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

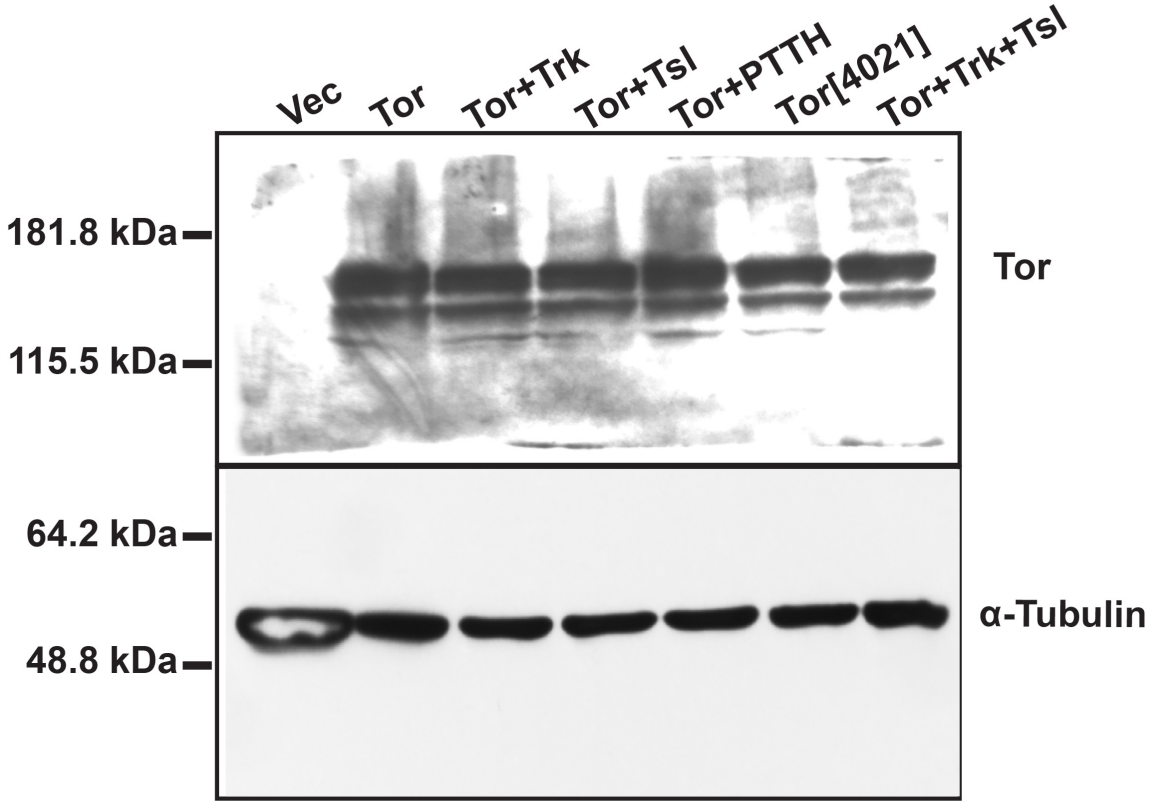

Fig. S1

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.

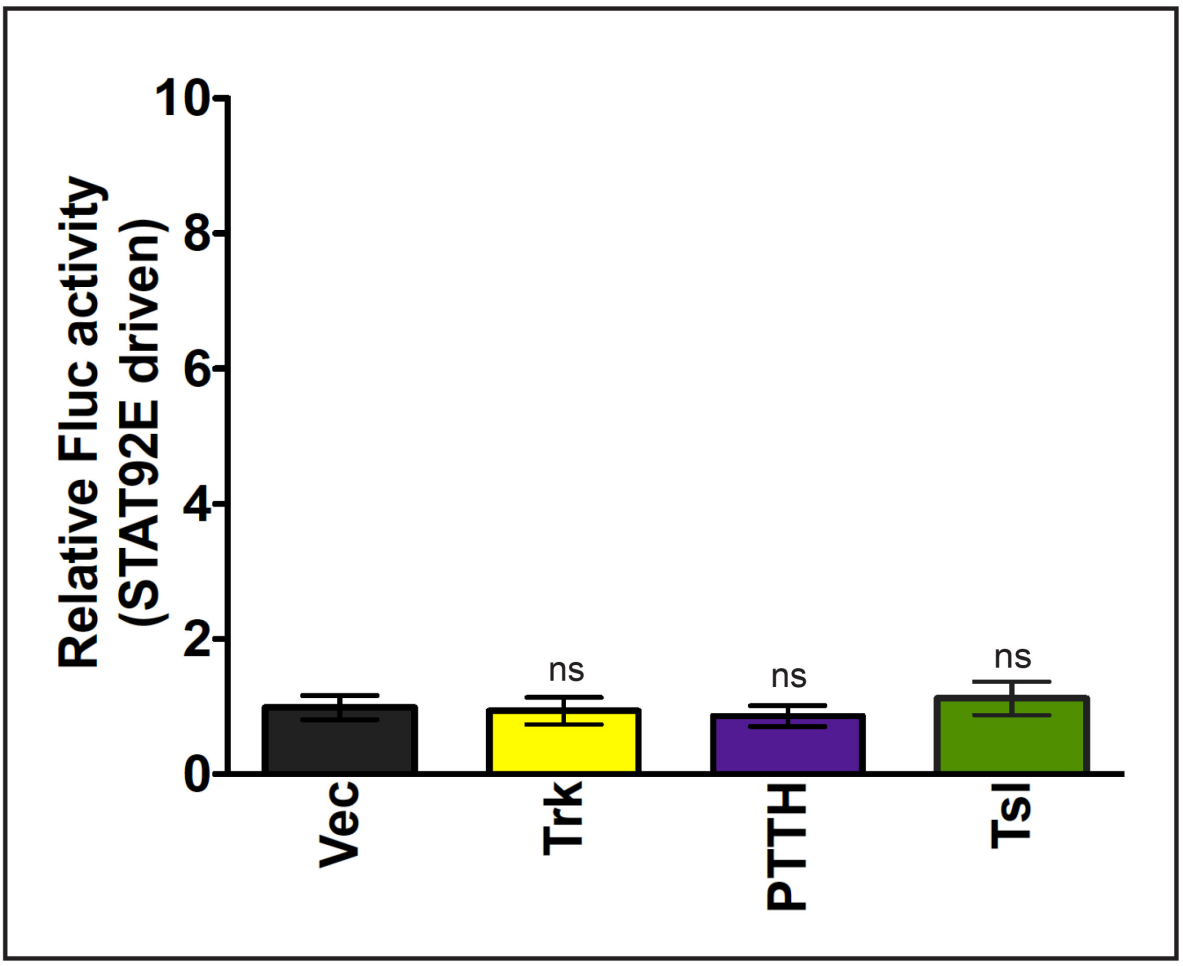

Fig. S2

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).

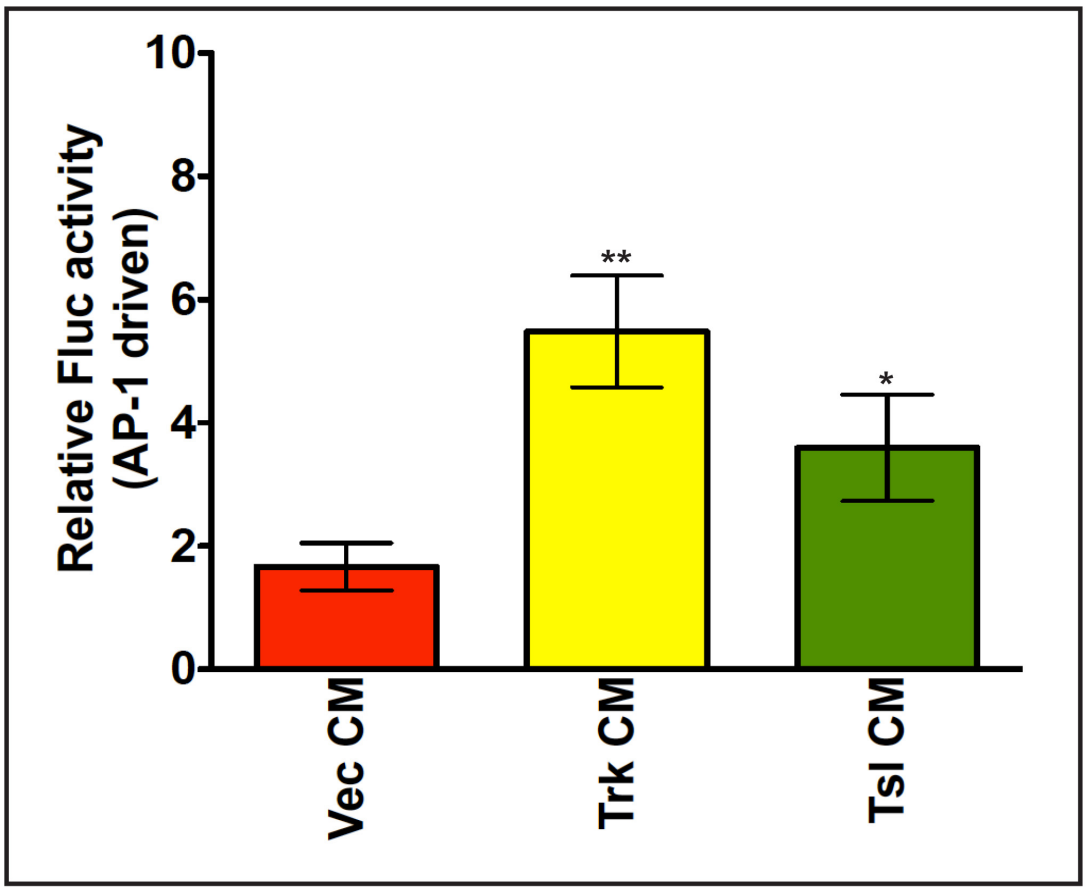

Fig. S3

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).

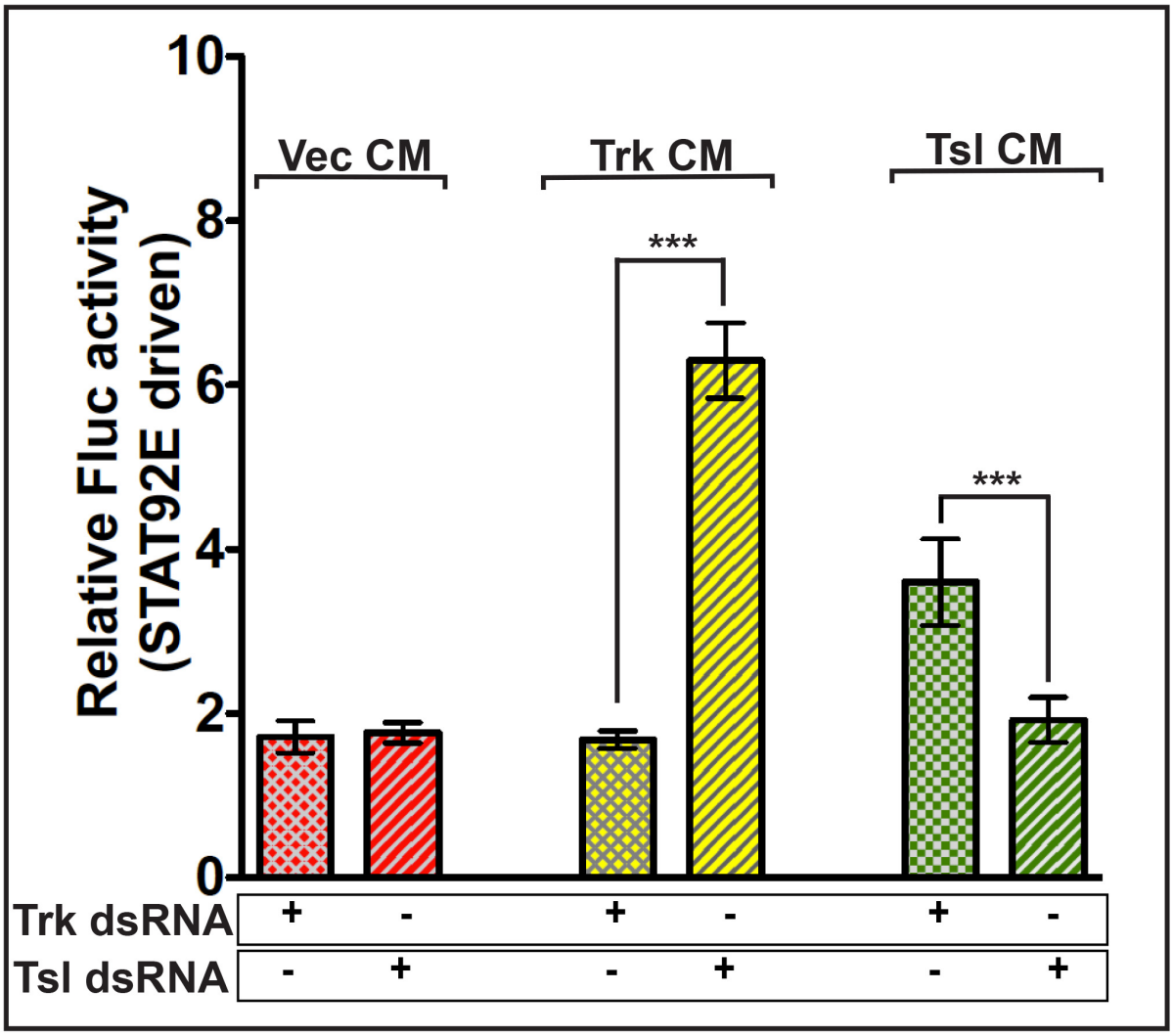

Fig. S4

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).

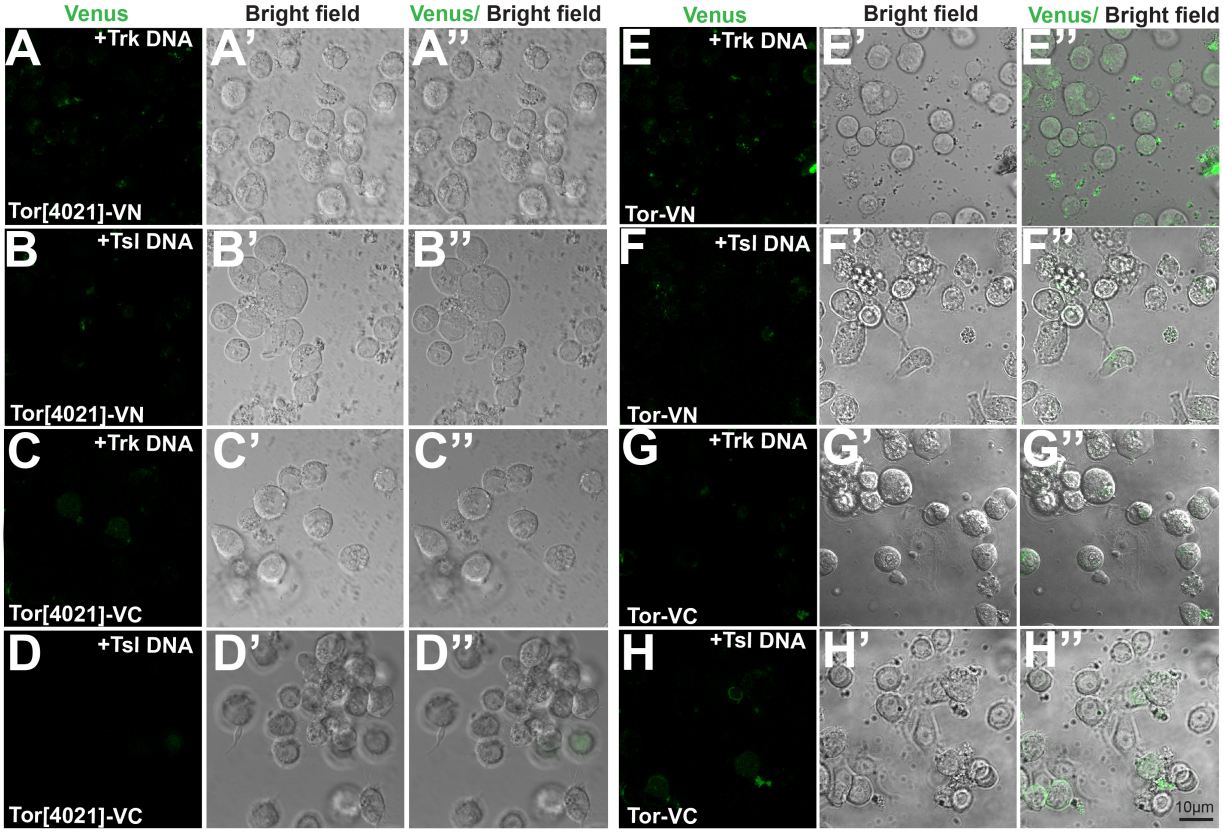

Fig. S5

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).

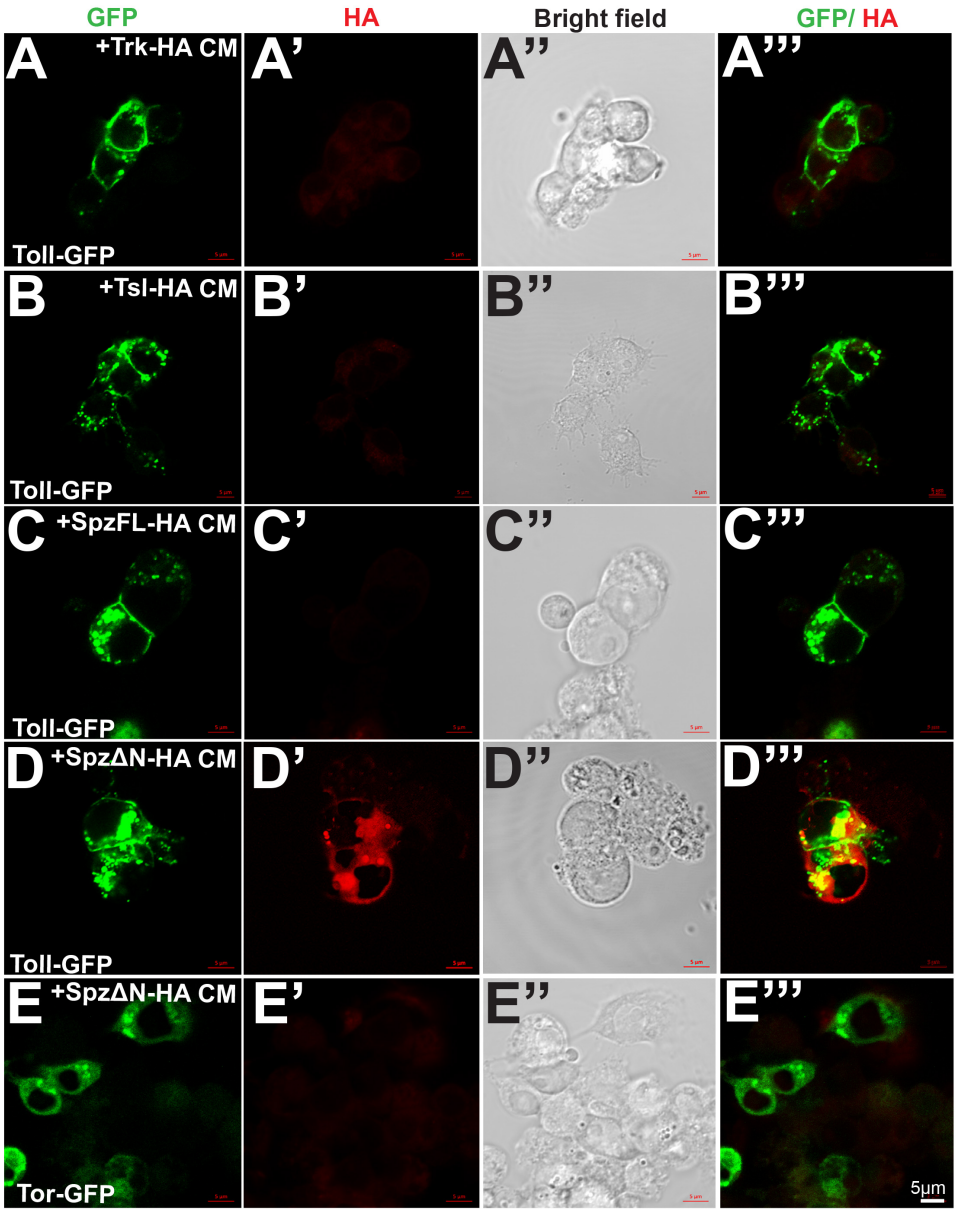

Fig. S6

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.

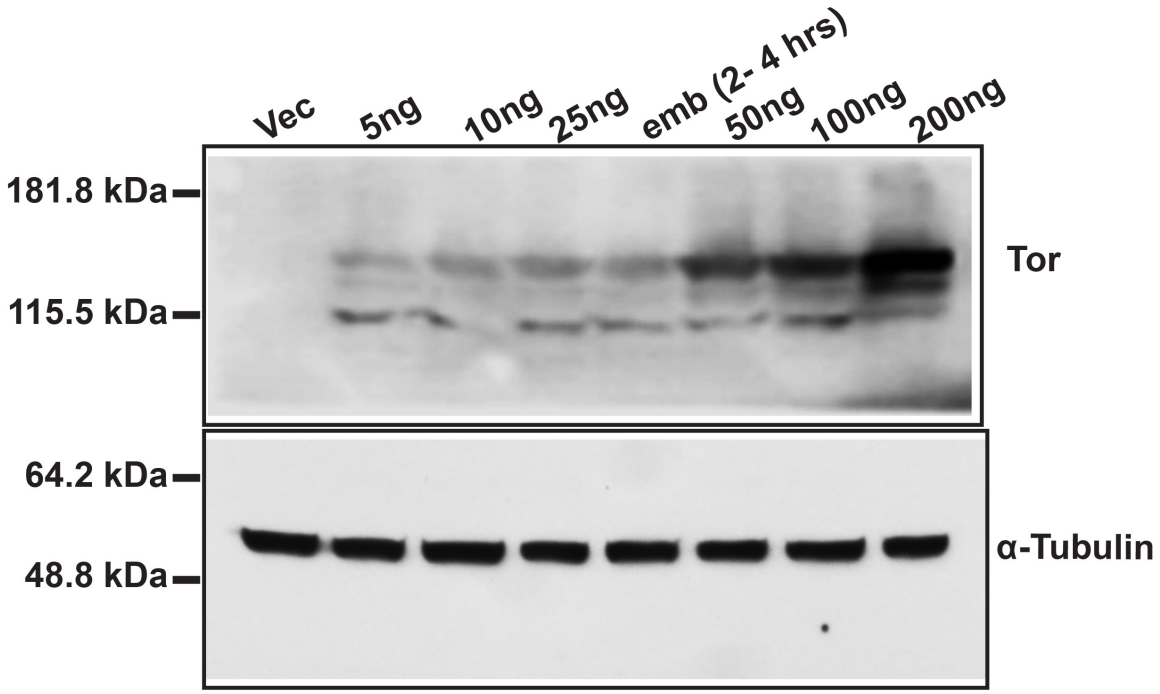

Fig. S7

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.

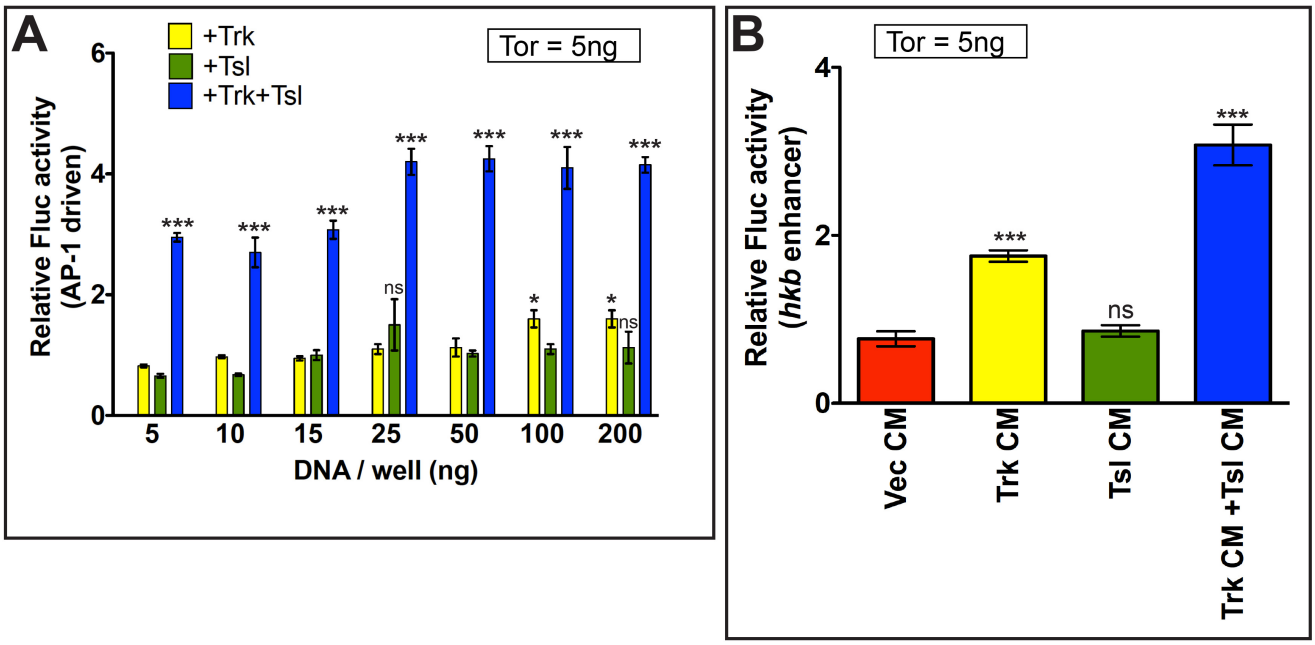

Fig. S8

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.

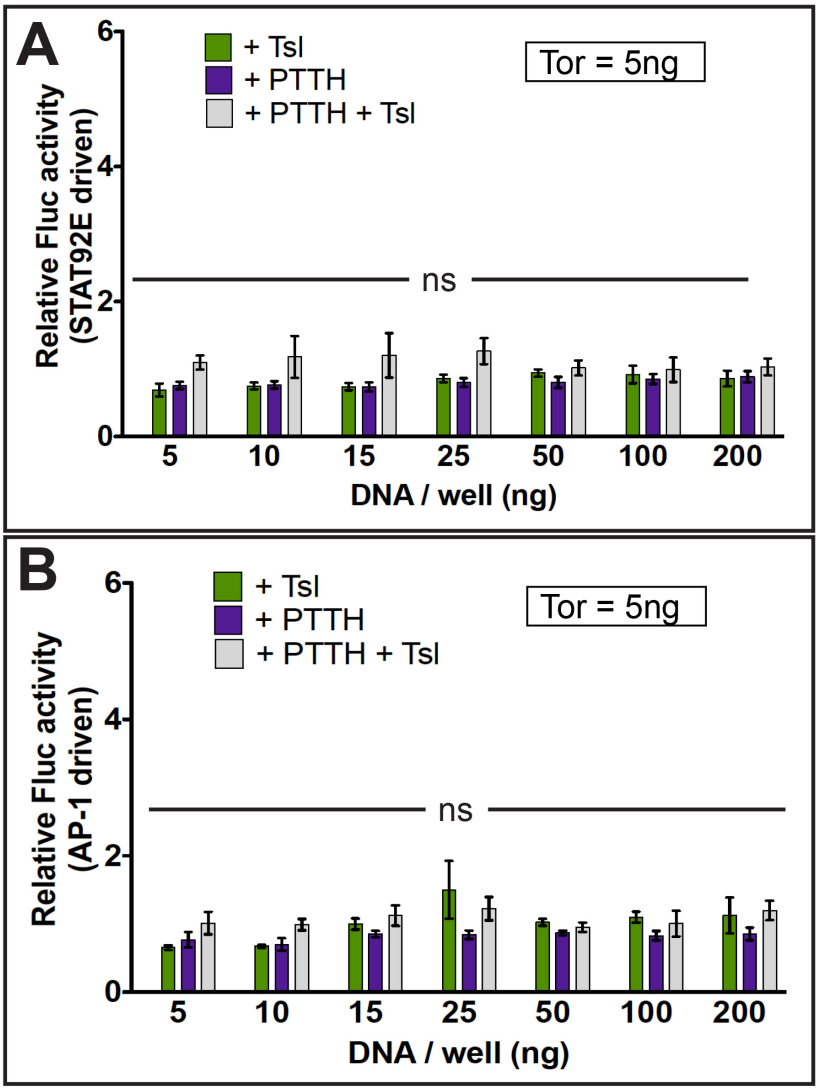

Fig. S9

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.