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RESEARCH ARTICLE

Reconstitution of Torso signaling in cultured cells suggests a role for both Trunk and Torso-like in receptor activation

Smita Amarnath, Leslie M. Stevens and David S. Stein*

ABSTRACT

Formation of the *Drosophila* embryonic termini is controlled by the localized activation of the receptor tyrosine kinase Torso. Both Torso and Torso's presumed ligand, Trunk, are expressed uniformly in the early embryo. Polar activation of Torso requires Torso-like, which is expressed by follicle cells adjacent to the ends of the developing oocyte. We find that Torso expressed at high levels in cultured *Drosophila* cells is activated by individual application of Trunk, Torso-like or another known Torso ligand, Prothoracicotropic Hormone. In addition to assays of downstream signaling activity, Torso dimerization was detected using bimolecular fluorescence complementation. Trunk and Torso-like were active when co-transfected with Torso and when presented to Torso-expressing cells in conditioned medium. Trunk and Torso-like were also taken up from conditioned medium specifically by cells expressing Torso. At low levels of Torso, similar to those present in the embryo, Trunk and Torso-like alone were ineffective but acted synergistically to stimulate Torso signaling. Our results suggest that Torso interacts with both Trunk and Torso-like, which cooperate to mediate dimerization and activation of Torso at the ends of the *Drosophila* embryo.

KEY WORDS: *Drosophila*, Terminal, Receptor tyrosine kinase, RTK, Membrane attack complex perforin, MACPF

INTRODUCTION

The receptor tyrosine kinase (RTK) Torso plays a crucial role in defining the terminal regions, the acron and telson, of the *Drosophila melanogaster* embryo (Nüsslein-Volhard et al., 1987; Sprenger et al., 1989; St. Johnston and Nüsslein-Volhard, 1992). Torso acts through the canonical Ras/Raf/MAP kinase (MAPK) pathway (Doyle and Bishop, 1993; Lu et al., 1993; Ambrosio et al., 1989; Brunner et al., 1994; Mishra et al., 2005), and in embryos from wild-type mothers two polar caps of phosphorylated MAPK are dependent upon Torso signaling (Gabay et al., 1997). Although Torso is activated only at the poles, it is uniformly distributed throughout the plasma membrane of the early embryo (Casanova and Struhl, 1989). The ligand for Torso in the embryo is thought to be Trunk (Trk), a secreted protein containing a cystine knot motif often found in secreted peptide growth factors (McDonald and Hendrickson, 1993; Casanova et al., 1995; Sun and Davies, 1995). *trk* and *torso* mRNAs are both expressed in the maternal germline (Sprenger et al., 1989; Casanova and Struhl, 1989) and mRNAs encoding both proteins are present in the early embryo at syncytial blastoderm stage, when the embryo is still a single cell. This raises the issue of how Torso and Trk interact productively only at the poles of the embryo.

A key component in determining the spatial specificity of Torso activation in the early embryo is the protein Torso-like (Tsl). Tsl is expressed during oogenesis in two groups of follicle cells that lie adjacent to the poles of the developing oocyte (Stevens et al., 1990; Savant-Bhonsale and Montell, 1993; Martin et al., 1994). Tsl is a secreted protein that carries a membrane attack complex/perforin (MACPF) domain found in a number of proteins known to oligomerize to form membrane pores (Ponting, 1999; Lukoyanova et al., 2016). Tsl becomes localized to the anterior and posterior regions of the vitelline membrane (VM) (Stevens et al., 2003): the inner layer of the eggshell that surrounds the developing embryo. In addition, Tsl has been detected in the membrane of the embryo at the anterior and posterior poles (Martin et al., 1994; Mineo et al., 2015). When *tsl* is ectopically expressed throughout the follicle cell layer, the resulting embryos exhibit phenotypes similar to those produced by the constitutively active *torso* gain-of-function alleles (Klingler et al., 1988; Savant-Bhonsale and Montell, 1993; Martin et al., 1994), suggesting that in wild-type embryos, Tsl determines where Torso is activated.

Trk exhibits similarity to Spätzle (Spz), another secreted cystine knot-containing protein (Casanova et al., 1995; Morisato and Anderson, 1994) that acts as the ligand for the Toll receptor in dorsal-ventral patterning of the *Drosophila* embryo. Spz is secreted into the perivitelline fluid surrounding the embryo as an inactive precursor (Stein and Nüsslein-Volhard, 1992; Schneider et al., 1994) that is cleaved to form an active ligand only on the ventral side of the embryo (LeMosy, 2006; Cho et al., 2010). Casali and Casanova (2001) identified several potential proteolytic cleavage sites in Trk and also reported that the expression of a 'pre-cleaved' C-terminal region of Trk activates Torso ectopically and does not require Tsl function. This led them to propose that Tsl controls Torso activation by mediating the cleavage of Trk into an active form only at the poles of the embryo. Henstridge et al. (2014) demonstrated that Trk does undergo processing in embryos, but at least some of the cleavage events are mediated by Furin proprotein convertases (Johnson et al., 2015) and are not Tsl dependent. Johnson et al. (2015) reported that secretion from the embryo of a fluorescent fusion protein containing N-terminal Trk sequences is enhanced by Tsl activity, leading them to propose that the role of Tsl is to promote secretion of Trk into the perivitelline fluid specifically at the two ends of the embryo.

Recently, it has been determined that Torso activation controls the initiation of metamorphosis at the end of the larval period (Rewitz et al., 2009) and the photophobic behavior exhibited by foraging and wandering larvae (Yamanaka et al., 2013). The ligand that activates Torso to regulate these two behaviors is prothoracicotropic hormone (PTTH) (Kawakami et al., 1990;

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Rewitz et al., 2009), a secreted cystine knot-containing peptide that is expressed in a bilateral pair of neurons in the brain. PTTH expressed in these neurons activates Torso in cells of the prothoracic gland (Siegmund and Korge, 2001; Rybczynski, 2005; McBrayer et al., 2007), in photoreceptive dendritic arborization neurons in the body wall of the larva (Xiang et al., 2010) and in cells of Bolwig's organ (Hassan et al., 2005; Mazzoni et al., 2005). Although the extent to which Tsl participates in the activation of Torso in the larval prothoracic gland remains controversial (Grillo et al., 2012; Johnson et al., 2013), transgenic expression of PTTH in the embryo activates Torso in a Tsl-independent manner, leading to a terminalizing phenotype similar to that produced by Torso gain-of-function mutations. This raises the possibility that for normal embryonic development to occur, a mechanism exists to prevent Trk and Torso from interacting productively except at the poles of the embryo, where spatially localized Tsl overcomes this mechanism either by facilitating the Trk/Torso interaction or by overcoming an inhibitory mechanism that prevents the interaction.

To better understand the mechanism of Torso activation and the role of Tsl in this process, we established conditions for ligand-mediated activation of Torso in a *Drosophila* cell culture-based system. Using multiple assays, we found that when Torso was expressed at high levels in these cells, its activity could be induced by Trk or Tsl acting independently. When Torso concentrations in the cultured cells were lower, at levels more similar to those present in the embryo, activation by Trk or Tsl alone was no longer observed. However, under these conditions, a strong synergistic effect was detected when Trk and Tsl were present together. In addition, both Trk and Tsl mediated dimerization of Torso receptors, and tagged versions of Trk and Tsl were taken up from conditioned medium by Torso-expressing cells. Our data support a mechanism in which both Trk and Tsl play a direct role in promoting Torso dimerization and activation.

RESULTS

Trk, Tsl and PTTH can activate Torso-dependent transcription of a STAT92E-responsive reporter gene in cultured *Drosophila* cells

To define the individual contributions of Trk and Tsl to Torso activation, we sought to establish conditions that would permit the characterization of ligand-dependent stimulation of Torso in a cultured cell system. We used the *Drosophila* S2R+ tissue culture line (Schneider, 1972; Yanagawa et al., 1998), which has been used effectively in other studies examining ligand-mediated receptor activation (Yanagawa et al., 1998; Sims et al., 2009). In our initial studies, we chose to use a STAT92E reporter as our measure of Torso stimulation. STAT92E is activated at the posterior pole of the embryo in response to Torso signaling and is required for the proliferation and migration of primordial germ cells in early embryos (Li et al., 2003). In these studies, we employed a firefly luciferase (Fluc) reporter system, together with multicistronic vectors directing the expression of various Torso constructs singly or together with Trk, Tsl or PTTH (Fig. 1, see also supplementary Materials and Methods). Western blot analysis demonstrated that when the same transfection conditions were used, the levels of Torso protein expressed by the cells were similar regardless of whether Torso was expressed alone or together with Trk, Tsl, PTTH or Trk and Tsl (Fig. S1).

In an initial test of the ability of activated Torso to induce expression of the STAT92E reporter, we expressed the reporter construct together with either wild-type Torso, constitutively active Torso[4021] (Klingler et al., 1988; Sprenger and Nüsslein-Volhard, 1992) or with the pAc5-STABLE2-Neo vector alone. The Torso

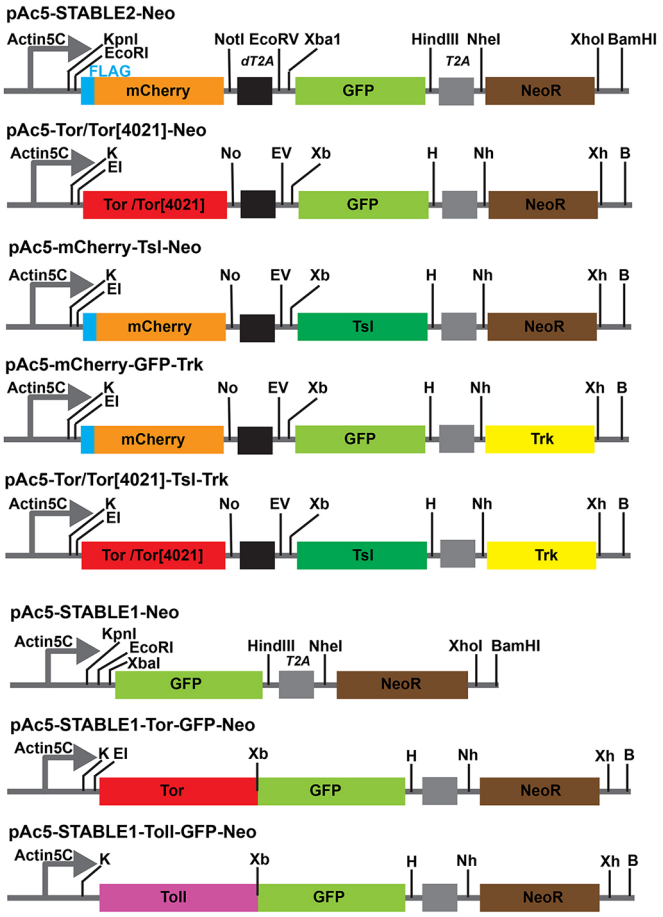

Fig. 1. Strategy for cloning the Torso (Tor), Tsl and Trk open reading frames into the multicistronic expression vectors pAc5-STABLE2-Neo and pAc5-STABLE1-Neo. (Top row) Schematic diagram of the multicistronic segment of pAc5-STABLE2-Neo (Gonzalez et al., 2011), which contains the *Actin5C* promoter, and *mCherry*, *GFP* and *Neo*^{R} genes, is shown. Restriction sites and the positions of the dT2A and T2A autocleavage sites (black and gray boxes, respectively) are shown. (Rows 2-5) Plasmid derivatives in which DNA fragments encoding wild-type Tor/Tor[4021], Tsl and/or Trk have been introduced. (Row 6) Schematic diagram of the bicistronic region of pAc5-STABLE1-Neo (Gonzalez et al., 2011), which carries the *Actin5C* promoter and *GFP* and *NeoR* open reading frames, with restriction sites indicated. (Row 7) The plasmid encoding a Tor-GFP fusion protein (pAc5-STABLE1-Tor-GFP-Neo), with the cloning sites that were used for the substitution displayed as well as other restriction sites that remain. pAc5-STABLE1-Toll-GFP-Neo, which encodes a Toll-GFP fusion protein, is shown in the last row. B, *Bam*HI; E1, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; Nh, *Nhe*I; No, *Not*I; Xb, *Xba*I; Xh, *Xho*I.

[4021] protein produced a strong level of induction, 6.5-fold over vector alone, indicating that Torso-mediated stimulation of STAT92E transcriptional activity is effectively detected in this tissue culture-based system (Fig. 2A). By contrast, expression of wild-type Torso was accompanied by only a modest, though significant, increase (~1.75-fold) in Fluc activity over vector alone. Expression of Trk, Tsl or PTTH alone, in the absence of co-expressed Torso, did not increase Fluc activity relative to the vector control (Fig. S2).

The co-expression of Trk with wild-type Torso led to a fourfold increase in Fluc activity relative to Torso alone (Fig. 2A). However, wild-type Torso co-expression with a Trk protein containing the loss-of-function mutation found in *trk*^{2} (Casanova et al., 1995) did not increase Fluc activity over that induced by Torso alone. (All subsequent fold increases in Fluc activity reported here are relative to

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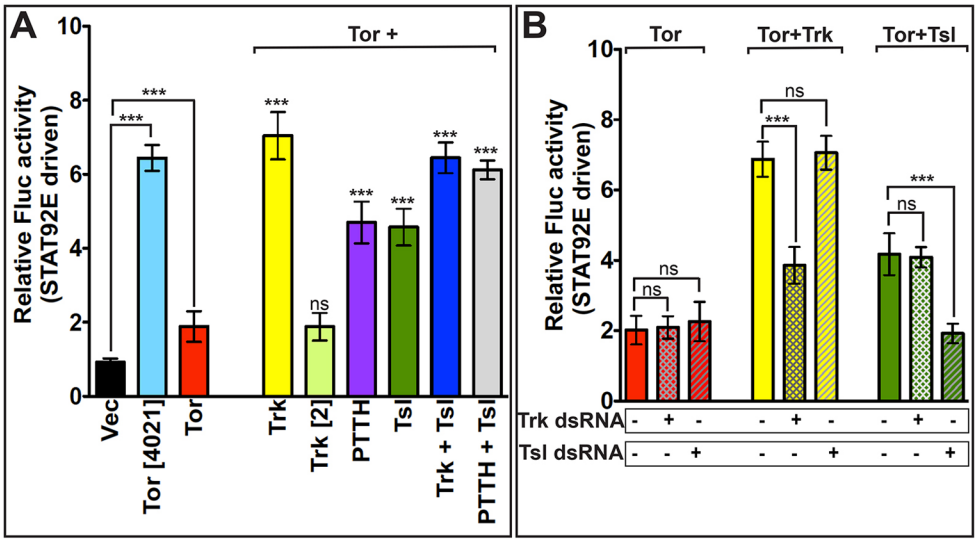

Fig. 2. Tor-dependent activation of STAT92E-driven Fluc activity is induced by co-expression of Trk, PTTH or Tsl. All S2R+ cells were co-transfected with the STAT92E-dependent Fluc reporter construct and the RNA PolIII 128 promoter-dependent R-luc control plasmid for transfection normalization. Relative Fluc activity of each sample is calculated with respect to cells expressing vector alone. (A) Samples shown on the left-hand side were transfected with either the pAc5-STABLE2-Neo vector control plasmid (Vec) (black), or with the vector encoding Tor[4021] (light blue) or wild-type Tor (red). Samples on the right-hand side were transfected with a plasmid encoding wild-type Tor and the following genes: Trk (yellow), Trk[2] mutant (light green), PTTH (purple), Tsl (dark green), Trk plus Tsl (dark blue) or PTTH plus Tsl (gray). (B) Trk and Tsl activation of Tor-mediated STAT92E-driven Fluc activity does not depend upon endogenous *trk* or *tsl* expression in S2R+ cells. Cells were transfected with Tor alone (red), Tor plus Trk (yellow) or Tor plus Tsl (green) and treated with no added dsRNA (solid bars), dsRNA targeting *trk* (cross-hatched bars) or dsRNA targeting *tsl* (hatched bars). In A, each data point is an average of three replicates, repeated seven times (n=7). Significance values for Tor and Tor[4021] are determined relative to vector alone. In all other cases, significance has been calculated relative to cells expressing Tor alone. In B, each bar represents an average of three replicates, repeated five times (n=5). Relative Fluc activity is displayed as mean±s.d., based on seven (A) or five (B) replicate measurements. Differences in values that are statistically significant are indicated above the bars. ***$P\leq0.001$. ns, not significant.

Torso alone unless specified otherwise.) We also tested the effect of co-expressing PTTH with Torso, which produced a 2.5-fold increase in Fluc activity. Surprisingly, co-expression of Tsl with Torso also led to a statistically significant 2.5-fold increase. However, when Tsl and Trk were simultaneously co-expressed with Torso, the 3.7-fold increase in Fluc activity was similar to that observed in experiments with only Torso and Trk, suggesting that an additional effect of Tsl is not detectable under these conditions. By contrast, when we co-expressed Tsl with PTTH and Torso, the 3.4-fold increase in Fluc activity was higher than that seen with just PTTH and Torso, possibly attributable to additive effects.

S2R+ cells have been reported to express *trk* (http://www.flyrnai.org/cgi-bin/DRSC_gene_lookup.pl), raising the possibility that some of the co-expression results described above were influenced by endogenous Trk protein. To address this issue, we generated dsRNA targeting the *trk* transcript and tested its ability to block Torso activation by co-expressed Tsl and Trk. For these experiments, we introduced Torso on a plasmid that was separate from the one bearing Trk, and confirmed that Fluc activity is stimulated when Torso and Trk or Torso and Tsl are introduced on separate plasmids (Fig. 2B). When dsRNA targeting Trk was included, the stimulation of Fluc activity induced by Tsl was unaffected, but the Trk-induced activity was substantially reduced. Similarly, the inclusion of dsRNA against Tsl interfered only with the ability of introduced Tsl to activate Torso. These results indicate that Tsl stimulation of Torso activity under these conditions does not depend on the presence of endogenous Trk protein.

Both Trk and Tsl direct Torso-dependent activation of the Ras/Raf/MAPK signaling pathway

To determine whether in S2R+ cells the Ras/Raf/MAPK pathway was being recruited in response to Torso activation, we used a Fluc reporter construct that is sensitive to the transcription factor AP-1 (Chatterjee and Bohmann, 2012), which has been shown to be regulated by MAPK in *Drosophila* (Peverali et al., 1996; Kockel et al., 1997; Ciapponi et al., 2001; Gritzan et al., 2002). Wild-type Torso alone led to a modest (two-fold) induction of AP-1-Fluc activity over vector alone, whereas Torso[4021] produced a larger (7.5-fold over vector alone) increase (Fig. 3A). Also consistent with our initial studies, we found that co-expression of Trk with Torso produced a strong (3.9-fold) increase in reporter activity over that elicited by Torso alone, whereas Torso co-expression with PTTH or Tsl resulted in more modest increases of 1.9 and 2-fold, respectively. Corresponding results were seen in a western blot analysis using an antibody directed against activated, doubly phosphorylated (p44/42) MAPK/pERK protein (Fig. 3B). Thus, activation of *Drosophila* Torso in S2R+ cells leads to the recruitment and activation of the Ras/Raf/MAPK pathway.

As an additional test of the ability of S2R+ cells to reflect the normal embryonic context of Tor-induced transcriptional regulation, we generated a reporter construct containing the transcriptional elements responsible for polar embryonic expression of the Torso target gene *hkb* (Häder et al., 2000). For reasons that are not clear, the Fluc activity produced by this reporter was modestly decreased by the introduction of Torso alone, relative to vector. Nevertheless, the fold increases in activity that were detected in response to Trk, PTTH and Tsl co-expression with Torso corresponded well with those described above for the STAT92E- and AP-1-Fluc reporter constructs (Fig. 3C).

Torso is activated by Trk and Tsl present in culture media

Our finding that Torso is activated when co-expressed with either Trk or Tsl suggests that both Trk and Tsl have the capacity to influence Torso receptor activation independently. As these

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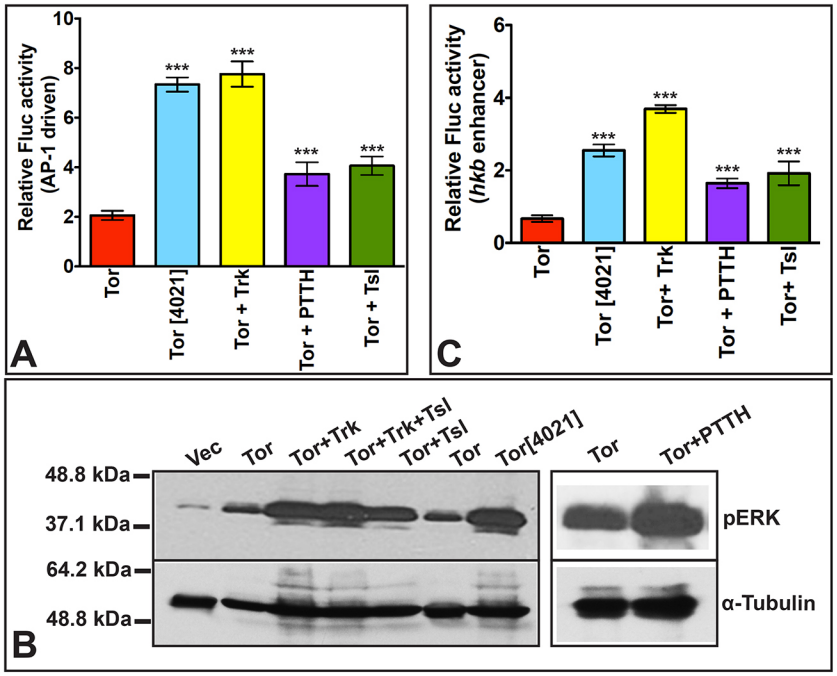

Fig. 3. Trk, PTTH and Tsl stimulate Tor activation of AP1- and *hkb* enhancer-driven Fluc activity and MAPK phosphorylation. (A,C) S2R+ cells were co-transfected with the R-luc transfection control plasmid and an AP-1-dependent Fluc reporter (A) or an *hkb* enhancer-driven Fluc reporter (C). The experimental plasmid(s) that each set of transfected cells received is shown below each bar. Data represent average±s.d. of three readings, repeated seven times (n=7). Statistical significance was determined with respect to relative Fluc activity in cells expressing Tor alone. ***P≤0.001. (B) Western blot analysis of extracts from S2R+ cells transfected with plasmids expressing the proteins shown at the top of each lane. Homogenates were divided in half and run on duplicate gels that were blotted and probed with either anti-phospho-p44/42 MAPK/pERK (top panels) or anti-α-Tubulin as a loading control (bottom panels).

studies relied upon co-expression, this raised the possibility that the effects that we observed were due to intracellular interactions, perhaps within the secretory pathway, that normally may not occur between these molecules in the embryo. To investigate this possibility, we tested whether exogenously added Trk or Tsl could induce Torso signaling by collecting conditioned medium (CM) from cells expressing Trk or Tsl and adding it to Torso-expressing cells. We found that CM from either Trk- or Tsl-expressing cells activated STAT92E- and AP-1-directed Fluc activity (Fig. 4, Fig. S3), suggesting that both Trk and Tsl are capable of independently activating Torso when supplied in the extracellular environment.

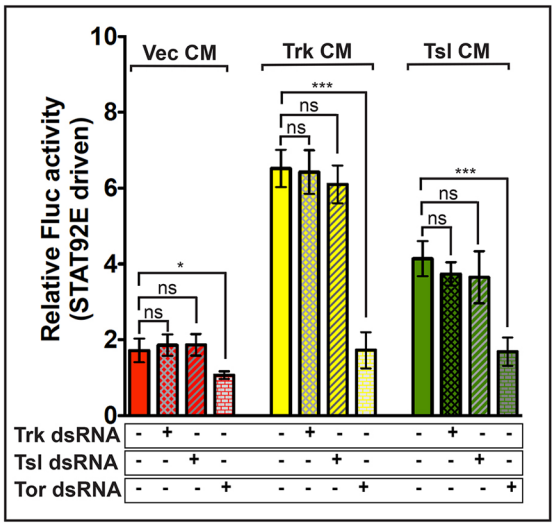

Fig. 4. CM from Trk- or Tsl-expressing cells induces Tor-dependent activation of STAT92E-driven Fluc activity. CM from cells transfected with vector (Vec) (red), Trk (yellow) or Tsl (green) was applied to S2R+ cells expressing Tor together with the STAT92E-Fluc and R-luc constructs. Some recipient cells were additionally treated with dsRNA targeting Trk (cross-hatched bars), Tsl (hatched bars) or Tor (brickwork bars). Each bar represents an average of three replicates, repeated five times (n=5)±s.d. Comparisons with statistically significant differences are indicated above the bars. *P≤0.05 (P=0.0102), ***P≤0.001. ns, not significant.

To address the possibility that the effect of Tsl CM was mediated by endogenous Trk in the Torso-expressing cells, we repeated the CM experiments while also exposing the recipient cells to dsRNA directed against Trk, Tsl or Torso. In these experiments, only dsRNA targeting Torso reduced the levels of Torso activation elicited by the addition of Tsl CM (Fig. 4). We similarly used dsRNA targeting Trk to rule out the possibility that endogenous Trk in the CM-producing cells was responsible for the effect of Tsl CM (Fig. S4). Taken together, these findings suggest that extracellular Trk and Tsl can independently stimulate Torso activation.

Both Trk and Tsl promote Torso dimerization

According to the prevailing model for RTK activation, most RTKs exist as monomers in a conformation that prevents dimerization, and ligand binding induces a conformational change that promotes receptor dimerization and subsequent cross-phosphorylation (Ullrich and Schlessinger, 1990; Lemmon and Schlessinger, 2010). To examine whether dimerization of Torso in response to Trk or Tsl could be detected in our system, we used bimolecular fluorescence complementation (BiFC) (Hu et al., 2002; Kodama and Hu, 2010; Tao and Maruyama, 2008; Shen and Maruyama, 2012), a method for visualizing interactions between proteins via dimerization-mediated complementation of split fluorescent proteins. We generated constructs to express wild-type and Torso [4021] sequences fused to either the N-terminal 155 amino acids (VN) or the C-terminal 84 amino acids (VC) of the fluorescent protein Venus.

Cells expressing only the VN or VC version of either wild-type Torso or Torso[4021] did not exhibit fluorescence, regardless of the addition of Trk or Tsl (Fig. S5). By contrast, cells expressing both Torso[4021]-VN and Torso[4021]-VC in the absence of either Trk or Tsl exhibited abundant fluorescence (Fig. 5A**)**. As Torso[4021] activation is ligand independent, this validates the use of BiFC to detect dimerization associated with receptor activation in this

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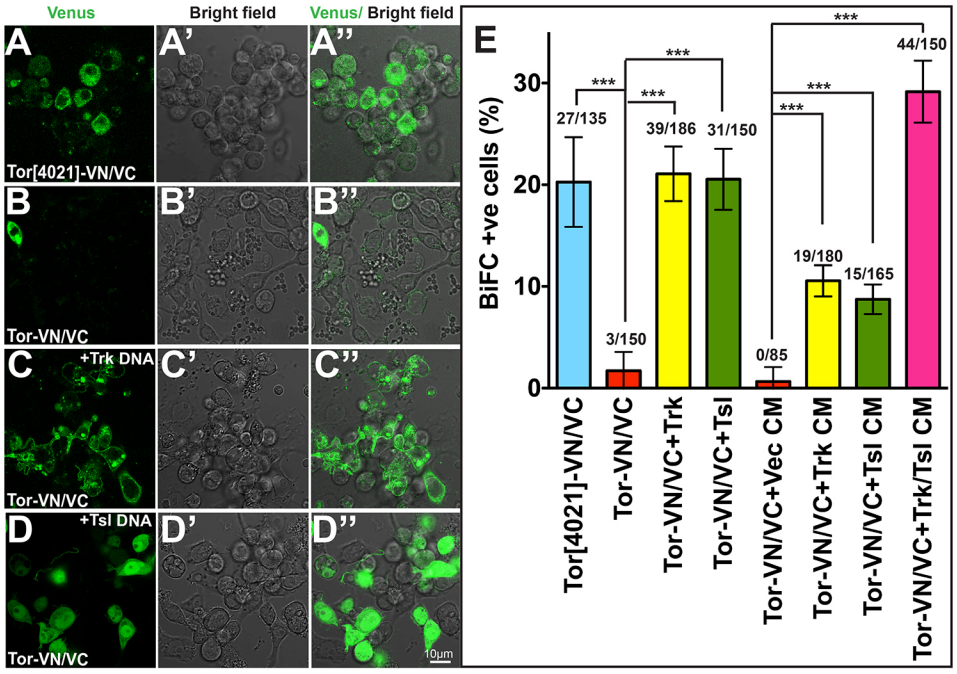

Fig. 5. Co-expression with Trk or Tsl leads to dimerization of Tor. (A-D″) Fusion proteins between Tor and the Venus protein N-terminus (VN) or C-terminus (VC) were co-expressed either alone or with Trk or Tsl. The appearance of Venus fluorescence indicates that dimerization of Tor receptors has occurred. The left column shows Venus fluorescence, the middle column shows bright-field images and the right column displays an overlay of the two. (A-A″) Co-expression of Tor[4021]-VN and Tor[4021]-VC. (B-D″) Co-expression of wild-type Tor-VN and Tor-VC in the absence of other introduced genes (B-B″), with Trk (C-C″) or with Tsl (D-D″). Scale bar: 10 μm. (E) Quantitation of the percentage of cells that exhibited bimolecular fluorescence complementation (BiFC) (see also Fig. 6). Data are mean±s.d. derived from three independent replicate experiments (n=3). The total number of cells counted is shown above each bar. ***P≤0.001.

system. When wild-type Torso-VN and Torso-VC were co-expressed, a small percentage (2.0%) of cells exhibited fluorescence (Fig. 5B,E), consistent with the low level of activation seen in our reporter assays when Torso was expressed alone. The co-expression of either Trk or Tsl with Torso-VN plus Torso-VC led to a significant increase (to 21.0% and 20.7%, respectively) in the percentage of fluorescent cells (Fig. 5C-E). A similar trend was seen in experiments in which CM from cells expressing Trk or Tsl was added to cells expressing both Torso-VN and Torso-VC (10.6% and 9.1% fluorescent cells, respectively) (Fig. 5E; Fig. 6A-C). Interestingly, the combination of Trk CM plus Tsl CM elicited the strongest response that we detected (29.3% fluorescent cells) (Fig. 5E; Fig. 6D). These observations suggest that Trk and Tsl induce Torso activation by promoting dimerization of the receptor.

Trk and Tsl bind S2R+ cells in a Torso-dependent manner

If the activation of Torso by Trk and Tsl in our cell-based assay reflects a direct interaction between these proteins and Torso, we would expect exogenously added Trk and Tsl to be detected on the surface of, or within, Torso-expressing cells. To test this, we expressed HA-tagged versions of Trk (Trk-HA) and Tsl (Tsl-HA) and confirmed that they were competent to induce STAT92E-mediated Fluc activity in Torso-expressing S2R+ cells (data not shown). We then collected CM from cells expressing the HA fusion proteins, applied it to Torso-expressing cells and processed the cells for immunohistochemical detection of the HA-tagged proteins. In these experiments, Torso was expressed as a GFP fusion protein (Torso-GFP) to allow identification of cells expressing detectable levels of the receptor. Many Torso-GFP expressing cells exposed to Trk-HA or Tsl-HA CM exhibited strong HA staining (Fig. 7C-D‴): 75.0% and 72.2%, respectively. Control cells expressing GFP without Torso sequences did not exhibit detectable HA staining after exposure to Trk-HA or Tsl-HA CM (Fig. 7B-B‴, data not shown), nor did Torso-GFP expressing cells that had been exposed to CM from cells expressing vector alone (Fig. 7A-A‴). As an additional control for the specificity of the interaction, we applied Trk-HA and Tsl-HA CM to cells expressing a GFP-tagged version of the *Drosophila* Toll receptor (Fig. S6A-B‴). No HA staining of these cells was detected, but when Toll-GFP-expressing cells were incubated with CM containing an HA-tagged version of processed active Spz (SpzΔN-HA), the ligand for Toll, HA immunofluorescence was observed (Fig. S6D-D‴). As

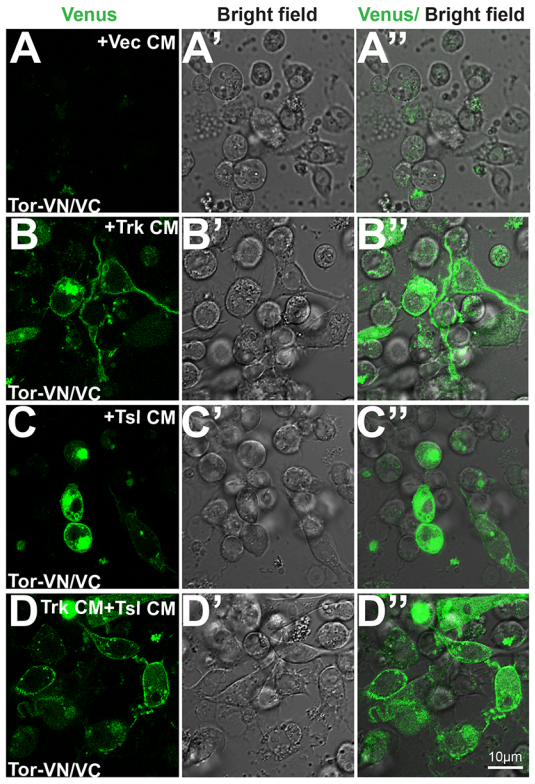

Fig. 6. Exogenously added Trk or Tsl induces dimerization of Tor. (A-D″) Cells co-expressing wild-type Tor-VN/Tor-VC were exposed to CM from cells transfected with vector alone (Vec) (A-A″) or cells expressing Trk (B-B″), Tsl (C-C″) or both Trk and Tsl (D-D″). The left column shows Venus fluorescence, the middle column shows a bright-field image and the right column displays an overlay of the two. Scale bar: 10 μm. (See Fig. 5E for quantitation.)

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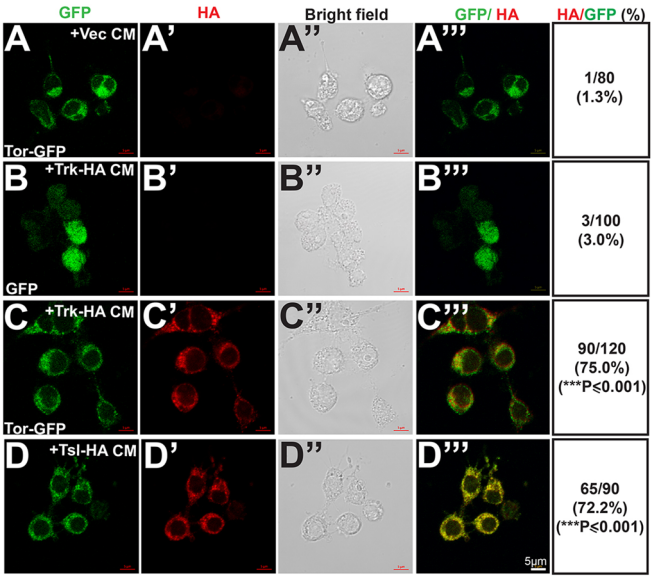

Fig. 7. Tor-expressing cells take up Trk and Tsl. (A-D‴) Cells expressing Tor-GFP (A-A‴,C-E‴) or GFP alone (expressed by pAc5-STABLE1-Neo) (B-B‴) were exposed to CM obtained from cells expressing vector alone (A-A‴), Trk-HA (B-C‴) or Tsl-HA (D-D‴). The cells were imaged for GFP fluorescence (A-D) and stained with anti-HA antibody (A′-D′). Bright-field images (A″-D″) and overlays of GFP and anti-HA staining (A‴-D‴) are also shown. For each study, the proportion of GFP- or Tor-GFP-expressing cells that also exhibited staining with anti-HA are shown in the last column. For each treatment, cells counted are the total of three independent experiments (n=3). Statistical significance was calculated relative to Tor-GFP-expressing cells treated with CM from vector control transfected cells. ***$P \leq 0.001$.

expected, HA-tagged, full-length unprocessed Spz did not bind to cells expressing Toll-GFP (Fig. S6C-C‴) and SpzΔN-HA did not bind to cell expressing Torso-GFP (Fig. S6E-E‴). Collectively, these data are consistent with the idea that Trk and Tsl both interact directly with the Torso receptor.

When Torso is present at low levels, Trk and Tsl act synergistically to activate the receptor

Our finding that Trk and Tsl are competent to activate Torso signaling independently is in stark contrast to the situation in wild-type embryos, where Trk and Tsl are both necessary to bring about Torso activation. The conditions that we used in the experiments described above were designed to ensure high transfection efficiency of multiple DNA components into individual cells. The resulting high levels of protein expression are unlikely to reflect the concentrations of Torso, Trk and Tsl present in *Drosophila* embryos. Indeed, our observation that expression of Torso alone produced a detectable increase in reporter activity suggests that the S2R+ cells were expressing levels of Torso considerably higher than that present in embryos, which we have confirmed by western blot analysis (Fig. S7).

To investigate whether the expression level of Torso in the S2R+ cells influenced the ability of Trk and Tsl to individually activate Torso, we carried out a series of transfections with successively lower amounts of multicistronic constructs encoding either Torso alone, Torso plus Trk, Torso plus Tsl or Torso plus both Trk and Tsl. At transfected DNA concentrations of 25 ng/well and below, neither Trk nor Tsl alone elicited detectable activation of Torso. However, the simultaneous addition of both Trk and Tsl led to a highly significant increase in Fluc levels (Fig. 8A). A similar finding was observed in a second experiment in which the Trk and/or Tsl DNAs ranged from 0.5 ng to 200 ng, while the Torso DNA concentration was kept constant at 5 ng/well (Fig. 8B), which we have found to produce Torso protein levels comparable with those seen in *Drosophila* embryos (Fig. S7). Although some Torso activation was detected with Trk alone, much higher levels were observed when Trk and Tsl were co-expressed, even at concentrations as low as 5 ng/well. Similar results were obtained using the AP-1-Fluc reporter (Fig. S8A).

To determine whether the synergistic effect of Trk and Tsl co-expression could be detected when they were expressed independently and presented to Torso from the extracellular environment, we expressed Trk and Tsl separately in transfected S2R+ cells and collected CM from these cells. We then tested the ability of the CM, at various folds of concentration, to induce Fluc activity in cells expressing Torso (5 ng/well) (Fig. 8C). In unconcentrated CM, neither Trk nor Tsl alone was capable of producing significant Torso activation. However, when unconcentrated CM from the Trk- and Tsl-expressing cells was combined 1:1 and applied to Torso-expressing cells, a marked increase in Fluc activity was observed. We observed a similar synergistic interaction between Trk CM and Tsl CM using the *hkb*-Fluc reporter (Fig. S8B). Taken together, these findings indicate that when Torso, Trk and Tsl are present at low levels, a situation that more closely approximates the conditions in the embryo, neither Trk nor Tsl can effectively activate Torso independently. However, when both are present in the extracellular environment they can act synergistically to induce receptor activation.

As described earlier, PTTH induced Fluc activity in S2R+ cells expressing high levels of Torso. By contrast, when Torso DNA was transfected at 5 ng/well, no amount of co-transfected PTTH DNA, up to 200 ng/well, was capable of activating Torso, as measured using the STAT92E (Fig. S9A) and AP-1 reporters (Fig. S9B). The co-expression of Tsl with PTTH also produced no activation under these conditions. A possible explanation for the discrepancy is that PTTH expressed in S2R+ cells may not be processed into its mature active form and consequently may be a relatively weak activator of Tor (see Discussion).

DISCUSSION

Previous models for Torso activation in the embryo have suggested that Tsl exerts its effects through Trk, either by promoting its processing (Casali and Casanova, 2001) or by facilitating its secretion at the poles (Johnson et al., 2015). Although our experiments in S2R+ cells do not necessarily reflect the situation in the embryo, our results suggest that Tsl is likely to play another role, possibly in addition to effects on Trk processing/secretion. First, when Torso is expressed at high levels, Tsl can activate Torso in the absence of Trk, and it can do so both when it is co-expressed in the same cells as Torso and when it is present in the extracellular medium. In these experiments, RNAi targeting of *trk* mRNA was employed to ensure that the effect of Tsl was not mediated through Trk. Although Tsl cannot activate Torso independently when Torso is present at low concentrations, Tsl and Trk can synergize to bring about Torso activation at concentrations of the two proteins that are insufficient to activate Torso on their own. In this set of experiments, the synergy between Trk and Tsl was seen both when the proteins were co-expressed together with Torso, and when they were expressed separately, collected in the medium and combined before presentation to Torso-expressing cells. Thus, in these experiments, Tsl enhanced the function of Trk that had already been secreted into the medium.

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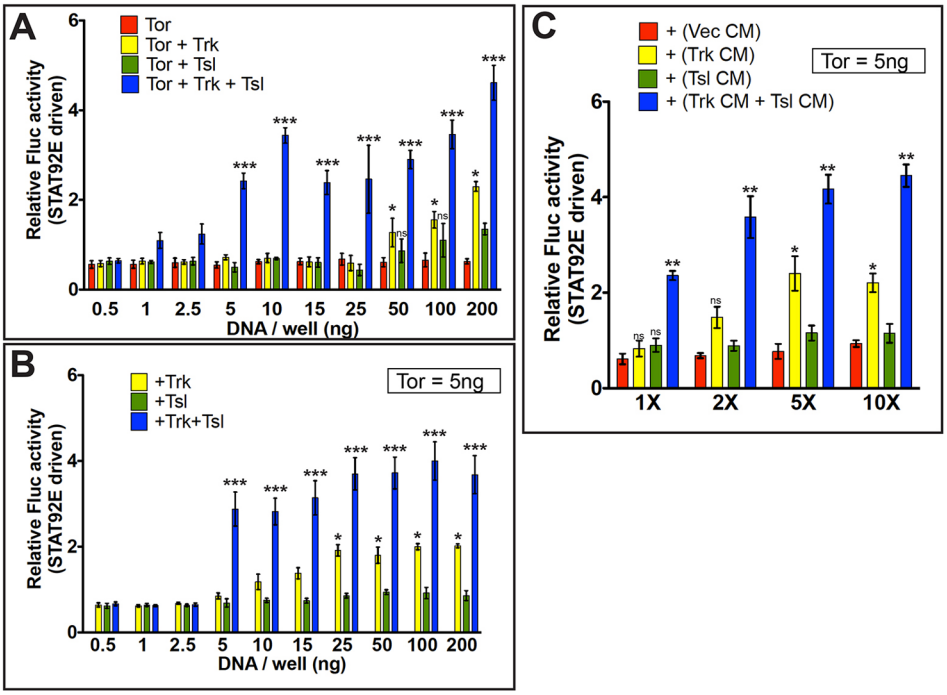

Fig. 8. Trk and Tsl synergize to activate Tor-dependent STAT92E-driven Fluc in cells expressing low levels of Tor. (A-C) S2R+ cells were co-transfected with STAT92E-Fluc/R-luc constructs and the level of STAT92E-directed Fluc expression was assessed. (A) S2R+ cells were additionally transfected with increasing amounts of plasmid DNA carrying Tor (red), Tor plus Trk (yellow), Tor plus Tsl (green) or Tor plus both Trk and Tsl (blue). (B) S2R+ cells were transfected with 5 ng of the Tor expression plasmid plus a concentration range of plasmids bearing Trk alone (yellow), Tsl alone (green) or Trk plus Tsl (blue). (C) S2R+ cells in all samples were transfected with 5 ng of the Tor expression plasmid and exposed to control CM from cells transfected with vector alone (red), CM from cells expressing Trk (yellow) or Tsl (green), or a 1:1 mixture of Trk and Tsl CM (blue). CM was either untreated (1×), or was concentrated by various amounts (2× to 10×). Data are mean±s.d. of three readings, repeated five times (n=5). Statistical significance was calculated with respect to relative Fluc activity for cells expressing Tor alone at the appropriate experimental concentration (0.5-200 ng/well) (A), Tor alone at 5 ng/well (B), or Tor alone (5 ng/well) plus control vector CM at the correct experimental concentration (1-10X concentrated) (C). (A) ***P=0.0010, *P=0.036. (B) *P=0.0202, ***P=0.0082. (C) **P=0.0086, *P=0.0421. ns, not significant.

It is imperative for normal embryonic development that Trk does not activate Torso ectopically. Thus, this pathway has evolved a mechanism in which Tsl function is required for Trk and Torso to engage in a productive interaction. As Tsl is localized to the poles of the embryo, this mechanism restricts Torso activation to the embryonic termini. Some insight into how Tsl might act can be gained by considering the structural differences between the two Torso ligands Trk and PTTH. Both are predicted to form a cystine knot structure, but there are important differences in the arrangement of these cysteine residues. PTTH has been shown to bind as a dimer to *Bombyx mori* Torso (Jenni et al., 2015). Although *Drosophila* Trk would also be expected to form a homodimer, the pattern of cysteine residues in Trk more closely resembles that of the Spz ligand, in which a different cysteine residue from the one used in PTTH participates in dimer formation (Hoffman et al., 2008). This and other structural differences between Trk and PTTH may significantly affect the affinity of the Torso/Trk interaction in comparison with Torso/PTTH interactions, bringing about a requirement for Tsl.

It was surprising that Trk and Tsl can independently activate Torso in cell culture when in the embryo both proteins are essential for Torso activation. In our experiments, independent activation of Torso by Trk or Tsl was most prominent when Torso was present at high levels, a condition that is likely to facilitate receptor dimerization. Indeed, when Torso was expressed alone we saw an almost twofold increase in reporter activity relative to vector control. Although some receptor tyrosine kinases remain in a monomeric state until binding of ligand leads to dimerization (Ullrich and Schlessinger, 1990; Lemmon and Schlessinger, 2010), others are present in an inactive dimeric state with a conformation in which the intracellular domains of the two proteins are unable to interact productively in the absence of ligand (Ward et al., 2007; Tao and Maruyama, 2008; Shen and Maruyama, 2012). Recently, it has been reported that *B. mori* Torso expressed in *Drosophila* S2 cells undergoes ligand-independent dimerization via disulfide linkages formed by cysteine residues present in the transmembrane domain (Konogami et al., 2016). Konogami et al. (2016) suggest that these disulfide bonds hold the dimerized Torso receptors in an inactive conformation that is released upon ligand binding. If Torso exists as an inactive dimer prior to ligand binding, a relatively small alteration in receptor conformation following the binding of Trk or Tsl might result in detectable receptor activation.

The situation is very different when Torso is present at low levels, which is more similar to the conditions in the embryo. Jenni et al. (2015) reported that the binding of the *B. mori* PTTH dimer to the first Torso monomer exhibited much higher affinity than the subsequent binding of the second Torso monomer required to complete the complex and allow signaling to occur. In this situation, the concentration of the receptor, rather than that of the ligand, determines the likelihood of an active complex forming. Thus, the requirement for both Trk and Tsl in the *Drosophila* embryo may reflect conditions in which Trk acts as the ligand for Torso, but due to negative cooperativity, Tsl is required to enhance the initial Trk/Torso interaction and/or facilitate the completion of Torso

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dimerization. It has not been determined whether the interaction of Trk and Torso in *Drosophila* exhibits negative cooperativity. However, even in the absence of this property, when Torso concentrations are low, Tsl may be necessary to locally concentrate Trk and/or to bind Trk in association with Torso. In this respect, Tsl may be functioning like Dally and Dally-like, *Drosophila* orthologs of vertebrate glypican proteoglycans, which act as co-receptors for the peptide growth factors Dpp (Fujise et al., 2003) and Wingless (Lin and Perrimon, 1999; Franch-Marro et al., 2005), and for FGF (Yan and Lin, 2007) and Hedgehog (Yan et al., 2010), respectively. Another intriguing possibility is that in the early embryo, under conditions of low Torso concentrations, Tsl promotes the formation of Torso dimers at the poles that are either inactive or only very weakly active. The presence of these pre-formed dimers could enhance the probability of Trk forming a productive interaction with Torso, which would be particularly important if the concentration of Trk is limiting, as has been reported (Sprenger and Nüsslein-Volhard, 1992). A similar mechanism has been proposed for the enhancement of VEGF-mediated activation of VEGFR2 by the transmembrane glycoprotein Emprin/CD147 (Khayati et al., 2015).

Alternatively, the role of Tsl may be dependent upon its affinity for membranes. Tsl has been detected in the plasma membrane at the two ends of the early embryo (Martin et al., 1994; Mineo et al., 2015) and Tsl bears a MACPF motif (Ponting, 1999), which is found in proteins known to become inserted into lipid bilayers (Lukoyanova et al., 2016). One possibility is that Tsl may modulate the local membrane environment in which Torso resides, perhaps by organizing lipid microdomains and thereby driving the formation of complexes competent to signal. This function would be similar to that of Caveolin, which interacts directly with the Insulin receptor (Kimura et al., 2002). Caveolin is involved in the formation of caveolae, lipid raft-containing microdomains in which Insulin receptor signaling is activated (Saltiel and Pessin, 2003; Strålfors, 2012).

The ability to control the expression levels of Torso, Trk and Tsl in cultured cells has allowed new insights into Torso activation to be obtained, specifically permitting conditions under which Tsl alone can activate Torso signaling and facilitate Torso dimerization. The ability of cells to take up Tsl-HA in a Torso-dependent manner provides strong evidence of a direct interaction between the two proteins. It remains possible that the novel observations we have made are limited to conditions in cultured cells and do not reflect the situation in the embryo. However, if these observations do extend to the control of Torso signaling in *Drosophila* embryos, direct regulation of receptor tyrosine kinase dimerization and activation would represent a novel function for a member of the MACPF class of proteins. This raises the possibility of other pathways in which Tsl influences the activity of Torso or other receptors in *Drosophila*, and of similar MACPF/receptor tyrosine kinase interactions operating in vertebrates.

MATERIALS AND METHODS

DNA constructs

DNA constructs encoding multicistronic messages expressing Torso, Tsl, Trk and/or PTTH were based on the vector pAc5-STABLE2-Neo (González et al., 2011) (Fig. 1), a gift from Rosa Barrio and James Sutherland (CIC bioGUNE, Technology Park of Bizkaia, Derio, Spain; Addgene, plasmid number 32426). Open reading frames introduced into this vector are transcribed as a single multicistronic message in which the individual open reading frames are separated by T2A cis-acting hydrolase elements that mediate the co-translational self-cleavage of the initial polyprotein translation product into individual proteins (Donnelly et al., 2001). During translation, cleavage of T2A sequences immediately upstream of secretory signal peptides results in the formation of free signal peptides that direct normal secretion of their associated peptides (de Felipe and Ryan, 2004). Detailed descriptions of the construction of these and other DNA constructs generated for these studies can be found in the supplementary Materials and Methods.

Cell culture, transfection and reporter assays

Drosophila S2R+ cells were obtained from the *Drosophila* Genome Research Center (DGRC) and cultured in Schneider's *Drosophila* medium (Gibco, #21720-024) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, #A31604-01) at 25°C in a humidified incubator.

S2R+ cells were seeded at 5×10^5 cells/well in a six-well plate and transfected the following day at a density of 10^6 cells in 1.8 ml medium. Transient transfection was carried out using Effectene (Qiagen, #301427) with a DNA:enhancer ratio of 1:8 and a total of 1.9-2.0 µg DNA per well in a six-well plate format for Fluc reporter assays. In the initial experiments at high Torso concentrations, 1.25 µg of the pAc5-STABLE2-Neo-based expression vector was used. For BiFC experiments, a total of 2-2.5 µg DNA per well was used. Typically, a 200 µl transfection mix was used per well containing 1×10^6/ml cells in 2 ml medium. All transfections were performed in triplicate. Forty-eight hours after transfection, the cells were harvested for reporter assays using Dual Glo reagents (Promega, #E2920). The luminescence was measured on a Luminometer (Berthold Technologies, Mithras LB 940) using the Mikrowin 2000 program. The relative luciferase units (RLUs) were calculated for each well as a ratio of Fluc to R-luc as described in the Promega literature accompanying the Dual Glo reagents. Relative Fluc activity was determined by dividing the average experimental RLU measurement for three wells of cells undergoing a particular experimental treatment by the average RLU value produced by three wells of cells transfected with vector alone during the same series of measurements. For all graphs, relative Fluc activity for cells transfected with vector control is therefore set to 1. Statistical significance in differences between cells receiving a particular experimental treatment was calculated using unpaired *t*-test with two-tailed *P* value after ascertaining that the data follows normal distribution using D'Agostino & Pearson, Shapiro-Wilk and Kologorov-Smirnov normality tests on Graphpad prism software. Each experiment was replicated at least five times in triplicate (n=5 for the purpose of determining standard deviation), accompanied by control measurements of activity directed by cells lacking Torso (pAc5-STABLE2-Neo) and by cells expressing Torso alone (pAc5-Tor [WT]-GFP-Neo), also both tested at least five times in triplicate.

RNA interference

dsRNAs used in these studies were generated by direct *in vitro* transcription of PCR products corresponding to sequences contained within target mRNAs. Each of the oligonucleotides used in these amplification reactions carried at their 5′ ends the sequence 5′ TAATACGACTCACTATAGGG3′, which corresponds to the bacteriophage T7 promoter. Using the PCR products thus generated, dsRNAs were generated using the Megascript T7 transcription kit (Ambion) according to the manufacturer's instructions. For assays in which RNAi was applied, 5 µl of dsRNA (3 µg/µl stock) were added to 3×10^6 ml cells/ml per well and incubated for 30 min at room temperature. Subsequently, the DNA transfection mix for the reporter assays was added. The reporter assays were carried out 72 h post-transfection. The sequences of oligonucleotides used for the generation of dsRNA can be found in the supplementary Materials and Methods.

Preparation of conditioned medium

For experiments using conditioned medium (CM), S2R+ cells were transfected as described above. The next day, medium was removed from the wells, the cells were rinsed once with PBS and fresh medium was added. Cells were then grown for two additional days. The cells were dislodged by pipetting once with a 5 ml pipette. The medium was then collected from the wells, the cells were pelleted and the supernatant (CM) used either unconcentrated or concentrated 2×, 5× or 10× using Amicon Ultra-15 columns (Ultracel-10 K, #UFC901008). In the experiment testing whether

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CM from Trk- and Tsl-expressing cells functions synergistically to activate Torso, unconcentrated Trk and Tsl CM were either mixed one to one with each other or with CM from cells transfected with vector alone. To make CM for this experiment, cells were transfected with 1 µg of one of the vectors pAc5-mCherry-GFP-Trk, pAc5-mCherry-Tsl-Neo or pAc5-mCherry-GFP-Neo.

Bimolecular fluorescence complementation

For bimolecular fluorescence complementation studies, the modified Torso constructs bearing the N- and C-terminal segments of Venus cloned in the pUASp vector (Rørth, 1998) were co-transfected with 1.2 µg of the plasmid pMT-Gal4 (Klueg et al., 2002), which enables copper-inducible expression. Expression of the Torso-VN/VC constructs was induced by addition of copper sulfate solution to a final concentration of 0.7 mM 6 h after transfection. Forty-eight hours post-transfection the cells were imaged on a Zeiss LSM 710 confocal microscope at 63× magnification. When Trk or Tsl were provided as DNA cloned on the pAc5-STABLE2-Neo vector, they were included in the transfection mix at 800 ng/well. When Trk or Tsl were presented in CM, the CM was added to the recipient cells 20 h after the transfection of pMT-Gal4.

Immunoblotting

Homogenates of cultured cells or embryos were subjected to SDS-PAGE, then transferred to nitrocellulose blotting membranes (Amersham Protran Premium, GE Healthcare Life Sciences), followed by western blot analysis using the following primary antibodies: rabbit monoclonal anti-phospho-p44/42 MAPK (1:2000, Cell Signaling Technology, #4370S), mouse anti-α-Tubulin (1:1000, Sigma, clone DM1A #T6199), rabbit polyclonal anti-Torso (1:4500, a kind gift from Frank Sprenger, University of Regensburg, Germany) (Sprenger and Nüsslein-Volhard, 1992). HRP-conjugated secondary antibodies produced in goat and directed against rabbit (1:5000, Jackson Labs, #111035003) and mouse (1:5000, Thermo Scientific, #31430) antibodies, respectively, were used. The blots were developed using Supersignal West Pico reagents (Thermo Scientific, #34080) and detected with X-ray film or using a Li-COR C-DiGit Blot Scanner. A more detailed description of the immunoblotting protocol can be found in the supplementary Materials and Methods.

Cell binding assays

S2R+ cells were transfected with pMT-Gal4, together with pUASp, or one of the pUASp-based constructs encoding HA-tagged versions of Trk, Tsl or Spz/SpzΔN, and plated on glass-bottomed six-well plates. Subsequently, $CuSO_4$ was added to these cells to induce the expression of Gal4 and thus the expression and subsequent secretion of the HA-tagged proteins. CM from these cells was collected and applied to recipient cells expressing Tor-GFP, Toll-GFP or GFP. These cells were subsequently fixed and processed for staining using a mouse monoclonal anti-HA antibody (Thermo Scientific, #26183, clone 2-2.2.14) (1:200 dilution) followed by Alexa 594-conjugated goat anti-mouse secondary antibody (Molecular Probes, #A11005) (1:250 dilution), then imaged directly in their wells using a Zeiss LSM 710 confocal microscope at 63× magnification. See also supplementary Materials and Methods.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

S.A., L.M.S. and D.S.S. initiated the study, designed the experiments and interpreted their results and wrote the manuscript. S.A. performed the experiments. D.S.S. contributed to the generation of DNA constructs used in these studies.

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Supplementary information

Supplementary information available online at
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SUPPLEMENTARY INFORMATION

Supplementary Materials and Methods

DNA constructs

pAc5-STABLE2-Neo carries genes encoding mCherry downstream of the Actin 5c promoter, followed by GFP and then neomycin phosphotransferase (NeoR) (Fig. 1). In our constructs, wild-type Torso or Torso[4021] replaced mCherry, Trk or PTTH replaced NeoR, and Tsl replaced GFP.

For the pAc5-STABLE2-Neo derivatives encoding either wild-type Torso (Tor) or Tor[4021], the oligonucleotides:
5'-CGCTCTGAATTCAAAATGCTTATTTTCTACGCGAA-3' and
5'-GGCTGTGCGGCCGCATTCAAAGGTTCTAGGTATAGCTCT-3' were used for high fidelity PCR reactions using wild-type *tor* or *tor*^{4021} cDNAs as templates. The resulting PCR fragments were purified, digested with EcoRI and NotI, and subcloned into similarly digested pAc5-STABLE2-Neo to replace the *mCherry* open reading frame and yield plasmids pAc5-Tor [WT]-GFP-Neo and pAc5-Tor[4021]-GFP-Neo.

For Trk, the oligonucleotides:
5'CGCTCTGCTAGCGCCAGCATGTTTCTGCGTATACTGT3'and
5'GGCTGTGGATCCCTAGTATAGCATAACACATT3' were used in high fidelity PCR amplification using a wild-type *trk* cDNA as template. The resulting PCR fragment was purified, digested with NheI and BamHI, and subcloned into similarly digested pAc5-STABLE2-Neo to replace NeoR and yield pAc5-mCherry-GFP-Trk. To generate pAc5-mCherry-GFP-PTTH, the two oligonucleotides:
5'CGCTCTGCTAGCAAAATGGATATAAAAGTATGGCGACTCC3' and
5'GGCTGTCTCGAGTCACTTTGTGCAGAAGCAGCCGG3' were used in high fidelity PCR amplification of the *ptth* cDNA. The resulting purified PCR fragment was digested with NheI and XhoI and ligated to similarly digested pAc5-STABLE2-Neo.

The Tsl coding sequence on a plasmid bearing the *tsl* cDNA was amplified using the two oligonucleotides:

5'CGCTCTTCTAGAAAAATGCGGTCGTGGCCTGGCCT3' and 5'GGCTGTAAGCTTTCGGGTGGGATGACTCTGCG3', digested with XbaI and HindIII and ligated to similarly digested pAc5-STABLE2-Neo to replace GFP and yield pAc5-mCherry-Tsl-Neo.

The same oligonucleotides and similar strategies, applied sequentially, were used to generate pAc5-STABLE2-Neo derivatives carrying combinations of the genes *tor*, *trk*, *ptth*, and/or *tsl* (e.g. pAc5-Tor-Trk-Tsl).

pAc5-Tor-GFP-Trk[2] encodes Tor together with Trk bearing the trk^2 mutation, C178S. The mutation was introduced by inverse PCR, using the two oligonucleotides: 5'pATGATCACTTTAATTcCAAGCCAAAATCATT3' and 5'pACCAACATTTTCTGGCAATA3' together with plasmid pAc5-Tor-GFP-Trk as a template. The amplification product was digested with DpnI followed by re-circularization by self-ligation.

In experiments in which the ability of Trk to mediate Tor oligomerization by bimolecular fluorescence complementation was tested, the GFP open frame was deleted from pAc5-mCherry-GFP-Trk by HindIII and XbaI digestion, followed by end-filling the cut DNA with Klenow enzyme and recircularization by self-ligation (pAc5-mCherry-Trk).

Reporter gene constructs used were 10XSTAT92-Fluc, a gift of Norbert Perrimon (Addgene plasmid number 37393) (Baeg et al., 2005) and the AP-1-dependent 4XTRE-Luciferase, a gift of Dirk Bohmann (Chatterjee and Bohmann, 2012). To generate the *hkb*-Fluc reporter construct, the two oligonucleotides: 5'GCCATTCCGGTACCGAATTCACGTTCGCTGGCCGAGTGGTTACC3' and 5'AAGCGATACTCGAGTCCTAAAAGATATCTGCTTTCTAGGG3' were employed in high fidelity PCR using *Drosophila* genomic DNA as a template to generate a 598 bp stretch of DNA from the *hkb* gene that is known to direct Tor-dependent transcription at the embryo termini (Häder et al., 2000). The purified PCR fragment was digested with the restriction enzymes KpnI and XhoI and ligated to similarly digested 4XTRE-Luciferase (Chatterjee and Bohmann, 2012), yielding plasmid *hkb*-Fluc. The R-luc expressing plasmid, *Renilla* luciferase-PolIII, was a gift of Norbert Perrimon (Addgene plasmid number 37380)(Nybakken et al., 2005).

Plasmids used for bimolecular fluorescence complementation were derived from plasmids pBiFC-VC155 and pBiFC-VN(I152L) (Kodama and Hu, 2010), both kinds gifts of Dr. Changdeng Hu. The two oligonucleotides:

5'GGCCATGGATCCCCGAATTCGGTCGACCGAGATC3' and
5'TCCTGCTCTAGATTACTTGTACAGCTCGTCCATG3'
were used for PCR-mediated amplification of a DNA fragment from pBiFC-VC155 encoding a carboxy-terminal fragment of Venus. Similarly, the two oligonucleotides:
5'GGCCATGGATCCCCGAATTCGGTCGACCGAGATC3' and
5'CCCCGCTCTAGATTAGGCGGTGAGATAGACGTTGTGGC3' were used for PCR-mediated amplification of a DNA fragment from pBiFC-VN155(I152L) encoding an amino-terminal fragment of Venus bearing the I152L mutation. Both PCR fragments were purified and digested with BamHI and XbaI and these purified fragments were individually ligated to similarly digested plasmid pUASp-Spz-GFP (Cho et al., 2010), yielding the two plasmids pUASp-Spz-VC and pUASp-Spz-VN, respectively. Subsequently, the two oligos:
5'GATCGAGCGGCCGCAAAATGCTTATTTTCTACGCGAAGTACGCATTTATC3' and
5'TCGATCGGATCCGCATTCAAAGGTTCTAGGTATAGCTCTTCC5' were used for PCR-mediated amplification using wild-type *tor* or tor^{4021} cDNAs as templates. The resulting PCR fragments were purified and digested with the restriction endonucleases NotI and BamHI. Both purified fragments were then individually ligated to NotI/BamHI-digested pUASp-Spz-VC and pUASp-Spz-VN, yielding the four plasmids pUASp-Tor-VC, pUASp-Tor-VN, pUASp-Tor[4021]-VC, and pUASp-Tor[4021]-VN.

For use in immunofluorescence experiments testing the ability of Tor to bind HA-tagged versions of Trk and Tsl, the *tor* open reading frame was cloned into the plasmid pAc5-STABLE1-Neo (Gonzalez et al., 2011), a gift of Rosa Barrio and James Sutherland (Addgene plasmid number 32425). The two oligonucleotides:
5'CGCTCTGAATTCAAAATGCTTATTTTCTACGCGAA3' and
5'GGCTGTTCTAGAATTCAAAGGTTCTAGGTATAGCTCT3' were used for a high fidelity PCR reaction using the wild-type *tor* cDNA as a template. The resulting PCR fragment was purified, digested with EcoRI and XbaI, and subcloned into similarly digested pAc5-STABLE1-Neo, yielding plasmid pAc5-STABLE1-Tor-GFP-Neo.

For similar binding studies, the *Toll* open reading frame was cloned into pAc5-STABLE1-Neo. The two oligonucleotides:
5'CGCTCTGGTACCAAAATGAGTCGACTAAAGGCCGC3' and

5'GGCTGTTCTAGATACGTCGCTCTGTTTGGC3' were used for high fidelity PCR using the wild-type *Toll* cDNA as a template. The resulting PCR fragment was purified, digested with KpnI and XbaI, and subcloned into similarly digested pAc5-STABLE1-Neo, yielding plasmid pAc5-STABLE1-Toll-GFP-Neo.

Plasmids encoding HA epitope-tagged versions of Tsl, Trk, PTTH and Spz were constructed as follows: Initially, the *tsl* coding sequences were introduced as an NcoI/EcoRI fragment into plasmid pSPBP4 (Driever et al., 1990) such that the initiation codon is present in the context of an NcoI site (CCATGG), yielding pSPBP4-Tsl. In this plasmid, the *tsl* coding sequences are present downstream of the *Xenopus* β-globin mRNA leader sequence, which directs abundant translation in *Drosophila* cells. Subsequently, site-directed mutagenesis was used to convert the *tsl* codons 351 and 352 (CCC ACC) to an XbaI site (TCTAGA), yielding pSPBP4-TslXba. A 109 bp XbaI fragment encoding three copies of the HA epitope (YPYDVPDVA) was then subcloned into XbaI digested pSPBP4-TslXba. A plasmid clone in which the fragment had inserted in the correct orientation and in frame with the *tsl* open reading frame was obtained and designated pSPBP-Tsl3xHA. The two oligonucleotides: 5'TTATGTATCATACGCGGCCGCTTTAGGTGACACTAT3' and 5'AATGCAGCTAGCGTCTAGCAGCGTAATCTGGAAC3' were then used for PCR amplification of a DNA fragment containing the *Xenopus* β-Globin leader, *tsl* open reading frame and 3xHA tag. This fragment was digested with the enzymes NotI and NheI and ligated to NotI/XbaI digested pUASp, yielding pUASp-Tsl-HA.

For the expression of HA-tagged Trk, the two oligonucleotides: 5'CTGTTTTTGCGGCCGCATGTTTCTGCGTATACTGTGTC3' and 5'ATGGCGTCTAGAGTATAGCATAACACATTCACAGC3' were used to amplify a DNA fragment encoding the *trk* open reading frame. This DNA fragment was digested with NotI and XbaI and ligated to similarly digested pUASp-Tsl-HA, yielding pUASp-Trk-HA, in which the *tsl* open reading frame was replaced by the *trk* open reading frame, positioned in frame with the DNA sequences encoding the three tandem HA epitopes.

For expression of HA-tagged PTTH, the two oligonucleotides: 5'GATCGAGCGGCCGCAAAATGGATATAAAAGTATGGCGACTCC3' and

5'CACATCTCTAGACTTTGTGCAGAAGCAGCCGGC3' were used to amplify a DNA fragment encoding the *ptth* open reading frame, which was digested with NotI and XbaI and ligated to similarly digested pUASp-Tsl-HA, yielding pUASp-PTTH-HA.

The construction of pUASp-Spz-HA, in which the *spz* open reading frame has been fused, in-frame with three tandem copies of the HA epitope, is described in Cho et al. (2010).

pUASp-SpzΔN-HA bears the Spz signal peptide fused directly to the amino terminal residue of the mature processed form of Spz, with three tandem copies of the HA epitope fused in-frame to the C-terminus of the ligand domain. To construct pUASp-SpzΔN-HA, the two oligonucleotides:
5'TAAGCCGCGGCCGCAAAATGATGACGCCCATGTGGATATCG3' and
5'TTTTCCTCTAGACCCAGTCTTCAACGCGCACTTG3' were used to amplify a DNA encoding the Spz signal peptide and ligand domain using the plasmid pUASp-SpzΔN-GFP (Cho et al., 2010) as a template. The purified amplification product was digested with NotI and XbaI and ligated to similarly digested pUASp-Tsl-HA.

RNA interference

Oligonucleotide primers used for the generation of dsRNAs are shown below.

For *trk*, the oligos:
5'TAATACGACTCACTATAGGGCTGCGCCGAGCTATCCACGCAGTCGCTGG3' and
5'TAATACGACTCACTATAGGGATCAAAATCAATTTATCGTTTATTCGAATG3'
were used to amplify a 510 bp fragment of the *trk* cDNA.

For *tsl*, the oligos:
5'TAATACGACTCACTATAGGGAGTTCTGCGAGAATCGGAGGCAACTG3' and
5'TAATACGACTCACTATAGGGCACTAGCCGATCGAATCTGGCCCAG3' were used to amplify a 507 bp fragment of the *tsl* coding sequence.

For *tor*, the oligonucleotides:
5' TAATACGACTCACTATAGGGCAGGAGCAAAATGCAATTGG and
5' TAATACGACTCACTATAGGGCCTGTTGGGCGATGTCTAGC were used to amplify a 468 bp fragment of the *tor* coding sequence.

These segments of the three genes and oligonucleotides were chosen based on amplicons listed by the *Drosophila* RNAi Screening Center as having few off-target effects (http://www.flyrnai.org/cgi-bin/DRSC_gene_lookup.pl).

Immunoblotting

Transfected S2R+ cells from 5-6 wells were washed once in Phosphate Buffered Saline (PBS), pelleted, and the pellets frozen at -70°C. The cells were lysed by pipetting in 50 µl of lysis buffer [50mM Tris pH 7.5, 150mM NaCl, 5mM EDTA, 1% NP40, complete EDTA free protease inhibitor cocktail (Roche #11873580001)]. Protein concentrations were measured using the Bradford Protein Assay reagent (Bio-Rad #500-0006). For analysis of phosphorylated MAPK, 300 µg of protein from each homogenate were boiled in sample buffer and then divided in half. Each half was loaded in one lane of two replicate SDS-polyacrylamide gels (10% Acrylamide:Bis). Following separation by electrophoresis and transfer to nitrocellulose blotting membranes (Amersham Protran Premium, GE Healthcare Life Sciences), one of the two blots was probed with Rabbit monoclonal anti-phospho-p44/42 MAPK (1:2000, Cell Signaling Technology #4370S) and the other probed with mouse anti-∝-Tubulin (1:1000, Sigma, clone DM1A #T6199). Following incubation and washes, HRP-conjugated secondary antibodies produced in goat and directed against rabbit (1:5000, Jackson Labs #111035003) and mouse (1:5000, Thermo Scientific #31430) antibodies, respectively, were applied to the two blots. The blots were developed using Supersignal West Pico reagents (Thermo Scientific #34080) and detected with X-ray film or using a Li-COR C-DiGit Blot Scanner.

For the analysis of Torso protein, homogenates of S2R+ cells expressing Torso were prepared as described above. For the preparation of embryonic extracts, 2-4 hour old embryos were collected, dechorionated and flash frozen in lysis buffer. Frozen embryo samples were dounced 3-5 times in lysis buffer, incubated at 100° C for 3 minutes, then centrifuged at 12,000 rpm for 5 minutes. Following addition of 4X Laemmli sample buffer to 1X concentration, the supernatant was boiled and loaded on an SDS-polyacrylamide gel (8% Acrylamide:Bis). 75 µg of protein from each homogenate was subjected to electrophoresis, followed by transfer to nitrocellulose. Following transfer, the blots were divided into upper and lower portions, at a position corresponding to that of the 65-kDa marker protein. The portion carrying the higher molecular weight proteins was probed with rabbit polyclonal anti-Torso (1:4500, a kind gift from

Frank Sprenger) (Sprenger and Nüsslein-Volhard, 1992) and the portion bearing the lower molecular weight proteins was probed with mouse anti-∝-Tubulin.

Cell binding assays

On day one, 10^6 S2R+ cells were transfected as described above with 1.20 μg pMT-Gal4 and 800 ng of either pUASp, or of one of the pUASp-based constructs encoding an HA-tagged version of Trk, Tsl, PTTH or Spz and plated on glass bottom 6-well plates. These were the CM-producing donor cells. Six hours after transfection, $CuSO_4$ was added to 0.7 mM to induce the expression of Gal4. On day two, medium was removed from the donor cells, they were washed with PBS and fresh medium containing 0.7 mM $CuSO_4$ was added. Donor cells were then grown for an additional 2 days. On day three, recipient cells were transfected with 500 ng of pAc5-STABLE1-Tor-GFP-Neo, pAc5-STABLE1-Toll-GFP-Neo or pAc5-STABLE1-Neo (vector alone control expressing GFP). On day four, CM was collected as described above from the donor cells that had been transfected on day one. Medium was removed from the recipient cells, which were then washed once with PBS, and CM was added to the recipient cells. On day five, CM was removed from the recipient cells, the cells were washed once with PBS, and then fixed with freshly made 4% paraformaldehyde in PBST (PBS + 0.1% TritonX) for 20 minutes at 25° C. Cells were then permeabilized with 0.5% TritonX in PBS for 20 minutes at room temperature and blocked with 5% fetal bovine serum (FBS) in PBST for 20 minutes at room temperature. Mouse monoclonal anti-HA antibody (Thermo Scientific #26183, clone 2-2.2.14), diluted 1:200 in PBS + 2.5% FBS, was added to the cells, which were then incubated overnight at 4° C with rocking. The following day, the anti-HA was removed and the cells washed with PBST three times for five minutes each at room temperature. Alexa 594-conjugated Goat anti-mouse antibody (Molecular Probes#A11005), diluted 1:250 in PBST + 2.5% FBS, was then added and the cells were incubated at room temperature for 2 hours in darkness. Antibody solution was then removed and the cells were washed in PBST three times for 5 minutes each at room temperature. Cover slips were added and the mounts sealed with nail polish. Cells were imaged directly in their wells using a Zeiss LSM 710 confocal microscope at 63X magnification.

Supplementary figures

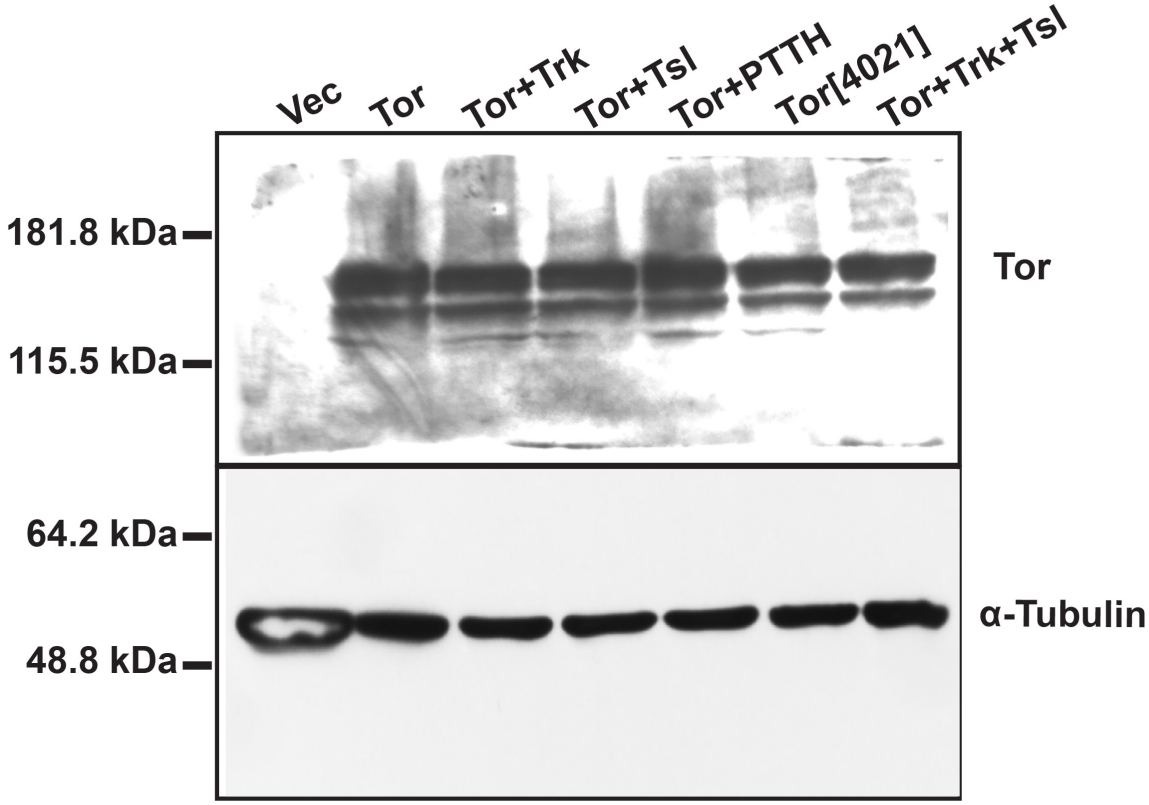

Fig. S1

Fig. S1. Multicistronic vectors express consistent levels of Tor in S2R+ cells.
Western blot analysis of homogenates of S2R+ cells transfected with a plasmid expressing Tor alone or together with other designated gene products. Lane labeled "Vec" is a homogenate of cells transfected with the parental vector pAc5-STABLE2-Neo. Top panel was probed with an antibody directed against Tor (Sprenger and Nüsslein-Volhard, 1992). The bottom panel shows a separate portion of the same blot probed with an antibody directed against α-Tubulin (Sigma) as a loading control.

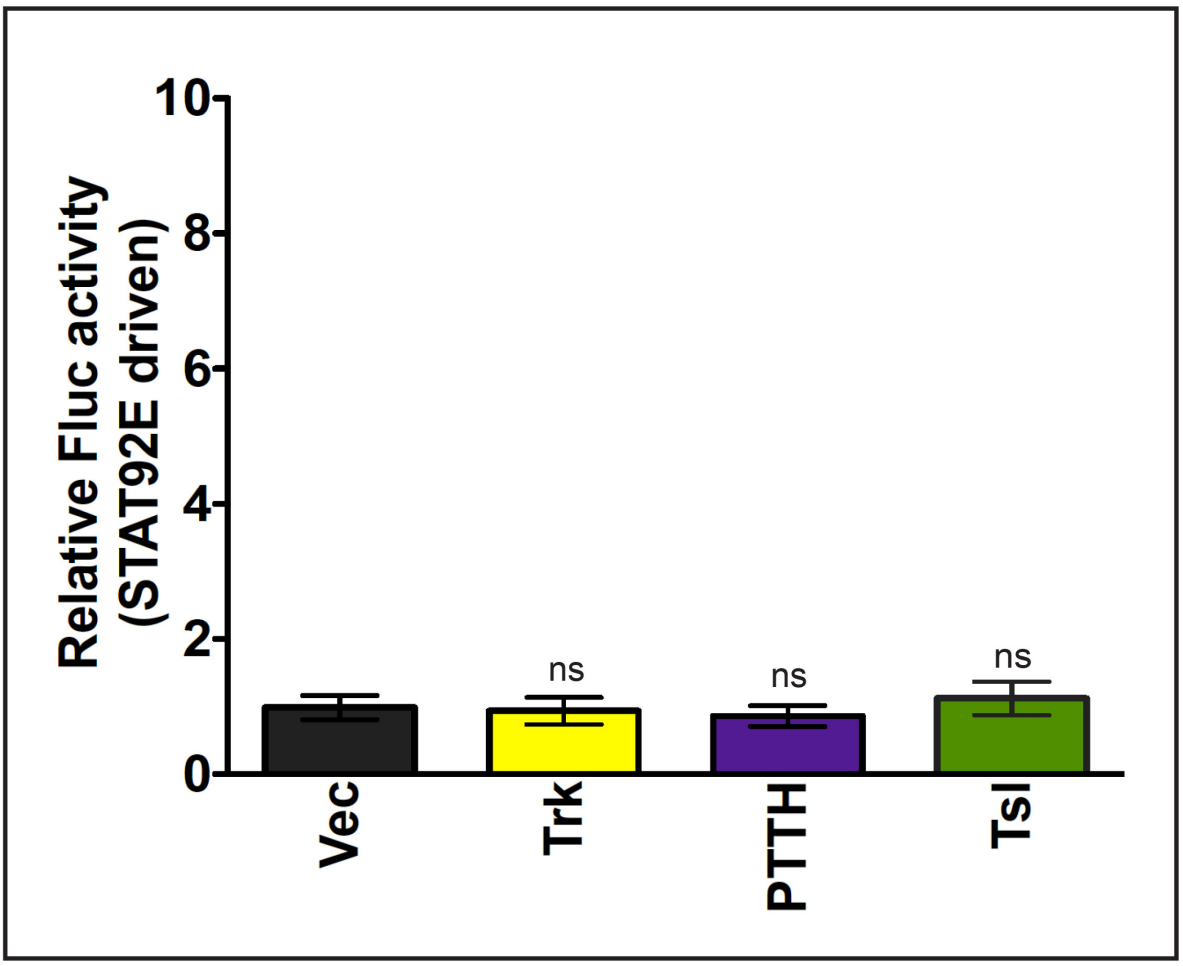

Fig. S2

Fig. S2. Trk, PTTH and Tsl have no effect on STAT92E-driven Fluc activity in the absence of Tor.

In all cases S2R+ cells were co-transfected with the STAT92E-dependent Fluc reporter construct and the RNA PolIII 128 promoter-dependent R-luc control plasmid for transfection normalization. Cells were additionally transfected with 1.25 ng/well Trk (yellow), PTTH (purple) or Tsl (green). Each bar represents the average of three replicates, repeated 5 times (n=5) +/- s.d. Statistical significance (P value = n.s.) has been calculated relative to parallel measurements of cells transfected with pAc5-STABLE2-Neo (Vec).

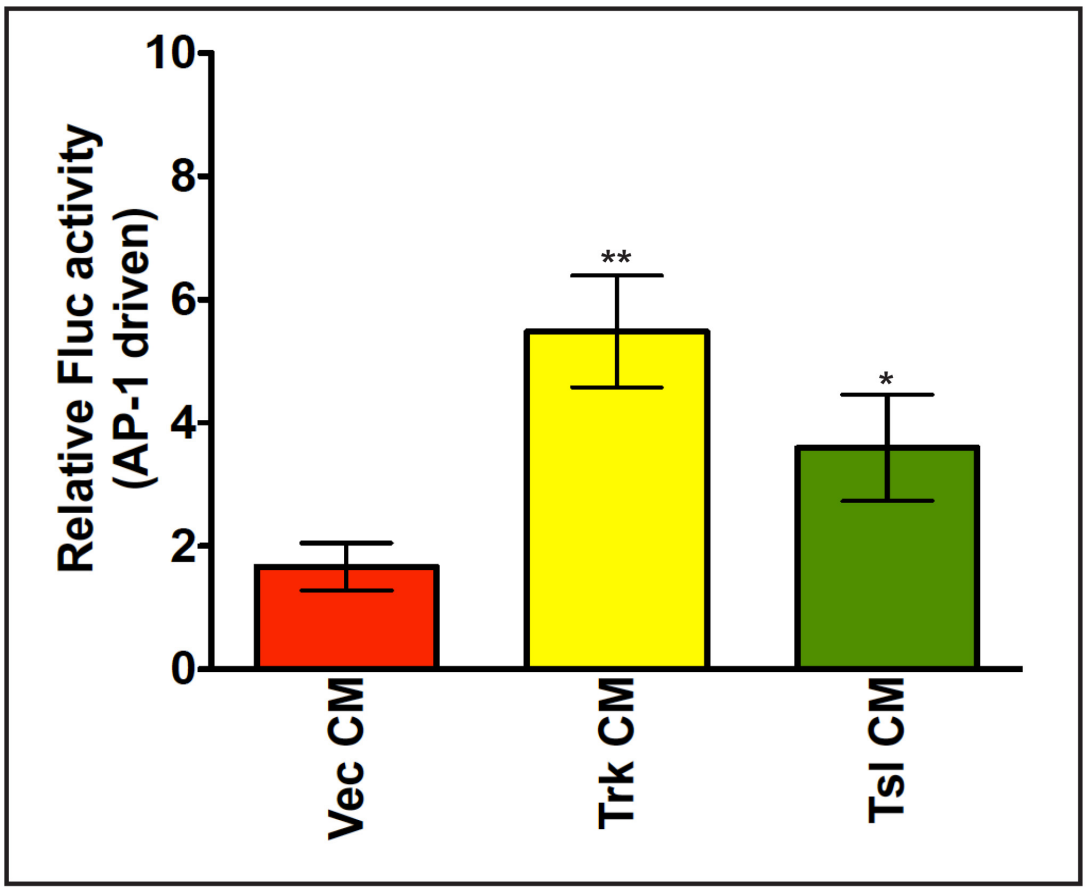

Fig. S3

Fig. S3. CM from Trk- or Tsl-expressing cells induces Tor-dependent activation of AP-1-driven Fluc activity.

CM from cells expressing vector alone (red), Trk (yellow) or Tsl (green) was applied to S2R+ cells expressing Tor together with the AP-1-Fluc and R-luc constructs. Each bar represents an average of three replicates, repeated 5 times (n=5) +/- s.d. P values, calculated relative to Tor-expressing cells treated with CM from control pAc5-STABLE2-Neo transfected cells: ** (P value =0.00902), * (P value = 0.0486).

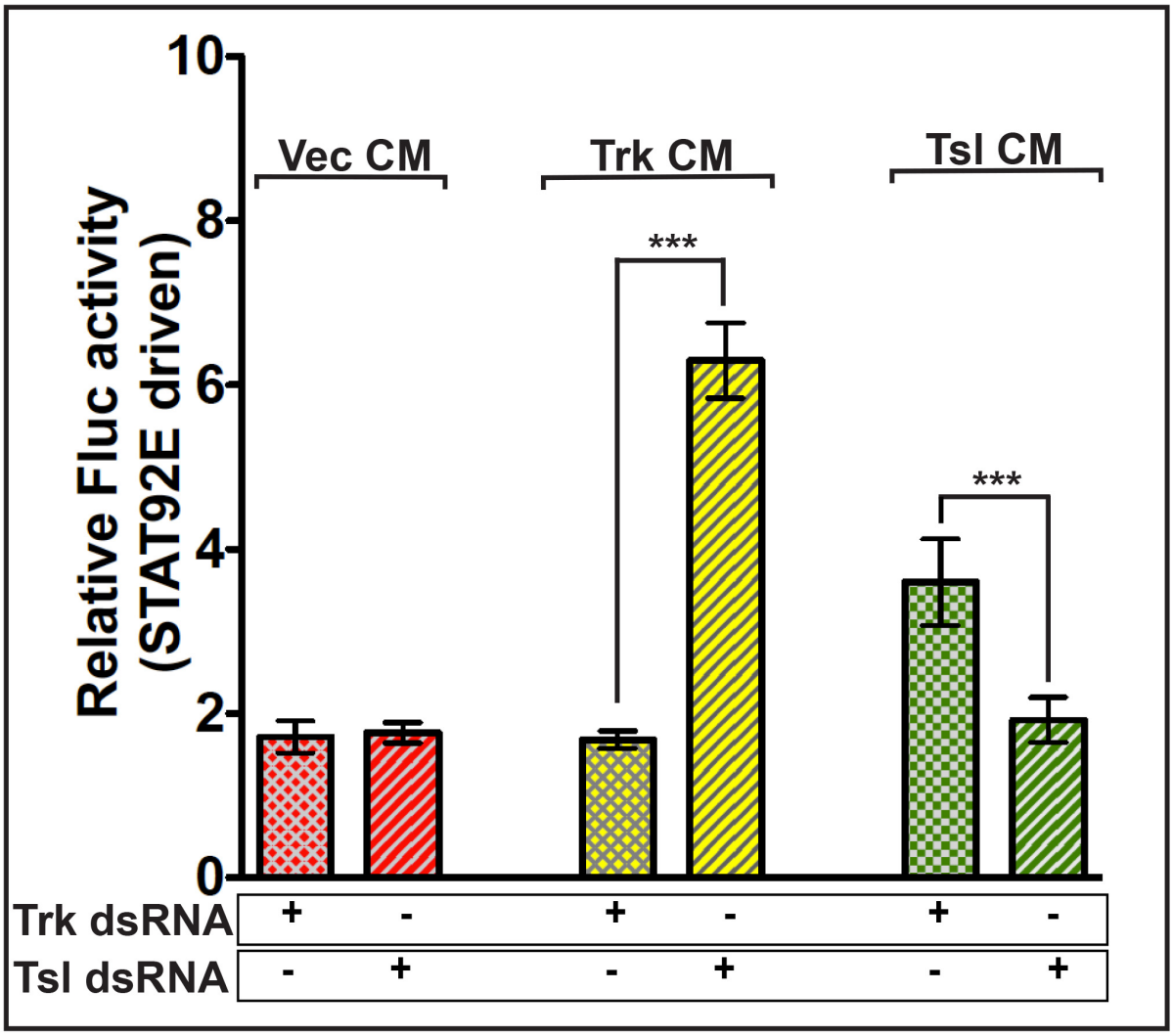

Fig. S4

Fig. S4. Tor-dependent activation of the STAT92E reporter is induced by Trk and Tsl CM produced in the absence of endogenous Tsl or Trk expression, respectively.

CM from cells transfected with vector control alone (red), Trk (yellow) or Tsl (green) was applied to S2R+ cells expressing Tor and the STAT92E-Fluc and R-luc constructs. CM-producing cells were additionally treated with dsRNA targeting Trk (cross-hatch pattern) or Tsl (diagonal line pattern). Each bar represents an average of three replicates, repeated 5 times (n=5) +/- s.d. Comparisons used to calculate P values are indicated above the bars. P values: *** (P value $\leq$0.001).

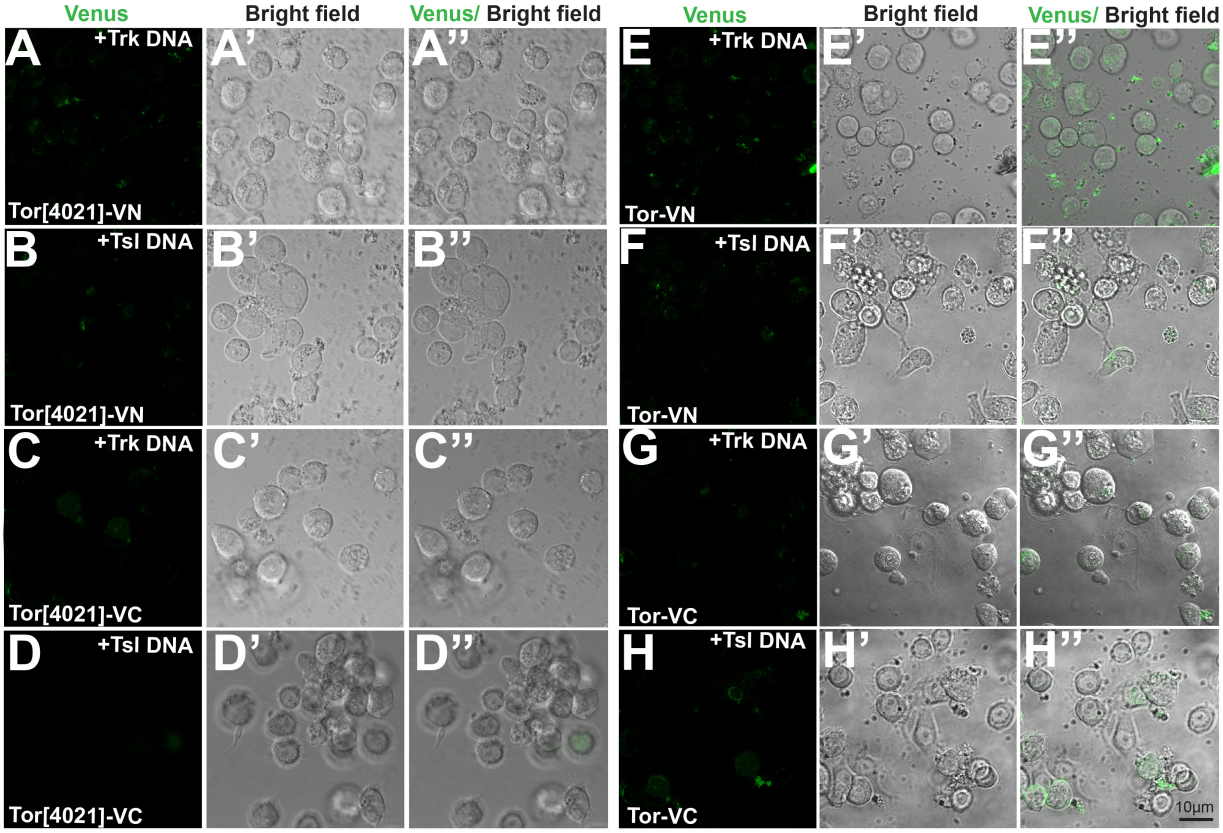

Fig. S5

Fig. S5. Trk and Tsl do not produce fluorescence complementation in cells separately expressing Tor[4021]-VN, Tor[4021]-VC, Tor-VN or Tor-VC.

Fusion proteins between wild-type Tor or Tor[4021] and Venus N-terminus (VN) or C-terminus (VC) were co-expressed with Trk or Tsl. When observed, the reconstitution of Venus fluorescence indicates that dimerization of Tor receptors has occurred. Left column shows Venus fluorescence, middle column shows a bright field image and the right column displays an overlay of the two. (A-A") Tor[4021]-VN plus Trk. (B-B") Tor[4021]-VN plus Tsl. (C-C") Tor[4021]-VC plus Trk. (D-D") Tor[4021]-VC plus Tsl. (E-E") Tor-VN plus Trk. (F-F") Tor-VN plus Tsl. (G-G") Tor-VC plus Trk. (H-H") Tor-VC plus Tsl. Each experiment was repeated three times (n=3).

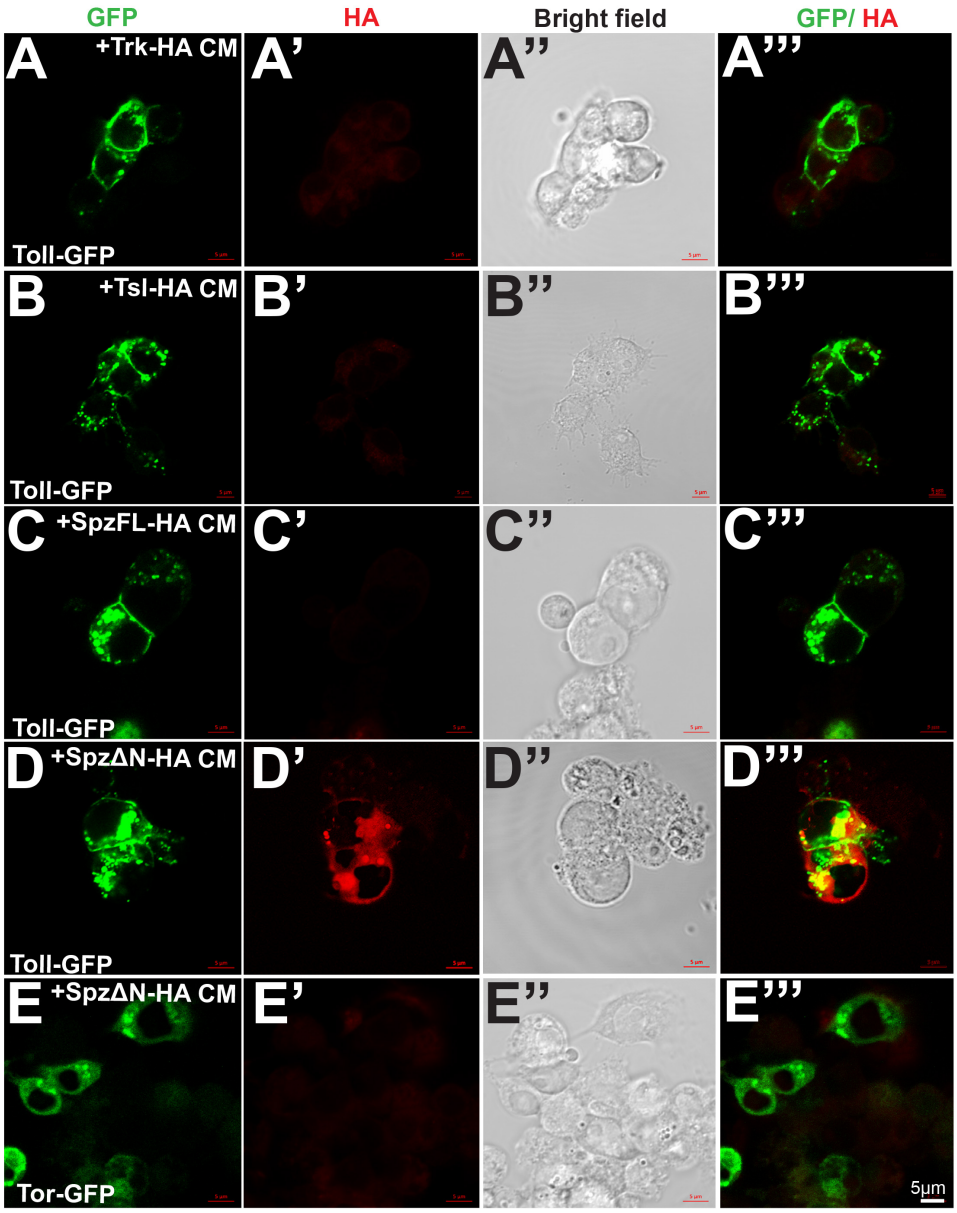

Fig. S6

Fig. S6. Uptake of HA-Tagged Trk and Tsl proteins is Tor-dependent.

Cells expressing Toll-GFP (Rows A-D) or Tor-GFP (Row E) were exposed to CM from cells expressing HA-tagged versions of Trk (A-A'''), Tsl (B-B'''), full-length Spz (SpzFL) (C-C''') or N-terminally deleted, active Spz (SpzΔN) (D-D''', E-E'''). The cells were imaged for GFP fluorescence (A-E) to identify Toll-GFP/Tor-GFP-expressing cells and stained with anti-HA antibody (A'-E'). Bright field images (A''-E'') and overlays of GFP and anti-HA staining (A'''-E''') are also shown.

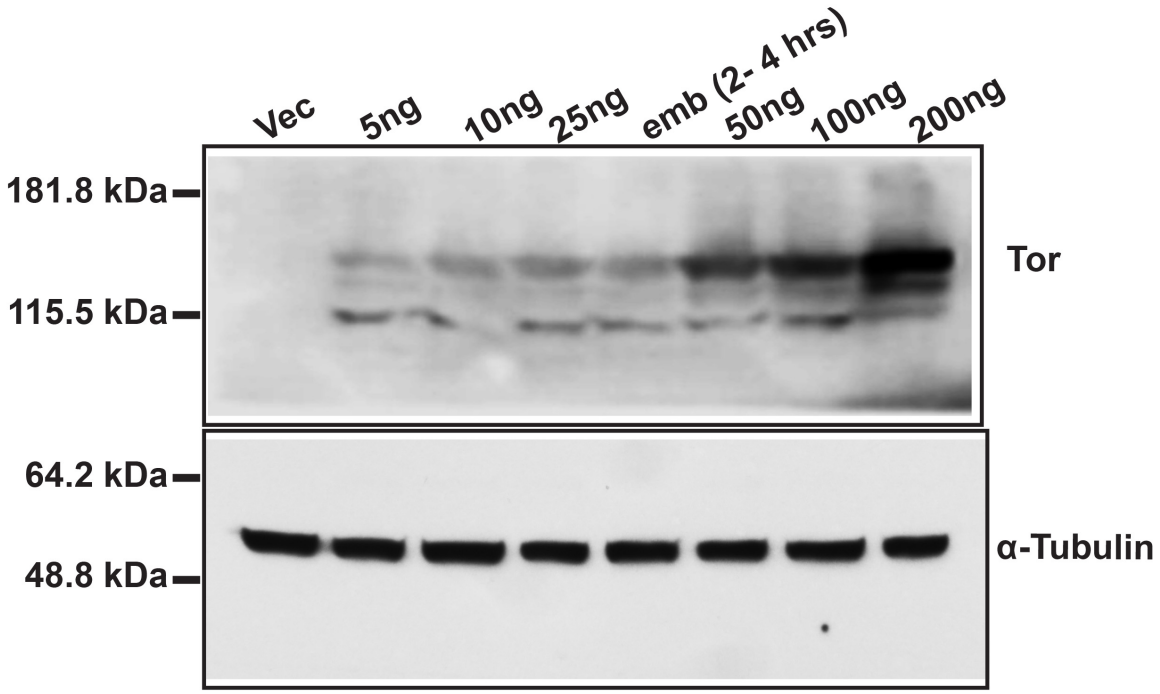

Fig. S7

Fig. S7. S2R+ cells transfected with 10-25 ng of pAc5-Tor-GFP-Neo express levels of Tor similar to those present in early *Drosophila* embryos.

Western blot analysis of extracts from S2R+ cells transfected with varying amounts of pAc5-Tor-GFP-Neo (shown at the top of the upper panel) or the vector alone (Vec) as well as an extract from 2-4-hr old wild-type embryos. 75 μg of protein were loaded into each lane. Top panel was probed with an antibody directed against Tor (Sprenger and Nüsslein-Volhard, 1992). The bottom panel shows a separate portion of the same blot probed with an antibody directed against alpha α-Tubulin (Sigma) as a loading control.

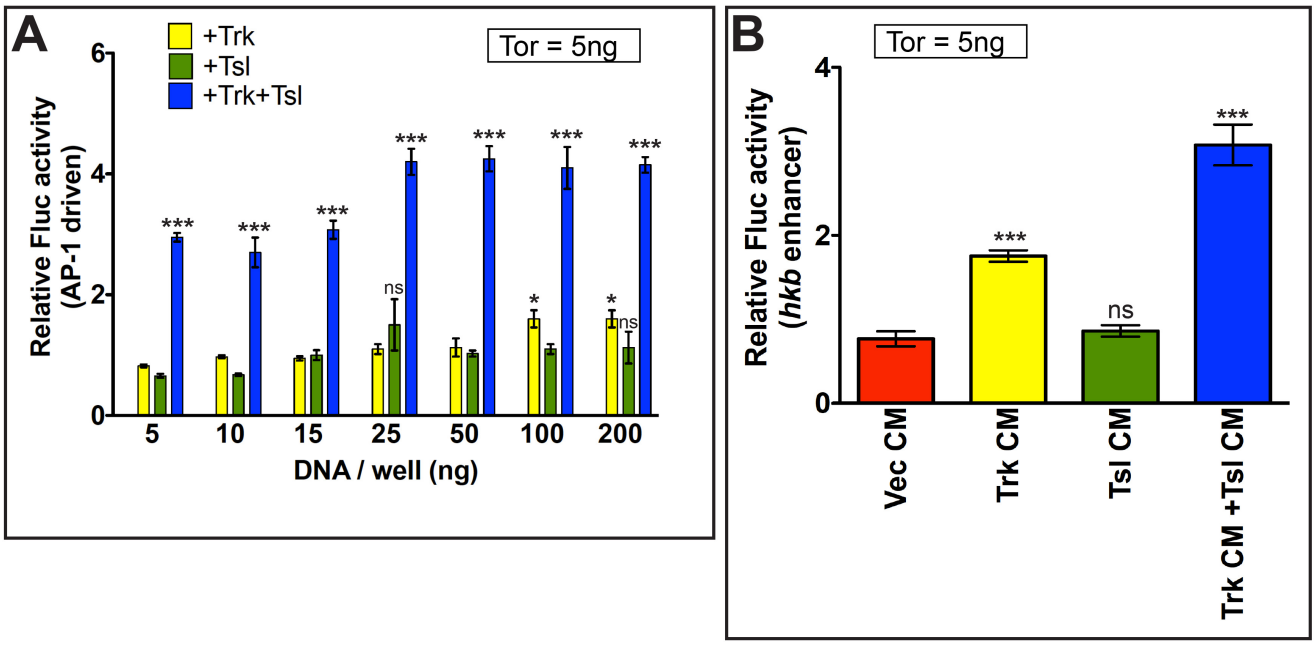

Fig. S8

Fig. S8. Trk and Tsl synergize to activate Tor-dependent AP1- and *hkb* enhancer-driven Fluc activity in cells expressing low levels of Tor.

(A) S2R+ cells were co-transfected with AP-1-driven Fluc reporter, R-luc reporter and 5 ng of the Tor expression construct. Cells were additionally transfected with a range (5-200 ng/well) of plasmid DNA encoding Trk (yellow), Tsl (green) or Trk plus Tsl (blue). (B) S2R+ cells were co-transfected with the *hkb* enhancer-driven Fluc reporter, R-luc reporter and 5 ng of the Tor expression construct. Cells were exposed to 5X-concentrated CM from cells expressing vector control (red), Trk (yellow) or Tsl (green), or to a 5X concentrated 1:1 mixture of Trk CM plus Tsl CM (blue). In both (A) and (B), each bar represents an average of three replicates, repeated 5 times (n=5) +/- s.d. In (A), P values have been determined relative to cells transfected with 5 ng/well Tor. In (B), P values have been determined relative to Tor-expressing cells exposed to CM from control vector transfected cells. P values: *** (P value $\leq$ 0.001). * (P value = 0.047). ns: not significant.

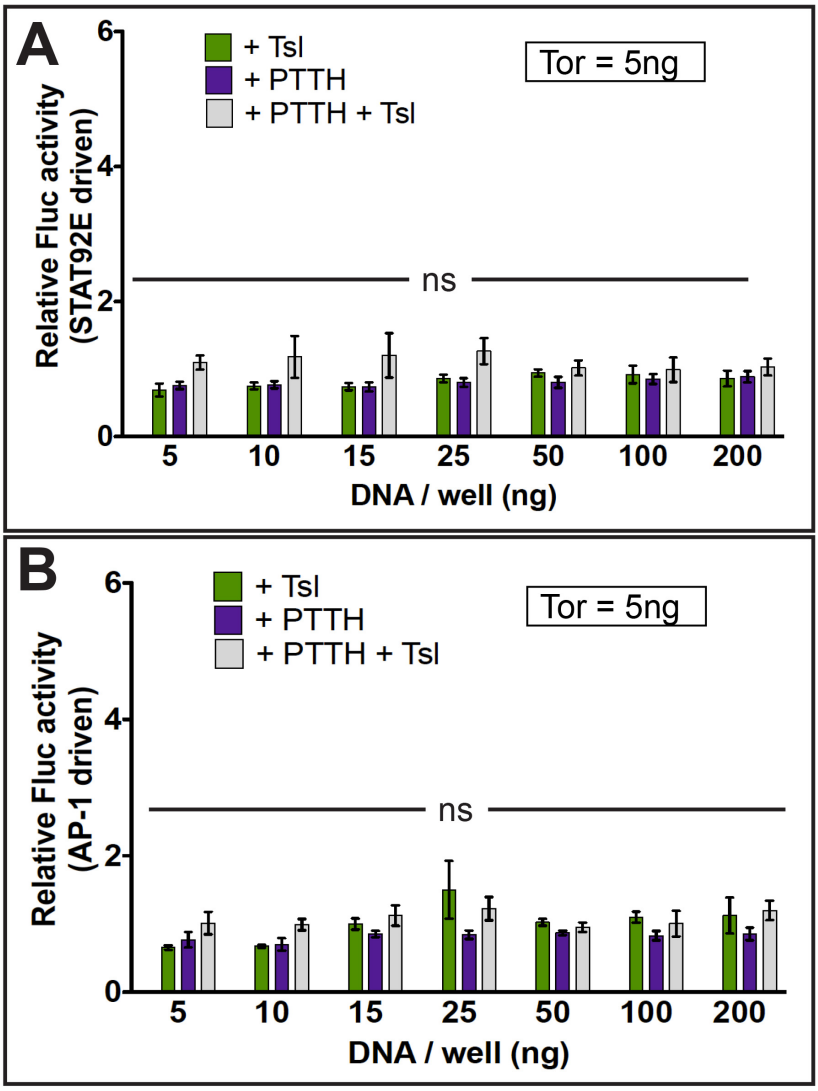

Fig. S9

Fig. S9. PTTH does not induce Tor-dependent STAT92E- or AP-1-driven Fluc activity in cells expressing low levels of Tor.

All samples were transfected with the R-luc transfection control plasmid, 5 ng of the Tor expression construct, and either the STAT92E-dependent (A) or AP-1-driven (B) Fluc reporter construct. Additionally, cells were transfected with increasing amounts (up to 200 ng) of expression vectors bearing Tsl alone (green), PTTH alone (purple) or Tsl plus PTTH (grey). Each bar represents an average of three replicates, repeated 5 times (n=5) +/- s.d. P values, all not significant (ns), have been determined relative to measurements of cells expressing Tor alone.