Localized surface activity of *torso*, a receptor tyrosine kinase, specifies terminal body pattern in *Drosophila*

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The subdivision of the *Drosophila* body into distinct terminal and central domains depends on the *torso* (*tor*) protein, a putative receptor tyrosine kinase that is active at both ends of the early embryo. We show that the *tor* protein is uniformly expressed along the surface membrane of early embryos despite its localized activity at both poles. Further, we present evidence that polarized activity of this protein depends on other terminal gene functions, one of which may be a localized extracellular ligand generated during oogenesis. Finally, using the temperature-sensitive gain-of-function mutation *tor*^{RL3}, we show that different levels of active *tor* protein can specify distinct portions of the terminal pattern. Thus, we argue (1) that *tor* functions as a ubiquitous surface receptor that is activated by a spatially restricted ligand, and (2) that localized activity of the *tor* kinase may generate one or more gradients of intracellular signals that control body pattern.

[Key Words: *Drosophila*; *torso* protein; terminal gene functions]

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Genetic analysis in *Drosophila* has identified many genes involved in specifying the body pattern (e.g., Lewis 1978; Nüsslein-Volhard and Wieschaus 1980; Schüpbach and Wieschaus 1986a; Nüsslein-Volhard et al. 1987). This process begins during oogenesis when a few localized determinants are laid down under the control of a small number of genes (Nüsslein-Volhard et al. 1987). After fertilization, these determinants organize the body pattern by directing the expression of subordinate regulatory molecules, the products of the gap, pair-rule, segment-polarity, and homeotic genes (for review, see Ingham 1988).

Distinct anterior and posterior determinant systems act in concert to control where most of the head, thoracic, and abdominal segments arise (Nüsslein-Volhard et al. 1987). Both depend on single morphogens (*bicoid* and *nanos*, respectively) that are initially localized at opposite ends of the embryo and then diffuse or are transported towards the middle forming opposing concentration gradients (Frohnhofer and Nüsslein-Volhard 1986, 1987; Lehmann and Nüsslein-Volhard 1986, 1987; Driever and Nüsslein-Volhard 1988a,b; Nüsslein-Volhard et al. 1987; Lehmann 1988). The terminal portions of the body are specified by a third determinant system (Nüsslein-Volhard et al. 1987). As in the case of the anterior and posterior systems, this system appears to depend on the localized activity of a single gene function, *torso* (*tor*) (Klingler et al. 1988).

The *tor* gene is only one of several terminal genes for which loss-of-function mutations cause the enlargement

of the central body segments at the expense of the terminalia (Perrimon et al. 1985, 1986; Degelmann et al. 1986; Schüpbach and Wieschaus, 1986a; Nüsslein-Volhard et al. 1987). It is unique, however, because gain-of-function mutations that cause the opposite phenotype—the expansion of the terminalia at the expense of the trunk segments—have also been isolated (Klingler et al. 1988; Strecker et al. 1989). In the most extreme cases, the enlarged head and tail meet in the middle of the embryo, and the trunk segments are eliminated (the splice phenotype; Schüpbach and Wieschaus 1989). Thus, presence or absence of *tor* activity can be viewed as defining alternative states of a developmental switch—‘on’ specifying terminal development; ‘off’ leading to central development by default. It follows that the normal subdivision of the embryo into distinct end and middle parts depends on *tor* being active at both poles but inactive or absent from the middle (Klingler et al. 1988).

The terminal system differs significantly from the anterior and posterior systems because it does not appear to be associated with a localized transplantable factor (Klingler et al. 1988). Instead, cytoplasm obtained from any position along the body appears to be able to restore the development of terminal structures when microinjected into *tor* mutant embryos. Hence, at present, the conclusion that *tor* activity is normally restricted to the poles rests solely on the genetic properties of the *tor* gene, particularly the reciprocal phenotypes caused by its gain- and loss-of-function mutations.

Recently, Sprenger et al. (1989) discovered that the *tor*

gene encodes a putative transmembrane protein that is structurally similar to a number of receptor tyrosine kinases. Moreover, these authors have found that the *tor* mRNA is ubiquitously distributed in maturing oocytes and early embryos. These findings suggest that *tor* protein may normally be expressed at the cell surface of the embryo and raise several questions about how the protein comes to be active only at the poles and about how it governs the specification of the end parts of the body.

Here we describe a series of experiments directed toward determining where the *tor* protein is expressed and how its distribution relates to its localized activity and the development of the terminalia. Our results establish that the *tor* protein is expressed in tight association with the surface membrane of early embryos and is ubiquitously distributed along the cell surface. Moreover, our data provide evidence suggesting that the protein exists in active and inactive forms and that transition to the active form requires an interaction with a localized factor present at both poles. Finally, we show that different levels of active *tor* protein are capable of dictating the development of different portions of the terminalia. Thus, our results support the proposal that the *tor* protein functions as a ubiquitous surface receptor that responds to a localized extracellular ligand and suggest that activated *tor* protein, or possibly its phosphorylated substrates, may organize body pattern by acting as gradient morphogens.

Results

Isolation of the tor gene and generation of antiserum directed against the tor protein

The subdivision of the early embryo into distinct domains of central and terminal pattern depends on localized *tor* activity (Klingler et al. 1988; Strecker et al. 1989; see Introductory section). Given the recent discovery that *tor* protein is likely to be a transmembrane receptor (Sprenger et al. 1989), it is reasonable to suppose that the protein is expressed at the surface of the early, syncytial embryo and that its activation involves an interaction with an extracellular ligand. Accordingly, either of two mechanisms could suffice for localized *tor* activity: (1) *tor* protein concentrated at both ends of the embryo may be activated by a ubiquitously distributed ligand or (2) *tor* protein expressed ubiquitously along the surface may be activated by ligand deposited only in the vicinity of the poles. To examine these possibilities, we cloned the gene, generated an antiserum against its encoded protein, and then used this antiserum to determine the distribution of *tor* protein in early embryos.

The *tor* gene was cloned by standard techniques using a revertible P-element-induced mutation (*tor*^{TC-17}) generously provided by T. Schüpbach (see Methods). The gene is entirely contained within an *Eco*RI fragment of about 11.5 kb that fully rescues the *tor* mutant phenotype when reintroduced into the genome. cDNAs homologous to the *tor* transcript were obtained. One of these encodes a polypeptide that begins at amino acid 68

in the *tor* protein, ends at around amino acid 600, and contains most of the putative extracellular domain, the transmembrane domain, and the first ~180 amino acids of the intracellular kinase domain (see Sprenger et al. 1989). This *tor* polypeptide was synthesized in bacteria and used to raise rat polyclonal antiserum (see Methods). The antiserum specifically detects *tor* protein, as indicated by the following three criteria: (1) wild-type embryos show consistent immunohistochemical staining in contrast to embryos derived from *tor*^{TC-17}/*tor*^{TC-17} females, which do not (e.g., Fig. 1A–C); (2) embryos derived from females with extra copies of the *tor*⁺ gene (see below) show correspondingly higher levels of immunohistochemical staining (e.g., Fig. 1E); and (3) some mutations that abolish *tor* function (e.g., *tor*^{PM}) show normal patterns of immunohistochemical staining (see Methods). This last piece of evidence eliminates the possibility that the antiserum recognizes a protein induced by *tor* rather than the *tor* protein.

Uniform expression of tor protein on the surface of early embryos

Wild-type embryos, or embryos derived from females carrying eight copies of the *tor* gene (see below), were assayed for *tor* protein expression using a variety of immunohistochemical detection systems (see Methods). These experiments provide information about the onset of *tor* protein expression, its distribution along the anteroposterior body axis, and its subcellular distribution, which we describe in turn.

We can first detect *tor* protein expression in wild-type embryos around the ninth nuclear division after fertilization, at which stage the nuclei have just arrived at the egg periphery. The level of protein then increases during the next several nuclear division cycles prior to cellularization of the blastoderm stage and the onset of gastrulation. Thereafter, it diminishes gradually over a period of 1–2 hr, after which we can no longer detect it. We also examined the time course of appearance in embryos derived from females carrying eight wild-type copies of the *tor* gene. Increasing the gene dosage allowed us to detect *tor* protein expression as early as the fourth or fifth nuclear cycle, suggesting that it is normally expressed in wild-type embryos at this stage. However, we still failed to detect *tor* protein expression in earlier embryos or in freshly laid eggs. Because *tor* mRNAs are known to be transcribed during oogenesis and to persist in the early embryo (Sprenger et al. 1989), our results suggest that the mRNA is not translated until after fertilization.

Throughout the period when we can detect *tor* protein expression, the protein appears to be associated with the cell surface (Fig. 1A–C; see below). Moreover, we find no evidence of localized expression of *tor* protein at the ends of the body, despite the genetic evidence indicating that *tor* activity is concentrated in these regions of the body. Indeed, in whole mount preparations (e.g., Fig. 1A), the staining intensity appears to be somewhat diminished at the poles and along the ventral side. This apparent decrease in signal intensity can be attributed at

Figure 1. *tor* protein expression in wild-type and mutant embryos. (A, B, and C) The pattern of *tor* protein expression in *tor*⁺ and *tor*^{TC-17} mutant embryos as revealed by immunohistochemistry (see Methods). *tor*⁺ and *tor*^{TC-17} embryos were fixed and stained together (the *tor*⁺ embryos were mutant for the *bcd* and *osk* gene functions and hence, could be readily distinguished from *tor*^{TC-17} embryos because they lacked pole cells; see Methods). An optical cross section of a *tor*⁺ embryo at the beginning of nuclear cycle 14 is shown in A. *tor* protein expression can be seen to be more concentrated at the cell periphery. Moreover, the protein is not localized at the poles, despite genetic evidence indicating that it acts selectively in the terminal regions. (Upper left) Arrow indicates a *tor*^{TC-17} embryo at the same stage; no staining is detectable. The surfaces of both these embryos are shown at higher magnification in B and C. Note that *tor* protein appears to be expressed in a lattice pattern; in double labeling experiments in which the nuclei are labeled independently, the lattice network outlines the positions of the nuclei. As shown in Fig. 2, the lattice pattern of expression reflects the higher density of membrane folded into grooves separating the nuclei. (D) An optical section of a mutant *trk* embryo stained for *tor* protein expression; the pattern of staining is similar to that of *tor*⁺ embryos (A). (E) Optical sections of a pair of embryos, the lower derived from a female carrying eight copies of the *tor*⁺ gene, the upper from a wild-type (two-copy) female; this micrograph is underexposed to give a better indication of the relative difference in *tor* staining intensity between the two embryos. (F and G) Different focal planes of a pair of embryos, one wild type (*top*) and the other derived from a female carrying two copies of the gain-of-function mutation *tor*^{RL3}. Both embryos developed at 25°C. Note that the level of *tor* protein expressed at the cell surface is reduced in the mutant relative to wild-type embryo; however, large abnormal intracellular vesicles appear throughout the mutant embryo, some of which express high levels of *tor* protein. These vesicles are readily observed in the peripheral egg cytoplasm, even in unstained preparations, as this region is usually free of yolk granules and other cytoplasmic inclusions. (H) Expression of *tor* protein in embryos derived from females heterozygous for a dominant gain-of-function mutation, *tor*^{D4021}, which is similar to wild type (A) and *trk* (D). (I and J) Images of *tor* staining at the anterior and posterior poles, respectively, obtained with the confocal scanning microscope (see also Fig. 2). Note that staining is continuous along the surface, although the level of staining appears higher dorsally (upper halves of each micrograph).

least in part to an optical artifact that results from the changing shape of the egg cell and the superimposition of signal in and out of the plane of focus; as it is less apparent when *tor* protein expression is monitored by confocal scanning microscopy (Fig. 1I,J).

When viewed in optical cross section by conventional microscopy (Fig. 1A), most of the *tor* protein appears to be associated with the cell surface, although the signal cannot be precisely localized because of the presence of signal in other focal planes. When the surface is viewed directly (Fig. 1B), *tor* protein staining exhibits a lattice-like pattern outlining the regularly spaced nuclei that lie just beneath the cell surface. To gain a more highly resolved image, we examined the distribution of *tor* protein using a confocal scanning microscope (Fig. 2). Under these conditions, we find that *tor* protein is ubiquitously distributed over the cell surface, the lattice pattern observed by conventional microscopy resulting from infoldings of the surface membrane that form around the perimeters of adjacent nuclei present just beneath the surface (Fig. 2; see also Turner and Mahowald 1976; Warn and Magrath 1982; Foe and Alberts 1983). We find no evidence for staining in the underlying nuclei or cytoplasm. Similar results are also obtained when embryos stained for *tor* protein by histochemical means are examined in 2–4- μ m plastic sections (data not shown).

Thus, *tor* protein is tightly associated with the external cell membrane of the early syncytial embryo; moreover, we find no evidence for localized expression of the protein at either end of the embryo. These results rule out the possibility that localized *tor* activity revealed by genetic experiments reflects a local distribution of the protein. Hence, one must posit that the protein is selectively activated at both poles, or alternatively that it is active everywhere, but that the substrates on which it acts are tightly restricted.

tor protein expression in gain-of-function mutant embryos

The existence of gain-of-function *tor* mutations that cause central portions of the body to develop into terminalia argues strongly for local activation of the *tor* protein, rather than a polarized distribution of its substrates (Klingler et al. 1988). Otherwise, it is difficult to see how such splice phenotypes could arise, since critical intermediates in the terminal signaling system would be absent in the middle of the embryo. Indeed, additional genetic experiments [Klingler et al. 1988, unpubl. (as cited in Sprenger et al. 1989)] confirm that these intermediate factors must be present in the middle of the embryo for gain-of-function mutations to cause the splice phenotype. For *tor* protein to be locally activated, it must be able to exist in active and inert states depending on the presence of other factors. Accordingly, gain-of-function *tor* mutations that cause ectopic activity in the middle of the embryo may relax the normal dependence of the protein on these factors, allowing it to function constitutively. Such gain-of-function mutations would not be expected to cause a change in the distribution or level of *tor* protein.

One gain-of-function mutation, *tor*^{D4021}, is a simple dominant allele; embryos derived from heterozygous females develop as if the *tor* protein is active indiscriminately throughout the body (Klingler et al. 1988). We cannot detect any differences between *tor* protein expression in embryos derived from wild-type or *tor*^{D4021}/+ females (Fig. 1H).

A second gain-of-function mutation, *tor*^{RL3} (Schübach and Wieschaus 1989), has unusual genetic properties (Klingler et al. 1988; Strecker et al. 1989). Females carrying two copies of the *tor*^{RL3} mutant allele give rise to splice embryos with greatly expanded terminal domains and few if any intervening body segments, even if they also carry a wild-type allele. This phenotype is temperature sensitive: mutant embryos develop almost normally at 17°C but show an extreme splice phenotype at 25–29°C. Females carrying only a single *tor*^{RL3} allele, whether alone or in the presence of a wild-type allele, generally give rise to normal larvae, although at 25–29°C, these larvae often show weak splice phenotypes such as the partial deletion of segments in the middle of the abdomen (Klingler et al. 1988; Strecker et al. 1989; data not shown). Hence, the level of ectopic *tor* activity caused by this mutation seems to depend critically on both temperature and the concentration of mutant protein (as determined by the number of copies of the mutant gene).

We find that embryos derived from *tor*^{RL3}/*tor*^{RL3} females at 25°C or 29°C (and hence having high levels of ectopic *tor* activity) generally express lower levels of *tor* protein at the cell surface (Fig. 1F,G). Both the yolk and peripheral cytoplasm, however, contain large vesicles, some of which show intense expression of the *tor* protein (Fig. 1F,G). These vesicles are not found in normal embryos and can also be observed in the peripheral cytoplasm of unstained mutant embryos using Nomarski optics. The number of abnormal vesicles, as well as the reduction in surface expression, is variable. We have not observed these vesicles in embryos derived from *tor*^{RL3}/+ or *tor*^{RL3}/– females, and they appear less frequently in embryos derived from *tor*^{RL3}/*tor*^{RL3} females at lower (17–22°C) temperatures. Thus, as in the case of *tor*⁴⁰²¹, the *tor*^{RL3} mutation appears to cause centrally located protein to become active rather than remain inert. However, this mutation has the unusual property that conditions that cause high levels of indiscriminate *tor*^{RL3} activity (i.e., high temperature and high concentration of mutant protein) also appear to be associated with a shift of the mutant protein from the cell surface toward membrane compartments within the cell.

Mutations in other terminal genes block localized activation of the tor protein

Several genes aside from *tor*, notably *trunk* (*trk*), *torso-like* (*tsl*), *female sterile (1) pole hole* [*fs(1)ph*] and *female sterile (1) Nasrat* [*fs(1)N*], are required during oogenesis for the development of terminal pattern at both ends of the developing embryo (Degelmann et al. 1986; Perimon et al. 1986; Schübach and Wieschaus 1986a;

Figure 2. *tor* protein expression at the cell surface of the early embryo. The expression of *tor* protein early in nuclear cycle 14 is shown in a series of optical sections obtained using a confocal scanning microscope. The first plane of focus [A] is just above the surface of the embryo; [B–G] A progression of focal planes obtained at 1- μ m intervals; [H] Optical section of the edge of the embryo. *tor* protein can be seen to be tightly associated with the surface of the embryo [B and C], as well as with infoldings of the membrane that partially separate adjacent nuclei [D–G]. Significant expression is not observed in the nuclei or in the underlying cytoplasm [H]. The embryo shown was derived from a female carrying eight wild-type copies of the *tor* gene; similar results were obtained with embryos from females with two copies, although the level of staining was much lower, reducing the resolution of the image.

Nüsslein-Volhard et al. 1987). That is, embryos derived from mutant females behave as if they lack *tor* activity. These other gene functions could be required for early expression of *tor* protein on the cell surface. Alternatively, they may be involved in activating *tor* protein, or in mediating the response of downstream functions to its activity. Mutations that block *tor* protein expression should be readily distinguishable from the other classes of mutations by antibody staining. We examined the distribution of *tor* protein in embryos derived from females that lack or have reduced function of the *trk*, *tsl*, *fs(1)ph*, and *fs(1)N* genes (see Methods for exact genotypes). In all cases (e.g., Fig. 1D), we find that *tor* protein expression in mutant embryos is indistinguishable from that of wild-type embryos.

Thus, the products of these other terminal genes appear to be required either for the local activation of *tor* protein, or for mediating the response to activated *tor* protein. These two possibilities are distinguishable because constitutive forms of the *tor* protein should restore terminal signaling in embryos in which the normal activation step is blocked; however, embryos unable to respond to active *tor* protein will remain so irrespective of mutations causing constitutive gain of *tor* activity.

In the case of the *trk* and *tsl* genes, we find that the loss-of-function phenotype can be overridden by the gain-of-function mutation *tor^{RL3}*. Specifically, embryos derived from doubly mutant *trk tor^{RL3}/trk tor^{RL3}* and *tor^{RL3}/tor^{RL3}*; *tsl/tsl* females at 25°C show the typical splice phenotype normally exhibited by embryos derived from singly mutant *tor^{RL3}/tor^{RL3}* females at the same temperature (see Methods for exact genotypes). As may be expected, embryos derived from *trk/trk* or *tsl/tsl* females that carry a single *tor^{RL3}* allele show a partial suppression of the terminal phenotype, depending on temperature (see Figs. 4 and 5 and below). We also find that a single *tor^{RL3}* allele similarly suppresses the terminal phenotypes caused by reduced function of the *fs(1)ph* and *fs(1)N* genes.

Thus, the *trk*, *tsl*, *fs(1)ph*, and *fs(1)N* gene functions all appear to be required for activation of the *tor* protein. Mutations in both the *fs(1)ph* and *fs(1)N* genes cause a variety of other pleiotropic phenotypes during oogenesis (Counce and Ede 1957; Perrimon et al. 1986), suggesting that these genes are not specifically involved in the activation of *tor* protein. However, this is not the case for mutations in the *trk* or *tsl* genes, suggesting that their products may play a more direct role.

Superabundance of tor protein does not alter terminal patterning

The foregoing results indicate that *tor* protein is initially inert when it is first expressed on the surface of the embryo, but that it is differentially activated at both ends by interactions with other signaling molecules, possibly the products of the *trk* or *tsl* genes. To gain a better understanding of how the normal distribution of *tor* activity is achieved, we examined the consequences of changing the total concentration of *tor* protein on the apparent distribution of active protein.

Conventional genetic techniques were used to generate flies with up to six copies of the *P(ry⁺, tor⁺)* genes introduced into the genome by P-element-mediated transformation (Methods). Embryos derived from females carrying a total of eight copies of the *tor* gene (two endogenous and six transduced genes) express much higher levels of *tor* protein, as assayed by immunohistochemistry (Fig. 1E). Nevertheless, such embryos give rise to normal larvae and adults, consistent with the conclusion that superabundance of *tor* protein has little if any effect on the distribution of active *tor* protein. However, the embryo can compensate for dramatic alterations of its initial fate map during the development of the larval body pattern (e.g., Berleth et al. 1988; Driever and Nüsslein-Volhard 1988b). Hence, we used two additional assays to test whether superabundance of *tor* protein causes any changes in the initial fate map. The first assay involved the expression of *fushi tarazu* (*ftz*). Periodic expression of the pair-rule gene *ftz* provides an early indication of the pattern of body segments and has proven to be a sensitive measure of changes in the initial fate map, particularly in the case of *tor* lack-of-function and gain-of-function mutations (Mlodzik et al. 1987; Klingler et al. 1988; Strecker et al. 1989; see also below and Fig. 5). As shown in Figure 3A–C, the patterns of *ftz* expression in embryos derived from females with one or eight copies of the *tor* gene are indistinguishable from each other, but clearly distinguishable from that of *tor⁻* embryos. The second assay involved *Krüppel* (*Kr*) expression in *bicoid oskar* (*bcd osk*) mutant embryos. The gap gene *Kr* is normally activated during the syncytial blastoderm stage in a precisely defined central domain. In *bcd osk* mutant embryos, which lack both the anterior and posterior determinant systems, the central domain of *Kr* gene expression is broader than in wild-type embryos (Gaul and Jäckle 1986). Further, under these conditions, the anterior and posterior boundaries of *Kr* expression depend primarily if not exclusively on the distribution of active *tor* protein, as indicated by the fact that triply mutant *tor bcd osk* embryos express *Kr* protein uniformly throughout the embryo (Fig. 3E). Embryos derived from *bcd osk* females carrying either two or six copies of the *tor* gene were generated by standard genetic means (see Methods) and then assayed for expression of *Kr* protein. All showed indistinguishable patterns of *Kr* expression (e.g., Fig. 3F,G), arguing strongly that the distribution of active *tor* protein remains unaffected despite the difference in *tor* protein concentration.

In the experiments described above, we assayed the distribution of *tor* activity only indirectly, by its influence on the patterns of expression of genes like *ftz* and *Kr*. Because our results were negative—changes in *tor* gene dosage had no apparent effect on the localized expression of these subordinate genes—it was necessary to test whether these assays are capable of detecting altered distributions of *tor* activity. Therefore, as a positive control, we asked whether we could detect evidence for an altered distribution of *tor* activity in embryos derived from females carrying a single copy of the *tor^{RL3}* gain-of-function mutation.

Figure 3. Superabundance of *tor* protein does not alter body patterning. The expression of the pair-rule gene *ftz* is shown in embryos derived from females carrying zero, one, and eight functioning copies of the *tor* gene in A, B, and C, respectively (all embryos are oriented with their anterior ends to the left, dorsal side up). Embryos derived from females with one and eight copies express the normal pattern of seven *ftz* stripes. In contrast, embryos derived from females with zero copies show an abnormal pattern of expression in which the most posterior (seventh) stripe is absent, and stripe six is broader than normal and shifted to the posterior pole. The expression of the gap gene *Kr* is shown in embryos derived from *bcd osk* females carrying zero, two, or six functioning copies of the *tor* gene in E, F, and G, respectively. Under these conditions (absence of both the anterior and posterior determinant systems), the pattern of *Kr* expression is determined solely by the activity of the terminal signaling system. As shown in E, *bcd osk* embryos lacking *tor* protein activity express *Kr* uniformly. However, embryos with two and six copies show similar patterns of central *Kr* expression. The expression of *Kr* protein was also examined in *bcd osk* embryos in which the only active *tor* protein derives from a single copy of the *tor^{RL3}* mutant allele. In embryos derived from *tor^{RL3}/tor^{PM}*; *bcd osk* females at 25°C, *Kr* protein is expressed in a broad central domain similar to that observed in *bcd osk* embryos with two or six copies (F and G), or a further abbreviated pattern of expression such as that shown in D, or not expressed at all. Hence these embryos behave as if the distribution of active *tor* protein has two components: the normal distribution found in *bcd osk* embryos as well as a low level of indiscriminate activity. In some of these embryos, the level of indiscriminate activity is sufficiently high to partially or completely abolish *Kr* expression in the central domain of the embryo. In embryos derived from *tor^{RL3}/+*; *bcd osk tsl* females at 25°C, *Kr* protein is expressed uniformly as in *bcd osk tsl* (E) and *tor*; *bcd osk* embryos, or in abbreviated patterns such as that shown in H, or not expressed at all. Hence, these embryos behave as if the *tor* activity derives solely from constitutive gain of function of the *tor^{RL3}* protein, *Kr* expression being partially or completely abolished when this level of activity reaches a critical threshold. The altered pattern of *Kr* expression that results from the presence of a single copy of the *tor^{RL3}* mutant allele serves as a positive control for the absence of any detectable effect of raising the gene dosage from two to six copies. Two additional points are noteworthy. First, in *bcd osk* embryos in which the central domain of *Kr* expression is partially repressed by ectopic *tor^{RL3}* function, we invariably observe that it is lost in the dorsal rather than the ventral half. Second, the number of pole cells is usually reduced or absent in embryos derived from females with eight copies suggesting that high levels of *tor* activity can interfere with determination of the germ line, an event which normally occurs before the major accumulation of *tor* protein.

As described previously (Klingler et al. 1988; Strecker et al. 1989) and above, *tor^{RL3}* protein behaves as if it is normally activated at the poles, but also as if it is constitutively active throughout the body, the level of constitutive activity being relatively low when the mother carries only a single copy of the mutant gene. As shown

in Figure 3D, the central domain of *Kr* expression is usually abbreviated or eliminated in embryos derived from *bcd osk* females that are also *tor^{RL3}/-* (unlike embryos derived from *bcd osk* females carrying up to six copies of the *tor⁺* gene; Fig. 3G). This change in the expression of *Kr* protein can be attributed solely to the constitutive activity of the *tor^{RL3}* protein. Embryos derived from *tor^{RL3}/+*; *bcd osk tsl* females, in which the local activation of both *tor⁺* and *tor^{RL3}* protein is blocked because of a lack of *tsl* gene function, usually fail to express any *Kr* protein or show only incomplete expression throughout the body (e.g., Fig. 3H). As described in the next section (see also Figs. 4 and 5), the periodic pattern of *ftz* expression is also altered under similar conditions.

Thus, we can readily detect minor changes in the distribution of *tor* activity that are attributable to a single copy of the *tor^{RL3}* mutation in the female. Hence, our failure to detect any evidence for such changes when the wild-type gene dosage is varied over an eightfold range indicates that the distribution of active *tor* protein is not closely coupled to the total concentration of *tor* protein. This result is most compatible with the notion that activation of the wild-type *tor* protein normally involves direct contact with a localized factor that is present in limiting quantity. Accordingly, the distribution of this factor would dictate the same distribution of active *tor* protein, even if the total concentration of *tor* protein is increased abnormally by changing the gene dosage.

Evidence for distinct responses to different levels of tor protein activity

As described in the introductory section, the distribution of active *tor* protein plays a determinative role in distinguishing terminal from central portions of the

Figure 4. Graded responses to incremental changes in the level of activity of *tor* protein. *trk tor^{RL3}/trk* females give rise to embryos in which the only *tor* activity is attributable to constitutive gain-of-function of the *tor^{RL3}* mutant protein. Moreover, the level of constitutive *tor^{RL3}* activity is temperature sensitive, increasing as the temperature rises. The cuticular phenotypes of larvae derived from such females at 29, 25, and 17°C are shown in A, B, and C; a larva derived from *trk/trk* females at 17°C is shown in D. All micrographs show lateral/ventral aspects of the posterior terminalia. In larvae that develop at 29°C (A), virtually all of the posterior terminal structures are present, including the anal tuft (t), anal sense organs (s), anal pads (p) (in more than 60% of the embryos), posterior spiracles (ps) (in 95% of the embryos), and all of the denticle bands associated with the posterior abdominal segments (A8 and A7) (in all the embryos). At somewhat lower temperatures (25°C; B), the most terminal structures (t, s, and p) appear in only 10% of the embryos, while the less terminal structures (ps, A8 and A7) remain at the back end. At 17°C (C), when *tor^{RL3}* activity is even further reduced, additional structures (ps and A8) are rudimentary or absent (the A8 denticle belt appears in only 30% of the embryos). Finally, in the absence of any *tor* function (D), all the terminal structures are absent and the A7 denticle belts appear in only 50% of the embryos.

body. However, the nature of this role is uncertain. In particular, it is not clear if the distribution of active protein organizes the pattern of terminal structures, or merely subdivides the embryo into terminal and central patterning domains. One way to distinguish these possibilities is to ask whether different levels of *tor* activity specify discrete portions of the terminal pattern. If such a relationship exists, it would argue that the distribution of active *tor* protein normally dictates the pattern of the terminalia. Conversely, the absence of such a relationship would suggest a simpler “switch” role in specifying terminal as opposed to central patterning.

To examine the relationship between the level of *tor* activity and the development of distinct portions of the terminalia, we made use of the unusual properties of the *tor^{RL3}* mutation. As described previously and above

Figure 5. Progressive shifts in periodic *ftz* expression caused by incremental change in the level of *tor* activity. (A, B, and C) Patterns of periodic *ftz* expression in embryos derived from *trk tor^{RL3}/trk* females at 29, 25, and 22°C (all embryos are oriented with their anterior ends to the left, dorsal side up). At 29°C, the activity of *tor^{RL3}* protein appears to be sufficiently high to allow the formation of all seven stripes, although the seventh stripe is unusually broad, extending toward the posterior pole. As both the temperature and level of *tor^{RL3}* activity decrease (B and C), stripe 7 is shifted progressively toward the posterior pole; at 22°C it is sometimes eliminated entirely. Similarly, stripe 6 is also shifted posteriorly. In the complete absence of *tor* function (e.g., Fig. 3A), stripe 6 is now positioned at the posterior pole. Note also that at 29°C, intermediate stripes, such as stripe 4 (A) are sometimes partially or completely eliminated. We attribute this aspect of the phenotype to the increasing level of inappropriate *tor^{RL3}* activity in the middle of the embryo that interferes with normal specification of the trunk segments.

(Klingler et al. 1988; Strecker et al. 1989; see also Fig. 3D,H), the *tor^{RL3}* mutation confers constitutive *tor* activity that is both temperature sensitive and independent of other terminal gene functions normally required for activating the wild-type protein (e.g., *trk* or *tsl*). Hence, in embryos derived from females that lack the *trk* gene function but carry a single copy of the *tor^{RL3}* mutation, the level of *tor* activity depends simply on temperature. Therefore, we examined the development of the posterior terminalia in embryos derived from *trk tor^{RL3}/trk* females at 17, 22, 25, and 29°C. At 17°C (when constitutive *tor* activity caused by the *tor^{RL3}* mutation appears to be minimal), the development of embryos derived from *trk tor^{RL3}/trk* females is similar although not identical to that of embryos derived from *trk/trk* females (i.e., both show loss of most or all of the terminal derivatives, although the loss of terminal structures is slightly less extreme in the progeny of *trk tor^{RL3}/trk* females). At progressively higher temperatures, however, (when constitutive *tor^{RL3}* activity increases), embryos derived from *trk tor^{RL3}/trk* females differentiate progressively more terminal structures [unlike their *trk/trk* counterparts, which show only a slight amelioration of the extreme *trk* phenotype (Schüpbach and Wieschaus 1986a)]. Thus, as the temperature rises from 17°C to 29°C, larvae derived from *trk tor^{RL3}/trk* females recover first abdominal segments 7, then 8, and then more terminal derivatives such as complete posterior spiracles, the anal pads, anal sense organs, and anal tuft (Fig. 4). This phenotypic rescue can also be observed at the level of *ftz* expression: As the temperature rises, the sixth stripe moves from the back end toward its normal position, the seventh stripe appears at the back end, and finally the seventh stripe shifts anteriorly toward its normal position (Fig. 5). At high temperature (25–29°C) we also observe deletions of middle abdominal segments (and their corresponding *ftz* stripes), presumably as a result of the rising level of inappropriate *tor* activity in the middle of the embryo.

Thus, under conditions in which the only *tor* activity results from temperature-sensitive constitutive activity of the *tor^{RL3}* protein, we find a direct correlation between temperature, the types of terminal structures specified, and the number and position of terminal *ftz* stripes. This correlation argues that different levels of *tor* activity can specify distinct elements of the terminal pattern.

Discussion

Prior genetic experiments provide compelling evidence that the *tor* gene function is active only at the poles of the early embryo where it directs the formation of terminalia as opposed to more central portions of the body pattern (Klingler et al. 1988; Strecker et al. 1989; see Introductory section). Conversely, its absence or inactivity in the middle of the embryo is essential if this region is to form the middle body segments rather than contribute to the terminalia. The recent discovery (Sprenger et al. 1989) that *tor* is likely to encode a receptor tyro-

sine kinase, suggests several possible mechanisms by which local *tor* activity is generated and the terminalia specified. The experiments described here argue strongly for particular mechanisms in each process.

Local activation of *tor*

Receptor tyrosine kinases contain an extracellular domain capable of binding particular ligands (e.g., EGF, PDGF) linked via a single transmembrane domain to an intracellular kinase domain (for review, see Yarden and Ullrich 1988; Williams 1989). In general, ligand binding induces activation of the kinase, which, in turn, triggers a variety of intracellular signaling pathways. As shown here, *tor* protein is expressed in tight association with the cell membrane of the early syncytial embryo, providing direct support for the proposal (Sprenger et al. 1989) that it functions as a surface receptor.

In principle localized *tor* activity may directly reflect a polarized distribution of the protein. However, we show that *tor* protein is ubiquitously expressed along the cell surface suggesting instead that its activity is locally triggered by contact with another factor present only at the poles. Other experiments support this conclusion and suggest that this ligand may be a localized component of the surrounding extracellular matrix secreted by the follicle cells.

First, we find that loss-of-function mutations in a number of other terminal genes block the activation of *tor* protein without altering its normal distribution. Hence, *tor* protein appears to be uniformly inert unless locally activated by a process that depends on these other gene functions. Of the four gene functions assayed, two, *tsl* and *trk*, appear to affect only the terminal signaling system, suggesting they play a specific role in this process.

Second, we show that the apparent distribution of active *tor* protein is not altered despite severalfold changes in the total concentration of *tor* protein. One simple interpretation of this result is that *tor* protein is normally present at levels that are saturating for a localized ligand necessary for its activation. Under these conditions, the distribution of active *tor* protein would directly reflect that of its putative ligand and remain unchanged if the concentration of *tor* protein were further increased.

Third, there is preliminary evidence that the *tsl* gene, which we show is required for *tor* activity, functions exclusively in follicle cells during oogenesis [H.G. Frohnhöfer and C. Nüsslein-Volhard, unpubl. (as cited in Sprenger et al. 1989)]. Hence, this gene may encode the putative *tor* ligand, or be involved in eliciting its local deposition. Our finding that *tor* protein is expressed during a restricted period just before and during the syncytial blastoderm stage suggests that interactions between *tor* and its putative ligand occur during this critical period, a conclusion that also agrees with the results of cytoplasmic rescue experiments (Klingler et al. 1988). Because proteins in the fluid layer between the early embryo and the surrounding vitelline membrane are free to diffuse (e.g., Warn and McGrath 1982), we suggest

that the putative ligand remains spatially localized during oogenesis and early embryogenesis because it is tethered to the vitelline membrane, an extracellular matrix secreted by the follicle cells. It is notable that specialized subsets of polar cells are positioned at either end of the sheet of follicle cells surrounding the developing oocyte (Brower et al. 1982); these cells could be responsible for locally depositing the ligand as the vitelline membrane is secreted.

Signal transduction by the *tor* protein

The case of the *tor*^{RL3} mutation presents a possible clue to the mechanism of signal transduction used by the *tor* protein. The *tor*^{RL3} protein behaves as if it is capable of both ligand-dependent and ligand-independent activation (e.g., Fig. 3D,H), the latter conditional on temperature and concentration. We suggest that this unusual behavior reflects a deviation from the normal mechanism of signal transduction in which ligand binding activates the intracellular kinase domain by inducing oligomerization of the extracellular domains (e.g., Williams 1989). Accordingly, the *tor*^{RL3} mutation may allow the protein to oligomerize in a ligand-independent fashion. Under these circumstances, the concentration of monomers, as well as their rate of diffusion in the bilayer (a function of temperature), are likely to influence the amount of oligomerization and hence the level of constitutive *tor* activity. If the shift of the protein from the surface into intracellular vesicles is a consequence of the abnormal function of the *tor*^{RL3} protein, then it could reflect high levels of ligand-independent oligomerization because it is known that receptor aggregation may trigger endocytosis (e.g., as in the case of the PDGF receptor; Yarden and Ullrich 1988; Williams 1989).

As suggested by Sprenger et al. (1989), activation of the *tor* protein is likely to lead to the phosphorylation of subordinate regulatory molecules, which, in turn, control the activation of zygotic segmentation genes that are responsible for generating terminal pattern. One intermediate in this putative signaling process appears to be *lethal(1)pole hole* [*l(1)ph*] (M. Klingler, unpubl. (as cited in Sprenger et al. 1989)], which is likely to be the *Drosophila* homolog of the *raf* oncogene (Nishida et al. 1988). It is notable that the *raf* protein is itself a serine/threonine kinase (Moelling et al. 1984). Hence, *tor* activity may trigger a cascade of spatially restricted kinase activities leading ultimately to the local activation of zygotic response genes, like the gap gene *tailless* (*tll*), which govern the development of the terminalia (Klingler et al. 1988; Strecker et al. 1989).

Gradient morphogens in the terminal signaling system

Localized *tor* activity is critical for specifying the portions of the body that generate terminal as opposed to central patterns. In this regard it is similar to both the *bcd* and *nos* morphogen systems in which localized activity normally determines the portions of the body that develop anterior and posterior segment patterns. It has

been shown recently, however, that these other morphogens play fundamentally different roles in their respective realms of action: The distribution of *bcd* exerts an instructive influence on the organization of anterior body pattern (Frohnhofer and Nüsslein-Volhard 1986, 1987; Driever and Nüsslein-Volhard 1988b; Driever et al. 1989; Struhl et al. 1989), whereas localized *nos* activity simply defines a region in which posterior body pattern can be specified by other morphogens (Hülskamp et al. 1989; Struhl 1989; Irish et al. 1989). Hence, it is important to ask whether localized *tor* activity merely specifies the portions of the body that will develop as terminalia or actively dictates the organization of terminal patterns within these domains.

We addressed this question by setting up a situation in which the only *tor* activity present derives from the temperature-sensitive constitutive activity of the *tor^{RL3}* protein. Using temperature to change the level of activity, we show that incremental increases in *tor* activity lead to the development of progressively more terminal structures at the posterior pole of the embryo (Fig. 4), as well as to corresponding shifts in the initial fate map (Fig. 5). These results provide evidence that the level of active *tor* protein can be causally related to the specification of particular portions of the terminal pattern and, hence, support the notion that the distribution of active protein plays an instructive rather than a permissive role in setting up the terminal pattern. It should be noted that all portions of the body do not invariably give rise to the same terminal structures in response to a given level of constitutive *tor* activity, presumably because of the activities of additional factors in more central portions of the body. However, higher levels of *tor^{RL3}* activity caused by increases in temperature or gene dosage are associated with the reduction or loss of middle trunk segments, and in the most extreme case with the formation of terminal structures in their place (the splice phenotype; Klingler et al. 1988; Strecker et al. 1989). Hence, when the level of ectopic *tor^{RL3}* activity is sufficiently high, these other factors appear to be suppressed or overridden thereby causing all parts of the body to differentiate similar terminal structures.

As discussed above, localized *tor* activity is likely to influence body patterning indirectly via its spatially restricted effects on the activation of one or more intracellular signaling molecules [e.g., *l(1)ph* and its substrates]. Since these locally activated signaling molecules could in principle diffuse within the cytoplasm, their distribution may not directly reflect that of active *tor* protein, but instead be more graded. Consequently, although it is possible that a gradient of active *tor* protein organizes terminal pattern, this need not be the case. Instead, a sharp step function of active *tor* protein at the surface could generate one or more gradients of phosphorylated intracellular substrates that specify the terminal pattern. Under these circumstances, different levels of constitutive *tor^{RL3}* activity would still reveal a causal relationship such as we observe, because they would trigger different levels of activity of these downstream factors. It is also notable that neither *l(1)ph* nor *tll* mutations

completely suppresses the development of terminalia, in contrast to *tor* mutations which do (Strecker et al. 1986; Klingler et al. 1988; Ambrosia et al. 1989). Hence, localized *tor* activity is likely to control the spatial activities of more than one intermediary signaling molecule and zygotic response gene, consistent with the notion that different levels of activity of *tor* or its target molecules dictate distinct molecular and morphological outcomes.

Methods

Mutant genotypes

The *tor^{TC-17}* mutation, a recessive loss-of-function allele of the *torso* gene induced by P–M hybrid disgenesis, was kindly provided by Trudi Schüpbach. The *tor^{RL3}* mutation was recovered as a female sterile mutation (Schüpbach and Wieschaus 1989) and subsequently shown to be an hypermorphic allele of the *tor* locus (Klingler et al. 1988). The *tor^{D4021}* mutation is another gain-of-function allele of *tor* recovered as a dominant female sterile mutation (Klingler et al. 1988).

The mutations *tor^{PM}*, *tsl¹⁰³⁵*, and *trk^{RA}* are apparent null alleles of the *tor*, *torsolike*, and *trunk* loci. The *fs(1)N¹²¹* and *fs(1)ph¹⁹⁰⁶* mutations are recessive alleles of the *female sterile (1)* *Nasrat* and *female sterile (1)* *pole hole* loci that cause phenotypes similar to that of *tor* loss-of-function mutations. Both of these mutant alleles are exceptional—the remaining mutations in these two loci generally cause more severe pleiotropic phenotypes, frequently blocking normal development of the egg (Perrimon et al. 1986). The *bcd^{E6}* and *osk¹⁶⁶* mutations abolish activity of the *bicoid* and *oskar* genes and were used in *cis* to inactivate both the anterior and posterior determinant systems (Frohnhofer and Nüsslein-Volhard 1986; Lehmann and Nüsslein-Volhard 1986; Nüsslein-Volhard et al. 1987; Struhl et al. 1989).

A chromosome carrying the *trk^{RA}* and *tor^{RL3}* mutations in *cis* was obtained by recombination as follows: *trk^{RA} pr / cn tor^{RL3}* females were outcrossed to males carrying the *CyO*, *pr cn²* balancer chromosome and putative *trk^{RA} pr cn tor^{RL3} / CyO*, *pr cn²* recombinant progeny selected on the basis of their purple cinnabar eye color. The presence of the *tor^{RL3}* mutant allele was readily confirmed by the gain-of-function mutant phenotype; the presence of the *trk^{RA}* mutation was confirmed by progeny tests in which it was recombined away from the *tor^{RL3}* mutation, again using the segregation of eye color phenotypes to identify potential recombinant progeny. Flies carrying the *tor^{RL3}* allele as well as the *tsl¹⁰³⁵*, *fs(1)N¹²¹* and *fs(1)ph¹⁹⁰⁶* mutations were generated by standard genetic crosses, using the presence or absence of appropriate balancer chromosomes to distinguish homozygotes from heterozygotes.

Cloning the *tor* gene

An EMBL4 phage library of genomic DNA was derived from flies homozygous for the revertible P-element-induced mutation *tor^{TC-17}* and screened with a P probe for homologous P sequences by standard means (Maniatis et al. 1982). Forty-five P element-positive phage clones were selected. Duplicate nitrocellulose filters containing these clones displayed as a grid were then hybridized with total, ³²P-labeled genomic DNA from wild-type embryos or embryos homozygous for a small deletion of the chromosome, *Df(2R)St1* (43B3–5; 43E18) (Ashburner et al. 1981) removing *torso* and neighboring loci (see Macdonald et al. 1986 for detailed protocol). Two clones appeared to hybridize

only to wild-type DNA. Hybridization of one of these, λ T54, in situ to the polytene chromosomes revealed that it mapped to the 43E region, which includes the *torso* locus. Appropriate probes from the λ T54 clone were used to obtain homologous DNAs from a wild-type (Oregon R) library leading to the isolation of phage clones covering a contiguous region of approximately 30 kb. Reverse Northern blotting experiments (Scott et al. 1983) were performed to identify sequences in this region that are transcribed during oogenesis and early embryogenesis. At the same time, Southern blotting experiments were performed on wild-type and *tor*^{TC-17} mutant DNAs to determine the location of the P element. These experiments showed that a P element was inserted just upstream of the start site of a transcript present only in oocytes and early embryos. This putative *tor* transcript is located within an 11.5-kb *Eco*RI fragment that contains all the sequences necessary and sufficient for providing normal *tor* function as shown by the fact that it fully rescues the *tor* mutant phenotype when returned to the genome by P-element-mediated germ line transformation. The restriction map of this *Eco*RI fragment is essentially identical to that of an *Eco*RI fragment containing the *tor* gene obtained independently by Sprenger et al. (1989).

Generation of antiserum

Several cDNA clones homologous to the *tor* transcript were obtained from an early embryo cDNA library kindly provided by Markus Noll. To raise an antibody against *tor* protein, different cDNAs were inserted into pET-3 translation vectors (Rosenberg et al. 1987) in each of the three possible reading frames. After IPTG induction of the *Escherichia coli* LysS strain carrying the plasmid and electrophoresis of its protein on an SDS–polyacrylamide gel, one of these constructs gave rise to a distinct protein band. After partial purification this protein was used to raise rat polyclonal antibodies.

The rat serum was preabsorbed with late (8–16 hr old) wild-type embryos and used to visualize putative *tor* protein by standard immunofluorescent and immunohistochemical techniques (see Macdonald and Struhl 1986). This antiserum fails to detect significant protein expression in embryos derived from homozygous *tor*^{TC-17} females confirming that it is indeed specific for the *tor* protein (see Fig. 1; we occasionally detect variable degrees of background staining in the yolk and pole cells of *tor*^{TC-17} mutant embryos—such staining is spatially distinct from bona fide signal).

Because of the possibility of fixation artifacts and the vagaries of the immunohistochemical and immunofluorescent staining methods employed, all analyses of *tor* protein expression in mutant genotypes were performed in strict parallel with wild-type controls as follows. Embryos of a given genotype were first mixed with roughly equal numbers of *bcd osk* mutant embryos of the same age and then dechorionated, fixed, and stained together. *bcd osk* embryos do not form pole cells, allowing them to be readily distinguished from embryos of the other genotype. As we are unable to detect any significant differences in *tor* protein expression in wild-type and *bcd osk* mutant embryos, we treat these embryos as wild-type controls.

Confocal scanning microscopy

Embryos were fixed and incubated with the primary rat antiserum to *tor* protein as usual and then washed to remove any unbound antibodies. They were subsequently incubated with a preabsorbed goat anti-rat immunoglobulin coupled to fluorescein isothiocyanate (FITC), washed, mounted in 50% glycerol with 2.5 mg/ml *p*-phenylenediamine added as an antibleaching

agent and analyzed under the argon ion laser beam of an MRC-500 confocal imaging system (Bio-Rad Microscience). Embryos from females with two or eight copies of the *torso* gene were examined. As in immunohistochemical preparations, we found the patterns of expression to be indistinguishable, although the level of expression was much higher in the case of eight-copy embryos.

Generation of flies with extra copies of the *tor* gene

An *Eco*RI fragment of about 11.5 kb was introduced into the germ-line by P-element-mediated transformation using the *P*(*ry*⁺) vector *Carnegie 20* (Rubin and Spradling 1983). Females homozygous for the *tor*^{PM} mutation, but carrying a single copy of this transduced gene give rise to phenotypically wild-type progeny confirming that the 11.5-kb *Eco*RI fragment contains in the intact *tor*⁺ gene.

Several independent transformant lines were recovered with *P*(*ry*⁺, *tor*⁺) insertions on the second or third chromosomes and shown to fully rescue the *tor* mutant phenotype in single copies. Two such insertions on the third chromosome were placed in *cis* by standard genetic techniques involving recombination and progeny testing. Flies homozygous for this third chromosome as well as for a second chromosome with another insertion carry a total of eight copies of the *tor*⁺ gene.

A *bcd*[−] *osk*[−] chromosome carrying a single *P*(*ry*⁺, *tor*⁺) insertion in *cis* was generated by standard genetic techniques taking advantage of the tight linkage of the *bcd osk* and *ry* loci. The presence of the insertion was confirmed by the ability of the chromosome to rescue the phenotype of the *torso*[−] mutations. Flies homozygous for this third chromosome as well as a second chromosome carrying another *P*(*ry*⁺, *tor*⁺) insertion carry six copies of the *tor* gene in a *bcd*[−] *osk*[−] background.

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Note added in proof

We determined recently that the lesion responsible for the *tor*^{RL3} gain-of-function phenotype maps to the extracellular domain of the protein. This is consistent with our proposal that the mutation causes a change in this portion of the molecule, which allows oligomerization in the absence of ligand. However, we only observe the apparent shift of protein from the cell surface to abnormal intracellular vesicles (Fig. 1F,G) in embryos derived from females homozygous for the original *tor*^{RL3}-bearing chromosome and not in embryos derived from females in which the second copy of the mutant gene is inserted on a P

element at a different location. Hence, the abnormal partitioning of *tor*^{RL3} protein shown in Figure 1 does not appear to be attributable to the lesion conferring ectopic gain-of-function, but rather to a second site mutation in another gene.

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Casanova and Struhl

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