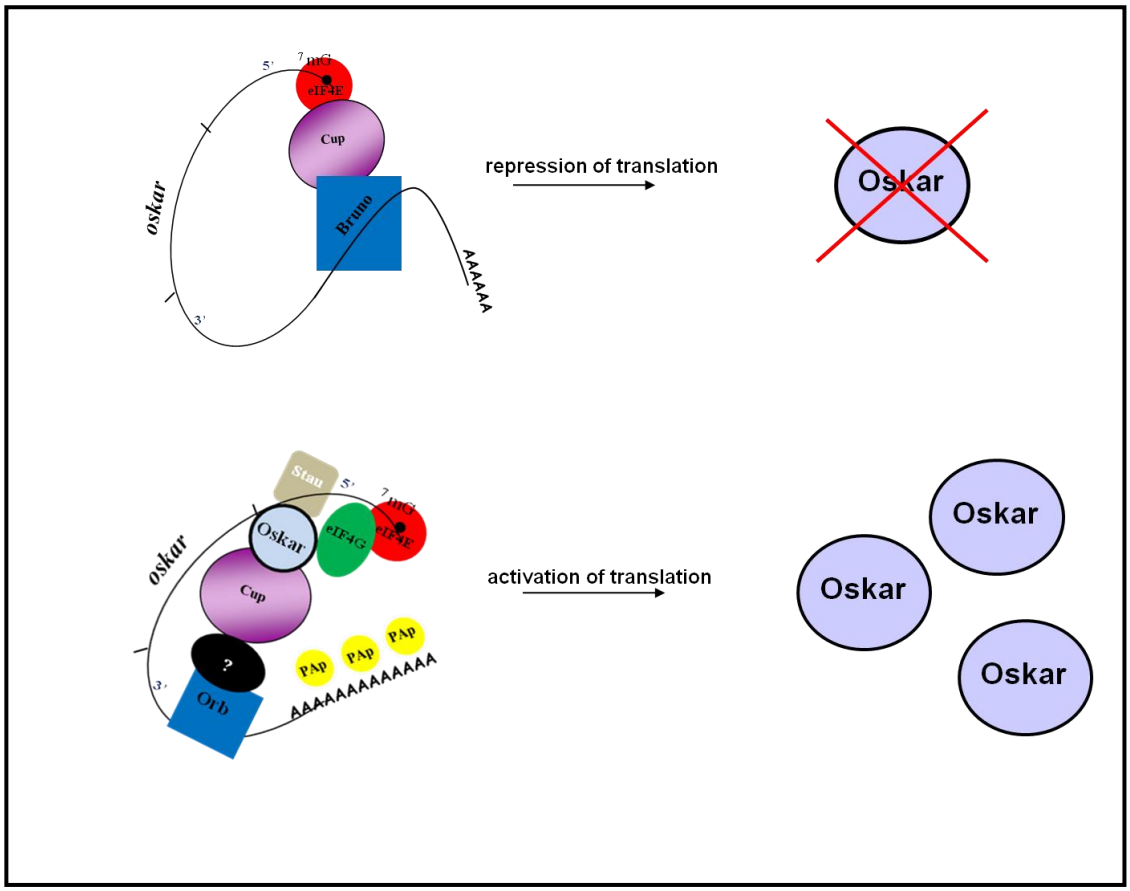


Figure 30. Hypothetic mechanisms of Cup during germ cell formation. (**A**) In wild-type embryos, Cup anchors and/or maintains germ plasm at posterior cortex, thus the process of germ plasm formation is properly activated and a correct germ cell number is produced. In heterozygous *cup* mutant embryos, germ plasm is destabilized and Stau-*osk* complexes appear not anchored to the posterior cortex and distributed in whole cytoplasm. (**B**) In this panel a probable role of Cup in *osk* translation is shown. (**Upper**) During the transport to the posterior pole of the oocyte, Cup represses *osk* translation via interaction with eIF4E and Bruno. (**Lower**) At the stage 10 of oogenesis, *osk* is localized at posterior pole and its translation starts: in this place, Cup forms a more stable complex with Osk, which in turn interacts with Stau and Vas, and a not identified factor that binds the 3'UTR, to stimulate Osk protein production.

Surprisingly, *osk* mRNA is increased in heterozygous *cup* mutant embryos. Since *osk* mRNA requires adequate Osk protein to remain tightly linked at the posterior cortex (Markussen et al., 1995; Rongo et al., 1995), the reduced amount of Osk protein observed in heterozygous *cup* embryos, should be not sufficient to maintain all *osk* mRNA to the embryonic pole and could stimulate, by positive feedback, *de novo osk* mRNA synthesis (**Figure 30A,B**).

Furthermore, I also cannot exclude a direct/indirect involvement of Cup in *osk* mRNA degradation (Lin et al., 2006) According to this latest hypothesis, it has been demonstrated the existence of dDcp1/Me31B on the *osk* mRNP complex that may contribute to the embryonic degradation of *osk* mRNA. One possibility is that an unknown factor, like Cup, can trigger the rapid degradation of *osk* mRNA at the maternal-zygotic transition in early embryogenesis, Instead of assembling a degradation complex de novo, the association of dDcp1/ Me31B with Cup may facilitate the degradation of *osk* mRNA during the maternal-zygotic transition (Lin et al., 2006) (**Figure 31**).

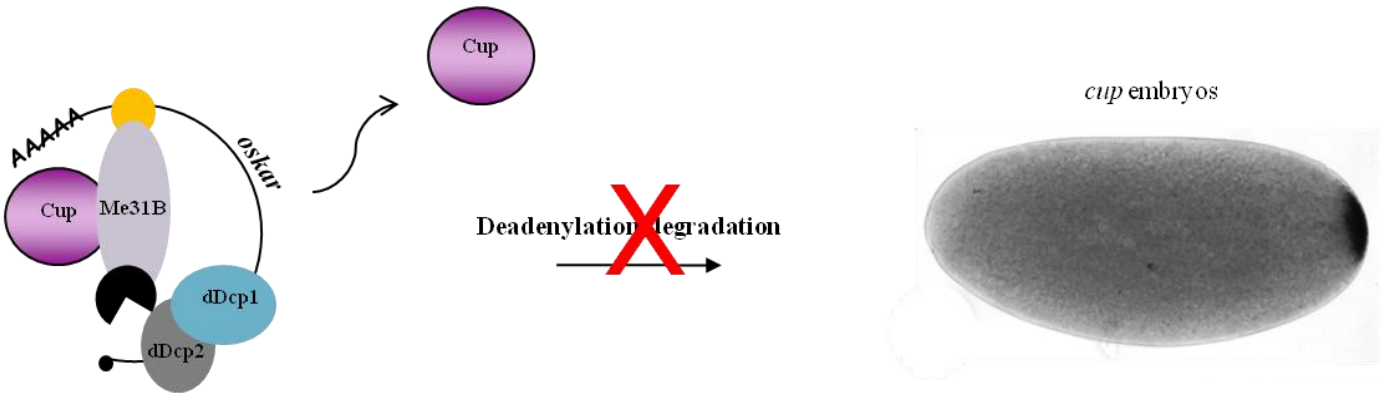


Figure 31. Cup could be involved in the *osk* mRNA degradation. Cup colocalizes with Me31B and dDcp1 in discrete cytoplasmic foci in nurse cells; dDcp1 is required for the degradation of several maternal mRNAs and is colocalized with dDcp2. In addition to dDcp1, both Me31B and the polysomal apparatus are also components of the *osk* mRNP complex in the oocyte. Both dDcp1 and Me31B are evolutionarily conserved decapping factors in P bodies and are present in *osk* mRNP at stage 10; moreover, it has been demonstrated that Cup, Me31B, Dcp1, Dcp2 are part of complex (Tritschler et al., 2008), Considering the results shown in this thesis, it is possible hypothesize that Cup could influences the status of *osk* mRNA, which can potentially shift from translation repression to degradation.

The findings that Cup has been found together with Osk when Osk is ectopically localized to the anterior pole of the embryos and that reducing *cup* copy number further decreases the total number of germ cells observed in heterozygous *osk* mutant embryos, strengthen the involvement of Cup in germ cell formation and/or in maintenance of their identity.

4. Does Cup play a role during germ cell maintenance?

Unlike Osk protein, both *cup* mRNA and protein have been detected within germ cells until the end of embryogenesis. These observations suggest that zygotic *cup* function in germ cell formation and maintenance are not limited to those carried out with Osk.

It has been demonstrated that specific features ensure the proper development of the germline: in fact, embryonic primordial germ cells are characterized by their transcriptional quiescence, mitotic arrest and extensive migration to the somatic gonadal sites.

In the past years, several studies have shown the essential role of *nanos* as a cell autonomous factor in the migration and transcriptional quiescence of embryonic primordial germ cells. Nos, a CCHC zinc-finger protein, has been identified as the critical factor both for pole cell differentiation and abdomen formation (Lehmann and Nüsslein-Volhard, 1991; Kobayashi et al., 1996; Forbes and Lehmann, 1998). Maternally transcribed *nos* mRNA is concentrated in the polar plasm at a late stage of oogenesis via the actions of *osk* and *vas*. After egg laying, it is translated in situ to form a Nos protein gradient with the highest concentration in the polar plasm (Ephrussi and Lehmann, 1992; Thomson and Lasko, 2004). The Nos gradient then specifies the abdomen by repressing the translation of maternal *hunchback* (*hb*) mRNA, which otherwise inhibits abdomen formation. Nos protein is only transiently present in the abdominal anlage, however, and becomes undetectable by the cellular blastoderm stage. In contrast, Nos protein in the polar plasm is incorporated into the pole cells and remains detectable throughout pole cell migration (Wang et al., 1994).

In the pathways leading to abdomen formation, Nos protein acts in concert with the RNA binding protein Pumilio (Pum), which is distributed ubiquitously in the embryo, to repress translation of maternal *hb* mRNA. Translational repression of *hb* is mediated by discrete target sites known as *nos* response elements (NREs) in its 3' UTR. Pum binds directly to the *hb* NREs in a sequence-specific manner, and the interaction of Nos with Pum is essential for the translational repression of *hb*. In pole cells, Pum, in a similar manner to Nos, is autonomously required for pole cell migration. Thus, Nos acts together with Pum to regulate germline specific events in pole cells by repressing the translation of specific transcripts in these cells.

One of the regulatory targets of both Nos and Pum in pole cells is maternal *cyclin B* (*cycB*) mRNA, which contains NRE-like sequences within its 3' UTR. This transcript is localized in the polar plasm and is partitioned into the pole cells, but its translation is repressed until the pole cells reach the gonads (Dalby and Glover, 1993). Consistent with this observation, pole cells cease mitosis at gastrulation and remain quiescent in the G2 phase of the cell cycle, whereas somatic cells continue to proliferate. Moreover, in embryo lacking either Nos or Pum, the migrating pole cells produce CycB, and are then released from G2 arrest and enter into mitosis. Furthermore, the induction of CycB in wild-type pole cells is sufficient to drive them from the G2 phase through mitosis and into G1. In addition, Nos and Pum bind *cycB* mRNA in NRE-dependent manner (Wharton et al., 1998; Sonoda and Wharton, 2001). These findings clearly demonstrate that Nos and Pum inhibit the transition from G2 to mitosis in migrating pole cells by repressing CycB production, and this leads us to speculate that the inhibition of sequential cell cycling has an important role in early germline development.

Additional target of Nos and Pum is head involution defective (*hid*) mRNA, which also contains an NRE in its 3' UTR and encodes a protein required for the induction of apoptosis. In the absence of Nos or Pum, migrating pole cells are eliminated by an apoptotic mechanism which is initiated at stage 9/10 in the developing embryo (Hayashi et al., 2004; Hayashi et al., 2005; Sato et al., 2007; Kobayashi et al, 2005).

In addition to their mitotic arrest and migration to the gonads, pole cells can be distinguished by their transcriptional regulation. Pole cells are transcriptionally quiescent until the onset of gastrulation, whereas transcription is initiated in the soma during the syncytial blastoderm stage. Consistent with this, RNA polymerase II (RNAP II) remains inactive in early pole cells. Furthermore, pole cells lack a subset of nucleosomal histone

modifications, such as methylated lysine 4 on histone H3 (H3meK4), which correlates well with transcriptional ability. Hence, the ability to express zygotic mRNA-encoding genes is suppressed only in pole cells in early embryo. Within pole cells, Nos is involved in maintaining transcriptional quiescence and is also required for the maintenance of a germline-specific chromatin status that correlates with transcriptional inactivity. In the absence of maternal Nos activity, somatic genes such as *fushi tarazu* (*ftz*), *even-skipped* (*eve*) and *Sex-lethal* (*Sxl*) are expressed ectopically in pole cells. In this instance, the phosphorylation of serine resides 2 and 5 in the carboxy-terminal domain (CTD) of RNAPII, both of which are required for transcriptional activation, and also the methylation of histone H3 on lysine 4 (H3meK4) are derepressed.

Another Nos target gene is represented by *impα2*, which is a Drosophila homologue of Importin α required for the nuclear import. At the blastoderm stage, Impα2 protein is distributed throughout the soma but not the pole cells, although impα2 transcripts are detectable in pole cells. In absence of Nos and Pum, Impα2 is ectopically expressed in pole cells, thus determining nuclear import of a transcriptional factors, like Ftz-F1, which in turn activates *ftz*. These data suggest that Nos and Pum repress somatic gene expression in pole cells by inhibiting nuclear import of transcriptional activators.

As far as Cup is concerned, the finding that homozygous *cup* mutant embryos display a further decrease of germ cell number in comparison with those heterozygous embryos (**Figure 28D,E**) supports the hypothesis an additional role of Cup during late embryogenesis. It has been demonstrated that Nos interacts genetically with Cup to promote normal development of the ovarian germline and it is also known that Cup, as well as Nos, acts as a translational repressor of selected mRNA. Among them, *cyclin A* transcript is translationally repressed by Cup, thus preventing that the mitotic cyclins are

inappropriately expressed (Sagimura and Lilly 2006), a very important aspect during germ cell development also.

Homozygous *cup* mutant embryos display a reduced number of germ cells and sometimes showed irregular shapes characteristic of apoptotic cells (**Figure 28D,E**).

According to these studies, it is possible hypothesize a scenario where Nos germline functions are carried out together with Cup to repress somatic differentiation and apoptosis and hence maintain germ cell fate.

Although these findings represent a very interesting assumption, whether or not *cup* zygotic function is concerned in the translational repression of specific mRNAs, different to *osk*, remains to be explored.

Materials and Methods

1. Culture of *Drosophila melanogaster.*

Drosophila is a typical holometabolous insect and its life can be divided into four stages: embryo, larva, pupa, and adult.

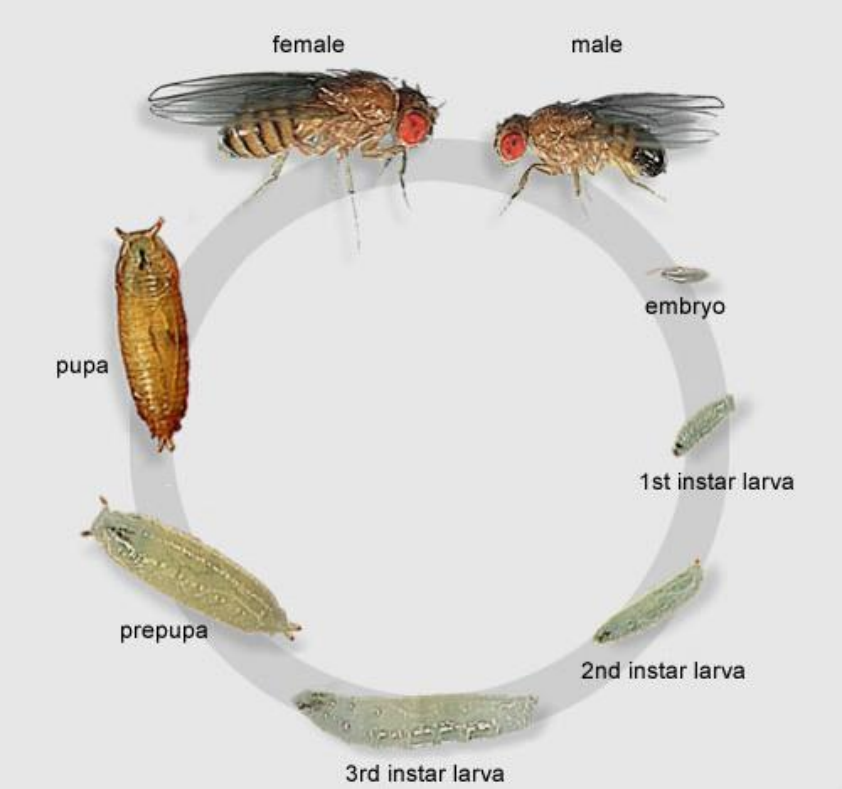


Figure 32. *Drosophila melanogaster* life cycle.

Drosophila stocks are usually maintained at 25°C or 18°C and are cultured in plastic vials, with hydrophobic cotton wool as stopper. A number of different recipes for fly food are in use. In the laboratory where I work the food is composed by yeast, maize meal, sugar, agar, Nipagin (methyl hydroxy benzoate).

For 2L of water:

- 100g of dry yeast
- 180g of maize meal
- 200g of sugar
- 15g of agar
- 5g of nipagin melted in ethanol 96%.

2. Egg chamber dissection.

To have egg chambers in each stage of development it is necessary to put the newly eclosed females with few males in a vial with some fresh yeast at 25°C for 1 or 2 days. After anesthetization, the flies were dissected using a drop of physiological medium (PBS1X). The ovaries were collected on ice in 1.5 ml eppendorf tube containing PBS 1X.

3. Immunostaining and Fluorescence Microscopy

3.1 Fix of egg chambers

In order to preserve the ovaries after dissection and maintain the egg chambers in a better condition, to avoid the cellular death and decomposition, after dissection it is very important to fix the ovaries following this protocol:

- change the PBS1X solution with a fix solution consist in: 200µl of PAF 4% (freshly prepared), 5% of DMSO and 600µl of heptane;
- put the eppendorf for 30' at room temperature on agitation,
- wash several times with PBT 0.1% (PBS1X + triton 0.1%).

Now the ovaries are ready to perform different experiments.

3.2. Immunofluorescence of whole-mount *Drosophila melanogaster* ovaries.

- Dissect ovaries in PBS1X
- Fix ovaries (200 µl PAF4%, 5%DMSO) + 600 µl heptane, 30' at RT
- Wash 3 times for 10' in PBS1X, Triton 0,1% at RT
- Permeabilize in PBS1X, Triton 1% + 3% bovine serum albumin o/n 4°C;
- Wash in PBS1X, Triton 0,1% at RT 1x10'
- Block with PBS1X, Triton 0,1% + 5% fetal bovine serum + 3% bovine serum albumin 1h at RT
- Incubate with primary antibody in the opportune dilution in PBS1X, Triton 0,1%
- Wash in PBS1X, Triton 0,1%at RT 3 times for 10'

- Incubate with secondary antibody (200 µl) in PBS1X, Triton 0,1% + 5% fetal bovine serum + 3% bovine serum albumin 2 h at RT (1:400)
- Wash 3 times for 10' in PBS1X, Triton 0,1% at RT
- Add 50% PBS 1X-glycerol

The following antibodies were used: rat anti-Cup (1:100, Verrotti and Wharton, unpublished), goat anti-Stau (1:100, S. Cruz), rabbit anti-Osk (1:2000, gift of A. Ephrussi), rabbit and rat anti-Vas (1:500, gift of P. Lasko). Nuage was visualized as described (Findley et al., 2003) and the primary antibodies were used at the following concentration: rat anti-Cup (1:25) and rabbit anti-Tudor (1:200, gift of P. Lasko).

For immunohystochemistry of wild type and mutant embryos, overnight or staged embryos were collected, dechorionated in bleach, fixed in 4% formaldehyde and stained as previously described in Giangrande et al. (1993). The following antibodies were used in order to detect the germ plasm and the germ cells in the embryos: rat anti-Cup (1:25), rabbit anti-Cup (1:50, Verrotti and Wharton, 2000), rat and rabbit anti-Vas (1:500, gift of P. Lasko), rabbit anti-Osk (1:1000, gift of A. Ephrussi) and goat anti-Stau (1:100, S. Cruz). F-actin was stained with Texas Red-X phalloidin (Millipore) according to Styhler et al. (1998).

Donkey anti-rabbit Alexa 488 and donkey anti-goat Alexa 559 (1:400, Molecular Probes), Cy3 conjugated donkey anti–rat (1:400, Jackson Labs), Cy3 conjugated donkey anti rabbit (1:800, Jackson Labs) and Cy5 conjugated donkey anti–rat (1:800, Jackson Labs) secondary antibodies were used according to manufacturer's instructions. DNA staining was performed using DAPI (Invitrogen) as previously described (Zappavigna et al., 2004).

Samples were analyzed on a Zeiss LSM510 Meta confocal microscope and quantified using ImageJ. For the quantifications, all images were taken with the same exposure/gain and a threshold was used so that ˜15% of the total signal was eliminated as background.

Whole mount *in situ* hybridization was performed as described (Bernardoni *et al*., 1999). Probes corresponding to either *bicoid* (*bcd*) or *oskar* (*osk*) coding sequences were detected using alkaline-phosphatase-conjugated sheep anti-digoxigenin antibody (Roche Diagnostics). Finally, the slides were analyzed using a conventional epifluorescence microscopy.

4. RNA probe labelling for *in situ* hybridisation

For *in situ* hybridisation experiments, anti-sense RNA probes were prepared with using DIG-Labelling Kit (Roche). As a template either plasmids containing corresponding cDNA, or direct PCR products amplified from genomic DNA with T7- containing primers, were used. The labelling was performed according to Roche Instruction Manual. The labelled probe was mixed with 10µl of 20mg/ml carrier tRNA (Sigma) with the final volume adjusted up to 200µl with DEPC treated water. RNA was precipitated by ethanol (salting agent 400mM LiCl) for at least 2h (or o/n) at -80^{o}C. The precipitated RNA was centrifuged at maximal speed for 15min, washed twice with 70% ethanol, vacuum-dried and dissolved in 100µl of DEPC-water for 30min at 37^{o}C.

The efficiency of labelling was tested on dot-blot. For that serial dilutions of the probes (1:10, 1:100, 1:1000) and control RNA (1ng/µl, 100pg/µl, 10pg/µl, 1pg/µl) were made. 1µl of the each – diluted controls, non-diluted and diluted probes – were spotted onto a piece of nylon membrane (Amersham). When fully dried, the nucleic acids were cross-linked to the membrane by UV-light for 2min. The membrane was washed in PBS 2x 5min, blocked with PBS supplemented with 10µg/ml bovine serum albumin and 5% sheep serum for 30min, and hybridised with anti-DIG alkaline phosphatase (AP)-conjugated antibody (1:5000 dilution, Roche) for 30min at room temperature. The membrane was washed 3x10

min in PBS and 2x 10min in AP-buffer. Colour reaction was developed in NBT/BCIP solution (20µl of stock solution (Roche) per 1ml AP-buffer) in dark.
The intensity of the experimental spots was compared with the control, and the approximate concentration of the labelled probe was estimated (suggested working concentration 1ng/ml).

Buffers:
PBS 130mM NaCl, 7mM Na_2HPO_4, 3mM NaH_2PO_4
AP-buffer 100mM NaCl, 100mM Tris-HCl pH 9.5, 0.1

5. Q/RT-PCR

Total RNA was prepared by crushing wild-type and mutant staged embryos in Trizol (Invitrogen) and the cDNAs were prepared using the Superscript VILO cDNA Synthesis Kit (Invitrogen). The semi-quantitative RT-PCR analyses for *cup*, *vas* and *actin 42A* were performed using the following primers: for *cup*, CGACACCCAATTGCTACTGC and GGCTGCAAGAGTCTGCTGG; for *vas, GTCGCCATTGGCATTGTAGG* and GTACGTCCAATGCGATGTACG; and for *actin 42A, GTGCTAAGTGTGTGCAGCG* and CTGGATGGCAACATACATGG. In each case, the primer pair flanks intron-coding sequence, such that amplification from contaminating genomic DNA yields a larger product (249 versus 322 for *cup*, 383 versus 505 for *vas* and 645 versus 491 for *actin* 42A). 50 µl PCR reactions were performed using standard conditions with the following protocol: 3 minutes at 98°C; 40 cycles of 1 minute at 94°C, 1 minute at 58°C, 1 minute at 72°C; and 7 minutes at 72°C. 10 µl aliquots were removed every 10 cycles and visualized by ethidium bromide staining following electrophoresis through agarose.

Real time PCRs were performed in 96-well thin-wall plates (Applied Biosystems) using an Applied Biosystems 7300/7500 Real Time PCR System according to the manufacturer's suggested procedure. The following primers, which spanning exon-exon junctions were used: for *osk*, AACAAATCTTGCACCGCTGGGC and GACTTGGCGTGGTGAGGCCTGA; for *bcd* AACGAGCAAGAAGACGACGCTACAGATCTTG and GCGAATAGCGTATTGCAGGGAAAGTATAGA; *rp49*, a ubiquitously expressed ribosomal protein mRNA, was used for normalization (GCTAAGCTGTCGCACAAA and TCCGGTGGGCAGCATGTG).

6. Protein extracts and Western blots

Total protein extracts from adult ovary pairs were prepared as described by Kim-Ha et al. (1995).

Wild-type and mutant staged embryos were collected and total protein extracts were prepared as follows. The volume of the embryos was estimated and an equal volume of lysis buffer [50 mM Tris HC1 (pH 7.5), 20 mM NaC1, 0.1% Triton X-100, 0.5 mM EDTA, 1 mM DTT, 1 mM PMSF, 1xEDTA free protease inhibitor cocktail (Roche, Basel, Switzerland) was added. Embryos were homogenized with a plastic pestle and centrifuged in a refrigerated microcentrifuge at 15,000 rpm for 10 min. Glycerol was then added to the supernatant to a final concentration of 10%, and extracts were stored at -70°C. Cup, Osk, Stau and Vas proteins were detected by Western blot using rat and rabbit anti-Cup antibody (1:5000), rabbit anti-Osk antibody (1:5000, gift of A. Ephrussi), rabbit

anti-Vas antibody (1:5000, gift of P. Lasko), goat anti-Stau antibody (1:500, S. Cruz) and mouse anti-α-tubulin antibody (1:2000, Sigma)

7. Interaction assays

Co-immunoprecipitation assays were performed as previously described (Grimaldi et al., 2007) except that protein A/G PLUS-Agarose beads (Santa Cruz) were bound to rabbit anti-Osk or rabbit anti-Vas antibodies. Rabbit pre-bleed serum was used as a negative control. RIPA buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 10 mM NaF, 1% NP-40, 1% sodium deoxycholate, 1 mM EGTA, 1 mM PMSF, 1 mM Na3VO4) were used. The samples were subjected to immunoblot detection using rat anti-Cup antibody, and rabbit anti-Osk or rabbit anti-Vas antibodies for the immunoprecipitation detection. For the yeast interaction assay, the AD-*cup* and DBD-*osk* fusions were used. Cup fragments bearing residues 29-962 and 2-1094 (Piccioni et al., 2009) were tested against a DBD-Osk fusion bearing residues 122-650 (a gift from P. Lasko).

8. *Drosophila* strains

Flies were raised at 25°C on standard sucrose/cornmeal/yeast food and the wild-type reference stock was Oregon R. *cup*^{8} was described in Keyes and Spradling (1997). *Df(2L)BSC187,* a chromosome bearing a complete deletion of *cup* gene confirmed by Western Blot (see Fig. S3E,F in the supplementary material), was provided by the Bloomington *Drosophila* Stock Center. Heterozygous *cup* mutant alleles were balanced

with Cyo, Twi Gal4, UAS GFP (abbreviated here as Cyo-GFP). Heterozygous *cup* mutant females carrying Cyo-GFP balancer were crossed with males of the same genotype and homozygous *cup* mutant embryos lacking of GFP expression were distinguished from those heterozygous *cup* mutant and homozygous-balancer embryos (GFP positive). Anyway, the presence of the balancer Cyo-GFP does not influence any phenotype, because I got the same results analyzing heterozygous mutant embryos carrying the *cup* alleles over wild-type chromosome. The latest embryos were obtained crossing the *cup* mutant females carrying Cyo-GFP balancer with wild-type males and heterozygous *cup* mutant embryos were selected by the lacking of GFP expression. For these reasons, heterozygous cup^{8} and *Df(2L)BSC187* mutant flies and embryos balanced with Cyo-GFP or Cyo alone are abbreviated into the manuscript as cup^{8}/+ and *Df(2L)BSC187*/+.

Wild type and mutant staged embryos were collected and the germ cells were counted after staining with anti-Vas antibody. 50 embryos of each maternal genotype described above were collected at cycle 14 (cellular blastoderm) and germ cells were counted three times for each embryo. The following mutant alleles and mutant combinations were used: osk^{54}/TM3, *Df(3R)p-XT103*/TM3 and osk^{84}/*Df(3R)p-XT103* (Lehmann and Nüsslein-Volhard, 1986), vas^{1} and vas^{PH165}(Schupbach and Wieschaus, 1986; Styhler et al., 1998). I also used the transgenic stock *osk-bcd* 3'UTR previously described in Ephrussi and Lehmann (1992).

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