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Control of cell fate determination by p21ras/Ras1, an essential component of torso signaling in Drosophila

Xiangyi Lu, Tze-Bin Chou, Nidhi Gupta Williams, Thomas Roberts, and Norbert Perrimon

1Howard Hughes Medical Institute, Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115 USA; 2Dana-Farber Cancer Institute, 3Department of Biological Chemistry and Molecular Pharmacology, and 4Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115 USA

Determination of cell fate at the posterior termini of the Drosophila embryo is specified by the activation of the torso (tor) receptor tyrosine kinase. This signaling pathway is mediated by the serine/threonine kinase D-raf and a protein tyrosine phosphatase corkscrew (csw). We found that expression of an activated form of Ras1 during oogenesis resulted in embryos with tor gain-of-function phenotypes. To demonstrate that p21ras/Ras1 mediates tor signaling, we injected mammalian p21ras variants into early Drosophila embryos. We found that the injection of activated p21ras rescued the maternal-effect phenotypes of both tor and csw null mutations. These rescuing effects of p21ras are dependent on the presence of maternally derived D-raf activity. In addition, wild-type embryos show a terminal-class phenotype resembling csw when injected with p21rasN17, a dominant-negative form of p21ras. Furthermore, we have analyzed the maternal-effect phenotype of Son of sevenless (Sos), a positive regulator of Ras1, and showed that embryos derived from germ cells lacking Sos' activity exhibit a terminal-class phenotype. Our study demonstrates that the Drosophila p21ras, encoded by Ras1, is an intrinsic component of the tor signaling pathway, where it is both necessary and sufficient in specifying posterior terminal cell fates. p21ras/Ras1 operates upstream of the D-raf kinase in this signaling pathway.

[Key Words: Drosophila; kinase; ras; Sos; signal transduction; pattern formation]

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p21ras plays crucial roles in cell proliferation and differentiation (for review, see Bourne et al. 1991). As a member of the GTPase superfamily, this protein binds guanine nucleotides and hydrolyzes GTP to GDP, thereby serving as a molecular switch by adopting a GTP-bound "on" state or a GDP-bound "off" state (for review, see Downward 1990). The proportion of GTP-bound active p21ras is increased in cells expressing oncogenic forms of tyrosine kinases or following the activation of epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptor tyrosine kinases (RTKs) (Gibbs et al. 1990; Qui and Green 1991). Microinjection of neutralizing anti-p21ras antibody or a dominant inhibitory mutant p21rasN17 blocks NIH-3T3 cell proliferation (Mulcahy et al. 1985; Feig and Cooper 1988a). Conversely, p21ras, a constitutively activated form of p21ras, transforms cells (Feig and Cooper 1988b). The p21ras-mediated NIH-3T3 cell transformation is partially inhibited by the expression of c-raf1 anti-sense RNA (Kolch et al. 1991). Recent studies have shown that p21ras is necessary and sufficient for nerve growth factor (NGF), fibroblast growth factor (FGF), and v-src-induced PC12 cell differentiation (Szeberenyi et al. 1990; Kremer et al. 1991). The activity of p21ras in PC12 cells is correlated with the phosphorylation states of Raf-1 kinase, mitogen-associated protein (MAP) kinases, and 85- to 92-kD ribosomal S6 kinase, suggesting the involvement of these serine/threonine kinases in signaling events downstream of p21ras (Thomas et al. 1992; Wood et al. 1992). Collectively, these studies have demonstrated a functional relationship among growth factor RTKs, p21ras, Raf-1 kinase, and MAP kinases (Bruder et al. 1992; Kyriakis et al. 1992; Williams et al. 1992).

Recent developmental studies in invertebrates have provided genetic evidence that p21ras mediates signal transduction of many RTKs. In Caenorhabditis elegans, the p21ras homolog, encoded by let-60, is essential in mediating signaling from let-23, an EGF receptor-like RTK, during vulval development (Beitel et al. 1990; Han and Sternberg 1990). In Drosophila, the p21ras homolog, encoded by Ras1, functions downstream of sevenless, a RTK related to the vertebrate c-ros protein, and possibly the Drosophila EGF receptor, a protein most homologous to the vertebrate EGF receptor and the neu oncoprotein (Simon et al. 1991). In this paper, we present evidence that p21ras also mediates signaling of torso (tor), a RTK structurally similar to the mammalian PDGF receptor, which functions in the terminal class pathway to
determine cell fates at the termini of the *Drosophila* embryo.

Developmental fate of early *Drosophila* embryos is initially set up by maternally deposited patterning molecules (St. Johnston and Nüsslein-Volhard 1992). At the anterior and posterior termini, formation of the acron and the telson, respectively, requires the activation of the *tor* signaling pathway. The *tor* RTK, which is uniformly distributed in the egg cell membrane (Casanova and Struhl 1989), is believed to be activated solely at the egg poles (Stevens et al. 1990). The current model (for review, see Perkins and Perrimon 1991; St. Johnston and Nüsslein-Volhard 1992) proposes that localized activation of *tor* triggers a phosphorylation cascade, which generates a graded morphogenetic signal that leads to the transcriptional activation of zygotic terminal genes such as *tailless* (*tll*) and *huckebein* (*hkb*). *tll* encodes a putative transcription factor of the steroid receptor superfamily [Pignoni et al. 1990] and *hkb* encodes a putative zinc finger transcription factor (H. Jäckle, pers. comm.). *tll* and *hkb*, expressed in overlapping domains within the terminal anlagen [Bronner and Jäckle 1991], further subdivide the terminal region by activating and/or repressing terminal-specific gene expression [Weigel et al. 1990; Steingrimsson et al. 1991].

This model is supported by many genetic and molecular studies, one of which is the isolation of gain-of-function *tor* mutations (*tor<sup>act</sup>*) (*tor<sup>act</sup>*). *tor<sup>act</sup>* gives rise to a phenotype that is complementary to that of the loss-of-function *tor* mutations (*tor<sup>loss</sup>*) (*tor<sup>loss</sup>*). *tor<sup>act</sup>* deletes the acron and telson in embryos derived from mutant mothers, whereas *tor<sup>loss</sup>* results in embryos with enlarged acron and telson regions, along with a concomitant reduction of thoracic and abdominal structures [also known as the “spliced” phenotype]. The repressed thoriac and abdominal segmentation in spliced embryos is caused by ligand-independent activation of *tor* ubiquitously in the embryo [Klingler et al. 1988; Strecker et al. 1989].

*D-raf* and *corkscrew* (*csw*) have been shown by genetic epistasis analyses to transduce *tor* signaling positively [Ambrosio et al. 1989b; Perkins et al. 1992]. *D-raf* encodes a serine/threonine kinase homologous to the mammalian Raf-1 kinase [Mark et al. 1987; Nishida et al. 1988]; and *csw* encodes a nonreceptor protein tyrosine phosphatase containing two SH2 domains. Null mutations in *tor* and *D-raf* have indistinguishable maternal-effect phenotypes, that is, deletion of the acron and the telson [Ambrosio et al. 1989a]. Unlike *D-raf*, *csw* null mutations do not eliminate all aspects of *tor* signaling.

Analysis of hypomorphic *csw* and *D-raf* double mutant combinations suggests that *csw* acts in concert with *D-raf* to increase *tor* signaling to a level sufficient for proper *tll* and *hkb* expression [Perkins et al. 1992].

Because *tor* is a RTK, we decided to analyze the potential role of the various ras proteins in the terminal class pathway. Three ras genes have been identified in *Drosophila* using mammalian v-ras as a probe [Neuman-Silberberg et al. 1984]. Ras1 is 75% identical to mammalian Ki/Ha ras (*p21<sup>ras</sup>*), whereas Ras2 and Ras3 are more related to the functionally distinct R-ras and Rap families of ras genes, respectively [Hariharan et al. 1991; J. Bishop, pers. comm.]. Using a combination of heat shock-induced expression, embryonic injection, and female mosaic analysis, this paper demonstrates that Ras1 and its positive regulator *Son of sevenless* (*Sos*) [Simon et al. 1991; Bonfini et al. 1992] are involved in the *tor* signaling pathway. Furthermore, our analyses demonstrate that *p21<sup>ras</sup>* activity operates upstream of the D-raf serine/threonine kinase and is necessary and sufficient in determining posterior terminal cell fates.

**Results**

**Expression of an activated form of Ras1, but not Ras2, during oogenesis, mimics the *tor<sup>act</sup>* embryonic phenotype**

To determine whether Ras1 plays a role in early embryonic development, we expressed a dominant-activated form of Ras1, Ras1<sup>Q13</sup> under the control of a heat shock promoter during oogenesis. Embryos derived from heat-shocked females have a low hatching rate of 2–5%. A percentage (30%) of the unhatched embryos has the spliced embryonic phenotype, resembling embryos derived from females carrying a *tor<sup>act</sup>* mutation (Fig. 1, cf. D, E, and F with B and C). Among these spliced embryos, we observed mutant embryos ranging from those missing only one abdominal segment (Fig. 1D) to those with more severe abdominal defects (Fig. 1E,F) and duplicated filzkörper material (Fig. 1F). The remaining 70% of the dead embryos showed defects in head involution, germ-band retraction, or dorsal/ventral patterning (data not shown). The phenotypic variability observed among embryos derived from the heat-shocked females may be a reflection of the stage of oogenesis at which the heat shock was delivered, where the different mutant phenotypes are likely the consequence of either the levels of expression of Ras1<sup>Q13</sup> or the specific maternal tissue affected (follicle cells or germ line). In contrast, expression of wild-type Ras1 during oogenesis did not lead to significant embryonic lethality, and the small number of dead embryos did not show the spliced embryonic phenotype (data not shown).

To test the specificity of the defects associated with the expression of Ras1<sup>Q13</sup> during oogenesis, we conducted a similar experiment using an analogous activating mutation in Ras2, Ras2<sup>Y12</sup> [Bishop and Corces 1988]. Although 60% of the progeny did not hatch when females were heat-shocked, the dead embryos showed head defects while segmentation and telson differentiation were normal (data not shown). Collectively, these results indicate that expression of activated Ras1, but not Ras2, during oogenesis is associated with a phenotype similar to that of spliced embryos and suggest that Ras1 may be a component of the terminal class pathway.

**Injection of p21<sup>ras</sup> protein rescues the *tor<sup>act</sup>* maternal-effect phenotype**

To test whether Ras1 mediates *tor* signaling, we injected a mammalian-activated p21<sup>ras</sup> protein, p21<sup>Y-ras</sup>, produced
in the baculovirus expression system, into early cleavage-stage embryos derived from homozygous torXRI females (referred to as torXRI embryos). torXRI contains a 10.5-kb deletion encompassing the tor gene and is both an RNA and protein null (Sprenger et al. 1989). torXRI embryos fail to develop telson structures at the posterior, that is, all structures posterior to abdominal segment 7 (A7), which includes A8, the anal pads, anal tuft, posterior midgut (PM), and the posterior spiracles along with their associated filzkörper material (Figs. 2A, 3C,D). When the p21Vras protein sample was injected posteriorly [0–5% egg length (EL); 0% is at the posterior pole], most cuticular elements posterior to A7 were rescued (Fig. 2B; Table 1A). In addition, some injected embryos (Fig. 2C) showed abdominal segmentation repression, possibly owing to diffusion of p21Vras into the central region of the embryos. In this assay no rescuing activity was associated with either the wild-type p21Cras or the control sample lacking p21Vras protein (see Table 1A). When p21Vras was injected anteriorly (95–100% EL) in torXRI embryos, the anterior head skeletal structures were very disrupted (see Discussion), the posterior structures were rescued and more abdominal segmentation repression was observed (data not shown). Similar results were obtained when the injections were done in the middle of the embryos.

To determine whether the injection of p21Vras into torXRI embryos is able to recover the PM cell fates, p21Vras was injected into torXRI embryos that carry the 1A121 enhancer trap marker. In wild-type embryos, 1A121 expresses lacZ in both the anterior midgut (AM) and PM during germ-band extension (Fig. 3A,B; Perrimon et al. 1991). In torXRI embryos, posterior 1A121 staining is missing owing to the absence of the PM (Fig.
Figure 2. Injection of p21\(^{v-ras}\) rescues the maternal-effect phenotype of tor\(^{ed}\) (tor\(^{XR1}\)) but not D-raf\(^{ed}\) [D-raf\(^{A7S}\)]. (A) A dark-field picture of the cuticular pattern elements present in a tor\(^{XR1}\) embryo. The head skeleton (indicated by a solid arrow) is truncated, and all structures posterior to abdominal segment 7 (A7) are missing. (B, C) Two tor\(^{XR1}\) embryos that have been injected with p21\(^{v-ras}\) at the posterior pole at stages 1–2 (Campos-Ortega and Hartenstein 1985). The embryo in B recovered the posterior spiracles, part of A8, anal pads, and anal tuft (a complete rescue of A8 was observed when a higher level of p21\(^{v-ras}\) was injected). The embryo in C differentiated most of the posterior terminal structures, but abdominal segmentation was severely repressed similarly to tor\(^{g}\) embryos (indicated by open arrow). Note that the head skeleton of embryos in B and C remains truncated (solid arrows). No rescuing effect is detected when D-raf\(^{A7S}\)-rescued (D, E) and D-raf\(^{A7S}\) null embryos (F) were injected with p21\(^{v-ras}\). The segment fusion in E is also detected in some embryos injected with the control protein sample.

3C, D). Following posterior injection of p21\(^{v-ras}\) into tor\(^{XR1}\) embryos, the PM expression of the 1A121 marker was restored indicating that the PM primodium was recovered (Fig. 3, cf. E and F with C and D, Table 1B).

p21\(^{v-ras}\) activates the tor signaling pathway

In tor\(^{XR1}\) embryos, the terminal genes tll and hkb are not expressed at the posterior terminus (Fig. 4, B1, B2, Bronner and Jäckle 1991), and there are only six stripes, rather than seven, of expression of the pair-rule gene fushi tarazu [ftz] (Fig. 4B3, Ambrosio et al. 1989a). The seventh ftz stripe is missing in tor\(^{XR1}\) embryos owing to the absence of tor signaling. To define the mechanism, and the stage during embryonic development at which p21\(^{v-ras}\) rescues the tor maternal-effect phenotype, we examined the expression of tll, hkb, and ftz in tor\(^{XR1}\) embryos injected with p21\(^{v-ras}\) posteriorly. Interestingly, the posterior expression of both tll and hkb was restored (Fig. 4, C1, C2), and the seventh ftz stripe was recovered (Fig. 4C3). These results indicate that activated p21\(^{ras}\) can activate the terminal-class signaling pathway in the absence of tor protein. Posterior injection of p21\(^{v-ras}\) in most cases did not affect the anterior tll and hkb expression patterns of tor\(^{XR1}\) embryos (Fig. 4, C1, C2). This result correlates with the absence of rescue of the anterior head defects present in these embryos (indicated in Fig. 2, B and C, by solid arrows).

D-raf activity is required for p21\(^{v-ras}\) to activate the terminal-class pathway

To determine whether p21\(^{v-ras}\) operates through D-raf to activate the terminal-class pathway, we injected p21\(^{v-ras}\) into eggs derived from females lacking D-raf activity into their germ line (referred to as D-raf embryos). These D-raf embryos show two phenotypic classes (Ambrosio et al. 1989a): (1) The D-raf null embryos have little cuticle differentiation and correspond to embryos that have received no maternal and no paternal D-raf gene activity; and (2) the D-raf-rescued embryos have a phenotype identical to that of tor\(^{XR1}\) embryos (Fig. 2A) and corre-
When p21TM was injected into wild-type embryos, anterior expression of either the pair-rule gene 

\[ \text{tor}^{X} \] or the D-raf serine/threonine kinase. This is in contrast to \[ \text{tor}^{X} \] and \[ \text{D-raf} \] embryos in which posterior \[ \text{tor} \] expression is eliminated completely. At the posterior terminus, \[ \text{csw} \] null embryos are only missing the PM, a structure derived from the most posterior part of the terminal anlagen. This PM primodium is defined by the posterior expression domain of \[ \text{hkb} \] [Weigel et al. 1990], which is not expressed at the posterior terminus of \[ \text{csw} \] null embryos [Fig. 5A; L. Perkins and N. Perrimon, in prep.]. To examine the relationship of \[ \text{csw} \] with respect to \[ \text{p21}^{\text{ras}} \], \[ \text{p21}^{\text{ras}} \] was injected into \[ \text{csw} \] null embryos posteriorly. In these injected \[ \text{csw} \] null embryos, posterior \[ \text{hkb} \] expression was recovered [Fig. 5B], as well as the PM primodium as visualized by the \[ \text{1A121} \] enhancer trap marker [Table 1B]. These results indicate that \[ \text{p21}^{\text{ras}} \] rescues the \[ \text{csw} \] maternal-effect phenotype, further supporting the notion that \[ \text{p21}^{\text{ras}} \] mediates \[ \text{tor} \] signaling.

**Injection of dominant-negative \[ \text{p21}^{\text{rasN17}} \] protein reduces \[ \text{tor} \] signaling**

The experiments described above indicate that activated \[ \text{p21}^{\text{ras}} \] can turn on the terminal-class pathway through the D-raf kinase in the absence of \[ \text{tor} \] RTK. However, these results do not demonstrate whether \[ \text{p21}^{\text{ras}} \] is a normal component of this pathway. To address this question, we injected a dominant-negative form of \[ \text{p21}^{\text{ras}} \], \[ \text{p21}^{\text{rasN17}} \], into the posterior poles of wild-type embryos. \[ \text{p21}^{\text{rasN17}} \] antagonizes wild-type \[ \text{p21}^{\text{ras}} \] by competitive binding to guanine nucleotide-releasing factor (GRF; Farnsworth and Feig 1991). If \[ \text{p21}^{\text{ras}} \] is necessary for \[ \text{tor} \] signaling, injection of \[ \text{p21}^{\text{rasN17}} \] should block, at least partially, \[ \text{tor} \] signaling and thus generate a terminal-class phenotype. To monitor the terminal cell fates in the injected embryos, we examined the expression pattern of the pair-rule gene \[ \text{ftz} \] [Hiromi and Gehring 1987]. In wild-type embryos, \[ \text{ftz} \] is expressed in seven evenly spaced stripes, with the seventh stripe located between 12% and 17% EL owing to the regulatory inputs from the terminal-class pathway [Casanova 1990; Weigel et al. 1990]. When \[ \text{p21}^{\text{rasN17}} \] was injected into wild-type embryos at the posterior pole (i.e., at 0–5% EL), a specific terminal class phenotype resembling that of \[ \text{csw} \] was ob-
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Table 1. Injection of p21ras and p21rasN17 into early Drosophila embryos

<table>
<thead>
<tr>
<th>Injection sample</th>
<th>Recipient</th>
<th>Number of developed embryos</th>
<th>Number with filzkörper</th>
<th>Number with PM</th>
<th>Number with seventh ftz stripe shifted</th>
<th>Number without PM</th>
<th>Percent rescue</th>
<th>Percent affected</th>
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A. Rescue of the filzkörper structures

B. Rescue of the PM

C. p21rasN17 reduces tor signaling

Injected into early cleavage-stage embryos were 1–3% egg volume of p21ras [0.05–0.1 mg/ml], p21rasN17 [2–3 mg/ml], p21ras [0.05–0.1 mg/ml], BSA [3 mg/ml], and control sample (see Materials and Methods) (stage 1–2, according to Campos-Ortega and Hartenstein 1985). The effects of the injected protein in embryos were examined at various developmental stages appropriate for visualization of the different markers (see text, Materials and methods).

[A] Embryos derived from homozygous torXR1 females (torXR1 embryos) were injected with p21ras, p21ras, and control protein samples at the posterior poles (i.e., 0–5% EL). Embryos derived from females carrying germ-line clones lacking D-raf activity (D-rafE75 embryos) were injected with p21ras at 0–5% EL. The injected embryos were allowed to develop at 18°C for 48 hr before cuticle preparation. Cuticles were scored for the presence of filzkörper structures. Uninjected embryos were collected overnight from the same batch of females used in the injection experiments. Of p21ras-injected torXR1 embryos, 51% developed either complete telson structures or just the filzkörper material, whereas p21ras-injected D-rafE75 embryos did not develop any structures posterior to A7. p21ras and the control sample did not show any rescuing activity. The level of the rescuing activity correlates with the amount of p21ras protein present in the sample (for details, see Materials and methods).

[B] Homozygous torXR1 females and females carrying germ-line clones deprived of csw activity (cswE75) were crossed to males carrying the 1A12I marker, which labels the PM primordium. Embryos derived from these crosses were injected with p21ras sample at 0–5% EL. The injected embryos were stained using X-gal at germ-band extension (stage 9–10), and the presence or absence of the PM was scored. cswE75 and torXR1 embryos do not develop the PM (Weigel et al. 1990; L. Perkins and N. Perrimon, unpubl.). However, following p21ras injection, 61% of cswE75 embryos and 38% of torXR1 embryos developed the PM. The rescued PM in cswE75 embryos had normal morphology. However, the PMs rescued in torXR1 embryos were malformed (Fig. 3E,F). This may reflect the fact that a higher amount of p21ras protein is required in torXR1 embryos to completely recover all terminal structures. Nevertheless, the results indicate that p21ras is sufficient to specify PM fate.

[C] Wild-type embryos derived from flies carrying the ftz-lacZ [4304] and the 1A12I marker were injected with p21rasN17 at 0–5% EL and 25–35% EL. Similar results were obtained with both types of injection. The injected embryos were stained for lacZ expression pattern using affinity-purified antibody. Of the p21rasN17 injected embryos, 81–90% showed the shifted seventh ftz stripe at the cellular blastoderm stage (stages 5–6) and lacked the development of the PM at germ-band extension (stages 9–10). BSA did not affect the ftz expression pattern when injected at similar concentrations.

served. First, in these injected embryos, the seventh ftz stripe was broadened and shifted posteriorly (Fig. 6, cf. B with A). Second, similar to torof and csw embryos, these injected wild-type embryos were commonly twisted during germ-band extension and missing the PM (Table 1C). Other embryonic morphogenetic events in these injected embryos appeared grossly normal. To confirm that the effect of p21rasN17 was not the result of a local disruption of the posterior pole of the embryo, a second set of injections that gave similar results was performed where p21rasN17 was injected at 25–35% EL. Injection of an unrelated protein such as BSA at a similar concentration as p21rasN17 does not affect ftz expression pattern (data not shown). The association of this specific terminal-class phenotype with the injection of p21rasN17 into wild-type embryos indicates that p21ras/Ras1 activity is required to determine terminal cell fates.

The maternal-effect phenotype of Sos resembles that of the D-raf mutations

A direct way to analyze the role of Ras1 in tor signaling
is to examine the maternal effect of Ras1 mutations following the production of germ-line mosaics. An efficient way to create such mosaics is to use dominant female sterile (DFS) mutations (Perrimon et al. 1984). Suitable DFS mutations that would allow us to examine the maternal effect of Ras1 are unfortunately not yet available; however, they exist for the analysis of mutations located on chromosomal arm 2L (T.B. Chou and N. Perrimon, in prep.), on which the Drosophila GRF encoded by Sos is located. Mosaic females carrying germ-line clones of Sos^{122}, a loss-of-function allele, produced two phenotypic classes of embryos when crossed to Sos^{+/+} males. [1] The Sos-rescued embryos [genotype Sos^{122}/+] corresponds to embryos that do not have maternal Sos^{+} activity but have received a copy of the Sos^{+} gene paternally. This class of embryos is missing all [Fig. 7A] or part of the A8/filzkörper structures [not shown]. [2] The Sos null embryos [genotype Sos^{122}/Sos^{122}] correspond to embryos that have received no maternal and no paternal Sos^{+} gene activities. These embryos show little cuticle differentiation [Fig. 7B]. These two classes of embryonic phenotypes are strikingly similar [although weaker; see Discussion] to those observed from females carrying germ-line clones of \textit{D-raf} mutations [Fig. 2D,F; Ambrosio et al. 1989b]. These results indicate that a reduction in the level of active Ras1 created by removal of maternal Sos^{+} activity is sufficient to cause a terminal-class phenotype.

Figure 4. \textit{p21r^ras} activates the \textit{tor} signaling pathway. The in situ hybridization patterns of \textit{tll} (A1, B1, C1), \textit{hkb} (A2, B2, C2), and X-gal staining of \textit{ftz-lacZ} (A3, B3, C3) in wild-type (A), \textit{torxR1} (B), and \textit{p21r^ras} injected \textit{torxR1} (C) embryos are shown. All embryos are oriented with the anterior to the left and dorsal up. The domains of \textit{tll} and \textit{hkb} expression are indicated as percent EL by numbers located above the arrowheads.

Discussion

The role of \textit{p21r^ras/Ras1} in \textit{tor}-mediated signaling

We have shown that expression of an activated Ras1 protein during oogenesis results in embryos with a spliced phenotype indistinguishable from those derived from mothers carrying \textit{tor} gain-of-function mutations. Similar experiments using an activated Ras2 protein do not lead to the same defects. Ras1 and Ras2 belong to the Ras2 and R-ras families of ras proteins, respectively. Although no specific function has been ascribed to Ras2, Ras1 is involved in sevenless, and possibly \textit{Drosophila} EGF, RTK signaling (Simon et al. 1991). Our results revealed that Ras1 and Ras2 are involved in distinct developmental processes and ruled out the possibility that Ras2 mediates signaling for \textit{tor}. The specific effect of activated Ras1 suggests that Ras1 may mediate tor signaling to specify terminal cell fates.

To test this hypothesis, we injected a mammalian-activated \textit{p21r^ras}, \textit{p21^ras} protein, into the posterior pole of \textit{torxR1} embryos during early cleavage stages. The injected embryos recovered cuticular elements posterior to A7, as well as the internal PM. In addition, wild-type embryos show a terminal-class phenotype when injected with \textit{p21r^rasN17}, a dominant-negative form of \textit{p21ras}. \textit{p21^rasN17} antagonizes wild-type \textit{p21ras} by competitive binding to GRF. This protein has been shown to selec-
Sos+ (GRF) activity in their germ line have a terminal-class phenotype. These results indicate that p21Ras/Ras1 is an intrinsic component of tor signal transduction.

Furthermore, we have shown that all the rescuing activity of p21ras as assayed by both the recovery of cuticular elements posterior to abdominal segment seven and posterior tll and hkb expression in tor+ embryos is absolutely dependent on the presence of D-raf activity. Studies in mammalian cells have shown that the activity of p21ras is correlated with the hyperphosphorylation of Raf-1 kinase and that v-ras-mediated cell transformation is partially inhibited by the expression of c-raf1 antisense RNA [see introductory section]. Our results demonstrate that the function of p21ras/Ras1 in the tor pathway is completely mediated through the D-raf kinase, providing the most convincing evidence that p21ras operates upstream of Raf-1 kinase. These results are also consistent with those of Dickson et al. (1992), who recently showed evidence that D-raf operates downstream of Ras1 in the sevenless pathway. The conserved roles of p21ras and Raf kinase in many signaling pathways provide additional support that many RTKs involved in diverse biological processes share common signaling molecules and mechanisms.

In conclusion, our experiments demonstrate that p21ras is a component of the tor signaling pathway where it operates upstream of D-raf and is both neces-

![Figure 5.](image-url) 

**Figure 5.** p21ras rescues hkb expression in csw null embryos. The expression pattern of hkb is shown in a csw+ embryo [A] and a p21ras injected csw+ embryo [B]. hkb is only expressed in a reduced domain anteriorly in A [cf. Fig. 5A with Fig. 4A2]. Following the injection of p21ras posteriorly, B has recovered the posterior hkb expression to a degree slightly greater than the wild-type domain [Fig. 4A2] and the anterior expression is extended back to near wild-type pattern. In some p21ras-injected csw embryos, the hkb posterior domain is only recovered to 2% EL while the anterior domain remains at an average of 93% EL [data not shown]. This result indicates that terminal gene expression is repressed within the central embryonic region by unknown mechanisms. A similar type of repression has also been detected for tll transcription in embryos expressing an activated c-raf1 gene under the control of the heat shock promoter hsp70 [A. Brand, X. Lu, and N. Perrimon, in prep.].

![Figure 6.](image-url) 

**Figure 6.** p21ras is an intrinsic component of the tor signaling pathway. Embryos derived from the 4304 stock were stained using an anti-β-gal antibody at late cellular blastoderm stages to visualize the ftz-lacZ expression pattern. A and B are oriented with the anterior to the left and dorsal up. [A] The wild-type ftz pattern, [B] the altered ftz pattern following injection of p21ras at 25% EL. Note that the seventh ftz stripe in B is expanded posteriorly.
p21<sup>ras</sup>/Rasl mediates torso signaling

bound Ras1, resulting in the activation of the D-raf kinase. In the developmental context of 1- to 2.5-hr *Drosophila* embryos, activation of the D-raf kinase leads to transcriptional activation of *tll* and *hkb* in specific terminal domains. Previous genetic studies have shown that *csw* potentiates *tor* signaling to a level sufficient for proper *tll* and *hkb* expression [Perkins et al. 1992]. Consistent with this role of *csw* in *tor* signaling, p21<sup>ras</sup> rescues the maternal effect of *csw*. Our results suggest that *csw* operates upstream of D-raf to increase the level of D-raf activation. However, the actual position of *csw* in this signaling pathway requires further investigation.

It is interesting that we have never observed a complete *tor*<sup>tot</sup> phenotype among wild-type embryos injected with p21<sup>rasN17</sup>, which blocks p21<sup>ras</sup> activation by binding to GRF/Sos competitively. This observation is consistent with the fact that many Sos-rescued embryos (genotype *Sos<sup>x122</sup>/+)
 have a phenotype weaker than that of *tor*<sup>x36</sup> embryos. There are a number of possibilities to explain these results. First, the Sos mutation used in our germ-line mosaic analyses, *Sos<sup>x122</sup>*
although a loss-of-function allele, may not be a complete null. Second, similar to the *sevenless* pathway, it is possible that a GAP protein operates in the *tor* pathway. Ras1 activity could still be increased above basal levels in Sos embryos via down-regulation of a ras–GAP activity, possibly encoded by GAP1 [Gaul et al. 1992]. Third, unlike other maternal terminal class genes, the terminal class phenotype associated with Sos may be paternally rescuable to some extent. Fourth, the existence of alternative pathway[s] that lead to the activation of either p21<sup>ras</sup>/Rasl or more downstream components remains open [Williams et al. 1992].

**tor signaling pathway update**

Figure 8 shows the updated terminal-class model that has incorporated our new findings. Localized activation of *tor* RTK at the embryonic termini leads to the activation of GRF/Sos, which increases the level of GTP-

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**Figure 7.** The maternal-effect phenotypes of the *Sos* gene. Two phenotypic classes of embryos are obtained from females carrying homozygous germ-line clones of *Sos<sup>x122</sup>*. The embryo in A is a *Sos*-rescued embryo (genotype *Sos<sup>x122</sup>/+). This class of embryos has phenotypes similar to that of *tor*<sup>x22</sup> embryos, which are missing most of the structures posterior to abdominal segment seven and exhibit anterior head defects (truncated head skeleton indicated by the arrow). The embryo in B is a *Sos* null embryo (genotype *Sos<sup>x122</sup>/Sos<sup>x122</sup>), which shows little cuticle differentiation.

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**Figure 8.** The updated terminal-class model. (→) Activation effects; (→) negative regulatory effects. It is unclear whether a ras–GAP activity is involved in the terminal-class pathway [for details, see discussion]. (Torso) Torso receptor tyrosine kinase; (csw) corkscrew protein tyrosine phosphatase; (GRF) guanine nucleotide-releasing factor; (Sos) son of sevenless; (GAP) GTPase-activating protein; (ras [Ras1]) p21<sup>ras</sup> GTPase; (D-raf) D-raf serine/threonine kinase.
About the terminal gradient mechanism

Previous studies have proposed that graded levels of tor signaling are required to direct pattern formation within the unsegmented terminal anlagen (0–20% EL) (Casanova and Struhl 1989; St. Johnston and Nüsslein-Volhard 1992). This gradient of tor activation is thought to be generated by diffusion of localized tor ligand originating from the poles (Stevens et al. 1990). However, we have observed that the posterior terminal structures were rescued at their normal posterior position even when p21ras was injected anteriorly or in the central region of torX; embryos. These results suggest that embryos devoid of tor receptor still possess an intrinsic polarity for tail formation, and this polarity arises in the embryo independently of tor activation at a level downstream of p21ras/Ras1. These observations are consistent with the recent model proposed by Sprenger and Nüsslein-Volhard (1992) that “activated torso might act more like a switch, triggering terminal development after a threshold level of torso activity is achieved,” and “gradient(s) of cytoplasmic molecules” would then be responsible for subdividing the terminal anlagen.

In contrast to the study of Sprenger and Nüsslein-Volhard (1992), who used injections of mRNAs coding for various mutated forms of the tor receptor, we have never observed the formation of centrally located ectopic posterior structures (i.e., filzk6rper material) following injection of p21ras. The differential instructive effects of tor mRNA and activated p21ras proteins may reflect their level and strength of interactions with the putative graded cytoplasmic molecules. This issue is likely to be clarified with the identification of additional components of the terminal system.

Materials and methods

Genetic strains

The DFS stock is C1/DX, y f/Y; Flp38/Flp38 females crossed to w ovoD1/v4 FRT105/Y; Flp38/Flp38 males (Chou and Perrimon 1992). Flp38 is the yeast site-specific recombinase that allows for excision of p21ras and p21ras proteins from the embryo independently of tor activation at a level downstream of p21ras. The differential instructive effects of tor mRNA and activated p21ras proteins may reflect their level and strength of interactions with the putative graded cytoplasmic molecules. This issue is likely to be clarified with the identification of additional components of the terminal system.

hs–Ras1 constructs

hs–Ras1 The 1.4-kb Ras1 cDNA (EF1 in pGM) (Neuman-Silberberg et al. 1984) was cut out by EcoRI and introduced into the P-element vector pCaSpeR–hs (Thummel et al. 1988; C. Thummel, pers. comm.) to generate three P[hs–Ras1]-independent transformant lines: hs–EF-m, hs–EF-m.5.2, and hs–EF-f12.

hs–Ras1Q1213 To generate an activated Ras1 gene, a G → A base mutation in Ras1 cDNA corresponding to amino acid residue Gly-13 changed to glutamic acid (Q-13) in Ras1 protein was generated by primer-directed polymerase chain reaction (PCR) mutagenesis. This mutation was found previously to be associated with one of the increase-of-function let-60 alleles, which causes multivulva phenotype in C. elegans (Beitel et al. 1990). Two P[hs–Ras1Q1213] transgenic lines, hs–9.4–M12 and hs–9.4–M8/Cyo, were analyzed and gave similar results.

Heat shock treatment of adult flies

Females carrying various heat shock constructs were heat-shocked at 35°C with three 10-min pulses at 20-min intervals. This heat shock treatment was repeated every 24 hr. Embryos were collected between each heat shock treatment, and the cuticles of unhatched embryos were prepared as described by van der Meer (1977). For females carrying P[hs–Ras1Q1213] and P[hs–Ras2V78] constructs, the embryos collected after the second heat shock treatment gave the best results.

Production of mosaic females using the FLP-DFS technique

D-raf embryos were obtained from females carrying germ-line clones of D-rafA15 using the FLP-DFS technique (Chou and Perrimon 1992). Germ-line clones of Sos220 were generated by X-ray treatment (Perrimon et al. 1984) using a DFS mutation, P[ovoD13], carried by a P-element transposon insertion on 2L (T.B. Chou and N. Perrimon, in prep.). Briefly, progeny from a cross between Sos220/SM6 virgin females and ovoD1 v4/Y, P[ovoD13]/CyO males were irradiated during the first-instar larval stage with 1000 rads (Torrex 120 X-ray machine, 100 kV, 5 mA, 3-µm aluminum filter) to induce germ-line clones. Under these conditions, ~2% of females with germ-line clones are recovered. P[ovoD13], Sos220/P[ovoD13] females carrying Sos220 homozygous germ-line clones were crossed to either +/Y; Sos220/Cyo or y/y; +/+ males and raised at 25°C. The presence of the ovoD1 mutation on the X chromosome is to ensure complete sterility of the ovoD1, v4/Y, +/+ females (T.B. Chou and N. Perrimon, in prep.).

Injection of p21ras, p21ras, and p21rasN17 proteins

The p21ras, p21ras, and p21rasN17 proteins were made in Spodoptera frugiperda (Sf9) cells (3 × 107) infected with recombinant baculovirus encoding p21ras or p21ras (Williams et al. 1992) according to standard procedures (Summers et al. 1987). Fifty to fifty-six hours postinfection, cells were washed twice in cold phosphate-buffered saline (PBS) solution and then lysed for 20 min in 300 µl of NP-40 buffer (135 mM NaCl, 20 mM Tris at pH 8.0, 1 mM MgCl2, 1 mM CaCl2, 10% glycerol, 1% NP-40) supplemented with phenylmethylsulfonyl fluoride (1 mm), aprotinin (0.15 µg/ml), and leupeptin (20 µg/ml). The lysate was centrifuged at 10,000g for 10 min to remove insoluble material. The cell lysates contain ~0.05–0.1 mg/ml of p21ras or p21ras proteins by Western immunoblotting. The control sample was made as lysed Sf9 cells without viral infection and contains no detectable amount of p21ras when assayed by Western Blotting. The p21rasN17 protein was expressed in Escherichia coli and purified to near homogeneity (2–3 mg/ml) (Farnsworth et al. 1991), kindly provided by Larry Feig, Tufts University School of Medicine, Boston, MA)

Injection of protein samples into Drosophila embryos was performed following standard DNA injection procedures (Spradling 1986). Approximately 1–3% egg volume of protein sample was injected into early cleavage-stage embryos [stage 1–2, ac-
according to Campos-Ortega and Hartenstein 1985). At a concentration of 0.05–0.1 mg/ml of p21ras protein, a complete rescue of telson structures was observed [Fig. 2B,C]. The amount of injected p21ras protein correlated with the rescuing activity observed. Reduction of p21ras protein present in the lysate by two- to threefold resulted in partial rescue of the torphenotype because only the dorsal straight spines and rudimentary spiracle structures were recovered in injected tor embryos (data not shown).

**X-gal antibody staining and in situ hybridization**

Whole-mount in situ hybridization with *tl* and *hkb* single-stranded DNA probes to *Drosophila* embryos was performed as described by Tautz and Pfeifle [1989], using PCR-derived single-stranded sense and anti-sense digoxigenin-labeled DNA probes (N. Patel, pers. comm.). To examine the expression of the *fix-1* or *A121* enhancer trap markers, virgin homozygous *tor* females or females carrying *D-raf* homozygous germ-line clones were crossed with males carrying these markers. For antibody staining, embryos derived from the above crosses were injected with p21ras protein and fixed at cellular blastoderm stage in 1% glutaraldehyde/PBS solution for 2 min, vitelline membranes were removed manually, and the embryos were incubated in anti-β-galactosidase (Boehringer Mannheim) at a dilution of 1:1000 and processed further using the Elite Kit (Vectastain). As an alternative, X-gal staining was performed as described previously by Bellen et al. [1989]. Stained embryos were photographed under Nomarski optics using a Zeiss Axioskop microscope.

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**References**


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