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# The molecular chaperone Hsp90 is a component of the cap-binding complex and interacts with the translational repressor Cup during *Drosophila* oogenesis

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ABSTRACT

In metazoa, the spatio-temporal translation of diverse mRNAs is essential to guarantee proper oocyte maturation and early embryogenesis. The eukaryotic translation initiation factor 4E (eIF4E), which binds the 5′ cap structure of eukaryotic mRNAs, associates with either stimulatory or inhibitory factors to modulate protein synthesis. In order to identify novel factors that might act at the translational level during *Drosophila* oogenesis, we have undertaken a functional proteomic approach and isolated the product of the *Hsp83* gene, the evolutionarily conserved chaperone Hsp90, as a specific component of the cap-binding complex. Here we report that Hsp90 interacts *in vitro* with the translational repressor Cup. In addition, we show that *Hsp83* and *cup* interact genetically, since lowering Hsp90 activity enhances the oogenesis alterations linked to diverse *cup* mutant alleles. Hsp90 and Cup co-localize in the cytoplasm of the developing germ-line cells within the germarium, thus suggesting a common function from the earliest stages of oogenesis. Taken together, our data start elucidating the role of Hsp90 during *Drosophila* female germ-line development and strengthen the idea that Cup has multiple essential functions during egg chamber development.

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## 1. Introduction

In eukaryotes, translation initiation is facilitated by the 7-methyl-guanosine cap structure, m$^7$GpppN, which is present on all mRNAs transcribed in the nucleus (Shatkin, 1976). The cap structure is recognized by the eukaryotic translation initiation factor 4E (eIF4E), which also directly binds the eukaryotic translation initiation factor 4G (eIF4G), a scaffolding protein aggregating with the 40S ribosome subunit through an interaction with the initiation factor eIF3 (Hershey et al., 1996). According to the closed loop model of translation (Wells et al., 1998), eIF4G, in turn, interacts with the poly(A) binding protein (PABP), bound to the poly(A) tail present at the 3′ end of the mRNAs, thus facilitating the recruitment of ribosomes for successive cycles of translation.

A widespread strategy to regulate eukaryotic translation relies on the perturbation of the eIF4E–eIF4G protein–protein interaction. Several proteins are able to interact with eIF4E, through a conserved eIF4E-binding motif (YxxxxLΦ, where $x$ is any residue and $\Phi$ is an aliphatic one), thus preventing its binding to eIF4G (Richter and Sonenberg, 2005). The eIF4E-binding proteins (4E-BPs) regulate the overall translation levels in cells by sequestering the majority of both free and cap-bound eIF4E (Karim et al., 2001). On the contrary, specific translational repressors, all containing eIF4E-binding motifs, exert their activity on select mRNAs by bridging eIF4E to sequence-specific RNA-binding proteins (Richter and Sonenberg, 2005; Vardy and Orr-Weaver, 2007).

*Drosophila* Cup is involved in translational repression of at least three specific mRNAs: *oskar* (*osk*) and *gurken* (*grk*) during oogenesis (Wilhelm et al., 2003; Nakamura et al., 2004; Clouse et al., 2008);

*Abbreviations:* eIF4E, eukaryotic translation initiation factor 4E; m$^7$GTP, 7-methyl-guanosine-triphosphate; m$^7$GpppN, m$^7$GTP cap structure; eIF4G, eukaryotic translation initiation factor 4G; eIF3, eukaryotic initiation factor 3; PABP, poly(A) binding protein; 4E-BP, eIF4E-binding protein; osk, oskar; grk, gurken; nos, nanos; UTR, untranslated region; EGTA, ethylene glycol tetraacetic acid; EDTA, ethylene diamine tetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PA, polyacrylamide; PAGE, PA-gel electrophoresis; nanoLC–MS/MS, nano-chromatography tandem mass spectrometry; HPLC, High Performance Liquid Chromatography; cDNA, DNA complementary to RNA; HEK293T, Human embryonic kidney 293T; FBS, fetal bovine serum; PMSF, phenyl-methylsulfonyl fluoride; Ig, immunoglobulin(s); AD, activation domain; DBD, DNA binding domain; otu, ovarian tumor; kDa, kilodalton(s).

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*nanos* (*nos*) in the early embryo (Nelson et al., 2004). All these functions have been linked to the ability of Cup to directly bind eIF4E and/or 3′-untranslated region (UTR) bound molecules. Furthermore, Cup appears to control the eIF4E phosphorylation status within the developing ovary (Zappavigna et al., 2004), thus modulating translation initiation efficiency.

In order to identify novel cap-binding factors that might be involved in translational control of specific mRNAs within the growing oocyte, we performed a functional proteomic analysis essentially based on the isolation of protein complexes by affinity chromatography on m$^7$GTP-Sepharose beads. Using mass spectrometry, we identified several proteins in these cap-binding complexes, including the product of the *Hsp83* gene (the evolutionarily conserved chaperone Hsp90). In *Drosophila*, the functions of Hsp90 have been extensively studied during male germ-line development (Castrillon et al., 1993; Yue et al., 1999). However, very little is known about Hsp90 functions in the ovary (Ding et al., 1993; Yue et al., 1999).

In this report, we show that Hsp90 binds the translational repressor Cup *in vitro*. Interestingly, *Hsp83* mutant alleles, similarly to *cup* mutant alleles, display a series of ovarian defects. We further demonstrate that a genetic interaction between *cup* and *Hsp83* occurs *in vivo*: reducing *Hsp83* activity specifically deteriorates the ovarian defects associated with different *cup* mutants. Cup and Hsp90 co-localize within the cytoplasm of developing female germ-line cells in the germarium and at later stages of oogenesis. Our results begin to shed light on Hsp90 functions during *Drosophila* female germ-line development and reinforce the concept that Cup has different essential roles during oogenesis (Piccioni et al., 2005).

## 2. Materials and methods

### 2.1. Cap-binding analysis

Cap-binding analysis was performed using m$^7$GTP-Sepharose beads (Amersham) according to Zapata et al. (1994), with the following modifications. Total protein extracts, derived from 10 wild-type ovary pairs, were pre-cleared in the presence of 30 µl of 50% slurry 4B-Sepharose beads (Amersham) for 1 h in binding buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 0.1% Tween-20) supplemented with a cocktail of protease inhibitors (Roche). The protein extracts were recovered by centrifugation (300×g for 5 min), added to 30 µl of 50% slurry m$^7$GTP-Sepharose beads and, then, gently mixed for 2 h. Beads were collected by centrifugation (300×g for 5 min), washed five times with binding buffer and one time with the same buffer, supplemented with 0.1 mM GTP, to eliminate non-specific bound proteins. Proteins bound to the cap structure were eluted by boiling in standard Laemmli buffer, supplemented with 100 mM DTT, separated by SDS-PAGE, and analyzed by mass spectrometry. All steps of the above experimental procedure were performed at 4 °C. Proteins bound to the 4B-Sepharose beads, during the pre-clearing step described above, were also eluted and analyzed as negative controls.

Protein bands, visualized by colloidal Coomassie, were excised from the gel, reduced, alkylated with iodoacetamide and digested with trypsin as previously described (Havlis et al., 2003). Peptide mixtures were extracted from the gel and analyzed by nano-chromatography tandem mass spectrometry (nanoLC–MS/MS) on a CHIP MS Ion Trap XCT Ultra equipped with a capillary 1100 HPLC system and a chip cube (Agilent Technologies, Palo Alto, CA). Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 400 to 2000 m/z) followed by MS/MS scans of the three most abundant ions in each MS scan. Raw data from nanoLC–MS/MS analyses were employed to query a non-redundant protein database using in house MASCOT software (Matrix Science, Boston, USA).

### 2.2. Co-immunoprecipitation assays

To generate pECFL-HA-Cup, an *Eco*RI-*Eco*RI *cup* cDNA fragment (encoding amino acids 2-1098) was inserted into the *Eco*RI site of the pECFL-HA vector. To generate pcDNA3-Flag-Hsp83, the *Bcl*I-*Bcl*I portion of the *Hsp83* cDNA (encoding amino acids 21–687) was inserted into the *Bam*HI site of the pcDNA3–Flag vector (Invitrogen). pMiranda–Myc is described in Piccioni et al. (2009).

Human embryonic kidney 293T cells (HEK293T) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% $CO_2$ atmosphere. Cells were transiently co-transfected with 1 µg of appropriate plasmids using FuGENE 6 (Roche Applied Science) in accordance with the manufacturer's instructions. Cells were lysed 48 h after transfection in lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0,1% Triton X-100, 40 mM β-glycerophosphate, 2 mM orthovanadate, 1 mM PMSF, 10% glycerol, and protease inhibitor cocktail). 1 mg of total cell lysate was pre-cleared with mouse IgG agarose beads (Sigma-Aldrich), and then incubated overnight with 40 µl of 50% slurry anti-Flag agarose beads (Sigma-Aldrich). After six washes in lysis buffer, at increasing ionic strength, immunocomplexes were eluted by incubating the beads with a Flag peptide (200 µg/ml) for 7 h at 4 °C. Samples were reduced by boiling in standard Laemmli buffer, supplemented with 100 mM DTT, and then subjected to SDS-PAGE, followed by immunoblotting with anti-HA or anti-Myc antibodies (Santa Cruz).

### 2.3. Drosophila strains

Flies were grown on standard sucrose–cornmeal–yeast food at 25 °C. Fly stocks were kindly provided by Dr. Stocker, Dr. Bopp, and by Bloomington *Drosophila* Stock Center. *Hsp83*$^{19F2}$ and *Hsp83*$^{e6A}$ alleles are described by Yue et al. (1999); *cup*$^{21}$ and *cup*$^{15}$ alleles by Keyes and Spradling (1997).

### 2.4. Ovary extracts

*Drosophila* ovarian protein extracts, derived from 3–5 day old females, were obtained by douncing in 150 mM NaCl, 1% NP-40, 50 mM Tris pH 8.0, 5% glycerol, supplemented with a cocktail of protease inhibitors (Roche).

### 2.5. Immuno-fluorescence and DNA staining

Whole ovaries were dissected, fixed, and incubated with antibodies as previously described (Gigliotti et al., 1998). Rabbit anti-Cup antisera were used at 1:200 dilution (Verrotti and Wharton, 2000); mouse anti-Hsp90 3E6 (Carbajal et al., 1990) was used at 1:50 dilution. Donkey anti-rabbit Alexa 488 and donkey anti-mouse Alexa 555 IgGs (Molecular Probes) were used as secondary antibodies at 1:400 dilution. DNA staining was performed using Hoechst 33258 (Sigma-Aldrich), as described by Zappavigna et al. (2004).

## 3. Results and discussion

### 3.1. Hsp90 is a component of the cap-binding complexes

In order to discover novel proteins involved in translational regulation during *Drosophila* oogenesis, we performed a cap-binding analysis. We used whole protein extracts from adult ovaries in affinity chromatography on m$^7$GTP-Sepharose beads (Zapata et al., 1994; Hernandez et al., 2005) and isolated approximately 30 candidates. These proteins were separated by SDS/PAGE and identified by liquid chromatography-tandem mass spectrometry (LC-MSMS; Fig. 1).

Several already known components of the active cap-binding complex could be detected (Table 1), namely: eukaryotic translation initiation factor 4E (eIF4E-1 and eIF4E-2; CG4035; Hernandez et al.,

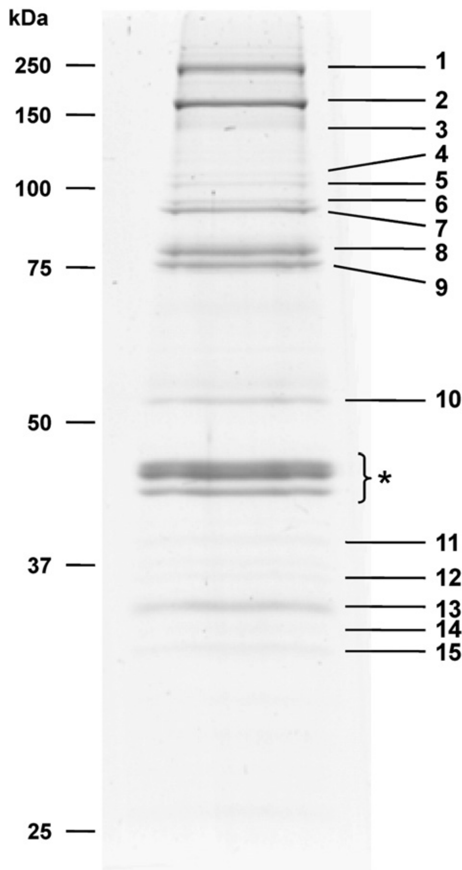


**Fig. 1.** Proteomics of *Drosophila* cap-binding complexes from adult ovaries. Whole protein extracts from *Drosophila* adult ovaries were used in affinity chromatography on $m^7$GTP-Sepharose beads. Bound proteins were separated by SDS/PAGE and individual protein bands (1–15) were identified by LCMS/MS analysis. The asterisk refers to vitellogenic proteins.

2005); eukaryotic translation initiation factor 4G (eIF4G; CG10811; Hernandez et al., 1998); Poly(A) binding protein (PABP; CG5119; Johnstone and Lasko, 2001); eukaryotic translation elongation factor 2b (EF-2; CG2238-PA; Grinblat et al., 1989); Suppressor of variegation 3–9 (eIF-2γ; CG6476-PB; Lasko, 2000); eukaryotic translation initiation factor 2α (eIF-2α; CG9946; Lasko, 2000); Trip1 (eIF-3β; CG8882; Lasko, 2000); Ribosomal protein S3 (RpS3; CG6779; Lyamouri et al., 2002); Ribosomal protein S3A (RpS3A; CG2168; Lyamouri et al., 2002); Ribosomal protein L5 (RpL5; CG17489; Coelho et al., 2005). We further isolated Cap-binding protein 80 (Cbp80; CG7035), which appears to replace eIF4E, for binding to the cap structure, during pre-mRNA maturation and nonsense-mediated mRNA decay processes (Maquat, 2004).

We could also identify specific factors whose involvement in translational repression mechanisms and/or localization of select mRNAs, during egg chamber development, had been either previously demonstrated or predicted (Table 1): Cup (CG11181; Piccioni et al., 2005); Maternal expression at 31B (Me31B; CG4916; Nakamura et al., 2001; Barbee et al., 2006); Trailer hitch (Tral; CG10686; Wilhelm et al., 2005; Barbee et al., 2006); Ypsilon schachtel (Yps; CG5654; Wilhelm et al., 2000; Mansfield et al., 2002); TER94 (CG2331; Ruden et al., 2000; Thomson et al., 2008); Rasputin (CG9412; Giot et al., 2003); Heterogeneous nuclear ribonucleoprotein at 87F (Hrb87F; CG12749; Giot et al., 2003); Heterogeneous nuclear ribonucleoprotein at 98DE (Hrb98DE; CG9983-PB; Giot et al., 2003).

Furthermore, we identified three members of the heat shock protein superfamily (Table 1): Glycoprotein 93 (Gp93; CG5520; homolog to human Endoplasmin, a member of the Hsp90 family); Heat shock protein 83 (Hsp83, hereafter called Hsp90; CG1242; Hartl, 1996); and Heat shock protein cognate 4 (Hsc70-4; CG4264; Perkins et al., 1990). Finally, three other factors, whose functional correlation with translational control is unclear, were found: α-Tubulin at 84B (α-Tub84B; CG1913; Hutchens et al., 1997), CG11148-PA, and CG18811.

As mentioned above, Hernandez et al. (2005) used a similar functional proteomic approach to identify proteins present in cap-binding complexes derived from *Drosophila* embryos. A comparison of the cap-binding analysis in embryos (Hernandez et al., 2005) and ovaries (this work) reveals the isolation of positive regulators of eukaryotic translation in higher percentage from embryos, but few of them (including eIF4E, eIF4G, PABP, Cbp80, Trip1 and RpS3) coincide. One possible explanation of this result is that the authors used 0–18 hour-old embryo extracts, a relatively broad developmental window when both maternally inherited and zygotically transcribed mRNAs are actively translated. On the contrary, during oogenesis several mRNAs (e.g. *nanos* and *oskar*) are known to be both negatively and positively regulated at the translational level to guarantee proper egg chamber development. Additional shared interactors isolated with the two affinity purification assays include α-Tub84B and two proteins putatively involved in RNA metabolism: Hsc70-4 (Dorner et al., 2006) and Hrb87F (Zu et al., 1996).

Our approach also identified Cup, a known negative regulator of translation and eIF4E-binding molecule, and a few of its interactors: Rasputin (Giot et al., 2003); Me31b (Giot et al., 2003; Wilhelm et al., 2005; Thomson et al., 2008); Tral (Wilhelm et al., 2005); Yps (Wilhelm et al., 2003). In addition, we isolated TER94 another protein involved in mRNA localization/translation (Thomson et al., 2008). It is interesting to notice that most of the available data from the literature point to *osk* (and/or *grk*) mRNA(s) as the primary molecular target of the above mentioned factors. It remains an open question if and how other mRNAs might be regulated by Cup at the translational level within the *Drosophila* ovary. However, a series of genetic data (Keyes & Spradling, 1997; Zappavigna et al., 2004) suggest that this might be true.

### 3.2. Effects of Hsp83 mutant alleles on oogenesis

Among the isolated components of the cap-binding complexes, we decided to focus our attention on Hsp90, a hub molecular chaperone

**Table 1**
Cap-binding proteins identified by mass spectrometry

| Bands | Proteins | Peptides[a] | % sequence coverage[b] |
|---|---|---|---|
| 1 | CG10811; eIF4G | 64 | 50 |
| | CG11148 | 15 | 14 |
| 2 | CG11181; Cup | 39 | 50 |
| 3 | CG18811 | 17 | 24 |
| 4 | CG2238; EF2 | 16 | 27 |
| | CG5520; Glycoprotein 93 | 13 | 18 |
| 5 | CG2331; TER94 | 10 | 18 |
| 6 | GC9412; Rasputin | 13 | 20 |
| | CG7035; Cbp80 | 11 | 18 |
| 7 | CG1242; *Hsp83* | 25 | 33 |
| 8 | CG10686; Trailer hitch | 5 | 11 |
| 9 | CG4264; Hsc70-4 | 36 | 66 |
| | CG5119; PABP | 11 | 25 |
| 10 | CG4916; Me31B | 21 | 46 |
| | CG6476; eIF-2γ | 8 | 21 |
| | CG5654; Ypslon schachtel | 9 | 33 |
| | CG1913; α-Tubulin 84B | 6 | 22 |
| 11 | CG12749; Hrb87F | 10 | 28 |
| | CG9946; eIF-2α | 6 | 19 |
| 12 | CG8882; Trip1 | 6 | 25 |
| | CG9983; Hrb98DE | 6 | 20 |
| 13 | CG4035; eIF4E-1 | 6 | 37 |
| 14 | CG6779; RpS3 | 15 | 58 |
| | CG17489; RpL5 | 11 | 33 |
| | CG2168; RpS3A | 9 | 35 |
| 15 | CG4035; eIF4E-2 | 6 | 29[a] |

[a] Total number of peptides used for identification.
[b] Percentage of sequence coverage for each identified protein.

(Young et al., 2001; Rutherford et al., 2007), since recent data from the literature show that it is involved in mRNA localization mechanisms during *Drosophila* early embryogenesis (Song et al., 2007). Subcellular mRNA localization often assures restricted protein synthesis (Seydoux, 1996; Johnstone and Lasko, 2001; St Johnston, 2005), thus Hsp90 might also act as a translational regulator of select mRNAs during oogenesis.

In order to investigate the role of Hsp90 during oogenesis, we first analyzed the ovarian phenotype of flies bearing diverse *Hsp83* mutant alleles. Most of the known *Hsp83* mutant alleles are homozygous lethal, however some are *trans*-heterozygous viable and display a sterile phenotype in males and either a sterile or a weakly fertile phenotype in females (Yue et al., 1999). We used the *Hsp83* alleles *e6A* and *19F2* (S592F and R48C respectively, according to their amino acid substitution) that cause, in *trans*-heterozygous combination, a dramatic reduced viability (<1%) and a full female sterility (Yue et al., 1999).

$Hsp83^{e6A}/Hsp83^{19F2}$ mutant female flies display a series of ovarian defects (Fig. 2). Most egg chambers (approximately 75%) are blocked at different developmental stages, not exceeding stage 9 (Fig. 2, panel A). The remaining egg chambers show a pronounced defect during the transfer of the nurse cell cytoplasm into the developing oocyte, a process named dumping and starting around stage 10B of oogenesis. In $Hsp83^{e6A}/Hsp83^{19F2}$ mutant egg chambers, the nuclei of the nurse cells do not degenerate at the end of the dumping process, as it normally occurs, but persist up to the latest stages of oogenesis, when the formation of the chorion culminates in the synthesis of the dorsal appendages (Fig. 2, panels B and C). Moreover, mutant mature eggs are smaller in size with altered dorsal appendages when compared to their heterozygous, wild-type counterparts (Fig. 2, panels D and E).

The oogenesis defects described above resemble a few of the diverse morphological alterations displayed by a number of *cup* alleles. In particular, the weakest group of *cup* alleles (class III) produces defective eggs reduced in volume, besides being characteristically shaped like cups (Keyes and Spradling, 1997).

### 3.3. Hsp90 and Cup co-localize in vivo and interact in vitro

In order to analyze the spatio-temporal expression pattern of the Hsp90 protein and to compare it with the expression pattern of Cup, we performed immuno-localization experiments on wild-type ovaries using either monoclonal anti-Hsp90 or polyclonal anti-Cup antibodies.

As shown in Fig. 3A (left panel), the Hsp90 protein is detected within the cytoplasm of both somatic and germ-line derived cells throughout oogenesis, thus suggesting a ubiquitous mode of action of Hsp90 during egg chamber development. On the contrary, the Cup protein is restricted to the cytoplasm of all germ-line cells and is

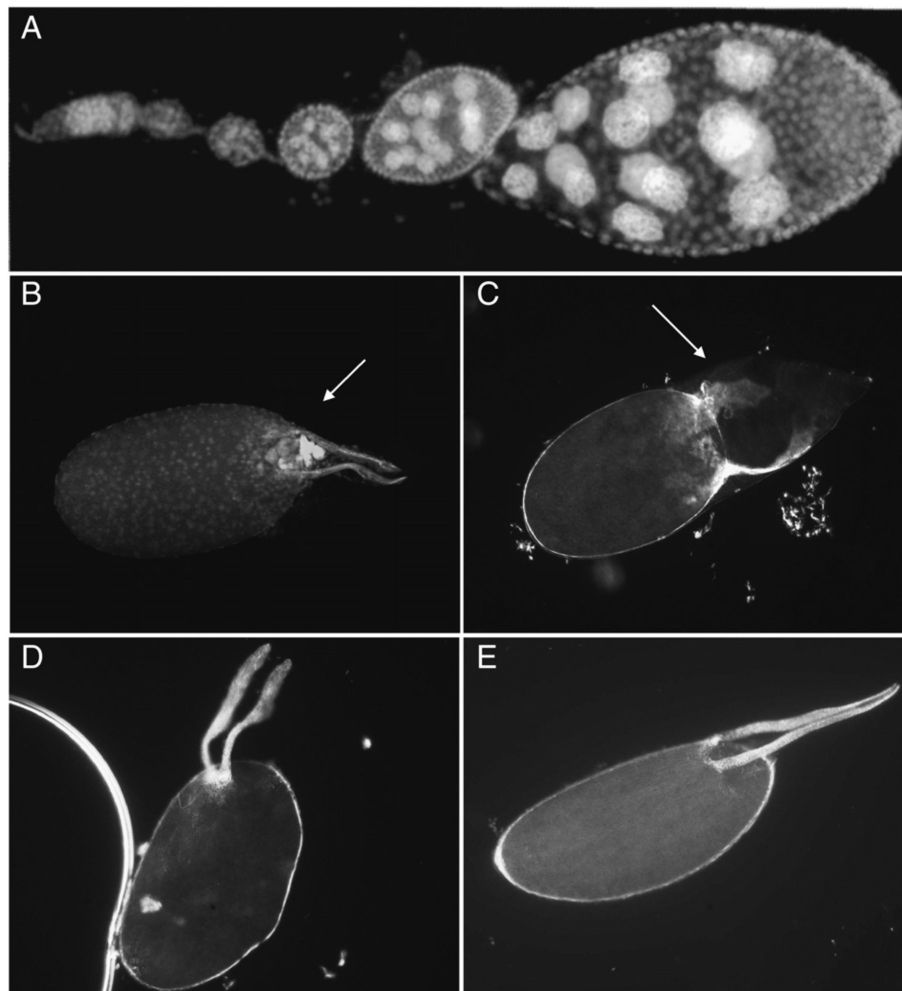


**Fig. 2.** Effects of *Hsp83* mutant alleles on oogenesis. $Hsp83^{e6A}/Hsp83^{19F2}$ females are weakly viable (<1%) and fully sterile (Yue et al., 1999). The majority of $Hsp83^{e6A}/Hsp83^{19F2}$ egg chambers (approximately 75%) are blocked in their development during mid-oogenesis (panel A). The remaining egg chambers show a pronounced defect during the dumping process (panels B and C). Hoechst-staining (panels A and B) and dark field (panel C) images are not on scale. In addition, dark field images show mutant mature eggs (panel D) smaller in size when compared to their heterozygous, wild-type counterparts (panel E); these two last pictures are on scale.

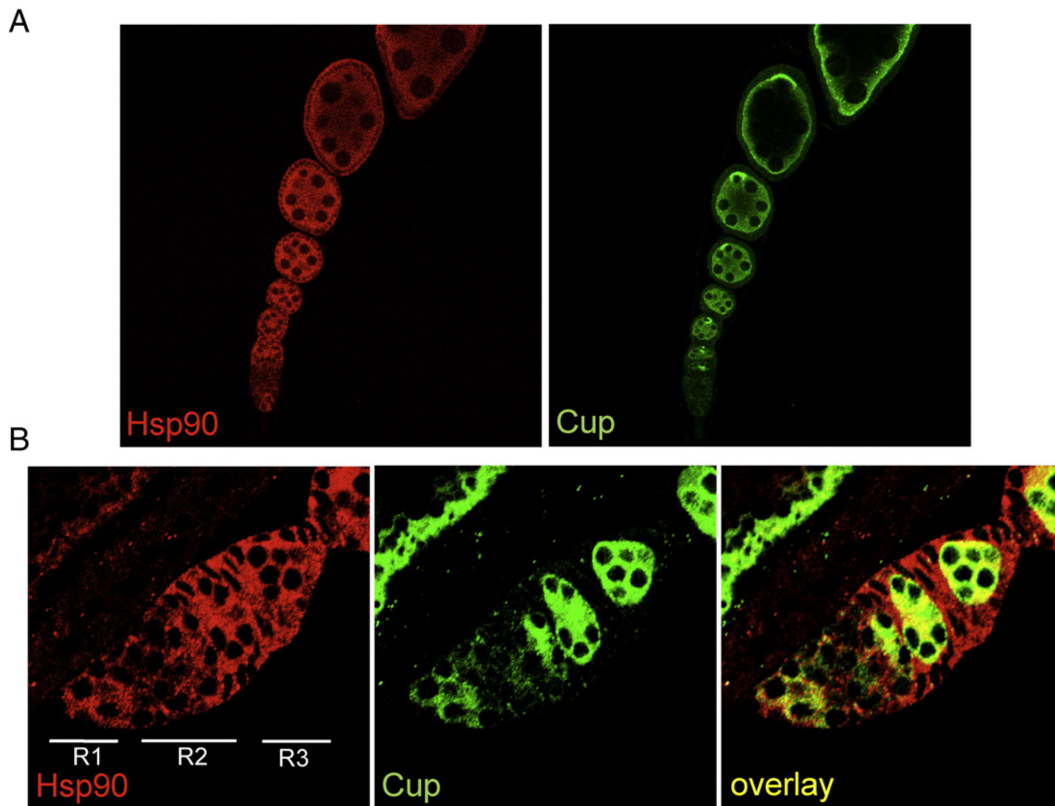


**Fig. 3.** Hsp90 co-localizes with Cup within wild-type germaria. Single confocal sections of wild-type egg chambers, doubly immunostained with anti-Hsp90 (red) and anti-Cup (green) antibodies, are shown. (A) Hsp90 (left panel) is distributed within the cytoplasm of both somatic and germ-line derived cells throughout oogenesis; Cup (right panel) accumulates at the posterior cytoplasm of developing oocytes (up to stage 8), around the nurse cell nuclei (in early egg chambers), and in the nurse cell cytoplasms; (B) Cup and Hsp90 show a similar dynamic profile of expression within the germarium. The two proteins co-localize in the cytoplasm of the germ-line stem cells (region 1, R1), are almost undetectable in the cystoblast cleavage stages (anterior portion of region 2, R2), and rise again to relatively high levels in the posterior portion of region R2 and in the germ-line maturing cysts (region 3, R3).

selectively enriched at the posterior of the developing oocyte up to stage 8 (Fig. 3B, right panel). Moreover, Cup surrounds the nurse cell nuclei in early egg chambers and by stage 10 most of the protein lies in the nurse cell cytoplasm. These observations indicate only that the distribution patterns of the two proteins overlap within selected area, including the oocyte posterior cytoplasm.

It is worth noticing, however, that both Hsp90 and Cup levels are specifically modulated in the initial phases of germ-line development, which occur within the anterior tip of the ovariole, called germarium, where germ-line stem cells divide asymmetrically to give rise to cystoblasts, successively developing into cysts (Fig. 3A; Keyes and Spradling, 1997; Verrotti and Wharton, 2000). This is better appreciated in double immunofluorescence experiments, performed using anti-Cup and anti-Hsp90 antibodies on wild-type ovaries (Fig. 3B). In region 1 (R1), both proteins are present in the germ-line stem cells and cystoblasts, become almost undetectable during cystoblast cleavage stages in the anterior portion of region 2 (R2), and rise again to relatively high levels in the posterior portion of R2 and in region 3 (R3), where the germ-line maturing cysts are located. The coincident spatio-temporal distribution of Cup and Hsp90, within stem cells, late cystoblasts, and maturing cysts of the germarium, suggests that the two proteins may play a common function at least during the earliest stages of oogenesis.

To demonstrate that a physical interaction occurs between Hsp90 and Cup, HEK293T cells were co-transfected with plasmids encoding HA–Cup and Flag–Hsp90 or Flag alone. As shown in Fig. 4A, the anti-Flag antibody co-immunoprecipitates HA–Cup when cells were co-transfected with HA–Cup and Flag–Hsp90 expressing vectors. On the contrary, the same antibody fails to co-immunoprecipitate HA–Cup

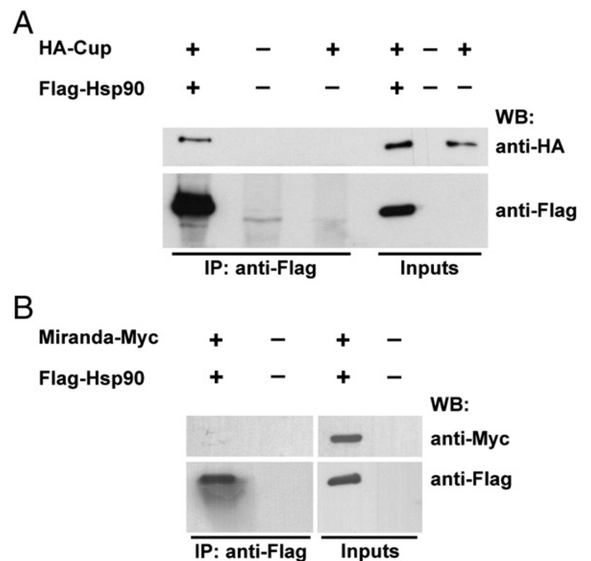


**Fig. 4.** Hsp90 associates specifically with Cup. Co-immunoprecipitation assays. (A) HEK293T-transfected cells expressing HA-Cup in the presence of Flag–Hsp83 or Flag alone. An anti-Flag antibody co-immunoprecipitates HA-Cup fusion protein specifically when cells are co-transfected with Flag–Hsp90 and HA–Cup expressing vectors (lane 1), but the same antibody fails to co-immunoprecipitate HA–Cup in a control reaction (lane 3). (B) HEK293T cells co-transfected with Flag–Hsp90 and Miranda–Myc expressing vectors. An anti-Flag antibody immunoprecipitates Flag–Hsp90 efficiently (lane 1, bottom panel), but does not co-immunoprecipitate Miranda–Myc (lane 1, top panel), All transfected cells show high-levels of expression of tagged proteins (input lanes).

when Flag alone and HA-Cup expressing vectors were co-transfected. In addition, to exclude the possibility that Hsp90 might act as a chaperone on some fraction of misfolded Cup, thus accounting for the biochemical interaction described above, we verified its binding ability on Miranda, a protein known to specifically interact with Cup (Piccioni et al., 2009). To this aim, we co-transfected HEK293T cells with plasmids encoding Miranda–Myc and Flag–Hsp90 and demonstrated that the anti-Flag antibody fails to co-immunoprecipitate Miranda–Myc (Fig. 4B).

Next, we wished to determine whether or not the interaction between Hsp90 and Cup might be direct. To test these possibilities, we performed a yeast two-hybrid assay using three different portions of the Hsp90 protein (corresponding to amino acids 21–687, 21–717, and 2–717) fused to the activation domain (AD) of GAL4 and full-length Cup (amino acids 2–1132) protein fused to the GAL4 DNA-binding domain (DBD). Although corrected expressed, none of these Hsp90 AD-fusions interacts with Cup in yeast (data not shown).

Altogether, these data demonstrate that Hsp90 is a component of the cap-binding complexes and a novel Cup-interactor, but also suggest that the interaction with Cup is indirect and consequently mediated by other factors. It is therefore likely that one or more of the proteins identified with the functional proteomic approach described above are responsible for this interaction.

### 3.4. Hsp83 interacts genetically with cup

Cup is a playmaker during female germ-line development: its interaction with *ovarian tumor* (*otu*) is required for the structure and function of germ-line chromosomes (Keyes and Spradling, 1997); the association with Nanos is necessary for the maintenance and survival

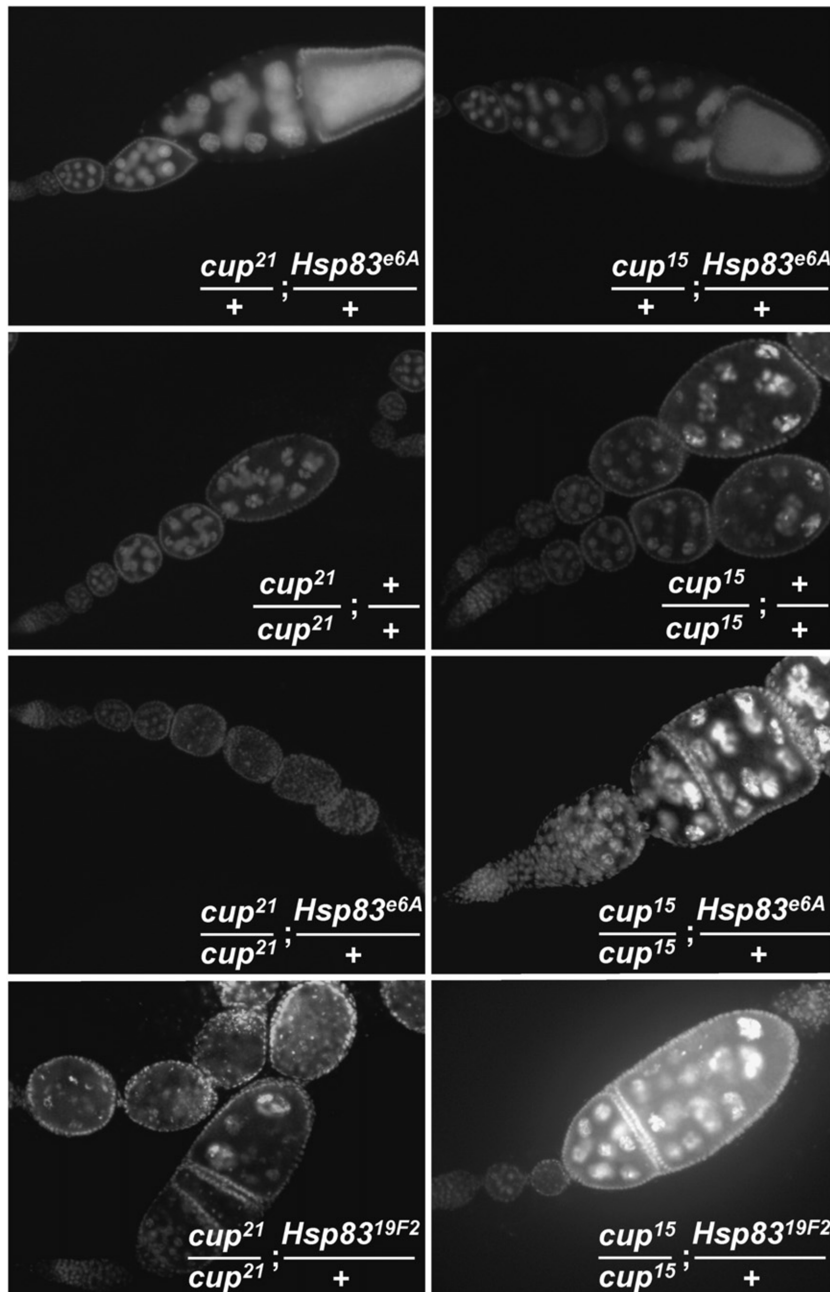


**Fig. 5.** *Hsp83* and *cup* interact genetically. Morphological analysis of homozygous *cup*$^{21}$ and *cup*$^{15}$ (as representative of the weak and intermediate class of *cup* alleles, respectively) mutant ovaries in combination with diminished Hsp90 activity, by using either *Hsp83*$^{e6A}$ or *Hsp83*$^{19F2}$ mutant allele. The panel displays fluorescence microphotographs of Hoechst-stained egg chambers to visualize nuclei. Note that all double heterozygous combinations behave as wild-types.

of female germ-line stem cells (Verrotti and Wharton, 2000); its interaction with *eIF4E* is required to control development and growth of ovaries (Zappavigna et al., 2004); it cooperates with Miranda to assure proper egg chamber development (Piccioni et al., 2009). Moreover, during oogenesis, Cup acts as a translational repressor directly associating with eIF4E and/or 3′-untraslated region (UTR)-bound factors, thus silencing *osk* and *grk* mRNAs (Whilhelm et al., 2003; Nakamura et al., 2004; Clouse et al., 2008).

However, the studies reported so far do not explain all the phenotypes shown by the different *cup* mutant alleles available, suggesting that Cup associates with multiple factors to accomplish its numerous roles. Our results suggest that Hsp90 is a novel Cup-interactor during *Drosophila* oogenesis. Thus, we asked whether or not *Hsp83* and *cup* might interact genetically during egg chamber development.

To address this question, we lowered the level of Hsp90 activity and looked for any modification of the ovarian phenotypes associated with different *cup* mutations. Whereas *Hsp83* mutant alleles (at least those used in this study) are homozygous lethal, *cup* mutant alleles are homozygous viable and grouped into three classes, based on the severity of their oogenesis defects (Keyes and Spradling, 1997). On the other hand, both *Hsp83* and *cup* alleles are fully recessive, thus behaving as wild-types when in heterozygous conditions.

In our studies, we used $cup^{21}$ and $cup^{15}$ as representative of classes III and II of *cup* alleles respectively. Class III includes the weakest *cup* alleles, which display characteristic cup-shaped mature eggs and dwarf oocytes; while the intermediate class II alleles show egg chamber growth arrest around stages 7–8 and abnormal chromatin configuration (Keyes and Spradling, 1997). The two *cup* mutant alleles were tested in combination with either $Hsp83^{e6A}$ or $Hsp83^{19F2}$ mutant alleles, previously shown to behave as *trans*-heterozygous female sterile.

On a morphological level, all *Hsp83* alleles tested exacerbate the oogenesis defects associated with both class III and class II *cup* alleles, as shown by Hoechst staining (Fig. 5). The reduction of *Hsp83* activity, in a $cup^{21}$ genetic background, results in earlier egg chamber growth arrest (around stages 4–5) and precocious degeneration of the nurse cell nuclei, which appear already altered at stages 2–3, when compared to homozygous $cup^{21}$ control ovaries. Moreover, $cup^{21}/cup^{21}$;*Hsp83*/+ germaria are often misshapen, with egg chambers either unbudded or fused, as underlined by the presence of supernumerary nurse cell nuclei (Fig. 5, left panels). Similar defects are also displayed by $cup^{15}/cup^{15}$;*Hsp83*/+ ovaries when compared to homozygous $cup^{15}$ controls (Fig. 5, right panels). 33% (75/227) of $cup^{21}/cup^{21}$;*Hsp83*/+ egg chambers stop development around stage 4; the remaining 67% (152/227) of these egg chambers are fused. On the contrary, only 12% (19/157) of $cup^{21}/cup^{21}$ control ovaries arrest their development around stage 4, and no significant fusion rate is observed. Comparable frequencies and types of defects are observed in $cup^{15}/cup^{15}$;*Hsp83*/+ egg chambers when compared to $cup^{15}/cup^{15}$ controls. Taken together, these results indicate a *bona fide* genetic interaction between *Hsp83* and *cup*. This genetic interaction is not allele-specific since two different *Hsp83* alleles deteriorate the ovarian phenotype displayed by two distinct *cup* alleles.

Finally, we tested whether or not the amount or distribution of the Cup protein are altered in $Hsp83^{e6A}/Hsp83^{19F2}$ mutant female ovaries. As demonstrated by Western blot of total ovary extracts, Cup protein levels were not reduced in *Hsp83* mutants when compared to their wild-type counterparts (data not shown). However, in 3–5% of stage 9 $Hsp83^{e6A}/Hsp83^{19F2}$ egg chambers Cup did not appear tightly localized to the posterior pole of developing oocytes.

The biochemical and genetic interactions we observed between Hsp90 and Cup suggest that the protein complex formed by these two proteins might mediate localization/translation of select mRNAs during egg chamber development. This hypothesis is corroborated by the finding that Hsp90 is required for mRNA localization during embryogenesis (Song et al., 2007). However, we cannot exclude that Hsp90 could also act as a hub molecular chaperone contributing to the proper structure and/or function of other proteins involved in localization/translation of specific mRNAs during oogenesis.

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