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RESEARCH REPORT

Accumulation of the *Drosophila* Torso-like protein at the blastoderm plasma membrane suggests that it translocates from the eggshell

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ABSTRACT

The eggshell serves as a depository for proteins that play an important role in early embryonic development. In particular, the *Drosophila* eggshell is responsible for transferring asymmetries from the egg chamber to specify the regions at both ends of the embryo through the uneven activation of the Torso (Tor) receptor in its membrane. This process relies on the restricted expression of the gene *torso-like* (*tsl*) in subpopulations of follicle cells during oogenesis and its protein accumulation at both poles of the eggshell, but it is not known how this signal is transmitted to the embryo. Here, we show that Tsl accumulates at the embryonic plasma membrane, even in the absence of the Tor receptor. However, during oogenesis, we detected Tsl accumulation only at the eggshell. These results suggest that there is a two-step mechanism to transfer the asymmetric positional cues from the egg chamber into the early embryo: initial anchoring of Tsl at the eggshell as it is secreted, followed by its later translocation to the egg plasma membrane, where it enables Tor receptor activation. Translocation of anchored determinants from the eggshell might then regulate the spatial and temporal control of early embryonic developmental processes.

KEY WORDS: *Drosophila*, MACPF domain, Plasma membrane, Torso, Torso-like, Vitelline membrane

INTRODUCTION

The *Drosophila* ovary provides the positional cues that specify the pattern of the future embryonic body plan. In particular, the transduction pathways specifying the dorso-ventral axis and the two embryonic end regions rely on asymmetries between the ovarian cells for the locally restricted activation of their corresponding receptors (reviewed by Stein and Stevens, 1991). In the case of the terminal regions, these are specified by the activity of the Torso (Tor) receptor tyrosine kinase, which is present throughout the entire blastoderm membrane but activated only at the poles. The precise mechanism of Tor activation is unknown, but it depends on the expression of *torso-like* (*tsl*) in subpopulations of follicle cells at both ends of the oocyte. In embryos from *tsl* mutant females, the Tor receptor is not activated and, conversely, ubiquitous expression of *tsl* in follicle cells leads to general activation of the Tor receptor throughout the embryo (reviewed by Furriols and Casanova, 2003).

Given that the *tsl*-expressing cells degenerate at the end of oogenesis and are not present at the time the Tor receptor is activated at early embryogenesis (Schupbach and Wieschaus, 1986; Sprenger et al., 1993), there must be a mechanism to ensure the transfer of the asymmetric positional cues from the egg chamber to the early embryo (Casanova and Struhl, 1993). This mechanism appears to be linked to eggshell proteins. A first indication pointing to this link came from the discovery that *fs(1)Nasrat* [*fs(1)N*] and *fs(1)polehole* [*fs(1)ph*; *fs(1)M3* – FlyBase], two germline genes required for eggshell biogenesis, have hypomorphic mutations that do not affect eggshell formation but prevent Tor receptor activation (Degelmann et al., 1990).

A further confirmation of this link came from the analysis of Tsl protein distribution (Stevens et al., 2003). Although Tsl was initially reported to accumulate at the embryonic poles, an observation taken as an indication consistent with Tsl being the ligand of the Tor receptor (Martin et al., 1994), it was not possible to replicate this result (Stevens et al., 2003). Instead, later experiments found that Tsl accumulated in laid eggs at the internal side of the vitelline membrane, the innermost layer of the eggshell (Stevens et al., 2003). Interestingly, Tsl extracellular accumulation is dependent on the *fs(1)N*, *fs(1)ph* and *closca* genes, indicating that the localisation of Tsl depends on eggshell proteins secreted from the oocyte (Jiménez et al., 2002; Stevens et al., 2003; Ventura et al., 2010). However, it remained an open question as to how the accumulation of Tsl at the eggshell could influence the embryo. Furthermore, the finding that Tsl harbours a membrane-attack complex/perforin domain (MACPF) (Ponting, 1999), a domain present in proteins involved in the formation of pores at the plasma membrane (for a review see Voskoboinik et al., 2006), made this observation difficult to reconcile with a function at the eggshell.

Many data have pointed alternatively to the trunk (*trk*) protein being the Tor ligand. *trk* RNA accumulates in the oocyte and its protein is likely to be secreted into the perivitelline fluid between the embryo and the eggshell (Casanova et al., 1995). Trk shares structural features with growth factors and its C-terminal fragment activates the Tor pathway even in a *tsl* mutant background (Casali and Casanova, 2001); however, Tsl still requires *trk* function to activate the Tor pathway (Furriols et al., 1998). These results prompted the notion that Tsl is involved in the cleavage of Trk, which would then behave as the ligand for the Tor receptor (Casanova et al., 1995). However, this notion for Tsl function has recently been challenged (Henstridge et al., 2014) (and see below).

Here, using a new anti-Tsl antibody (Grillo et al., 2012) and an alternative fixation procedure, we show that Tsl accumulates at the blastoderm plasma membrane, even in the absence of the Tor receptor, thereby ruling out that this accumulation is indicative of Tsl being a Tor ligand (Martin et al., 1994). Furthermore, during oogenesis, we detected Tsl accumulation only at the vitelline membrane. These results suggest that there is a two-step mechanism through which the Tsl asymmetric positional cue is transferred from the egg chamber into the early embryo: initial anchoring of Tsl at the

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vitelline membrane as the protein is secreted by the follicle cells, followed by its later translocation to the oocyte plasma membrane, where it would enable Tor receptor activation.

RESULTS AND DISCUSSION

By means of a haemagglutinin-tagged Tsl construct (Tsl-HA), we previously reported that Tsl is found in the cytoplasm of a specialised group of follicle cells surrounding the oocyte and extracellularly between the follicle cells and the oocyte (Jiménez et al., 2002). We have now been able to generate an anti-Tsl antibody (Grillo et al., 2012) that reproduces the previously unveiled Tsl-HA pattern (Fig. 1A). We have further proved the specificity of the antibody by the absence of signal in females homozygous for tsl^{604} (Fig. 1B), a P-element-induced mutation in the promoter that eliminates *tsl* function (Martin et al., 1994).

Nevertheless, this antibody was unable to reveal any specific signal in embryos upon standard chemical fixation or by electron microscopy (data not shown). However, when embryos were instead subjected to heat-fixation (Tepass, 1996; Tanentzapf et al., 2007), we detected a clear Tsl accumulation at both embryonic poles (Fig. 1C-E), which was also specific to Tsl because it was absent in embryos from tsl^{604} females (Fig. 1F) and was found throughout the embryo upon ectopic expression of *tsl* in the follicle cells (Fig. 1G). Note that upon *tsl* ectopic expression, we also observed high levels of Tsl within embryos. We then analysed the requirements for Tsl accumulation at the embryonic poles. We first concluded that embryonic Tsl accumulation does not reflect receptor binding, as Tsl also accumulates in embryos without the Tor receptor (Fig. 1H). Similarly, Tsl accumulation at the embryonic poles was also independent of *trk* (Fig. 1I).

How can the previously reported accumulation of Tsl at the vitelline membrane in laid eggs be reconciled with Tsl accumulation at the plasma membrane? There is a time lapse between *tsl* expression in the follicle cells by stage 8 (Savant-Bhonsale and Montell, 1993; Martin et al., 1994) and Tor receptor accumulation in early embryogenesis (Schupbach and Wieschaus, 1986; Casanova and Struhl, 1993; Sprenger et al., 1993), when the follicle cells are no longer present. Our findings suggest that the mechanism that ensures that the spatial asymmetry in the ovary is transmitted to the embryo to allow restricted activation of the Tor receptor (Casanova and Struhl, 1989) and consists of the initial anchoring of Tsl, as it is secreted, into the vitelline membrane, followed by its later translocation to the embryonic plasma membrane.

To support this hypothesis, we then assessed the precise localisation of Tsl protein during oogenesis. Although Tsl-HA is detected extracellularly (Jiménez et al., 2002), the exact compartment of Tsl accumulation has never been analysed. We have found now that domain containing extracellular Tsl overlapped with that of the vitelline protein Nasrat (Fig. 2A). This observation is consistent with Tsl accumulation at the vitelline membrane in laid eggs (Stevens et al., 2003) and fits well with the requirement of Nasrat for Tsl extracellular accumulation (Jiménez et al., 2002; Stevens et al., 2003). As expected, Nasrat did not overlap with the Yolkless (Yl) receptor (Schonbaum et al., 2000) (Fig. 2B), an oocyte plasma membrane protein (we were unable to simultaneously examine the Tsl and Yl patterns as both antibodies are derived from rat). We obtained the same results for Polehole (Fig. 2C), a protein closely related to Nasrat (Jiménez et al., 2002). Of note, our experiments also showed variable patterns of vitelline membrane proteins, as determined by confocal microscopy. Thus, for example, the locations of Nasrat and VM32E, which have been assigned to the vitelline membrane by electron microscopy (Andrenacci et al., 2001) and identified as eggshell proteins by proteomics (Fakhouri et al., 2006), often do not overlap when viewed by confocal microscopy (Jiménez et al., 2002). This observation might indicate heterogeneity within the vitelline membrane or differences in antibody accessibility or recognition caused by sample processing and/or distinct fixation procedures used for confocal microscopy or for electron microscopy. Similarly, we also observe different patterns for Tsl and VM32E by confocal microscopy (Fig. 2D). In summary, these results point to the initial anchoring of Tsl at the vitelline membrane, which is probably mediated by interactions with the products of the *fs(1)N*, *fs(1)ph* and *closca* genes (Jiménez et al., 2002; Stevens et al., 2003; Ventura et al., 2010).

We then examined how early Tsl protein accumulates at the plasma membrane. As Tsl is detected in very young embryos (Fig. 2E), we addressed whether Tsl accumulation at the plasma membrane is linked to fertilisation; however, Tsl was also present in the plasma membrane of unfertilised eggs (Fig. 2F). Therefore, we conclude that Tsl accumulates at the plasma membrane between late oogenesis and fertilisation. In this period, the most prominent changes are related to egg activation, the conversion of the oocyte into an egg capable of supporting embryogenesis (Sartain and Wolfner, 2013). Although in higher organisms fertilisation is coupled to egg activation (Sartain and Wolfner, 2013), in *Drosophila*, as in many insects, these two events are independent, and egg activation takes place as the oocytes pass through the oviducts before becoming fertilised (Heifetz et al., 2001). While passing through the oviduct, eggs swell, causing an increase in their Ca^{2+} concentration, which in turn triggers many physiological events including resumption of meiosis of the female pronucleus, translation of maternal mRNAs and crosslinking of the vitelline membrane (Sartain and Wolfner, 2013).

However, a direct analysis of Tsl accumulation at the oocyte plasma membrane just before egg activation could not be performed because it is not technically possible to obtain properly heat-fixed eggshell-free oocytes. We also reproduced the egg activation process *in vitro* (Endow and Komma, 1997; Page and Orr-Weaver, 1997) but were not able to readily separate the oocytes from their vitelline membranes. Finally, we examined mutants for *sarah* (*sra*), the gene encoding an inhibitor of calcineurin, a Ca^{2+}-dependent phosphatase, in which egg activation begins but does not progress. In *sra* mutants many events of egg activation fail, but the cross-linking of the vitelline membrane components is apparently normal (Horner et al., 2006; Takeo et al., 2006). Eggs from *sra* females (see Materials and Methods) showed Tsl accumulation in plasma membranes (Fig. 2G), thus indicating that this process is not dependent on *sra*.

We also examined whether any of the existing *tsl* point mutations specifically impairs a particular step in Tsl accumulation. For tsl^{1}, tsl^{2} and tsl^{3} and tsl^{5} point mutants (Savant-Bhonsale and Montell, 1993), we found that Tsl protein accumulated at the plasma membrane (data not shown), indicating that these mutations render the protein non-functional without interfering with its secretion, anchorage or translocation. Of note, we observed a reduction in Tsl levels at the plasma membrane in tsl^{3} mutants; however, tsl^{3} does not appear to specifically affect accumulation of Tsl at the plasma membrane as the protein fails to remain localised to the vitelline membrane in tsl^{3} mutants (unpublished results in Stein et al., 2008). Besides its role in terminal patterning, *tsl* plays an additional role in regulating timing at pupariation (Grillo et al., 2012; Johnson et al., 2013). In this regard, Tsl-HA can replicate *tsl* function to regulate the timing of pupariation but cannot function in terminal patterning (Johnson et al., 2013). We thus examined the accumulation of Tsl-HA and found that, in spite of properly accumulating at the egg

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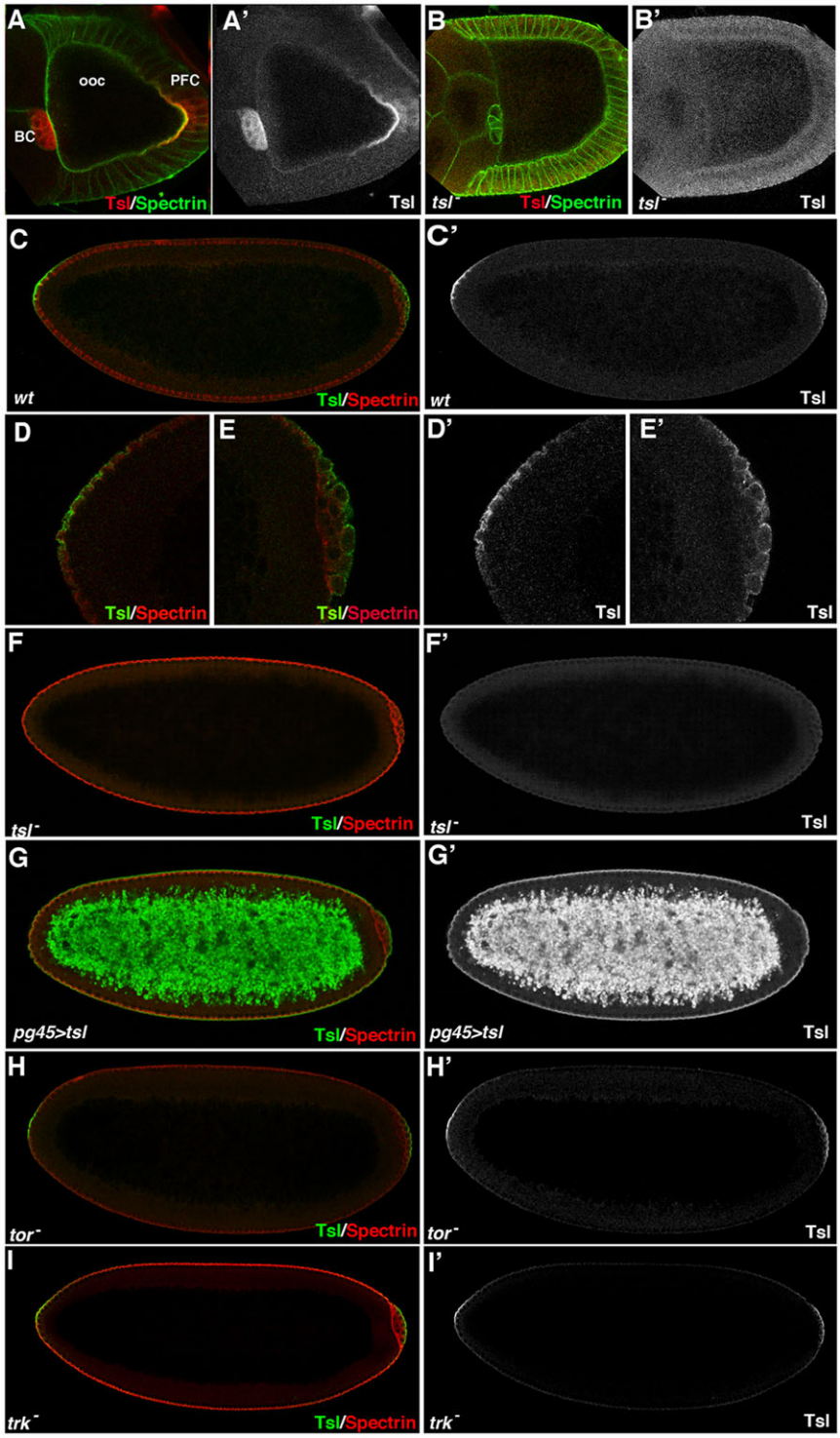

Fig. 1. Torso-like accumulates at the poles of the embryonic plasma membrane. (A) Confocal section of a stage 10 egg chamber. (A′) Same image in the red channel; Tsl is detected in border cells (BC), posterior follicle cells (PFC) and extracellularly between the oocyte (ooc) and the follicle cells. (B) Confocal section of a tsl^{604} stage 10 egg chamber. (B′) Same image in the red channel. The gain is enhanced in the confocal microscopy to make the absence of Tsl clearer. (C) Projection of confocal sections of a blastoderm. (D,E) Magnifications at the anterior D and the posterior poles E. (C′-E′) Same images as C-E in the green channel. (F) Projection of confocal sections of a tsl^{604} blastoderm. (F′) Same image in the green channel. (G) Projection of confocal sections of a blastoderm from a *tsl*-overexpressing female. (G′) Same image in the green channel; Tsl is around all of the plasma membrane. Note the high levels of Tsl inside the embryo. (H) Projection of confocal sections of a tor^{XR} blastoderm. (H′) Same image in green channel. (I) Projection of medial confocal sections of a trk^{RI53} blastoderm. (I′) Same image in the green channel. (C-I) Anti-Tsl antibody immunostaining is shown in green and anti-Spectrin immunostaining in red.

chamber, where it overlapped with the Viteline membrane-like (Vml) protein (Zhang et al., 2009) (Fig. 3A,B), it failed to accumulate in the embryonic plasma membrane (Fig. 3E). We confirmed these results with the anti-Tsl antibody, which recognised Tsl-HA in the ovaries (Fig. 3C,D) but not at the embryonic plasma membrane (Fig. 3F). These results are thus consistent with a functional relevance for Tsl accumulation at the plasma membrane for terminal patterning.

The plasma membrane accumulation of Tsl clearly fits with it harbouring an MACPF domain (Ponting, 1999), which is present in proteins that associate with the plasma membrane to form membrane pores (for a review, see Voskoboinik et al., 2006) and, particularly interesting for the Tor pathway, are involved with the delivery of proteolytic enzymes (Bolitho et al., 2007). As a cleaved form of Trk activates the Tor receptor, bypassing Tsl function (Casali and Casanova, 2001), the notion has emerged that Tsl is involved in the specific cleavage of Trk (Casanova et al., 1995). However, MACPF proteins are usually involved in the entry of enzymes for intracellular proteolysis, whereas the Trk cleavage is thought to occur extracellularly. Indeed, Trk has been recently

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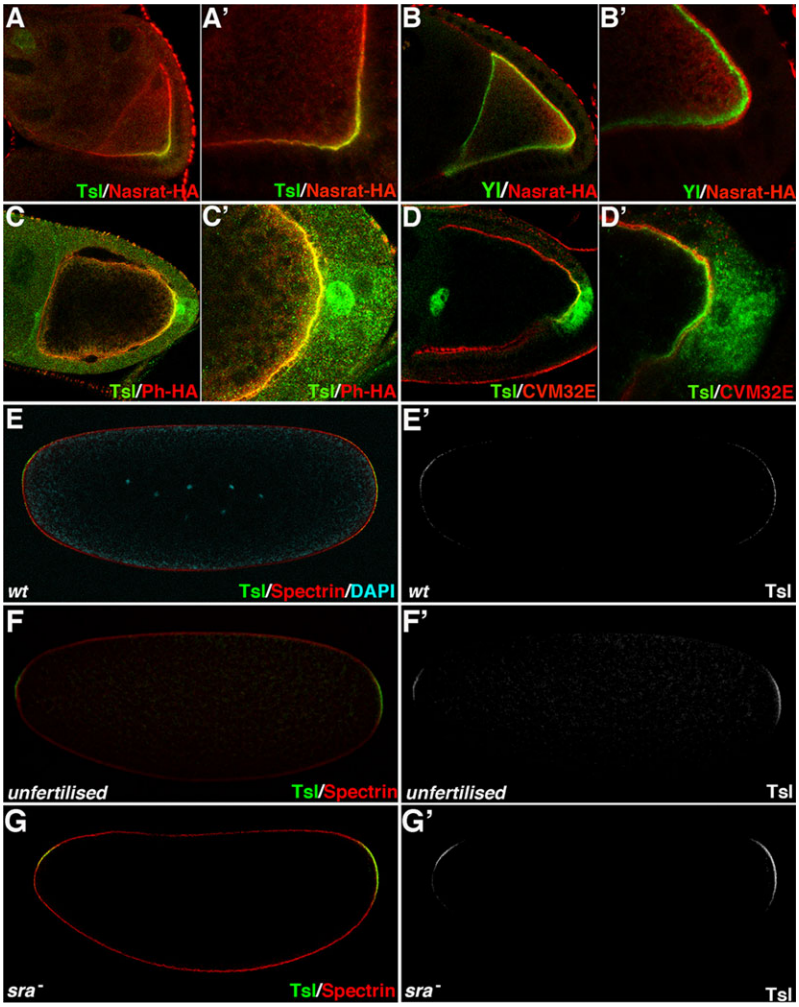

Fig. 2. Torso-like accumulation in development. (A) Confocal section of a stage 9 egg chamber expressing Nasrat-HA and immunostained with anti-Tsl and anti-HA antibodies. (A′) A magnification of A showing the colocalisation. (B) Confocal section of a stage 9 egg chamber expressing Nasrat-HA immunostained with anti-HA and anti-Yl antibodies. (B′) A magnification of B showing their different patterns. (C) Confocal section of a stage 10 egg chamber expressing Polehole-HA immunostained with anti-Tsl and anti-HA antibodies. (C′) A magnification of C showing their colocalisation. (D) Confocal section of a stage 10 egg chamber immunostained with anti-Tsl and anti-cVM32E antibodies. (D′) A magnification of D showing their different patterns. (E) Projection of confocal sections of a third mitotic division embryo immunostained anti-Tsl and anti-Spectrin antibodies and DAPI. (E′) Same image as E in the green channel. (F) Projection of confocal sections of an unfertilised egg immunostained with anti-Tsl and anti-Spectrin antibodies. (F′) Same image as F in the green channel. (G) Projection of confocal sections of an embryo from an sra^{A426}/sra^{KO} female immunostained with anti-Tsl and anti-Spectrin antibodies. (G′) Same image as G in the green channel.

shown to go through multiple proteolytic cleavages, although in a *tsl*-independent manner (Henstridge et al., 2014). Whether there is an as yet unidentified proteolytic step that is indeed *tsl*-dependent or whether Tsl is involved in a different process, the fact remains that a cleaved form of Trk produced in the oocyte does not require *tsl* function to activate the Tor receptor, whereas the full-length Trk protein does (Furriols et al., 1998; Casali and Casanova, 2001). It is also worth noting that anchorage of MACPF proteins depends on Ca^{2+} (Voskoboinik et al., 2005), which is also required for egg activation (Sartain and Wolfner, 2013). Thus a change in Ca^{2+}

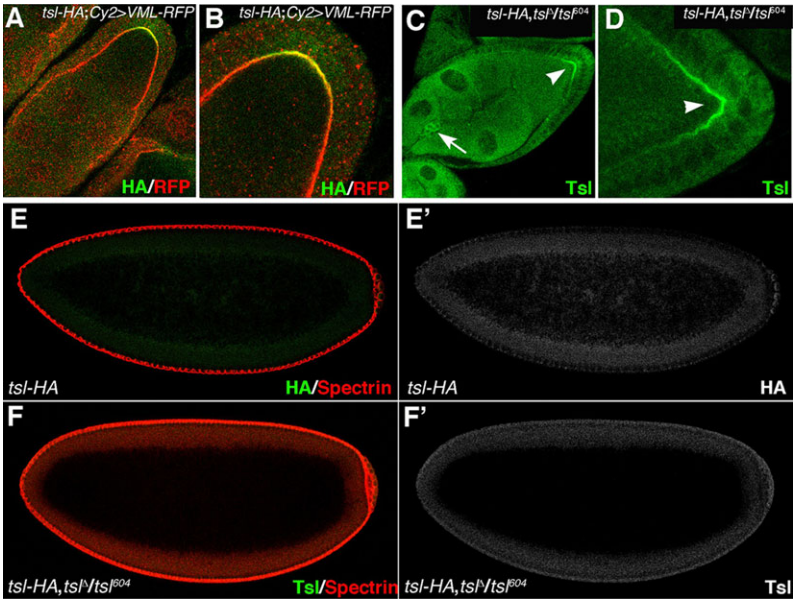

Fig. 3. Tsl-HA fails to accumulate at the embryonic plasma membrane. (A) Confocal section of a stage 9 egg chamber expressing Tsl-HA and UAS-RFP-VML under the Cy2Gal4 driver immunostained anti-HA and anti-RFP antibodies. (B) A magnification of A showing the colocalisation. (C) Confocal section of a tsl^{Δ}/tsl^{604} stage 9 egg chamber expressing Tsl-HA; anti-Tsl antibody immunostaining shows its normal accumulation in the border cells (arrow) and extracellularly at the posterior pole (arrowhead). (D) Confocal section of a similar stage 9 egg chamber showing the extracellular Tsl-HA accumulation in more detail (arrowhead). (E) Projection of confocal sections of a blastoderm expressing Tsl-HA immunostained with anti-HA and anti-Spectrin antibodies. (E′) Same image as E in the green channel. (F) Projection of confocal sections of a tsl^{Δ}/tsl^{604} blastoderm expressing Tsl-HA, immunostained with anti-Tsl and anti-Spectrin antibodies. (F′) Same image as F in the green channel.

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concentration might act to trigger Tsl translocation, linking it to egg activation and eggshell crosslinking (Fig. 4). It would be interesting to sort out whether a translocation event could also occur for Nudel, a *Drosophila* protease secreted by the follicle cells and which is involved in eggshell crosslinking and dorsoventral patterning (Hong and Hashimoto, 1995; LeMosy and Hashimoto, 2000). Although Nudel accumulates at the embryonic plasma membrane (LeMosy et al., 1998), it has been found at the oocyte plasma membrane by confocal microscopy (LeMosy et al., 1998) but is defined as an eggshell component by proteomics (Fakhouri et al., 2006).

It was proposed that Tsl anchoring in the vitelline membrane serves to keep Tsl restricted to the poles from oogenesis to early embryogenesis (Stevens et al., 2003). Our observations suggest that it acts to control developmental timing: Tsl accumulation at the plasma membrane and *tor* RNA translational control under a shared trigger (egg activation) should allow the simultaneous presence of Tsl and the Tor receptor at the embryonic plasma membrane, and thus the timely activation of the Tor pathway. Thus, translocation of determinants from the eggshell might serve as a general mechanism to provide spatial and temporal control of early embryonic developmental processes.

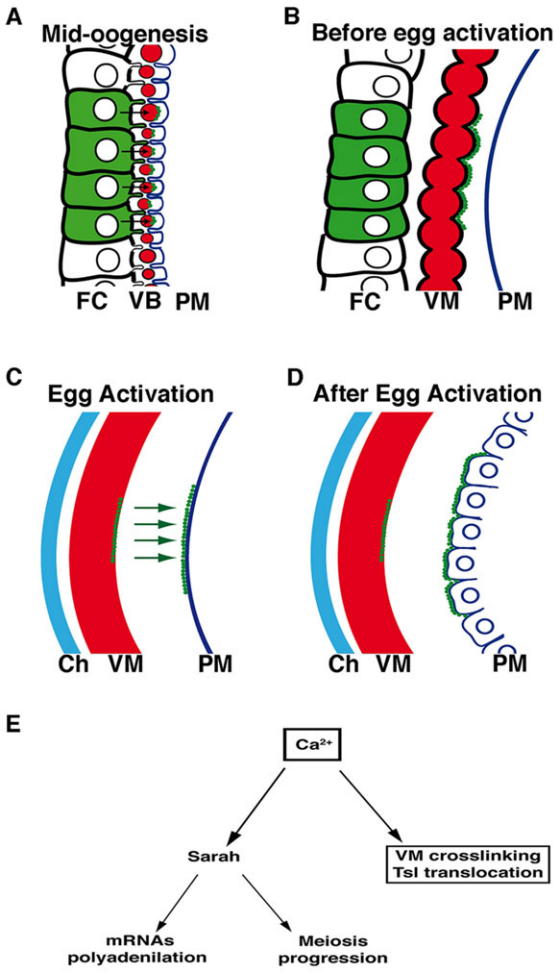

Fig. 4. A two-step model for the transfer of Tsl from follicle cells to the embryo. (A-D) Tsl is secreted from the follicle cells (FC) and incorporated into the vitelline bodies (VB), which fuse and form the vitelline membrane (VM). Upon egg activation, Tsl translocates to the oocyte plasmatic membrane (PM) and afterwards it is found both at the embryonic VM and PM. Ch, chorion. (E) Egg activation events. See text for details.

MATERIALS AND METHODS

Fly strains

The *Drosophila* stocks used are described in FlyBase. We used the heteroallelic combination sra^{A426}/sra^{KO}.

Antibodies and immunostaining

Heat fixation was as described previously (Tepass, 1996; Tanentzapf et al., 2007) with minor modifications (embryos were dechorionated for 2 min, E-wash solution was 100 mM NaCl, 0.3% Triton X-100 and cooling was by adding four volumes of ice-cold E-wash). Afterwards, embryos were devitellinized in heptane-methanol and collected in methanol. Ovaries were dissected as described previously (Furriols and Casanova, 2014) and heat fixed as described above. For *in vitro* egg activation, we followed published protocols (Endow and Komma, 1997; Page and Orr-Weaver, 1997). We used antibodies against Tsl 1/50 (Grillo et al., 2012), Yl 1/500 (Schonbaum et al., 2000), HA (12CA5 1/100 and 3F10 1/300, Roche), Spectrin (3A9, DSHB; 1/5), CVM32E 1/150 (Andrenacci et al., 2001), RFP (ab62341, Abcam; 1/300) and secondary antibodies (Jackson ImmunoResearch; 1/300). Confocal images were obtained with a Leica SPE microscope and analysed with Fiji software.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.M. and M.F. performed the experiments. A.M., M.F. and J.C. conceived and designed the experiments and analysed the data. J.C. wrote the paper.

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