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Cristina Ottone, Silvia Gigliotti, Angela Giangrande, Franco Graziani, Arturo Verrotti Di Pianella. The translational repressor Cup is required for germ cell development in Drosophila. Journal of Cell Science, 2012, 10.1242/jcs.095208 . hal-03447894

HAL Id: hal-03447894
https://hal.science/hal-03447894

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

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Accepted 6 March 2012
Journal of Cell Science 125, 3114–3123
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doi: 10.1242/jcs.095208

Summary

In *Drosophila*, germ cell formation depends on inherited maternal factors localized in the posterior pole region of oocytes and early embryos, known as germ plasm. Here, we report that heterozygous *cup* mutant ovaries and embryos have reduced levels of Staufen (Stau), Oskar (Osk) and Vasa (Vas) proteins at the posterior pole. Moreover, we demonstrate that Cup interacts with Osk and Vas to ensure anchoring and/or maintenance of germ plasm particles at the posterior pole of oocytes and early embryos. Homozygous *cup* mutant embryos have a reduced number of germ cells, compared to heterozygous *cup* mutants, which, in turn, have fewer germ cells than wild-type embryos. In addition, we show that *cup* and *osk* interact genetically, because reducing *cup* copy number further decreases the total number of germ cells observed in heterozygous *osk* mutant embryos. Finally, we detected *cup* mRNA and protein within both early and late embryonic germ cells, suggesting a novel role of Cup during germ cell development in *Drosophila.*

Key words: Oogenesis, *Drosophila*, Germ cells, Cup, Oskar

Introduction

In *Drosophila*, germ cell differentiation begins during oogenesis, when maternal germ-line determinants, produced by the nurse cells, are transported and then localized in the oocyte. During late oogenesis, a specialized cytoplasm, termed germ plasm, is assembled to the posterior of the oocyte. The germ plasm contains polar granules which are associated with germ-line specific ribonucleoprotein (RNP) determinants, like Osk, Vas, and Tudor (Tud) proteins (Frohnhöfer et al., 1986; Saffman and Lasko, 1999).

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 (Riechmann and Ephrussi, 2001). The posterior determinant Osk directs formation of the germ-cell lineage and abdominal embryonic structures (Ephrussi and Lehmann, 1992). To achieve this aim, the first key event is the accumulation of *osk* mRNA at the posterior of the oocyte. *osk* transcripts are produced by the nurse cells and enclosed within dynamic RNP particles containing specific localization and translation factors, such as Y14, eIF4AIII, Barentsz (Btz), and HRP48 as well as exon junction complex (EJC) components, Mago nashi (Mago) (Hachet and Ephrussi, 2001; Huynh et al., 2004; Newmark and Boswell, 1994; van Eeden et al., 2001; Yano et al., 2004).

The RNA-binding protein Staufen (Stau) and several microtubule motor proteins are then 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). Moreover localization-dependent translational control processes are essential to confine protein synthesis in space and time. Specifically, 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 eIF4G, thus repressing the translation of unlocalized *osk* mRNA (Wilhelm et al., 2003; Nelson et al., 2004). Consequently, lack of Cup protein promotes premature *osk* mRNA translation at stages 4–7 (Nakamura et al., 2004).

Following localization, *osk* mRNA can be translated to produce 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 Vas, Tud and Nanos (Nos) (Ephrussi et al., 1991; Kim-Ha et al., 1991; Vanzo and Ephrussi, 2002). 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 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).

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In this report, we show that Cup physically interacts and colocalizes with Osk protein in the germ plasm during oogenesis. Furthermore, both *cup* RNA and protein 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 germ plasm assembly through accumulation and/or maintenance of these maternal germ-line determinants. As a consequence, embryos laid by heterozygous *cup* mothers show defects in germ plasm. In these embryos *osk* mRNA/Stau particles reach the posterior pole as in wild-type embryos, but a significant amount of these complexes are not properly anchored to the cortex and appear mislocalized. Consistent with the translational 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. Consequently, there is a reduced germ cell number is found in heterozygous *cup* mutant embryos. Finally, we show that *cup* and *osk* interact genetically, since reducing *cup* gene copy number further decreases the number of germ cells detected in heterozygous *osk* mutant embryos. Taken together, our 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

Cup is a component of the germ plasm

Emerging evidences suggest that Cup is a germ plasm component. First, Cup accumulates at the posterior pole of stage 10 oocytes, where it colocalizes with Osk (Wilhelm et al., 2003) and Stau proteins (Piccioni et al., 2009). Second, Thomson et al. performed immunoprecipitation experiments to identify novel polar granule components, associated with Vas and Tud, and isolated several P body related proteins, including Cup (Thomson et al. 2008). Third, Thomson et al. hypothesized 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). Finally, we previously demonstrated that Cup colocalizes with Stau at the posterior pole of stage 10 oocytes (Piccioni et al., 2009).

To further extend these observations, we 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 colocalize 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 Fig. 1, Cup colocalizes with Stau, Osk and Vas proteins at the posterior pole of stage 10 oocytes.

Cup associates with Osk and Vas

In order to test whether Cup physically interacts with germ plasm components other than Stau (Piccioni et al., 2009), wild-type ovary extracts were immunoprecipitated with anti-Osk and anti-Vas antibodies (Kluger et al., 2010) and the precipitates analyzed by western blot using an anti-Cup antibody. As shown in Fig. 2A,B, Cup, consistently and specifically, co-precipitates with either Osk or Vas. In addition, two different Cup fragments interact directly with Osk in yeast two hybrids assay (Fig. 2C). This latest result suggests that the 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.

During the organization of the germ plasm, germ-line components are hierarchically localized and anchored to the posterior pole of the mature oocyte (Thomson and Lasko, 2005). To assess the impact of the pathway controlling germ plasm organization on Cup distribution, we analyzed Cup localization at the posterior cortex of oocytes derived from *osk* and *vas* alleles. In both wild-type and osk^{54}/*Df(3R)p-XT103* stage 9–10A oocytes, similar to Stau protein localization (St Johnston et al., 1991; Kim-Ha et al., 1991; Vanzo and Ephrussi, 2002) (Fig. 3A,E), Cup accumulates at the posterior cortex (Fig. 3B,F). In contrast to wild-type controls, Cup (Fig. 3D,H) and Stau (St Johnston et al., 1991) (Fig. 3C,G) detach from the posterior pole of osk^{54}/*Df(3R)p-XT103* stage 10B oocytes, thus resulting in a significant reduction (50%) of posteriorly localized proteins (quantification of fluorescent signals is shown in Fig. 3I).

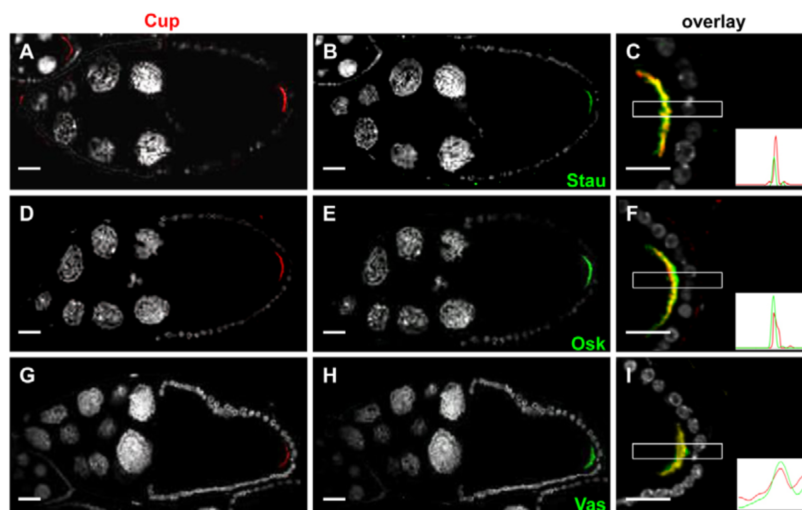

Fig. 1. Cup localizes to the germ plasm. Single confocal section of wild-type stage 10 egg chambers to visualize Cup (shown in red; **A**,**D**,**G**) and Stau, Osk and Vas (shown in green; **B**,**E**,**H**, respectively). Scale bars: 20 µm in all panels. (**C**,**F**,**I**) Enlarged view of stage 10 oocytes shows the colocalization of Cup with Stau (C), Osk (F) and Vas proteins (I); the insets in C, F and I show the intensity profile across the posterior pole oocyte (region of white square) and confirm the overlapping localization. Scale bars: 10 µm.

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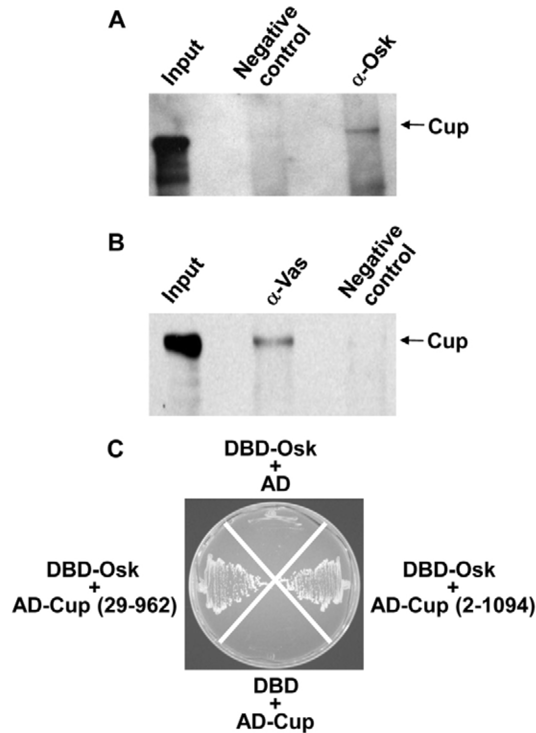

Fig. 2. Cup associates with Osk and Vas. (**A**,**B**) Co-immunoprecipitation assay. Ovary extracts were immunoprecipitated by rabbit anti-Osk and rabbit anti-Vas antibodies, and the precipitates analyzed by western blotting using rat anti-Cup antibody. Cup consistently and specifically co-precipitated with Osk (A) and Vas (B). Rabbit pre-bleed serum was used as 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 medium lacking His and containing 3 mM 3-aminotriazole.

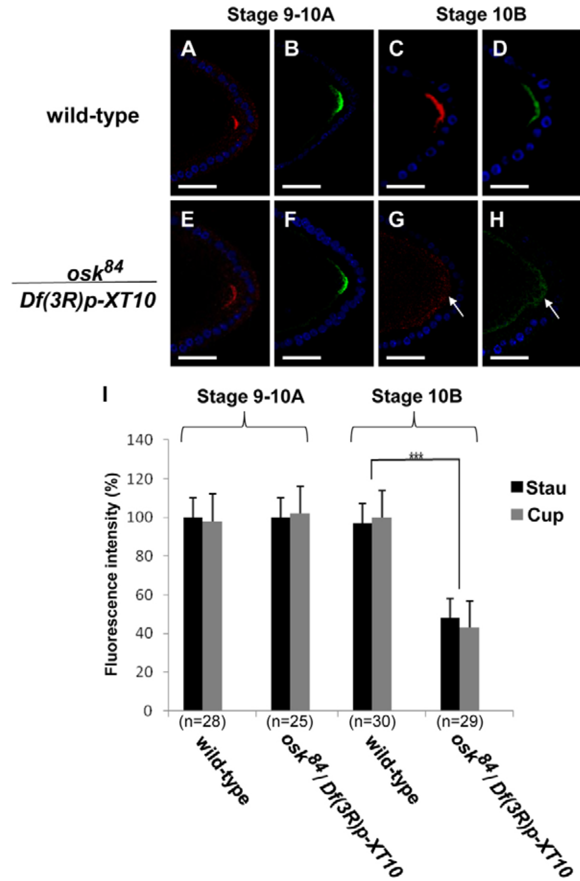

Fig. 3. Cup posterior localization is affected in *osk* mutant oocytes. (**A–H**) Wild-type and *osk* nonsense mutant oocytes (*osk*^{54}/*Df(3R)p-XT103*) stained with anti-Stau (shown in red; A,E,C,G) or anti-Cup (shown in green; B,F,D,H) antibodies. During stages 9–10A, Stau and Cup are present at the posterior pole of both wild-type and *osk*^{54}/*Df(3R)p-XT103* 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*^{54}/*Df(3R)p-XT103* ovaries, Cup, as well as Stau (H and G, respectively), detached 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) were stained with DAPI. Each panel shows a single stained oocyte. Scale bars: 10 μm in all panels. (**I**) Quantification of the effect shown in the upper panel: comparison of fluorescent signal intensities showed that the reduction of Stau and Cup is specific and significant: ***$P<0.001$, Student's *t*-test.

Homozygous *vas*^{1} females undergo normal oogenesis and lay a similar number of eggs as wild-type flies but the developing embryos lack abdominal segments (Lasko and Ashburner, 1990). 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, but most arrested at stage 10, only a small number of oocytes are able to reach stage 14 and complete oogenesis (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 (Fig. 4B,C; quantification of fluorescent signals is shown in 4F). Moreover, about 20% ($n=69$) of stage 10 *vas*^{1} egg chambers display a punctuate distribution of Cup at the posterior pole (Fig. 4D). This Cup distribution pattern is also observed at the posterior pole of the few stage 10 *vas*^{PH165} oocytes (Fig. 4E), where *osk* mRNA signal is greatly reduced and diffused (Styhler et al., 1998).

Our 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, this analysis also shows that any destabilization of germ plasm assembly, as occurs in *vas* mutants, alters Cup posterior localization. Taken together, our data indicate that Cup is a novel component of the germ plasm.

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

Homozygous *cup* mutants are sterile, their ovaries display a wide range of defects, and *osk* mRNA is precociously translated around stage 4; in contrast, heterozygous *cup* mutants are fertile, egg chambers develop normally (Keyes and Spradling, 1997; Piccioni et al., 2009), and *osk* mRNA is translated at stage 8 at the posterior pole, as in wild type. To investigate the role of Cup protein during germ plasm assembly, we 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)BSC*^{187}/+ egg chambers, Osk protein is

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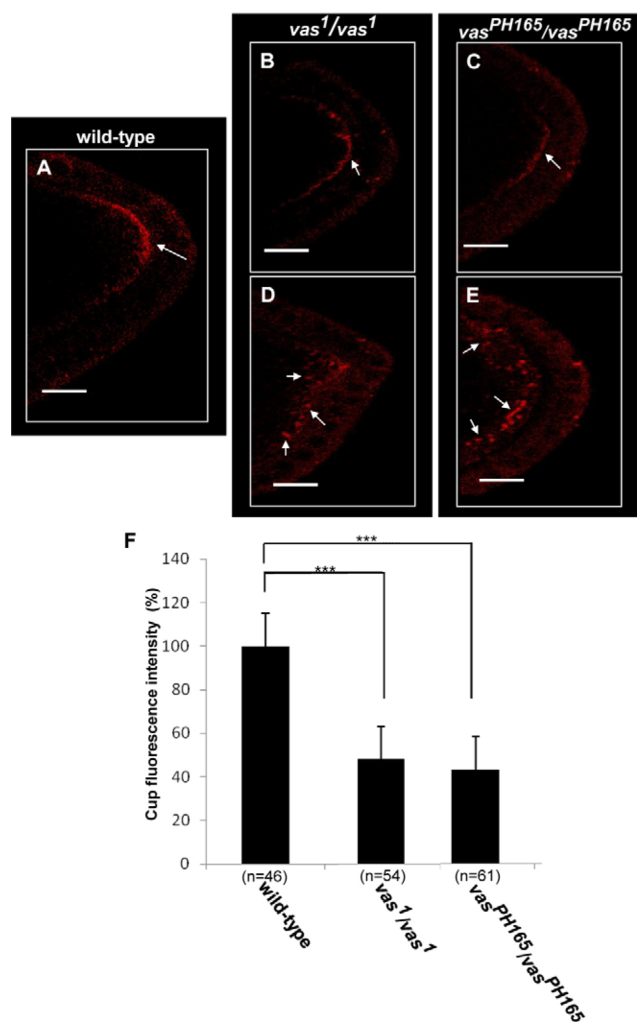

Fig. 4. Disruption of Cup localization in *vas* mutant oocytes. (**A–E**) Wild-type and homozygous *vas* mutant oocytes (*vas*^{1} and *vas*^{PH165}) were stained with anti-Cup antibody. At stage 10, 80% of homozygous *vas* mutant ovaries showed a posterior enrichment of Cup protein, correctly localized to the cortex of the oocyte (B,C) but at a reduced level compared with that in wild-type (A). In the remaining 20% of homozygous *vas* mutant oocytes, Cup signals were detected at numerous sites of punctuate fluorescence around the posterior cortex, indicating that Cup is not stably anchored to the posterior pole (D,E). Scale bars: 10 µm in all panels. (**F**) Quantification of the effect shown in A and E: comparison of fluorescent signal intensities showed that the reduction of Cup is specific and significant: ***$P<0.001$, Student's *t*-test.

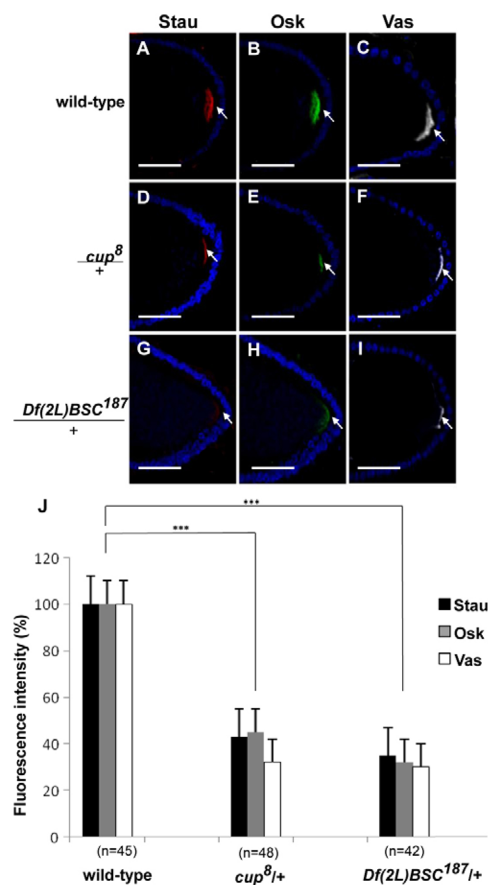

Fig. 5. Cup promotes the accumulation of the germ plasm determinants at the posterior pole of the oocyte. (**A–I**) Stage 10 wild-type and heterozygous *cup* mutant egg chambers (*cup*^{8}/+ and *Df(2L)BSC*^{187}/+) stained with anti-Stau (shown in red; A,D,G), anti-Osk (shown in green; B,E,H) or anti-Vas (shown in grey; C,F,I) antibodies. Compared to wild-type oocytes, Stau, Osk and Vas protein levels were 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 stained with DAPI. Each panel shows a single stained stage 10 oocyte. Scale bars: 10 µm in all panels. (**J**) Quantification of fluorescent signals indicates that the decrease of Stau, Osk and Vas is significant: ***$P<0.001$, Student's *t*-test.

normally localized, coincident with Stau accumulation at the posterior pole (data not shown).

Later in oogenesis, when the first steps of germ plasm assembly takes place and Osk anchoring becomes crucial (Ephrussi et al., 1991; Vanzo et al., 2007), we detected a significant reduction of localized Stau, Osk and Vas proteins at the posterior cortex of heterozygous *cup* stage 10B oocytes, compared to the wild type (Fig. 5).

Although normal levels of *osk* mRNA were detected by *in situ* hybridization experiments and no Osk was not observed in immunostaining experiments at stages 4–7 (data not shown), we could not rule out the possibility that the defects in pole plasm assembly observed in *cup* heterozygous flies are due to altered transport of *osk* mRNA to its posterior localization In order to provide more compelling evidence that the reduction of localized Osk (and as a consequence of Stau and Vas) observed when *cup* activity is reduced, is not due to ectopic translation of *osk* mRNA in transit from the nurse cells, we increased the *osk* gene dosage in the *cup* heterozygous females. We reasoned that if Cup is the limiting factor for pole plasm assembly/maintenance, excess of *osk* should not rescue the pole plasm defects. On the contrary if the diminution in localized Osk is due to altered *osk* mRNA transport coupled with its ectopic translation, increase in *osk* copy number should rescue pole plasm defects. Results are shown in supplementary material Fig. S3A,B,E; confirming that Cup is the limiting factor for pole plasm assembly and/or maintenance. In agreement with this finding, we observed significant reduction of localized Osk also when the dosage of the *orb* gene, which is known to promote *osk* mRNA translation (Chang et al., 1999), was decreased in *cup*^{8}/CyO females (supplementary material Fig. S3C–E).

In addition, western blot analysis on total protein extracts derived from cup^8/+ and *Df(2L)BSC*^{187}/+ ovaries revealed levels of Stau, Osk and Vas comparable to that of the wild type (Fig. 6). 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.

Moreover, Cup does not colocalize with F-actin at the posterior pole of stage 10 oocytes (data not shown), suggesting that Cup mediates the anchoring of germ plasm determinants at the 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).

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.

cup gene products are expressed in germ cells

Given the new role of Cup in germ plasm assembly, we asked whether Cup may exert an important, yet unknown function during germ cell determination. To this aim, we analyzed *cup* gene product distribution during *Drosophila* embryogenesis, by

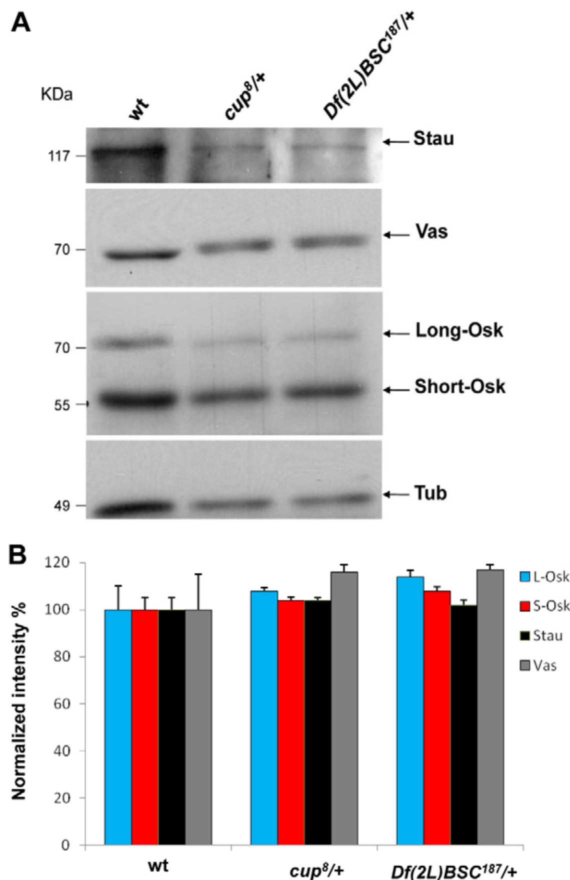

Fig. 6. Stau, Vas, and Osk protein levels in heterozygous *cup* mutants. (**A**) Western blot detection of Osk, Stau and Vas in total extracts of wild-type and heterozygous *cup* mutant ovaries. α-tubulin was used as loading control. (**B**) Densitometry analysis of the western blot bands, which shows that the levels of Osk, Stau and Vas (normalized to α-tubulin) in heterozygous cup ovaries are similar to those in wild-type ovaries.

in situ hybridization and immunohistochemistry on wild-type embryos at different developmental stages. In stage 2, *cup* mRNA and protein are uniformly distributed anyhow the whole embryo (Fig. 7A,B). During blastoderm formation (stage 4), *cup* mRNA and protein are concentrated at the posterior pole to become incorporated into newly formed germ cells (Fig. 7D,E). Subsequently, *cup* gene products accumulate specifically in the pole cells at stage 10, when they migrate through the posterior midgut primordium, and during stage 14, when the germ cells reach their final destination (Fig. 7G,H,J,K). *cup* gene product expression during whole embryogenesis was confirmed by semi-quantitative RT-PCR and western blotting analyses (supplementary material Fig. S1). Interestingly, Cup has the same sub-cellular localization within developing germ cells as displayed by Vas a well known germ cell marker (Fig. 7M–R).

To further demonstrate that Cup tightly associates with Osk during germ plasm assembly, we examined whether or not Cup is recruited by Osk to the ectopic site in embryos carrying the *os-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 Fig. 8, anterior germ cells of *osk-bcd* 3′UTR embryos show expression of Cup protein, simultaneously with Osk. However, both proteins also maintain their posterior localization.

Taken together, our 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.

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, we performed immunostaining experiments on heterozygous *cup* mutant embryos (0–2 hours AEL). Consistent with the results obtained during egg chamber development (Fig. 3), cup^8/+ and *Df(2L)BSC*^{187}/+ mutant embryos, screened as described in Materials and Methods, show a reduced amount of localized Osk, Stau, and Vas at the posterior pole (9A–I; quantification of fluorescence signals is shown in Fig. 9J). Moreover, Stau is diffusely distributed throughout the whole cytoplasm of these embryos (Fig. 9E,H). Accordingly, western blot analyses on total embryo extracts (0–2 hours) show a significant reduction of Osk and Vas protein levels in heterozygous *cup* mutants when compared to wild-type controls (supplementary material Fig. S2A,C,D). On the contrary, Stau levels appear unchanged (supplementary material Fig. S2B,D), indicating that Stau protein is not properly localized but not degraded, as has been shown in *cappuccino* and *spire* mutants (St Johnston et al., 1991). In addition, heterozygous *osk* mutant embryos (0–2 hours), obtained from two different alleles (osk^{54} and *Df(3R)p-XT103*), show a consistent reduction of the total amount of Cup protein (supplementary material Fig. S2E,F), supporting our scenario, where Cup plays a crucial effect on Osk protein accumulation and vice versa *in vivo*.

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

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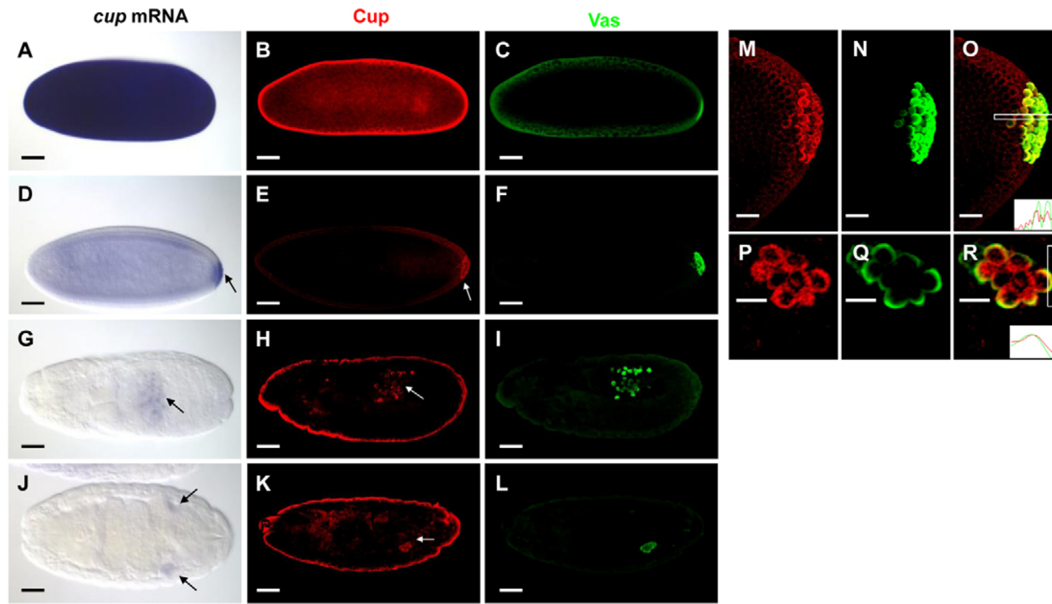

Fig. 7. ***cup* gene products are expressed in germ cells during embryogenesis.** (**A–L**) At stage 2, *cup* mRNA and protein are expressed in whole embryos (A,B). At cellularization of the embryo (stage 4), *cup* gene products accumulate in the germ cells (D,E, arrows), which are marked with anti-Vas antibody (C,F,I,L). Specific *cup* mRNA and protein signals localize within germ cells in migration (stage 10; G,H, arrows), and also during stage 14, when the germ cells reach their final destination (J,K, arrows). Scale bars: 50 µm. (**M–R**) Magnification of Vas-positive cells (N,Q) within stage 4 embryos stained with anti-Cup antibody (M,P). Scale bars: 20 µm (M–O); 10 µm (P–R). The insets in the O and R show the intensity profile across the posterior pole of the embryo and inside a single germ cell, respectively (regions indicated by white rectangles in main images) and confirm the overlapping localization. The *in situ* hybridization images are adapted from FlyExpress and the Berkely Drosophila Genome Project resources.

osk mRNA expression and localization are affected in embryos derived from *cup* heterozygous mothers

To understand whether or not the reduction of Osk protein, described above, is due to alteration of *osk* mRNA levels, we performed *in situ* hybridization experiments on 0–2 hours embryos. In both wild-type and heterozygous *cup* mutant embryos, we detected, in addition to the expected *osk* mRNA signal at the posterior pole (Fig. 10B,D,F), a gradient of mRNA extending from the posterior to the whole cytoplasm of the embryos (Fig. 10D,F). Accordingly, quantitative RT-PCR experiments (Fig. 10G,H, panel on the right) reveal a significant increase in total *osk* mRNA levels in heterozygous *cup* mutant embryos (0–2 hours) when compared to those in the wild type. By contrast, the total amount of *bcd* mRNA (Fig. 10G,H, panel on the left) and its localization pattern remain unchanged (Fig. 10A,C,E), demonstrating that the reduction of Cup activity specifically affects *osk* mRNA accumulation and localization.

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

In *Drosophila* embryos, germ cell number depends on correct 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 $Df(2L)BSC^{187}$) 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 (Fig. 11A–C). This number is further decreased in homozygous *cup* mutant embryos (Fig. 11D,E). These results appear to be a direct consequence of maternal germ plasm determinant reduction observed in heterozygous *cup* mutant ovaries and early embryos.

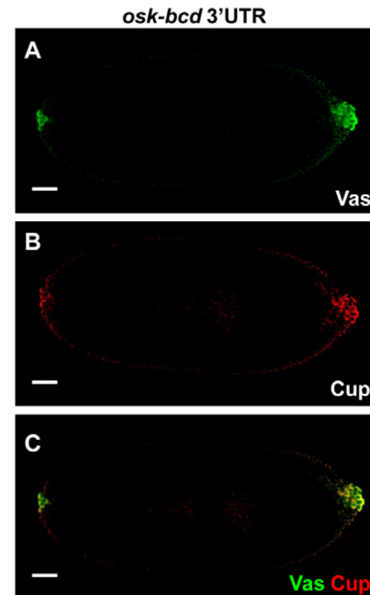

Fig. 8. **Cup localizes within anterior germ cells of *osk-bcd* mutant embryos.** Germ cell staining of cellular blastoderm-stage embryos laid by wild-type females expressing the *osk-bcd* 3′UTR. (**A**) Germ cells were stained with anti-Vas antibody. (**B**) Cup localizes in ectopic, anterior germ cells. Note that Cup-specific signal is also concentrated into the posterior germ cells. (**C**) Merged image. Scale bars: 50 µm in all panels.

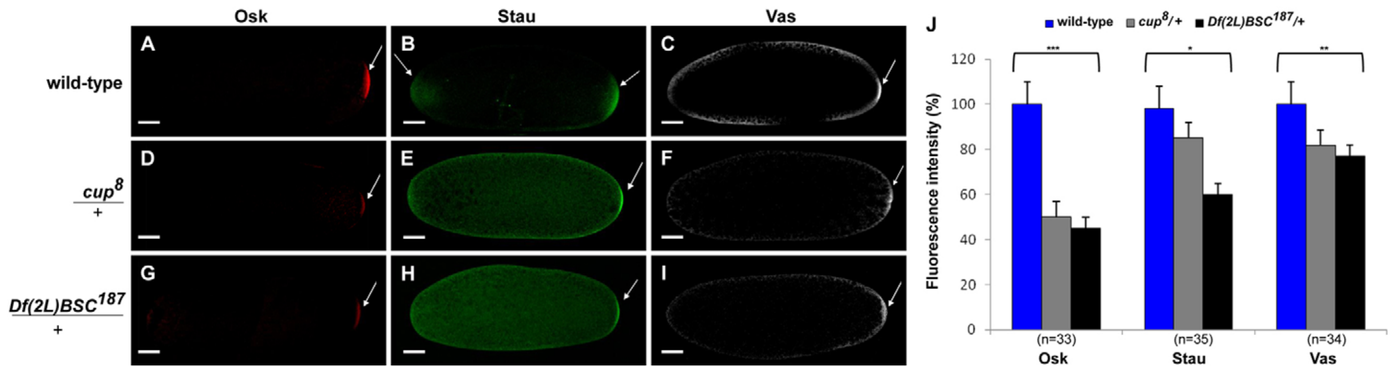

Fig. 9. Osk, Stau and Vas levels are reduced in heterozygous *cup* mutant embryos. (**A–I**) Wild-type and heterozygous *cup* mutant pre-blastoderm embryos (*cup*^{8}/+ and *Df(2L)BSC*^{187}/+) stained with anti-Osk (shown in red; A,D,G), anti-Stau (shown in green; B,E,H) or anti-Vas (shown in grey; C,F,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 types of heterozygous *cup* mutant embryos (E,H). Scale bars: 50 µm in all panels. (**J**) Quantification of the effect shown A–I. Comparison of fluorescent signal intensities showed that the reduction of Osk, Stau and Vas is specific and significant: ***$P<0.001$; **$P<0.01$, *$P<0.05$, Student's *t*-test.

To demonstrate whether *cup* and *osk* genetically interact, we further analyzed double heterozygous *cup-osk* mutant embryos: whereas *osk*^{54}/+ (Ephrussi et al., 1991; Lehmann and Nüsslein-Volhard, 1986) and *cup*/+ heterozygous mutant embryos display an average of 23/25 germ cells (Fig. 11B,C,F), double heterozygous *cup* and *osk*^{54} mutant embryos have only an average of 12 germ cells (Fig. 11G,H). Quantification of germ cell average number is shown in Fig. 11I.

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 offspring 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 weak increase of the grandchildless phenotype in comparison to that observed in the offspring of heterozygous *osk* females (Fig. 11J).

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

Discussion

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 (*cappuccino*, *spire*, *par-1*, *mago nashi*, *barentz*, *stau*, *tsunagi*, *rab11* and *valois*) (St. Johnston et al., 1991; Wellington et al., 1999; Mohr et al., 2001; Hachet and Ephrussi, 2001; van Eeden et al., 2001; Dollar et al., 2002; Anne and Mechler, 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 result in the recruitment of all factors, such as Vas, Tud and Stau proteins and *nanos*, *germ less* mRNAs (Mahowald, 2001), necessary for the establishment of functional germ-line structures. Posterior anchoring of Osk requires the functions of Vas (Breitwieser et al., 1996) Tud (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 regulate F-actin dynamics, which in turn are necessary to attach 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* (Wilhelm et al., 2003; Nakamura et al., 2004), *gurken* (Clouse et al., 2008), and *cyclinA* (Sugimura and Lilly, 2006), during early oogenesis.

Our results establish that Cup is also a novel germ plasm component. First, Cup colocalizes with Osk (Wilhelm et al., 2003; Nakamura et al., 2004), Stau (Piccioni et al., 2009), and Vas (this work; Fig. 1) at the posterior pole of stage 10B oocytes. Second, biochemical evidence indicates that Cup interacts with Stau (Piccioni et al., 2009), Osk and Vas (Fig. 2). 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 the interaction with the Osk protein, which 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 (Figs 3, 4), demonstrating that Osk and Vas are essential to achieve a correct localization of Cup at the posterior cortex of stage 10 oocytes. Our study suggests that the presence of Cup, Osk, Stau and Vas are required for a correct germ plasm assembly. Moreover, several immunoprecipitation 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 our results suggest that Cup plays at least an additional role at stage 10 of oogenesis. Cup, besides repressing translation of

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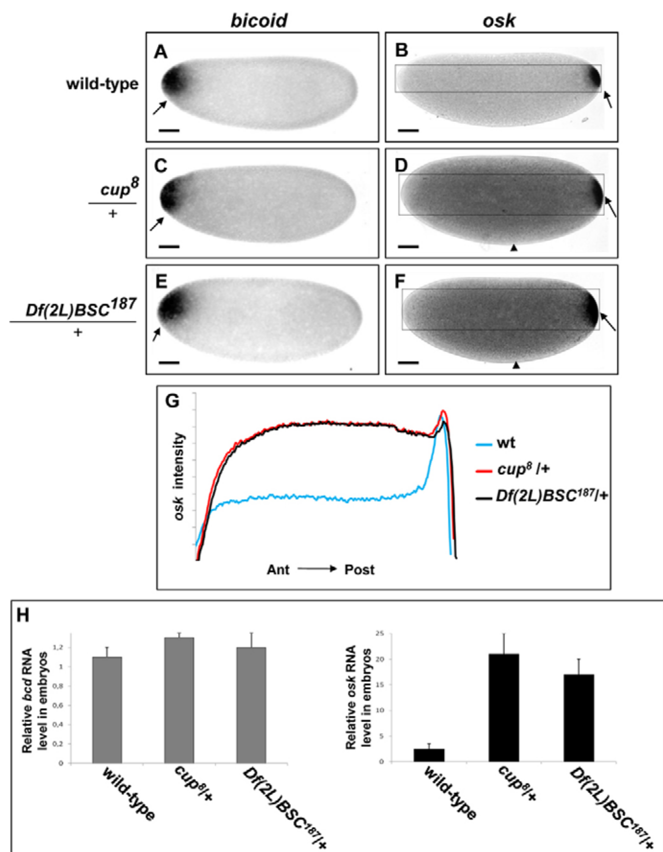

Fig. 10. *osk* mRNA levels are affected in heterozygous *cup* mutant embryos. (**A–F**) *In situ h*ybridization experiments on wild-type and heterozygous *cup* mutant pre-blastoderm embryos (cup^8/+ and $Df(2L)BSC^{187}$/+). *osk* mRNA is localized at the posterior pole of heterozygous *cup* mutant (D,F), as in wild-type embryos (B). Note that *osk* mRNA signal is also distributed throughout the whole cytoplasm of heterozygous *cup* mutant (D,F) but not in wild-type embryos (B). In contrast, *bicoid* (*bcd*) mRNA is correctly localized at the anterior pole of heterozygous *cup* mutant, as in wild-type embryos. Scale bars: 50 µm in all panels. (**G**). Intensity profiles across the embryos of the indicated genotypes. The regions within the black rectangles in B–F were analyzed and the relative intensity profile from the anterior to posterior pole is shown. Note that in *cup* mutants *osk* is distributed along the whole embryo. (**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 embryos compared with the level in wild-type pre-blastoderm embryos (right panel). In contrast, total *bcd* mRNA levels were unchanged in both wild-type and *cup* mutant pre-blastoderm embryos (left panel).

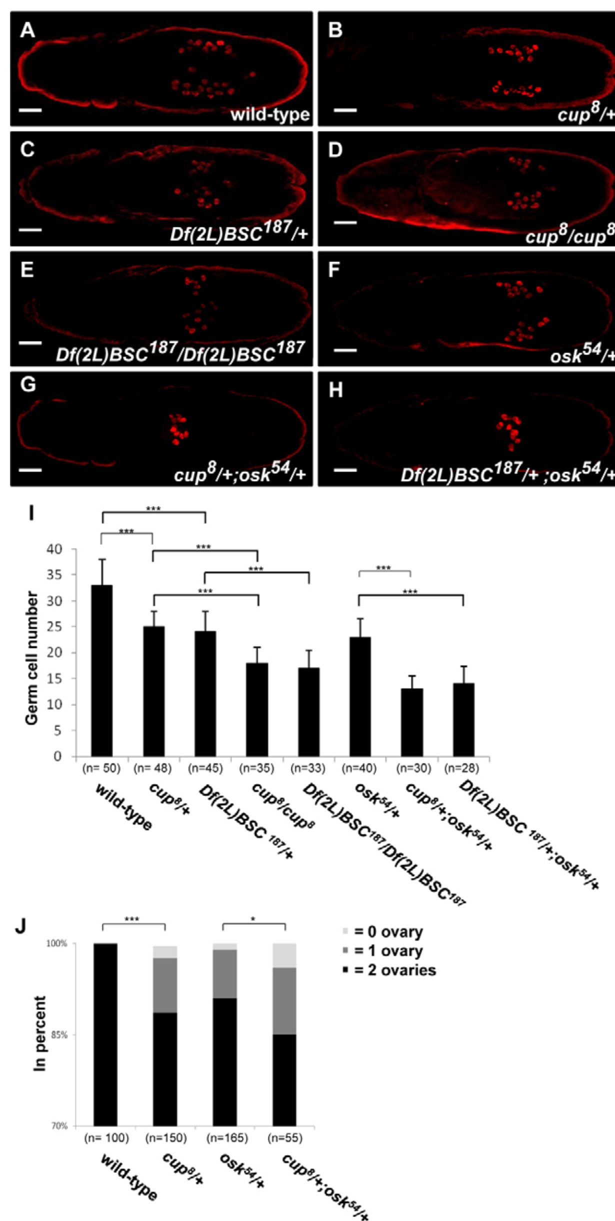

Fig. 11. *cup* assures germ cell development and interacts genetically with *osk*. (**A–I**) Germ cells of stage 11 embryos of the indicated genotypes were stained with anti-Vas antibody (A–H) and counted (I). The number of germ cells in heterozygous *cup* mutant embryos (cup^8/+ and $Df(2L)BSC^{187}$/+; 24 cells on average; B,C) was reduced 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,E). The number of germ cells in osk^{54}/+; cup^8/+ and osk^{54}/+; $Df(2L)BSC^{187}$/+ (12 cells on average; G,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. The grandchildless phenotype was stronger in cup^8/+ than in wild-type females (***$P<0.001$; 2). osk^{54}/+; cup^8/+ females showed a weak but significant grandchildless phenotype when compared to osk^{54}/+ females (* statistically significant at $P<0.05$; 2).

unlocalized *osk* mRNA (Nakamura et al., 2004), is necessary to anchor and/or maintain Stau, Osk and Vas at the posterior cortex (Fig. 5). 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.

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. Surprisingly, *osk* mRNA is increased in heterozygous *cup* mutant embryos. Since *osk* mRNA requires sufficient 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 at the embryonic pole and could stimulate, by positive feedback, *de novo osk* mRNA synthesis. We also cannot exclude a direct/indirect involvement of Cup in *osk* mRNA degradation and/or deadenylation (Lin et al., 2006; Igreja and Izaurraide, 2011).

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 idea that Cup is involved in germ cell formation and/or in maintenance of their identity.

Unlike Osk protein, we detected both *cup* mRNA and protein within germ cells until the end of embryogenesis. These observations suggest that zygotic *cup* functions, during germ cell formation and maintenance, are not limited to those carried out in combination with Osk. The finding that homozygous *cup* mutant embryos display a further decrease of germ cell number, in comparison with heterozygous embryos, supports this hypothesis. Whether or not *cup* zygotic function is involved in the translational repression of specific mRNAs, different from *osk*, remains to be explored.

Materials and Methods

Immunostaining and fluorescence microscopy

Fixation and antibody staining of hand-dissected ovaries were carried out as previously described (Gigliotti et al., 1998). The following antibodies were used: rat anti-Cup [1:100 (Verrotti and Wharton, 2000) and 1:200 (Keyes and Spradling, 1997)], goat anti-Stau (1:100; Santa Cruz), rabbit anti-Osk (1:2000; gift from A. Ephrussi), rabbit and rat anti-Vas (1:500; gift from P. Lasko). For immunohistochemistry of wild-type and mutant embryos, overnight or staged embryos were collected, dechorionated in bleach, fixed in 4% formaldehyde, and stained as previously described (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), rabbit anti-Osk (1:1000) and goat anti-Stau (1:100). F-actin was stained with Texas Red-X phalloidin (Millipore) as described previously (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 manufacturers' 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 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 previously (Bernardoni et al., 1999). Probes corresponding to either *bicoid* (*bcd*) or *osk* coding sequences were detected using alkaline-phosphatase-conjugated sheep anti-digoxigenin antibody (Roche Diagnostics). Finally, the slides were analyzed using conventional epifluorescence microscopy.

Quantitative 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*, 5′-CGAC-ACCCAATTGCTACTGC and 5′-GGCTGCAAGAGTCTGCTGG-3′; for *vas*, 5′-GTCGCCATTGGCATTGTAGG-3′ and 5′-GTACGTCCAATGCGATGTACG-3′; and for *actin 42A*, 5′-GTGCTAAGTGTGTGCAGCG-3′ and 5′-CTGGATGGC-AACATACATGG-3′. In each case, the primer pair flanked 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 processed using standard conditions: 3 min at 98℃; 40 cycles of 1 min at 94℃, 1 min at 58℃, 1 min at 72℃; and 7 min at 72℃. 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, with spanning exon–exon junctions were used: for *osk*, 5′-AACAAATCTTGCACCGCTGGGC-3′ and 5′-GACTTGGCGTGGTGAGGCC-TGA-3′; for *bcd* 5′-AACGAGCAAGAAGACGACGCTACAGATCTTG-3′ and 5′-GCGAATAGCGTATTGCAGGGAAAGTATAGA-3′. *rp49*, a ubiquitously expressed ribosomal protein mRNA, was used for normalization (5′-GCTA-AGCTGTCGCACAAA-3′ and 5′-TCCGGTGGGCAGCATGTG-3′).

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, 1× EDTA free protease inhibitor cocktail (Roche)] 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 stored at −70℃. Cup, Osk, Stau and Vas proteins were detected by western blot using the following antibodies: rat and rabbit anti-Cup (1:5000), rabbit anti-Osk (1:5000), rabbit anti-Vas (1:5000), goat anti-Stau (1:500) and mouse anti-tubulin (1:2000, Sigma).

Interaction assays

Co-immunoprecipitation assays were performed as previously described (Grimaldi et al., 2007) except that protein A/G PLUS-Agarose beads (Santa Cruz) and 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 Na_3VO_4) were used. The samples were subjected to immunoblot detection using rat anti-Cup and rabbit anti-Osk or rabbit anti-Vas antibodies. Rabbit pre-bleed sera were used as negative controls.

Yeast interaction assays were performed as previously described (Verrotti and Wharton, 2000). The Gal4 transcriptional activation domain (AD) was fused to *cup* fragments, encoding residues 29–962 and 2–1094 (Piccioni et al., 2009), and tested against a Gal4 transcriptional DNA-binding domain (DBD) fused to an *osk* segment encoding amino acids 122–650.

Drosophila strains

Flies were raised at 25℃ on standard sucrose/cornmeal/yeast food. cup^8 was described in Keyes and Spradling (Keyes and Spradling, 1997) and Verrotti and Wharton (Verrotti and Wharton, 2000). $Df(2L)BSC^{187}$, a chromosome bearing a complete deletion of the *cup* gene, 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 GFP expression, were distinguished from heterozygous *cup* mutant and homozygous *CyO* embryos (GFP positive). It is worth noting that the presence of the *CyO*–GFP balancer does not influence any phenotype, because identical results were obtained with the above mentioned *cup* alleles over wild-type chromosomes. The heterozygous *cup* mutant embryos, resulting by crossing *cup*/*CyO*–GFP mutant females with wild-type males, were selected for the lack of GFP expression. In the text, cup^8 and $Df(2L)BSC^{187}$ flies, balanced with CyO–GFP or CyO alone, are referred to as $cup^8/+$ and $Df(2L)BSC^{187}/+$, respectively.

Wild-type and mutant staged embryos were collected and the germ cells counted upon staining with anti-Vas antibody. 50 cycle 14 embryos (cellular blastoderm), for each maternal genotype described above, were collected and germ cells counted three times for each embryo. osk^{54}/TM3, Df(3R)p-XT103/TM3, the transgenic stock *osk-bcd* and P (osk^+ FA, ry^+)/CyO; ry/TM3, were obtained by A. Ephrussi; vas^1/CyO and vas^{PH165}/CyO by P. Lasko; $orb^{343}hd^{19}$G/TM3 by P. Schedl.

Acknowledgements

We would like to thank P. Lasko, A. Ephrussi, P. Schedl, T. Schupbach, P. MacDonald and D. St. Johnston for *Drosophila* stocks and antibodies; the DIM facility, in particular F. Formiggini at Ceinge for imaging microscopy. We also thank T. Russo for helpful discussions and M. Clements for critical reading of the manuscript. We greatly appreciate helpful comments from the anonymous reviewers.

Funding

This work was supported by Merit/Futuro in Ricerca-FIRB [grant number RBNE08LN4P_002 to Umberto di Porzio]; Institut National

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de la Santé et de laRecherche Médicale; Centre national de la recherche scientifique; Université de Strasbourg; Hopital de Strasbourg; Association pour la Recherche sur le Cancer [grant number SL2008 n 3173 to A.G.]; Institut National du Cancer [grant title UBICAN to A.G.]; and Agence Nationale de la Recherche (grant title ANR BLANC Epinest).

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.095208/-/DC1

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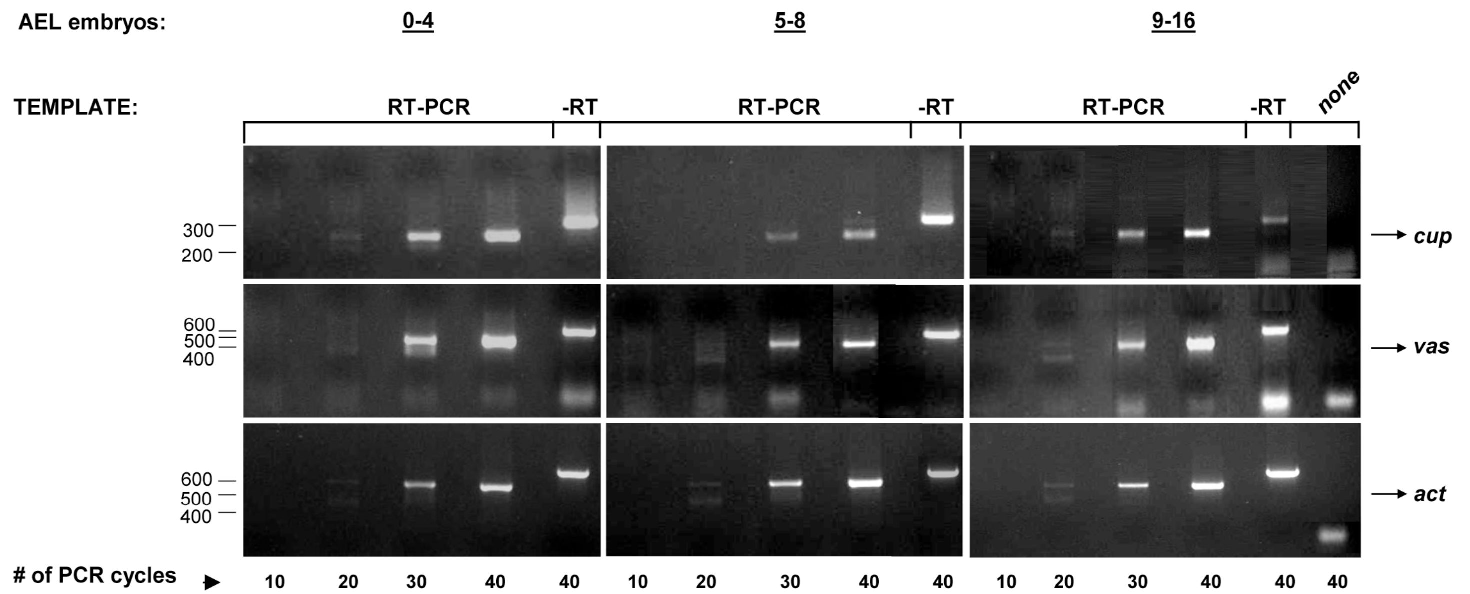

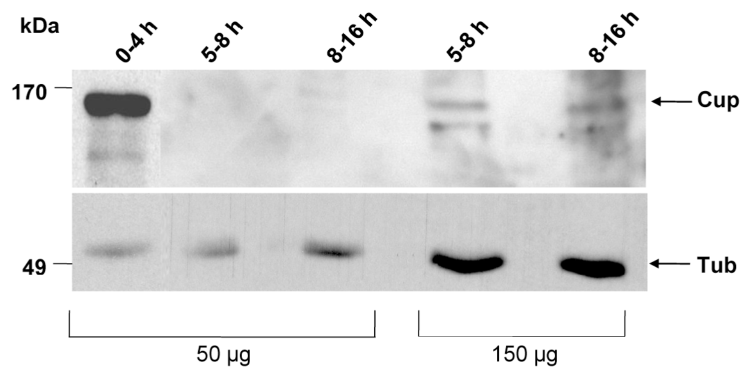

Fig. S1. *cup* gene products are detected in wild-type embryos. (A) The agarose gel displays RT-PCR products amplified with *cup* (top), *vas* (middle) and *actin42A*-specific primers (bottom). Total RNAs were isolated from wild-type embryos [0–4, 5–8 and 9–14 hrs after egg laying (AEL), respectively] 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, and 9–14 hrs AEL) were analyzed by Western blot using an anti-Cup antibody. Note that the amount of Cup protein present in 5–8 and 9–14 hrs AEL embryos is very low compared to the amount of Cup within 0–4 hrs embryos.

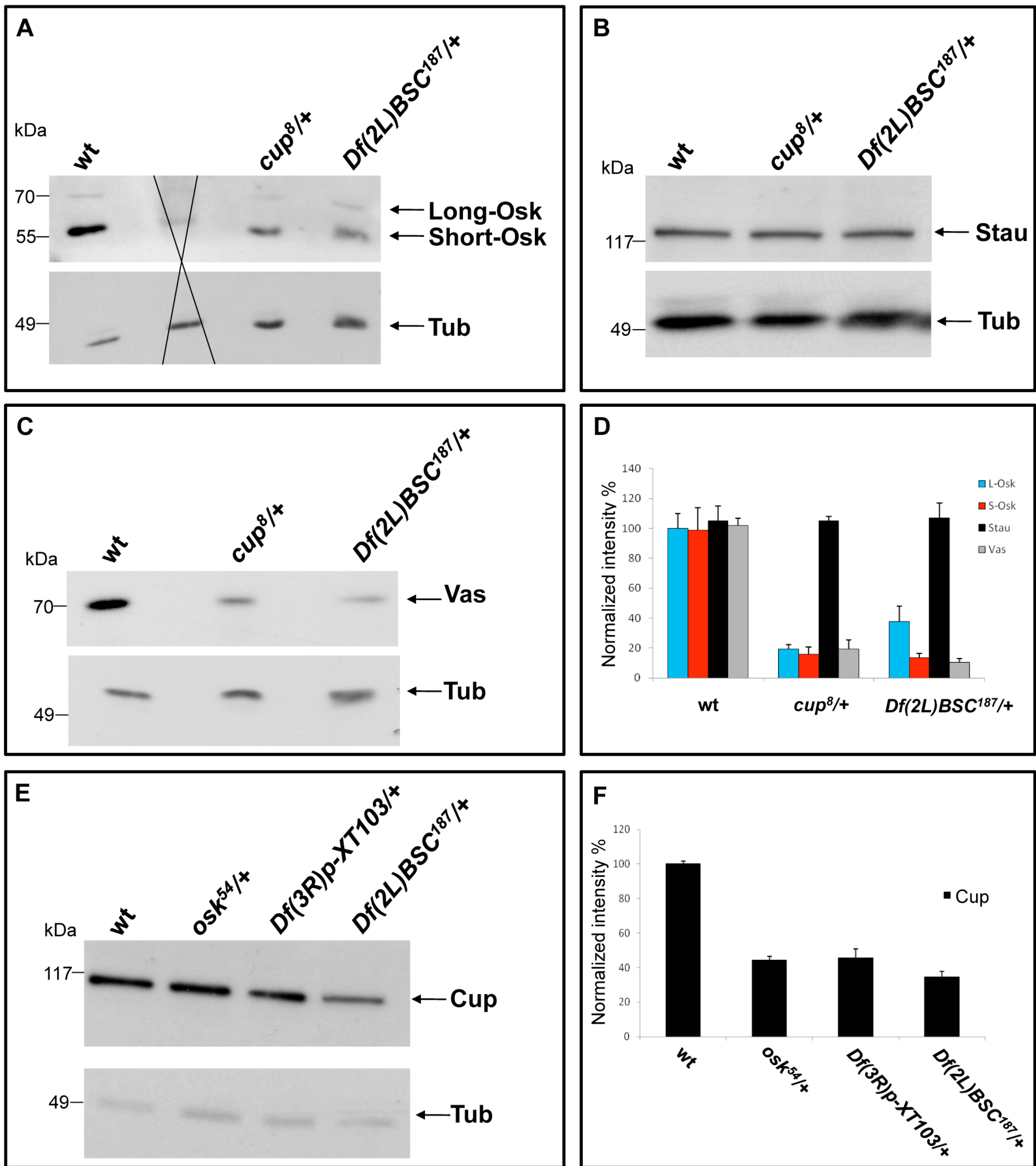

Fig. S2. Western blot analyses of germ cell components in *cup* heterozygous embryos. (**A–C**) Western blot detection of Osk (A, full membrane where the lanes to be considered are indicated), Stau (B) and Vas (C) protein in extracts of wild-type and heterozygous *cup* mutant (*cup*^{8}/+ and *Df(2L)BSC*^{187}/+) pre-blastoderm 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 shows that the levels of L- and S-Osk isoforms as well as Vas (normalized to α-Tubulin) were considerably reduced in the heterozygous *cup* mutant compared to those in wild-type embryos (arrows, A and C respectively; quantification is shown in D). On the contrary, the total level of Stau protein was unchanged (arrow, B; quantification is shown in D), confirming that the fluorescence signal observed in whole cytoplasm of the heterozygous *cup* mutant embryos probably represented the amount of Stau protein not correctly localized and not degraded (Fig. 9E,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*) pre-blastoderm embryos. We also used protein extract obtained from heterozygous *Df(2L)BSC*^{187} pre-blastoderm 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 α-Tubulin) were appreciably reduced in the heterozygous *osk* mutant compared to wild-type embryos (arrows, E; quantification, F).

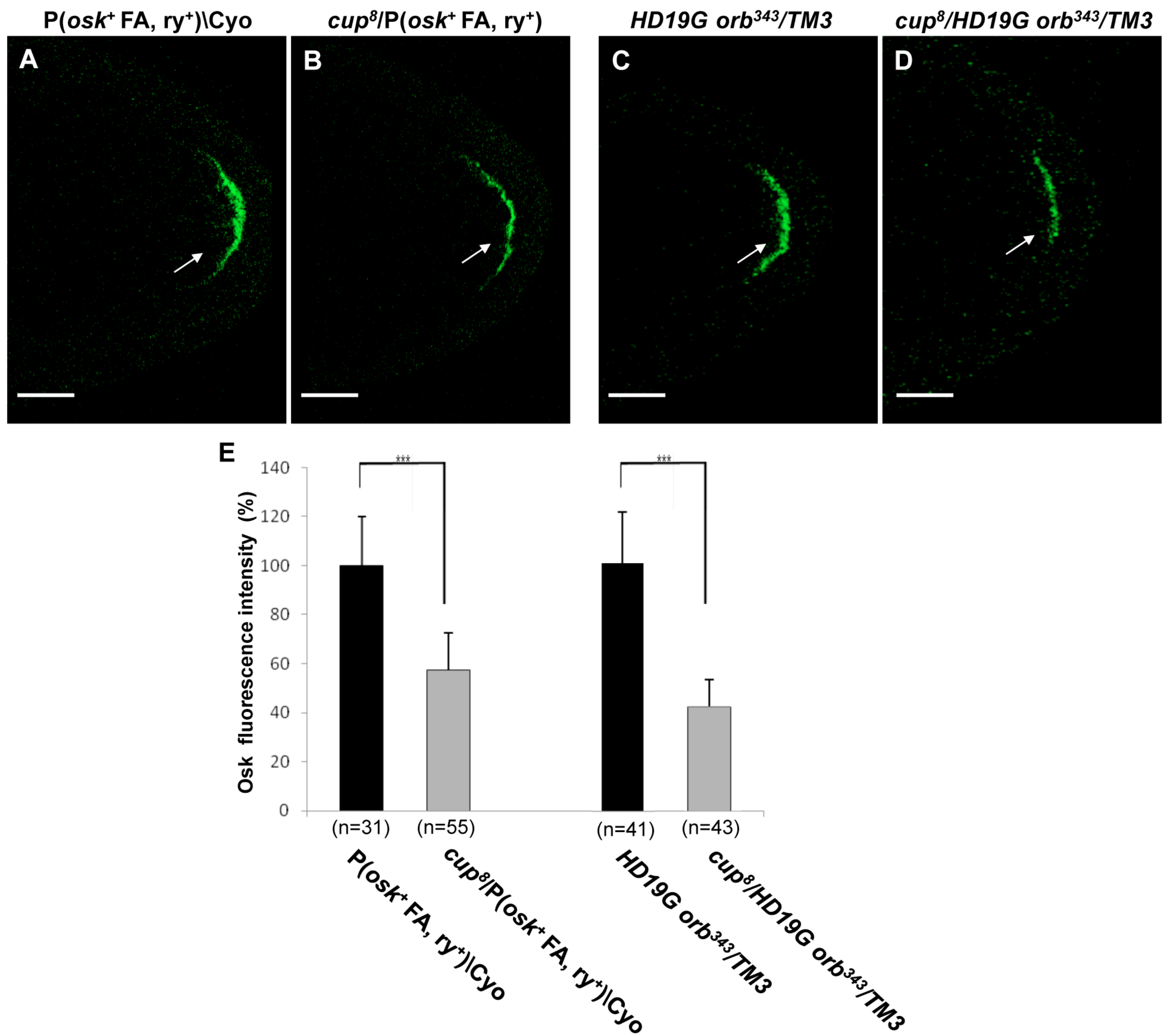

Fig. S3. Increase of *osk* gene dosage and decrease of *orb* gene dosage in *cup^8/Cy* O mutant do not rescue the pole plasm defects. (A–D) Stage 10 egg chambers from *P(osk$^+$ Fa, ry$^+$/CyO* (A); *cup^8/ P(osk$^+$ Fa, ry$^+$* (B); *orb^{343}hd19G/TM3* (C); *cup^8/+; orb^{343}hd19G/*+(D) stained with anti-Osk antibody. The staining at posterior pole is very similar to that observed in Fig. 5. (E) Quantification of fluorescent signals indicates that the decrease of Osk is significant at $P<0.001$ (***); Student's *t* test. n indicates the number of stage 10 egg chambers examined.