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MUTANT SCREEN REPORT

Genome-Wide Screen for New Components of the *Drosophila melanogaster* Torso Receptor Tyrosine Kinase Pathway

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ABSTRACT Patterning of the *Drosophila* embryonic termini by the Torso (Tor) receptor pathway has long served as a valuable paradigm for understanding how receptor tyrosine kinase signaling is controlled. However, the mechanisms that underpin the control of Tor signaling remain to be fully understood. In particular, it is unclear how the Perforin-like protein Torso-like (Tsl) localizes Tor activity to the embryonic termini. To shed light on this, together with other aspects of Tor pathway function, we conducted a genome-wide screen to identify new pathway components that operate downstream of Tsl. Using a set of molecularly defined chromosomal deficiencies, we screened for suppressors of ligand-dependent Tor signaling induced by unrestricted Tsl expression. This approach yielded 59 genomic suppressor regions, 11 of which we mapped to the causative gene, and a further 29 that were mapped to <15 genes. Of the identified genes, six represent previously unknown regulators of embryonic Tor signaling. These include *twins* (*tws*), which encodes an integral subunit of the protein phosphatase 2A complex, and *α-tubulin at 84B* (*αTub84B*), a major constituent of the microtubule network, suggesting that these may play an important part in terminal patterning. Together, these data comprise a valuable resource for the discovery of new Tor pathway components. Many of these may also be required for other roles of Tor in development, such as in the larval prothoracic gland where Tor signaling controls the initiation of metamorphosis.

KEYWORDS
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The first events that define the major axes and termini of the early *Drosophila* embryo are governed by genes expressed in the mother (St Johnston and Nüsslein-Volhard 1992). Patterning of the termini is achieved by spatially localized activation of the receptor tyrosine kinase (RTK) Torso (Tor) and signal transduction via the highly conserved Ras/mitogen-activated protein kinase (MAPK) cascade. This pathway leads to the transcriptional derepression of key zygotic target genes including *tailless* (*tll*) and *huckebein* (*hkb*) to drive terminal cell fate specification (Brönner and Jäckle 1991). Since its discovery, the Tor pathway/terminal patterning system has been a valuable paradigm for understanding fundamental aspects of how RTK signaling is controlled in space and time [for a review, see Li (2005)].

Despite much interest, however, several key features of the terminal patterning system remain unclear. These include the mechanism by which Tor is activated by its ligand Trunk (Trk) only at the ends of the embryo. A significant body of work suggests that Tor activation is controlled by Torso-like (Tsl), a protein localized to the early embryo termini (Savant-Bhonsale and Montell 1993; Martin *et al.* 1994). For example, Tor signaling is not activated in the absence of Tsl (Sprenger *et al.* 1993), and unrestricted expression of Tsl induces ubiquitous Tor signaling (Savant-Bhonsale and Montell 1993; Martin *et al.* 1994).

Tsl is a member of the perforin-like superfamily of proteins best characterized in terms of their membrane pore-forming roles in vertebrate immune defense (Rosado *et al.* 2008; Law *et al.* 2010; Dudkina *et al.* 2016). Current hypotheses for Tsl function include: the control of Trk proteolysis and activation (Casanova *et al.* 1995; Casali and Casanova 2001), Trk secretion (Johnson *et al.* 2015), and facilitation of Tor

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dimerization or the binding of Trk to Tor (Amarnath *et al.* 2017). For each of these possibilities, it is highly likely that additional pathway components remain undiscovered; their identification could provide valuable insights into the mechanism controlling localized Tor activation.

A number of screening approaches have previously been used to identify genes involved in terminal patterning. The terminal class genes were first discovered in early mutagenesis screens that identified many components of the major developmental pathways required for embryonic patterning (Schupbach and Wieschaus 1986, 1989; Nusslein-Volhard *et al.* 1987). However, a major limitation of this approach was that the maternal effect of a given allele could only be examined if homozygous mothers were viable. Zygotic lethal maternal effect screens and dominant modifier screens have been used to overcome this problem (Strecker *et al.* 1991; Perrimon *et al.* 1996; Bellotto *et al.* 2002; Li and Li 2003; Luschnig *et al.* 2004). Unlike the saturating whole-genome mutant screens, however, zygotic lethal screens are low-throughput and hence were performed on a smaller scale. Furthermore, this approach precludes the detection of genes that perform critical roles during oogenesis and prior to pattern formation. By contrast, dominant modifier screens are not limited in this regard and have been successfully performed using the phenotype produced by *tor* gain-of-function alleles to find new genes that act downstream of Tor (Strecker *et al.* 1991; Li and Li 2003). However, use of these alleles does not permit the discovery of potential interactors involved in generating the active Tor ligand (*i.e.*, acting upstream of *tor*).

Since we were interested in the events upstream of Tor, we conducted a genome-wide screen to identify suppressors of the phenotype produced upon ectopic *tsl* expression. This screen has the ability to identify new pathway components that function either upstream or downstream of *tor* and led to the identification of six genes that had not previously been associated with embryonic terminal patterning. In addition, we performed detailed mapping for a further 48 genomic suppressor regions that may contain novel regulators of ligand-dependent Tor signaling.

MATERIALS AND METHODS

Drosophila stocks

The following stocks were used: w^{1118} (BL5905), *P{GawB}c355* (called *c355-Gal4*, from BL3750), *P{tubP-GAL80^{ts}}* (from BL7018), *UAS-tsl* (Johnson *et al.* 2013), *nos-Cas9* (CAS-0001), and *P{nos-ΦC31}* lines (TBX-0002 and TBX-0003; Kondo and Ueda 2013), and the Bloomington *Drosophila* Stock Centre (BDSC) deficiency kit (Cook *et al.* 2012). All nonkit deficiency lines used for mapping are listed in Table 2, and alleles tested are given in Supplemental Material, Table S2. All flies were maintained on standard media at 25° unless otherwise stated.

Screening crosses

Autosomal deficiencies or mutant alleles were tested by crossing virgin females from the screen line (*c355-Gal4*; *Gal80ts*; *UAS-tsl*) to deficiency males. X-chromosome deficiencies or mutant alleles were tested by crossing deficiency females to males from the screen line. In all cases, crosses were performed at 22° and cultures shifted to 29° ~92 hr post egg lay. From each cross, at least 10 F1 females carrying the deficiency chromosome and screening transgenes were placed in a vial containing apple juice agar supplemented with yeast paste and allowed to mate with w^{1118} males.

Cuticle preparations and scoring

Adults were allowed to lay for 24 hr, and embryos were left to develop for a further 24 hr. Embryos were then dechorionated in 50% (v/v) bleach and mounted on slides in a mixture of 1:1 (v/v) Hoyer's solution: lactic acid. Slides were incubated overnight at 65° and imaged using dark field optics (Leica). Three consecutive cuticle preps were performed for each deficiency; if suppression was observed, the deficiency was retested at least twice. Suppression strength was qualitatively assessed based on the number of embryos showing central segment gain and the number of segments gained compared with controls (screen line crossed to w^{1118}).

Immunostaining and imaging

Ovaries were dissected in phosphate-buffered saline (PBS) while on ice and fixed in 4% paraformaldehyde in PBS for 1 hr. Ovaries were then washed five times in PBS with 0.1% Triton-X (PTx) followed by blocking in 5% goat serum in PTx and incubation with anti-Tsl (1:500) overnight at 4°. Anti-Tsl was raised in a rabbit against a peptide consisting of the C-terminal 18 residues of Tsl and affinity purified (Genscript). We note that this antibody does not detect endogenous Tsl and only recognizes overexpressed Tsl in fixed tissue. Ovaries were then washed five times with PTx and incubated with anti-rabbit Alexa568 (1:500; Thermo Fisher Scientific) secondary antibody in PTx. Following washing in PTx, ovarioles were further dissected and mounted on slides in VectaShield (Vector Laboratories). Single confocal sections of stage nine egg chambers were captured on a Nikon C1 confocal microscope at 20× magnification.

Generation of Rab3-GEF^5

Transgenic flies expressing two guide RNAs targeting Rab3-GEF (535: 5′-ATC GGT TCG GGA TAG TCT TC-3′; 5244: 5′-AGT CAG GAG CGT GAT ATG AT-3′) were made by cloning annealed guide sequence oligos into the pBFv-U6.2 vector (Kondo and Ueda 2013), followed by genomic integration via ΦC31-mediated transgenesis (*attP40* and *attP2* landing sites). These flies were crossed to the Cas9 source, and single lines carrying deletions between the two guide RNA target sites were established. The genomic deletion in line five (coordinates: X:15,097,551–15,102,259 inclusive, *Drosophila melanogaster* release 6.18) removes 4708 bp and is predicted to truncate the Rab3-GEF protein (PC isoform) at residue L546 (of 2084 amino acids) and add a short out-of-frame C-terminal extension of 12 residues. We note that these flies are homozygous viable and fertile.

Data and reagent availability statement

Data and reagents are available upon request. Table S1 contains a list of the BDSC deficiency kit deficiencies that do not suppress the ectopic Tsl phenotype. Table S2 lists the alleles of genes within suppressor regions that were screened.

RESULTS

Normally, *tsl* expression is restricted to subpopulations of follicle cells located at the anterior and posterior ends of the developing oocyte (Savant-Bhonsale and Montell 1993; Martin *et al.* 1994). Expressing *tsl* ectopically in all follicle cells (*c355-Gal4*; *UAS-tsl*, Figure 1) causes a highly consistent maternal embryonic lethal cuticle phenotype whereby the terminal regions are expanded and all central segments are lost (known as *spliced*, Savant-Bhonsale and Montell 1993; Martin *et al.* 1994). Importantly, Tor activation in this scenario is ligand dependent and thus may also be sensitive to loss of genes that function upstream of *tor*, such as those involved in ligand generation and trafficking, in addition to those that act downstream.

In order to conduct an F1 suppressor screen using this background, we first needed to overcome the maternal sterility associated with ectopic *tsl* expression. This was necessary to enable the screening line to be

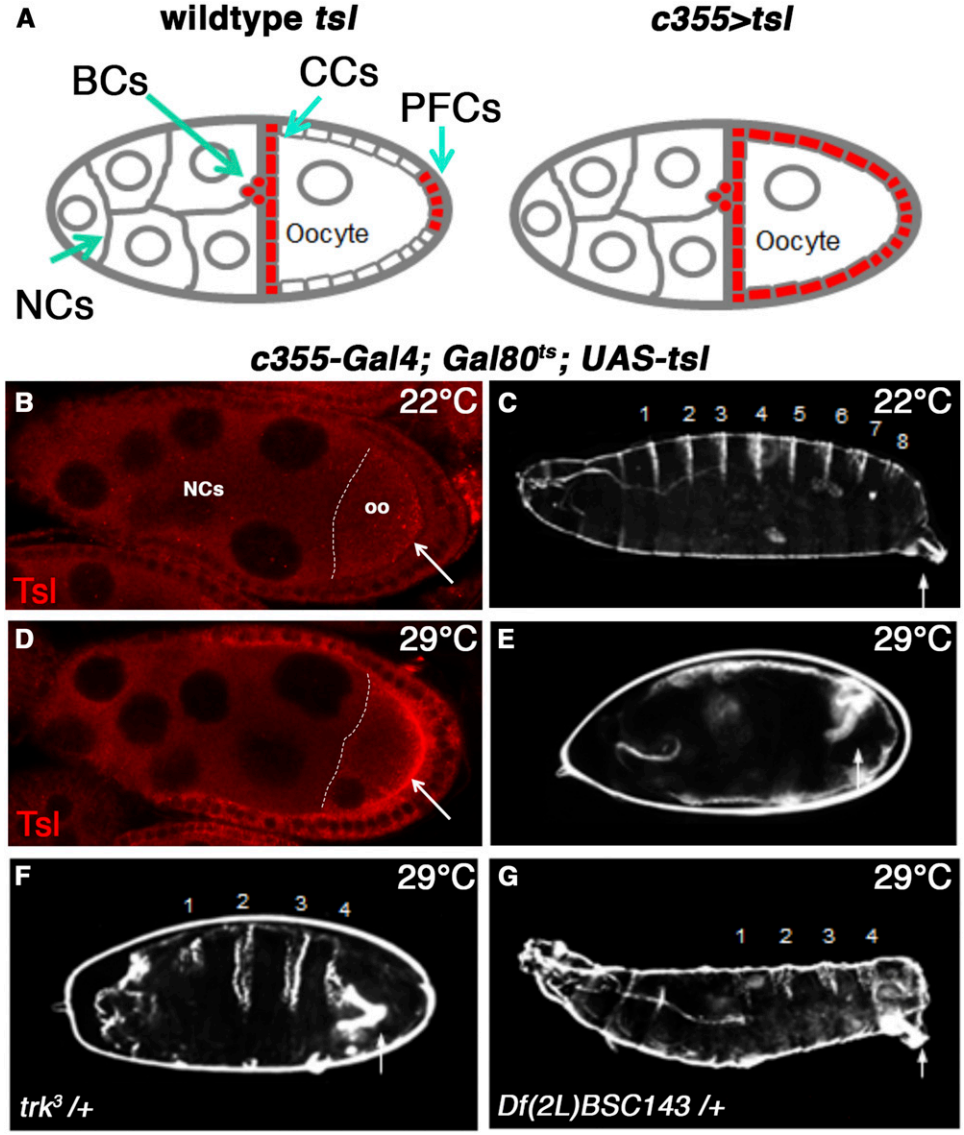

Figure 1 Validation of the ectopic Tsl suppressor screen. (A) Schematics of stage 10 egg chambers showing the endogenous *tsl* expression pattern (red, left) and ectopic pattern used for screening (*UAS-tsl* driven by *c355-Gal4*, right). (B) A stage-eight egg chamber from a screening line female raised at 22° and immunostained for Tsl. No ectopic Tsl expression is observed in the perivitelline space (open arrow). These flies produce viable offspring with wild-type cuticles containing eight abdominal segments (numbered) and a filzkörper [closed arrow, (C)]. (D) An egg chamber from a screening line female raised at 29° showing strong ectopic expression of Tsl. (E) These flies produce embryos with the lethal *spliced* phenotype (no abdominal segments and expanded termini). Suppression of ectopic *tsl* phenotype (gain of multiple abdominal segments) is observed upon introduction of either one copy of the amorphic *trk* allele, trk^3 (F) or *Df(2L)BSC143*, which deletes one copy of *trk* (G). Anterior is to the left. BCs, border cells; CCs, centripetal follicle cells; NCs, nurse cells; oo, oocyte; PFCs, posterior follicle cells.

crossed directly to chromosomal deficiencies and mutant alleles, and to examine embryos produced by the offspring. We achieved this using the temperature-sensitive (ts) repressor of Gal4, $Gal80^{ts}$ (McGuire *et al.* 2004). When females (*c355-Gal4*; $Gal80^{ts}$; *UAS-tsl*) were raised at 22°, $Gal80^{ts}$ efficiently prevented ectopic maternal *tsl* expression (Figure 1B), and females produced embryos with wild-type cuticles (Figure 1C). Raising females at 29°, either from the third instar larval stage or by shifting adults raised at 22° to this temperature, fully restored ectopic *tsl* expression (Figure 1D) and induced the *spliced* phenotype (Figure 1E).

To test whether this line was suitable for a suppressor screen, we introduced one copy of a strong hypomorphic allele of *trk* (trk^3), a terminal class gene known to be limiting with respect to Tor signaling (Sprenger and Nusslein-Volhard 1992). This strongly suppressed the ectopic *tsl* phenotype, as evidenced by restoration of multiple central segments in almost all embryos (Figure 1F). A similar degree of suppression was observed using one copy of a chromosomal deficiency that deletes a genomic region including *trk* (*Df[2L]BSC143*; Figure 1G). Taken together, these data suggest that the ectopic *tsl* phenotype is sensitive to *tor* pathway gene dosage and may serve as a discovery tool for new pathway components, including genes that act upstream of *tor*.

To identify regions of the genome that contain novel Tor pathway genes, we performed a screen using the BDSC deficiency kit. This is a collection of 467 lines each containing a genomic deletion with molecularly defined breakpoints, collectively covering ~98% of euchromatic genes in *D. melanogaster* (Cook *et al.* 2012). Of the 467 lines, data were obtained for 429 lines, equating to ~90% of the genome (Table 1). The remaining lines were mostly deficiencies on the X chromosome that were problematic owing to poor culture health. The screen identified 65 deficiencies that consistently suppressed the ectopic *tsl* phenotype to varying degrees (Table 2), and 363 deficiencies that showed no suppression (Table S1). Most of the observed suppressor deficiencies were moderate in strength (gain of at least one segment, 44 deficiencies in the primary screen), whereas 17 were scored as strong suppressors (gain of multiple segments), and one was a weak suppressor. Very strong suppression (gain of multiple segments and partially restored viability) was observed for three deficiencies, *Df(1)ED7005* and *Df(1)ED7289*, neither of which span previously known *tor* pathway genes, and *Df(2L)BSC143*, which

spans *trk*. We note that seven suppressor deficiencies overlap in the regions they delete, and hence these may represent a single suppressor region. After such cases were accounted for, we estimated there to be at least 59 individual suppressors (hereafter referred to as suppressor regions) across the three major chromosomes (Table 1). To reduce the sizes of the regions and further narrow the list of candidate suppressor genes, mapping was conducted using additional available deficiency lines (Table 2). This markedly reduced the number of candidate genes, leaving 15 genes or fewer in 29 of the regions and five genes or fewer in nine of the regions.

We next wanted to identify the genes responsible for the observed suppression in each of these regions. To do this, we used available known loss-of-function and potential loss-of-function mutant alleles, as well as mutant alleles that we generated (*Rab3-GEF*^{5}, this study; and *trk*$^{\Delta}$, Henstridge *et al.* 2014), and tested whether they too could suppress the ectopic *tsl* phenotype. In total, we tested mutant alleles of 88 genes (Table S2) and successfully identified the causative suppressor gene in 10 of the regions (in addition to *trk*, Table 3). Four of these genes—*Ras oncogene at 85D* (*Ras85D*), *Son of sevenless* (*Sos*), *tailless* (*tll*), and *C-terminal Binding Protein* (*CtBP*)—have known roles in terminal patterning (Pignoni *et al.* 1990; Simon *et al.* 1991; Lu *et al.* 1993; Cinnamon *et al.* 2004) and thus further validate the screening approach. In each of these cases, the strength of suppression caused by the original deficiency was moderate to strong, and this matched well with the alleles we tested. Notably, many deficiencies that include other previously identified terminal class genes were not identified as suppressors. This suggests that these genes may not be dosage limiting for the ectopic *tsl* phenotype (Table 4). The remaining six genes that we identified had no previously known involvement in terminal patterning and thus represent potentially novel regulators of the Tor pathway. These genes are: *Tetraspanin 3A* (*Tsp3A*), *Ribosomal protein S21* (*RpS21*), *RpS26*, *Protein disulphide isomerase* (*Pdi*), *αTub84B*, and *tws*. As suppressors of the ectopic *tsl* phenotype, our findings suggest that these genes are haploinsufficient with respect to unrestricted Tor signaling.

DISCUSSION

We were initially surprised by the large number of suppressor regions detected in our screen. A previous screen performed using the *tor*^{Y9} gain-of-function allele found only 26 suppressor regions (Li and Li 2003). Although this study used a different deficiency kit with cytologically defined breakpoints and larger deleted regions, the difference between these figures could also reflect differences in our approaches. Our approach is ligand dependent and also sensitive to dosage-limited genes that act either upstream or downstream of *tor*. It is therefore possible that the additional suppressor regions we detected contain genes required for generation of the active Tor ligand. In addition, because our screening strategy relied upon Gal4/UAS-driven expression of a *tsl* transgene, genes required for production of ectopic Tsl (*e.g.*, those involved in protein translation) could act as suppressors. Indeed, the suppressor genes *RpS21* and *RpS26* that we identified may be haploinsufficient in this regard. Both genes encode crucial structural constituents of the ribosome and are required for protein translation (Marygold *et al.* 2007). Despite this, five of the 11 genes that we identified in our screen are known terminal patterning genes. Thus, many of the suppressor regions remaining are likely to house genes relevant to Tor signaling rather than suppressors of Gal4-driven *tsl* expression. A brief discussion of each identified suppressor gene in the context of this screen is given below.

Table 1 Summary of the ectopic *tsl* suppressor screen

Chromosome	BDSC Kit Deficiencies Tested/Total	Suppressor Deficiencies	Minimum Number of Suppressor Regions
1	71/91	17	13
2	187/190	15	14
3	165/180	33	32
4	6/6	0	0
Total	429/467	65	59

The proportion of the BDSC deficiency kit screened and number of suppressor deficiencies are shown by chromosome. Minimum number of suppressor regions accounts for deficiencies that overlap and may therefore represent a single suppressor deleted by both deficiencies.

Tetraspanin 3A

Tsp3A is one of 35 known tetraspanin genes in the *Drosophila* genome (Fradkin *et al.* 2002). Members of the tetraspanin superfamily are characterized by the presence of four transmembrane segments, and associate with each other and other transmembrane proteins to carry out cellular functions such as migration, fusion, adhesion, immune response, and intracellular vesicle trafficking [for a review, see Zhang and Huang (2012)]. However, the mechanism of tetraspanin function remains poorly understood (Zhang and Huang 2012). The allele of *Tsp3A* (*Tsp3A*^{e03287}) responsible for suppression of the ectopic *tsl* phenotype is homozygous viable and fertile, and exhibits no terminal patterning defects (data not shown). This could possibly be explained by functional redundancy between *Tsp3A* and other tetraspanin-encoding genes during normal development, and *Tsp3A* dosage sensitivity in our screening background. In this regard, *Tsp26A* and *Tsp86D* are known to act redundantly with *Tsp3A* in the promotion of Notch signaling for ovarian border cell migration (Dornier *et al.* 2012). Whether tetraspanins including Tsp3A have a similar role at the embryo plasma membrane for a Tor signaling factor will be interesting to determine.

Protein disulphide isomerase

Pdi encodes one of the two major protein disulphide isomerases found in *Drosophila*. The primary function of these enzymes is to form and break disulfide bonds between cysteine residues as proteins fold in the endoplasmic reticulum (ER, Freedman 1989; Noiva and Lennarz 1992). In our screen, *Pdi* was identified as a strong suppressor and thus may play an important part in terminal patterning. How might it achieve this? The Pdi-like protein Windbeutel (Thioredoxin/ERp29 superfamily; Konsolaki and Schupbach 1998) is required for the trafficking of Pipe through the ovarian follicle cell ER to assist in localizing the signal for dorsoventral patterning (Sen *et al.* 2000). Thus, Pdi may be essential for the correct folding and/or transport of one or more key Tor signaling components. This could include genes that function at the level of the ligand, receptor, or downstream pathway components.

α-Tubulin at 84B

α- and β-Tubulin proteins are the major structural constituents of the eukaryotic microtubule (MT, Nogales 2001). *D. melanogaster* has three α- and four β-Tubulin-encoding genes; however, no other tubulin genes were found to be suppressors of the ectopic *tsl* phenotype (*βTub85D* and *βTub97EF* were not tested). MTs are cylindrical organelles essential for many cellular processes, including intracellular transport, signal transduction, mitosis, and maintenance of cell shape (Ludueñna 1998; Gundersen and Cook 1999; Nogales 2001).

Table 2 Genomic regions that contain suppressors of the ectopic maternal Tsl phenotype

Suppressor Region	BDSC Kit Deficiency	Strength of Suppression	Additional Deficiencies Tested	Strength of Suppression	Suppressor Region Coordinates (Estimated)	Number of Genes in Suppressor Region
1	*Df(1)svr*	+++	*Df(1)ED6396*	+++	X:493,529–523,630	*CG17896*, *CG17778*, *svr*, *arg*, *elav*
	Df(1)BSC530	+++				
2	*Df(1)ED6443*	+++	*Df(1)Exel6223*	+++	X:769,982–841,105	8
			Df(1)BSC534	n.s.		
3	*Df(1)ED411*	++	*Df(1)ED6579*	++	X:2,589,210–2,636,213	14
			Df(1)ED6584	n.s.		
4	*Df(1)ED6712*	++			X:3,432,535–3,789,615	24
5	*Df(1)ED6727*	++			X:4,325,174–4,911,061	56
6	*Df(1)BSC867*	++	*Df(1)BSC882*	n.s.	X:7,015,408–7,041,515	*CG12541*, *CG14427*
7	*Df(1)BSC622*	+++			X:7,908,547–7,955,978	11
	Df(1)C128	+++				
8	*Df(1)ED7005*	++++	*Df(1)ED429*	n.s.	X:10,071,922–10,086,426	*CR45602*, *ZAP3*, *CG2972*, *CG2974*, *RhoU*
			Df(1)BSC822	++		
			Df(1)BSC712	+++		
9	*Df(1)ED7161*	++	*Df(1)Exel6244*	n.s.	X:12,007,087–12,750,866 excluding X:12,463,585–12,547,951	58
10	*Df(1)BSC767*	++			X:13,278,810–13,591,554	39
11[a]	*Df(1)ED7217*	++			X:13,642,083–14,653,944	111
	Df(1)ED7225	++				
	Df(1)ED7229	++				
12	*Df(1)ED7289*	++++	*Df(1)BSC310*	n.s.	X:15,089,556–15,125,750	*Rab3-GEF*, *CG9702*, *Cyp4s3*, *drd*
13	*Df(1)ED7331*	+++	*Df(1)BSC706*	+++	X:15,450,255–15,484,792	12
14	*Df(2L)JS31*	+++			2L:2,830,265–2,868,633	10
	Df(2L)BSC692	+++				
15	*Df(2L)BSC295*	++	*Df(2L)BSC218*	++	2L:4,197,800-*Art2* (incl.)	9
			Df(2L)M24F-B	n.s.		
16	*Df(2L)ED7853*	+	*Df(2L)Exel8013*	++	2L:4,979,299–5,000,943	*CG8892*, *CG34126*, *Rtnl1*
			Df(2L)Exel6010	n.s.		
			Df(2L)Exel9062	n.s.		
			Df(2L)Exel8012	n.s.		
			Df(2L)BSC182	n.s.		
			Df(2L)BSC811	n.s.		
			Df(2L)Exel7021	n.s.		
17	*Df(2L)BSC143*	++++	*Df(2L)Exel7046*	n.s.	2L:10,260,017–10,276,871	8
			Df(2L)BSC827	+++		
18	*Df(2L)BSC812*	++	*Df(2L)BSC147*	n.s.	2L:13,800,829–13,819,589	*CAH1*, *Adat1*, *CG16865*, *CG16888*, *Sos*
			Df(2L)BSC691	n.s.		
			Df(2L)Exel7059	++		
19	*Df(2L)ED793*	++	*Df(2L)Exel8033*	n.s.	2L:14,013,641–14,300,969	17
			Df(2L)Exel6035	n.s.		
20	*Df(2L)Exel8038*	++	*Df(2L)BSC256*	n.s.	2L:18,320,008–18,444,727	7
			Df(2L)BSC149	n.s.		
21	*Df(2L)ED1303*	++	*Df(2L)Exel7077*	n.s.	2L:19,753,324–19,918,015	47
			Df(2L)ED1315	n.s.		
			Df(2L)BSC258	n.s.		
22	*Df(2R)BSC595*	++			2R:10,462,874–11,197,412	76
23	*Df(2R)ED2426*	++	*Df(2R)Exel7135*	n.s.	2R:15,262,942–15,373,060	12
24	*Df(2R)BSC355*	++	*Df(2R)BSC406*	++	2R:17,484,828–17,518,127	7
25	*Df(2R)ED3791*	++			2R:20,870,855–21,215,223	69
26	*Df(2R)BSC787*	++	*Df(2R)BSC598*	n.s.	2R:22,678,681–22,729,367	10
			Df(2R)Exel6079	n.s.		
27	*Df(2R)BSC136*	++	*Df(2R)BSC660*	n.s.	2R:23,949,444–23,992,124	19
			Df(2R)BSC770	++		
			Df(2R)BSC601	++		
28	*Df(3L)ED50002*	++			3L:1–123,924	7
29	*Df(3L)BSC371*	++	*Df(3L)BSC372*	+	3L:5,366,062–5,558,677	18
			Df(3L)Exel6105	++		
			Df(3L)ED210	n.s.		
			Df(3L)BSC387	n.s.		

(continued)

Table 2, *continued*

Suppressor Region	BDSC Kit Deficiency	Strength of Suppression	Additional Deficiencies Tested	Strength of Suppression	Suppressor Region Coordinates (Estimated)	Number of Genes in Suppressor Region
30	*Df(3L)BSC410*	++	*Df(3L)BSC438*	++	3L:5,777,085–5,926,648	25
			Df(3L)BSC411	n.s.		
			Df(3L)Exel7210	n.s.		
			Df(3L)BSC884	n.s.		
31	*Df(3L)BSC816*	++			3L:8,639,081–8,745,326	14
32	*Df(3L)BSC113*	++			3L:9,384,075–9,423,491	10
33	*Df(3L)ED4457*	++			3L:10,363,951–11,096,989	85
34	*Df(3L)ED4502*	++	*Df(3L)BSC737*	n.s.	3L:13,346,618–13,417,155	14
			Df(3L)BSC614	++		
			Df(3L)Exel6119	n.s.		
35	*Df(3L)ED217*	+++	*Df(3L)BSC837*	+++	3L:15,098,731–15,151,057	18
			Df(3L)BSC578	n.s.		
			Df(3L)BSC441	n.s.		
36	*Df(3L)ED4674*	+++	*Df(3L)Exel6130*	++	3L:16,780,123–16,806,648	*CG9706, eIF3e, CG9674*
			Df(3L)ED4606	n.s.		
			Df(3L)ED4685	n.s.		
			Df(3L)Exel9002	n.s.		
			Df(3L)Exel9003	n.s.		
			Df(3L)Exel9004	n.s.		
37	*Df(3L)ED4710*	+++			3L:17,612,170–17,795,144	29
38	*Df(3L)ED4858*	++	*Df(3L)BSC830*	++	3L:19,929,242–20,103,877	26
			Df(3L)BSC446	n.s.		
39	*Df(3L)BSC223*	+++	*Df(3L)BSC284*	++	3L:21,916,420–21,955,091	6
			Df(3L)Exel6137	+++		
40	*Df(3R)BSC633*	++	*Df(3R)ED7665*	n.s.	3R:7,080,388–7,090,527	*Ref1, Dpck, Tailor, CR45907, alphaTub84B*
41	*Df(3R)ED5339*	++	*Df(3R)BSC507*	+++	3R:9,259,246–9,352,375	13
			Df(3R)Exel6152	n.s.		
42	*Df(3R)BSC476*	+++	*Df(3R)Exel6153*	++	3R:9,513,020–9,550,705	9
43	*Df(3R)Exel6155*	++			3R:9,928,791–10,089,458	12
44	*Df(3R)BSC621*	+++			3R:10,111,458–10,144,754	8
	Df(3R)ED5474	+++				
45	*Df(3R)ED5514*	++			3R:11,200,280–11,230,695	12
46	*Df(3R)BSC486*	++	*Df(3R)Exel8157*	++	3R:13,012,713–13,051,785	12
			Df(3R)ED5610	n.s.		
47	*Df(3R)ED5664*	++	*Df(3R)ED10557*	++	3R:14,986,411–15,094,157	7
			Df(3R)ED10556	++		
			Df(3R)ED10555	n.s.		
			Df(3R)BSC750	n.s.		
48	*Df(3R)Exel7328*	++			3R:16,009,418–16,157,456	19
49	*Df(3R)BSC748*	++	*Df(3R)Exel6176*	n.s.	3R:16,977,724–17,149,107	23
50	*Df(3R)BSC650*	++	*Df(3R)Exel6178*	++	3R:18,196,430–18,274,622	22
			Df(3R)BSC682	++		
51	*Df(3R)BSC517*	++	*Df(3R)BSC141*	n.s.	3R:20,031,267–20,594,880	56
52	*Df(3R)Exel6272*	++			3R:21,060,603–21,112,334	11
53	*Df(3R)ED6085*	++	*Df(3R)ED6090*	n.s.	3R:22,026,005–22,042,224	8
			Df(3R)BSC804	++		
54	*Df(3R)BSC619*	++	*Df(3R)ED6096*	n.s.	3R:23,258,092–23,279,758	*wda, CG13827, orb*
			Df(3R)BSC803	n.s.		
			Df(3R)ED6105	++		
			Df(3R)Exel9012	n.s.		
55	*Df(3R)BSC461*	+++	*Df(3R)BSC679*	++	3R:25,092,692–25,137,846	15
			Df(3R)BSC493	++		
56	*Df(3R)BSC501*	++	*Df(3R)Exel6212*	++	3R:29,215,175–29,288,231	15
57	*Df(3R)BSC620*	++	*Df(3R)BSC861*	++	3R:29,877,104–29,934,580	14
58	*Df(3R)ED6346*	++			3R:30,794,955–31,011,935	20
59	*Df(3R)BSC793*	+++			3R:31,311,048–31,458,140	14

Suppressor strength and the molecular coordinates of the suppressor regions are given when possible. Suppression was scored qualitatively based on segment gain and consistency; weak (+), moderate (++), strong (+++), and very strong (++++). Mapping was conducted using a combination of the BDSC deficiency kit and additional molecularly defined deficiencies. The number of genes within each candidate region is indicated; in cases where there are five or less, the gene symbols are provided. n.s., no suppression was observed.

[a] Region likely contains multiple suppressor loci.

Table 3 Genes identified as suppressors of the ectopic *tsl* phenotype

Suppressor Region	Suppressor Gene	Allele(s) Tested	Strength of Suppression
3	***Tetraspanin 3A***	*Tsp3A*^{e03287}	++
14	***Ribosomal protein S21***	*RpS21*^{03575}	++
		RpS21^{k16804a}	++
17	*Trunk*	*trk*^{3}	++++
		trk$^{\Delta}$	++++
18	*Son of Sevenless*	*Sos*$^{34Ea-6}$	+++
20	***Ribosomal protein S26***	*RpS26*^{KG00230}	+++
35	***Protein disulfide isomerase***	*P{lacW}l(3)j2A2*^{jA2A}	+++
40	***α-Tubulin at 84B***	*αTub84B*^{5}	++
42	*Ras oncogene at 85D*	*Ras85D*^{e1B}	++
44	***twins***	*tws*^{02414}	++
46	*C-terminal binding protein*	*CtBP*^{03463}	++
58	*tailless*	*tll*^{49}	+++

Mutant alleles used to identify the gene as a suppressor are indicated. The suppression score is listed with the allele identified as a suppressor for this region. Suppression was scored qualitatively based on segment gain and consistency; moderate (++), strong (+++), and very strong (++++). Bolded genes have no previously known involvement in terminal patterning.

Interestingly, members of the MAPK signaling cascade, including the extracellular signal regulated kinases, were first identified as MT-associated protein kinases (Reszka *et al.* 1995; Morishima-Kawashima and Kosik 1996). These interactions are thought to regulate kinase activity and permit intracellular protein transport for efficient signal transduction (Parker *et al.* 2014). One possibility, therefore, is that *αTub84B* is important for promoting MAPK signaling downstream of Tor.

twins

tws encodes one of three different B subunit classes of the heterotrimeric serine/threonine phosphatase complex known as protein phosphatase 2A (PP2A, Mumby and Walter 1993; Walter and Mumby 1993). PP2A is composed of a scaffold A subunit, a regulatory B subunit, and a catalytic C subunit that together form the active holoenzyme (Janssens and Goris 2001). The B subunits provide temporal and spatial specificity for PP2A activity (Zolnierowicz *et al.* 1994; Janssens and Goris 2001; Seshacharyulu *et al.* 2013).

tws has been shown to have roles in a wide variety of cellular processes in *Drosophila*, including cell fate determination (Mumby and Walter 1993), cell cycle regulation (Walter and Mumby 1993), centriole amplification (Brownlee *et al.* 2011), and neuroblast proliferation and self-renewal (Chabu and Doe 2009). Most notably, *tws* has been identified as a suppressor of a constitutively active form of Sevenless, an RTK with strong homology to Tor (Maixner *et al.* 1998). It is possible that Tws acts similarly downstream of Tor in terminal patterning. The maternal role of *tws* has previously been investigated in three different studies; however, the results of these are conflicting. Germline clone analyses using the *tws*^{j11C8} allele by Bajpai *et al.* (2004) showed that embryos arrested prior to segmentation, whereas Perrimon *et al.* (1996) observed patterning defects resulting in the variable deletion of segments using the *tws*^{02414} allele that we used here. Finally, Bellotto *et al.* (2002) reported terminal defects associated with *l(3)*^{S027313}, a lethal but unmapped *P*-element insertion. This insertion was later mapped to the *tws* locus (Deak *et al.* 1997), thus further supporting the idea that *tws* is essential for terminal patterning.

Conclusion

We have performed a genome-wide suppressor screen for genes that act downstream of *tsl* in embryonic terminal patterning, and identified several new Tor pathway regulators. Many of the suppressor regions where the causative gene(s) are yet to be identified have been mapped to a tractable number of candidate genes. Thus, the generation of new loss-of-function alleles for candidate genes in these suppressor regions will permit the rapid discovery of new genes involved in Tor signaling. This will be particularly informative with respect to unanswered questions, including how the Tor ligand is generated and, more broadly, how RTK signaling is spatially controlled and transduced.

Table 4 Terminal class genes not detected as suppressors of the ectopic *tsl* phenotype

Terminal Class Gene(s)	Loss-of-Function Phenotype	Reference
closca	Loss of termini	Ventura *et al.* (2010)
fs(1)polehole	Loss of termini	Jimenez *et al.* (2002)
fs(1)Nasrat	Loss of termini	Jimenez *et al.* (2002)
Furin 1 and *Furin 2*	Loss of termini	Johnson *et al.* (2015)
torso-like	Loss of termini	Stevens *et al.* (1990)
torso	Loss of termini	Sprenger *et al.* (1989)
corkscrew	Suppressor of *tor* GOF	Perkins *et al.* (1992)
downstream of receptor kinase	Partial loss of termini	Simon *et al.* (1991)
huckebein	Loss of posterior midgut	Weigel *et al.* (1990)
sprouty	Suppresses *torso* GOF	Casci *et al.* (1999)
bicoid	Loss of anterior	Driever and Nusslein-Volhard (1988)

Terminal class gene names and their previously reported loss-of-function phenotypes are given. Note that only genes known to encode positive regulators of *tor* signaling are shown (*i.e.*, those expected to be suppressors) and the relevant reference is given for each.

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