Holes in the Plasma Membrane Mimic Torso-Like Perforin in Torso Tyrosine Kinase Receptor Activation in the *Drosophila* Embryo

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ABSTRACT Receptor tyrosine kinase (RTK) pathways play central roles in development, and, when abnormally activated, they can lead to pathological conditions, including oncogenesis. Thus, RTK activation, mediated by ligand binding, is under tight control, a critical step being the conversion of an inactive precursor into the active form of the ligand. A variety of mechanisms have been shown to be involved in this conversion; however, little attention has been paid to how mechanical phenomena may impinge on this process. Here we address this issue by studying Torso, an RTK activated at both poles of the *Drosophila* embryo at the blastoderm stage. Torso activation is induced by a cleaved form of Trunk, a growth factor-like protein, but it also requires the accumulation of the Torso-like (Tsl) protein at both ends of the blastoderm. Tsl is the only known protein in *Drosophila* bearing a membrane attack complex/perforin (MACPF) domain—a motif present in proteins involved in pore formation at cell membranes. However, while different hypotheses have been put forward to account for the function of Tsl in Torso receptor activation, little is known about its molecular role and whether it indeed contributes to membrane pore formation. Here, we show that mechanically induced holes in the *Drosophila* embryo can substitute for Tsl function. These results suggest that Tsl is required for an exchange between the interior of the *Drosophila* embryo and its surrounding milieu and that mechanically induced cell injuries may contribute to abnormal RTK activation.

KEYWORDS torso; torso-like; RTK; *Drosophila*

ACTIVATION of the Torso receptor tyrosine kinase (RTK) at both poles of the *Drosophila* embryo is induced by a cleaved form of Trunk, a growth factor-like protein, but it also requires the accumulation of the Torso-like (Tsl) protein at both poles (Furriols and Casanova 2003). Tsl is the only known *Drosophila* protein bearing a membrane attack complex/perforin (MACPF) domain—a motif present in proteins involved in pore formation at cell membranes (Ponting 1999). However, little is known about its molecular role and whether it indeed contributes to membrane pore formation. Previous studies addressing whether other perforins might substitute Tsl in Torso (Tor) signaling have so far proven unsuccessful (T. Johnson, personal communication), probably due to the specific features of Tsl trafficking between the ovarian follicle cells, the eggshell, and the blastoderm plasma membrane (Stevens *et al.* 2003; Mineo *et al.* 2015).

In an alternative approach, we examined whether Tsl function might be substituted by mechanically induced holes. Embryos derived from *tsl* mutant females (hereafter referred to as *tsl* mutant embryos) do not show activation of the Tor receptor and therefore do not develop full mouth structures at the anterior end and lack the terminal abdominal structures at the posterior end (Stevens *et al.* 1990; Sprenger *et al.* 1993). Among the latter, they lack a pair of conspicuous protruding spiracles, known as the filzkörper (Figure 1, A and B). However, when pierced at the posterior pole at the blastoderm stage with a sharpened capillary similar to the ones used to generate transgenic flies (*Materials and Methods*), some mutant embryos for either tsl^{604}, a *P*-element mutation that eliminates Tsl protein (Martin *et al.* 1994; Mineo *et al.* 2015) or hemizygous embryos for tsl^{Δ}, a small deletion removing the *tsl* coding region (Johnson *et al.* 2013), develop some terminal structures that are absent in *tsl* mutants (Figure 1C and Table 1), an indication of Tor pathway activation. It is not easy to score the complete set of terminal structures

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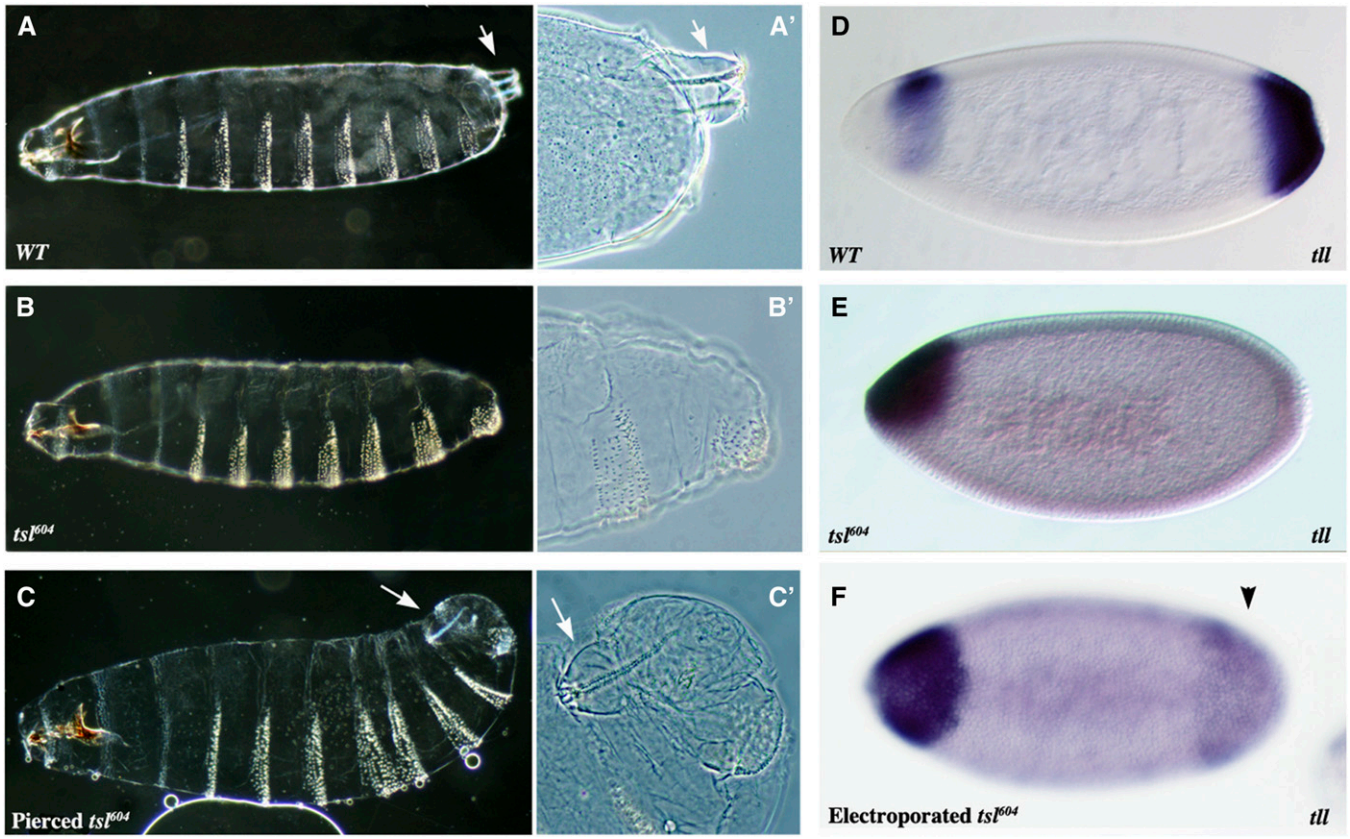

Figure 1 (A–C) Dark field images of wild type (A), *tsl*^{604} (B) and pierced *tsl*^{604} (C) embryos; posterior spiracles and filzkörpers are present in (A and C) (arrows) but absent in (B). (A′–C′) Magnification in phase contrast of the posterior end of embryos in (A–C); posterior spiracles and filzkörpers are present (A′ and C′) (arrows) but absent in (B′). (D) *tll* expression assessed by whole mount *in situ* hybridization in a wild type embryo. (E) *tll* expression in a *tsl*^{604} embryo. (F) *tll* expression in an electroporated *tsl*^{604} embryo; although very few *tsl*^{604} embryos may occasionally show a bit of signal at their posterior pole, the *tll* posterior expression shown in (F) was never observed in nonelectroporated *tsl*^{604} embryos (note that the embryo in (F) is in a more dorsal position than those in (D and E)). In this and all figures, anterior is to the left and dorsal is up.

in the pierced embryos as they develop in an oil medium, and many of them do not exit the vitelline membrane. Thus, we decided to rely on the spiracles and filzkörpers—very conspicuous structures that we can precisely score even inside the vitelline membrane of pierced embryos. We think that, in many cases the rescue may not be complete although in some embryos we could also detect the tuft, a more terminal structure in origin associated with higher levels of torso activity. We note that single piercing with very fine needles did not induce terminal structures, while multiple piercing with the same needles did (Table 1). This observation suggests that a minimal hole size or damage is required to bypass Tsl function.

In the above experiments, piercing embryos produced both holes and plasma membrane bending. Thus, either of the two phenomena (bending or holes) might trigger Tor activation. To distinguish between these alternatives, we pushed *tsl* mutant embryos with needles without piercing (*Materials and Methods*). While similar membrane bending was induced, embryos did not generate terminal structures (Table 1), thereby indicating that piercing, and not bending, was responsible for Tor receptor activation. We also examined other approaches to induce holes and found that low-capacitance electroporation of dechorionated embryos (Kamdar *et al.* 1995) (see *Materials and Methods* for details) also gave rise to some rescue of *tsl* mutant embryos, albeit with a very low efficiency (2% of *tsl*^{604} embryos, $n = 392$; Figure 2A).

To corroborate that piercing of embryos triggers *bona fide* Tor receptor activation, we pierced *tor* mutant embryos (under the same conditions that rescued *tsl* mutant embryos) and found that they did not develop any terminal structure (Figure 2C and Table 1); thus, generation of terminal structures upon embryonic piercing is Tor dependent. We also checked the expression of *tailless* (*tll*), a gene whose transcription at the posterior embryonic pole is elicited by the Tor pathway (Weigel *et al.* 1990) and found posterior accumulation of *tll* transcripts in some pierced *tsl* mutant embryos. However, embryo piercing also led to holes in the embryonic eggshell, thereby preventing eggshell removal using standard protocols. As a consequence, we obtained poor quality staining with *in situ* hybridization of pierced embryos. We therefore proceeded to examine *tll* expression by *in situ* hybridization in electroporated *tsl* mutant embryos, finding that some also show a posterior accumulation of *tll*

Table 1 Percentage of embryos with filzkörper

Maternal genotype	%	*n*
tsl^{604}/tsl^{604}	0.13	2319
tsl^{Δ}/Df(3R)$caki^{X\text{-}313}$	0	564
Posterior piercing with broken needles		
tsl^{604}/tsl^{604}	18	171
tsl^{Δ}/Df(3R)$caki^{X\text{-}313}$	5	263
tor^{XR}/tor^{4}	0	227
$fs(1)ph^{1901}$ HM	7	102
trk^{4}/Df(2L)BSC827	0	565
trk^{3}/Df(2L)BSC827	0	237
trk^{4}/trk^{3}	0	122
Posterior piercing with very fine/unbroken needles		
tsl^{604}/tsl^{604} (single piercing)	0	85
tsl^{604}/tsl^{604} (triple piercing)	11	128
Posterior pressure with blunt-ended needles		
tsl^{604}/tsl^{604}	0	93
Lateral piercing with broken needles		
tsl^{604}/tsl^{604}	4	244

n, number of embryos that survived injection and developed cuticle.

transcripts never observed in *tsl* mutant embryos (Figure 1, E and F). All together, these results show that piercing of early embryos can substitute, at least partially, for Tsl function in Tor pathway activation.

Tsl provides the spatial cue for Tor receptor activation (Savant-Bhonsale and Montell 1993). In fact, Tsl protein accumulates exclusively at the poles in the blastoderm (Mineo *et al.* 2015), the precise location where the Tor receptor is normally activated, in spite of Tor being present throughout the entire blastoderm membrane (Casanova and Struhl 1989) (Figure 3). Given this observation, we examined whether Tor activation by piercing was restricted to the pole regions. Thus, we pierced *tsl* mutant embryos laterally in their middle regions. In this case, rescue of the terminal structures was significantly reduced: not only fewer embryos had terminal structures, but these were much less developed (Figure 2B and Table 1). Notably, in all cases, terminal structures appeared at the poles and not in the middle regions of the embryos. This observation might point to the "spread" of the effect of the piercing in the middle region of the embryo, which would activate Tor at the poles. However, previous experiments showed that uniform levels of Tor receptor activity throughout the embryo facilitate a transduction response at the poles rather than in the middle region of the embryo (Casanova and Struhl 1989). This response is probably due to interactions with regionally expressed gap genes. Furthermore, it is important to note that at this stage the whole embryo is a syncytial blastoderm with no cell divisions as yet. Therefore, the results of the piercing experiments in the middle regions are compatible with either the local generation of an output downstream of the Tor receptor that then spreads and triggers a response at the poles, or with the generation of an upstream signal that spreads and activates the Tor receptor only at the poles (Stevens *et al.* 2003).

Accumulation and function of Tsl at both embryonic poles depend on a group of three proteins (Nasrat, Polehole, and Closca) related by sequence and required for eggshell integrity (Degelmann *et al.* 1990; Jimenez *et al.* 2002; Ventura *et al.* 2010; Mineo *et al.* 2017). Eggs without any one of these proteins collapse (Degelmann *et al.* 1990; Ventura *et al.* 2010). However, specific mutant variants for each of these proteins ensure eggshell formation, thus allowing embryogenesis to proceed, but impair Tor receptor activation due to their effect on *tsl* function (Jimenez *et al.* 2002; Ventura *et al.* 2010). Consequently, such embryos [*i.e.*, embryos from $fs(1)ph^{1901}$ females] lack terminal structures. However, as for *tsl* mutant embryos, embryos from $fs(1)ph^{1901}$ females developed terminal structures when pierced at the posterior pole (Figure 2D and Table 1). Together with the observation that Polehole, Nasrat, and Closca are required for proper Tsl accumulation, our results also suggest that their only role in Tor receptor activity is to enable Tsl function.

As mentioned above, and in addition to the requirement of Tsl function, Tor receptor activation is induced by a cleaved form of the growth factor-like protein Trunk (Trk) (Casanova *et al.* 1995; Casali and Casanova 2001; Henstridge *et al.* 2014; Johnson *et al.* 2015). Thus we examined whether piercing-induced activation of Tor still required Trk function. Indeed, mechanically induced piercing did not generate terminal structures in hemizygous embryos for either trk^{3}, a mutation that introduces a premature stop codon, or trk^{Δ}, a small deletion that removes the entire *trk* coding sequence (Casanova *et al.* 1995; Henstridge *et al.* 2014) (Figure 2E and Table 1). Thus, also in this regard, mechanical induction of holes also mimics Tsl function, as the function of Tsl protein in triggering Tor receptor activity in the *Drosophila* embryo absolutely requires the presence of Trk protein.

These results are at odds with some recent results in cell culture experiments suggesting that Tsl might activate the Tor receptor in a Trk-independent manner (Amarnath *et al.* 2017). The results from those experiments were surprising because wild-type activation of the Tor receptor is completely Trk-dependent in the *Drosophila* embryo: *trk* mutant embryos lack terminal structures, thereby indicating that Tsl alone is unable to activate the Tor receptor. A parameter likely to differ between the two experimental settings might be the overall amount of Tsl protein accumulated at the cell membrane. We therefore explored whether high amounts of Tsl protein might induce Trk-independent Tor receptor activation in the embryo. To this end, using the GAL4/UAS system (Brand and Perrimon 1993), we induced high levels of *tsl* expression. In particular, we used TubGal4 (Lee and Luo 1999) to drive *tsl* expression in all follicle cells, in contrast to the wild type situation, where only few follicle cells at both ends of the oocyte express *tsl* (Stevens *et al.* 1990; Savant-Bhonsale and Montell 1993). Under these circumstances, high levels of Tsl protein accumulate in the whole embryo plasma membrane (Mineo *et al.* 2015). As previously reported, high general expression of *tsl* in an otherwise wild-type background gave rise to embryos with a general activation of the Tor receptor (100% of the embryos, $n = 194$; Figure 4A). However, the same high general expression of *tsl* did

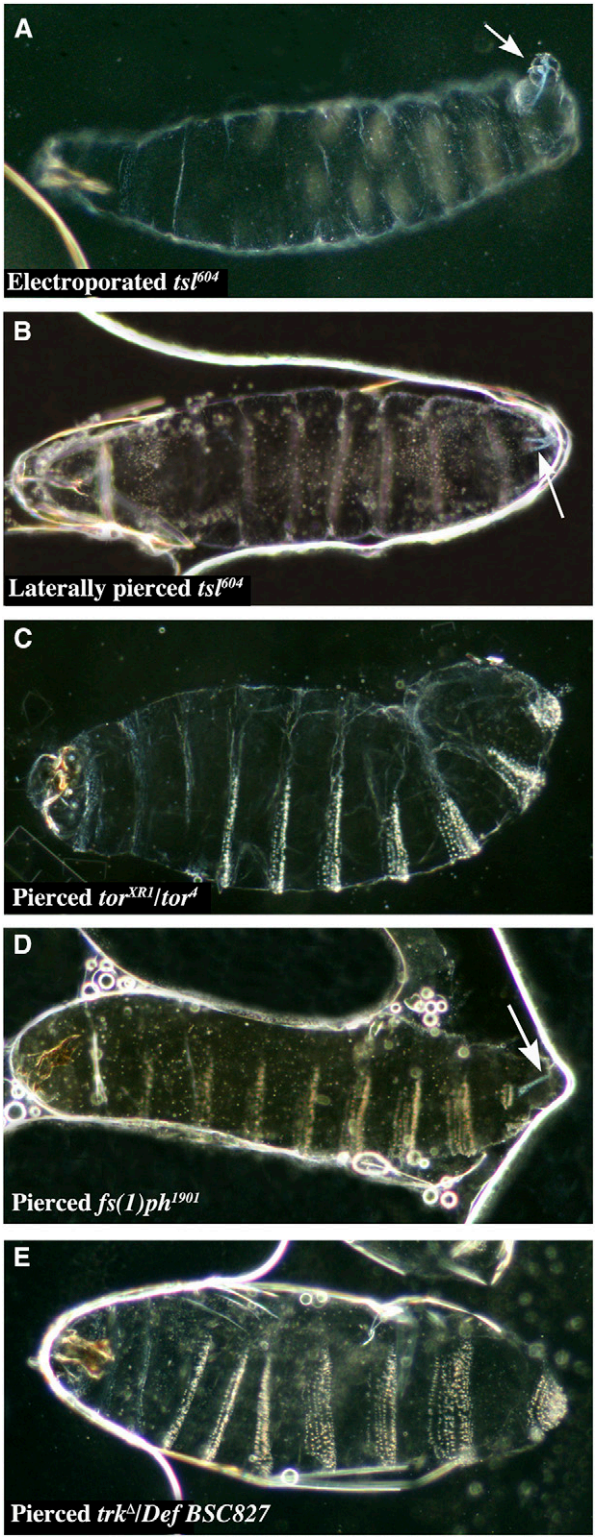

Figure 2 Dark field images of *tsl*^{604} mutant embryos upon electroporation (A) or lateral piercing (B), and *tor*^{XR1}/*tor*^{4} (C), *fs(1)ph*^{1901} (D) and *trk*$^{\Delta}$/Df(2L)BSC827 (E) mutant embryos upon posterior piercing; posterior spiracles with filzkörpers or just filzkörpers are present in (A, B, and D) (arrows) but not in (C and E). For a comparison with wild-type and *tsl*^{604} embryos see Figure 1, A and B respectively.

not lead to Tor receptor activation in *trk* mutant embryos (*Materials and Methods* for exact genotypes) (100% of the embryos, $n = 350$; Figure 4B). Thus, even when present in high amounts, Tsl is fully dependent on Trk function for Tor receptor activation in the *Drosophila* embryo, its *in vivo* setting.

In summary, our results show that holes in the embryonic membrane can substitute for Tsl function or make Tsl function dispensable. In this regard, we note that holes produced by needle piercing are likely to differ greatly in size from pores induced by a MACPF protein such as Tsl. In spite of these differences, these results support the proposed notion that Tsl is involved in a mechanism that enables exchange between the interior of the blastoderm and its surrounding extracellular environment—an exchange somehow allowing the Trk protein to activate the Tor receptor.

Some models have been put forward for the role of Tsl in Tor receptor activation. In each of these models, Tsl acts in a distinct step of the process, from the production of Trk inside the blastoderm to the binding of a Trk active form to the Tor RTK at the exterior side of the blastoderm cell membrane. One of the models proposes that Tsl is required for the cleavage of Trk to generate the active form of the ligand (Furriols and Casanova 2003). This model arose from the observations that C-terminal truncated forms of Trk were able to activate the Tor receptor, and that this activation was Tsl-independent (Casali and Casanova 2001). According to this model, Tsl would enable an extracellular cleavage of Trk around the poles of the embryo, where it would then activate the Tor receptor. However, these experiments were based mainly on the injections of RNA encoding such truncated forms in *tsl* mutant embryos, and we have now demonstrated that the mere physical injection can rescue *tsl* mutants. Thus, while our results do not rule out the requirement of Tsl for Trk cleavage, this hypothesis would require additional confirmation. An alternative model posits that Tsl is required for the export of an already truncated form of Trk from the interior of the blastoderm to the extracellular environment (Johnson *et al.* 2015). According to this model, Trk would be cleaved uniformly in the interior of the whole blastoderm but secreted by a Tsl-mediated process only at the poles, then activating the Tor receptor (Johnson *et al.* 2015). Indeed, these two models are not necessarily mutually exclusive as secretion of Trk might be linked to its processing. In this regard, while our results do not support a Trk-independent role of Tsl in Tor receptor activation, they are consistent with both a secretory and/or processing role for Tsl function.

Many RTK pathways are activated by ligands that need to be secreted and/or cleaved to reach and bind their receptors (Sopko and Perrimon 2013). As mentioned before, these are tightly regulated steps. Our results indicate that, in the case of the Tor RTK, some of these regulatory events can be recapitulated by piercing a hole in the cell membrane. This finding suggests that a critical step in this process involves the permeabilization of this membrane. These results also suggest that mechanically induced cell injuries may contribute to the abnormal activation of RTKs.

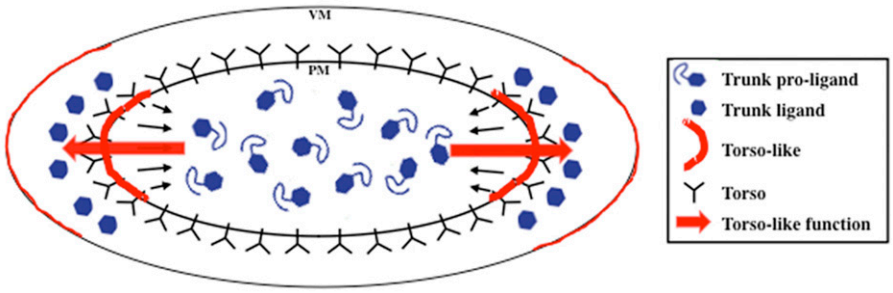

Figure 3 Scheme of the terminal system. Tsl protein accumulates exclusively at the poles of the blastoderm plasma membrane, the precise location where the Tor receptor is normally activated (small arrows). The mechanism of Tsl action is not well known although its function is required for the Trunk proligand inside the embryo to become an active ligand in the space between the plasma membrane (PM) and the eggshell (VM, viteline membrane).

Materials and Methods

Fly strains

We used the following *Drosophila* stocks: *yw* flies as wild type, *fs(1)ph*^{1901} (*fs(1)M3*^{1901} in FlyBase), *tor*^{4}, *tor*^{XR1}, *trk*^{3}, *trk*$^{\Delta}$, *tsl*^{604}, *tsl*$^{\Delta}$, P{tubP-GAL4}LL7, P{UAS-*tsl*.S}, Df(2L)BSC827 and Df(3R)caki$^{X\text{-}313}$. Details can be found in flybase (http://flybase.org).

Embryo piercing

Early embryos (30–45 min) were collected and dechorionated for 2 min in bleach, aligned on peach juice agar, transferred to a coverslip containing 50 μl of tape glue, desiccated with silica gel (orange 13767; Sigma-Aldrich) for 10 min, and covered with halocarbon oil (Voltalef 10S). Microinjection needles were generated from glass capillaries (1 × 90 mm, GD-1 Narishige) using a standard micropipette puller. Piercing was induced by the very fine needles out of the puller, which did not induce terminal structures, or by needles broken to an appropriate aperture and sharpness by gently pushing against the edge of a coverslip while monitoring the process with the microscope. To exert mechanical pressure, needles were broken at the same aperture as in other injections but their tips were blunt in order to avoid piercing the embryo.

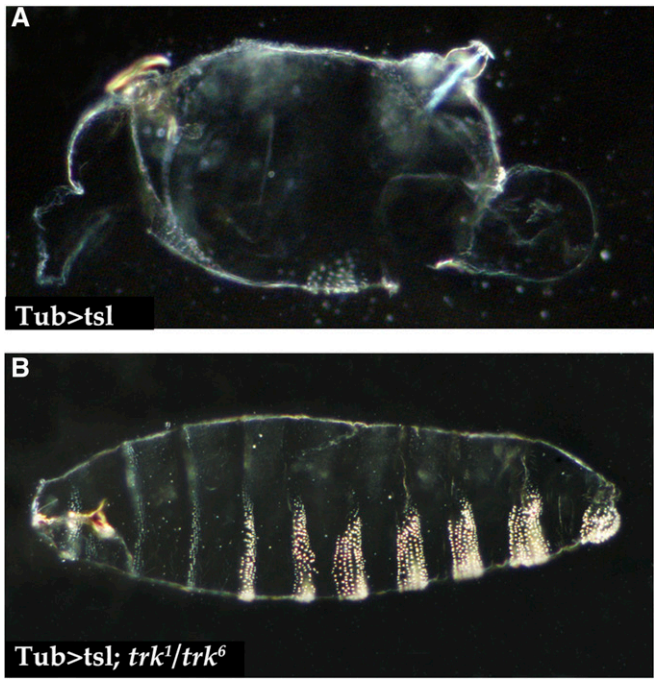

Figure 4 Dark field images of embryos upon general expression of *tsl* with a tubulin-GAL4 driver in otherwise wild-type (A) or *trk*^{1}/*trk*^{6} mutant (B) backgrounds. The embryo in (A) displays a *tor* dominant phenotype (known as "spliced") (Klingler *et al.* 1988) in which many central segments are deleted. The embryo in (B) has the typical terminal phenotype associated with the absence of Tor activation. For a comparison with wild-type embryo see Figure 1A.

Preparation of embryonic cuticles and in situ hybridization

For cuticle preparations, embryos 24–48 hr after injection were mounted in Hoyer:lactic (1:1) upon removal of excess halocarbon oil in excess, and incubated at 50–60° overnight. Photographs were taken in a Zeiss Axioskop microscope. For *in situ* hybridization, embryos were washed with heptane, then collected from the heptane and devitellinised in heptane-methanol 1:1. However, pierced embryos do not devitellinise properly and were thus collected from the heptane-methanol interphase. *In situ* hybridization was performed according to Tautz and Pfeifle (1989). A *tll* probe was generated from a tll cDNA as described in de Las Heras *et al.* (2009).

Electroporation of Drosophila embryos

Electroporation was performed according to Kamdar *et al.* (1995) with some modifications. We used the Bio-Rad Gene Pulser II (model 165–2106) with a capacitance extender plus (model 165–2108). Embryos were collected 45 min after egg laying, dechorionated with bleach for 2 min, thoroughly rinsed with distilled water in a basket, and placed with a paintbrush in a cuvette with a 0.2 cm electrode gap containing 800 μl of the electroporation buffer. Best results were obtained with five pulses of 0.2, 1.25 or 2.5 Kv at low capacitance (1 μF); in the three conditions filzkörpers formed in 2–3% of *tsl*^{604} embryos.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the manuscript are represented fully within the manuscript.

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