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Control of cell fate determination by p21^{ras}/Ras1, an essential component of *torso* signaling in *Drosophila*

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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 *p21^{ras}/Ras1* mediates *tor* signaling, we injected mammalian p21^{ras} variants into early *Drosophila* embryos. We found that the injection of activated p21$^{v\text{-}ras}$ rescued the maternal-effect phenotypes of both *tor* and *csw* null mutations. These rescuing effects of p21$^{v\text{-}ras}$ 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 p21^{rasN17}, a dominant-negative form of p21^{ras}. 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* p21^{ras}, 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. p21^{ras}/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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p21^{ras} 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 p21^{ras} 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-p21^{ras} antibody or a dominant inhibitory mutant p21^{rasN17} blocks NIH-3T3 cell proliferation (Mulcahy et al. 1985; Feig and Cooper 1988a). Conversely, p21$^{v\text{-}ras}$, a constitutively activated form of p21^{ras}, transforms cells (Feig and Copper 1988b). The p21$^{v\text{-}ras}$-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 p21^{ras} 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 p21^{ras} 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 p21^{ras} (Thomas et al. 1992; Wood et al. 1992). Collectively, these studies have demonstrated a functional relationship among growth factor RTKs, p21^{ras}, 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 p21^{ras} mediates signal transduction of many RTKs. In *Caenorhabditis elegans*, the p21^{ras} 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 p21^{ras} 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* oncogene (Simon et al. 1991). In this paper, we present evidence that p21^{ras} 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 overlaping 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^{gof}). tor^{gof} gives rise to a phenotype that is complementary to that of the loss-of-function *tor* mutations (tor^{lof}). tor^{lof} deletes the acron and telson in embryos derived from mutant mothers, whereas tor^{gof} 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 thoracic 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* ($p21^{ras}$), 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 $p21^{ras}$ 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^{gof} *embryonic phenotype*

To determine whether *Ras1* plays a role in early embryonic development, we expressed a dominant-activated form of *Ras1*, $Ras1^{Q13}$ 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^{gof} 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, germband 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 $Ras1^{Q13}$ 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 $Ras1^{Q13}$ during oogenesis, we conducted a similar experiment using an analogous activating mutation in *Ras2*, $Ras2^{V12}$ (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 $p21^{v\text{-}ras}$ *protein rescues the* tor^{lof} *maternal-effect phenotype*

To test whether Ras1 mediates *tor* signaling, we injected a mammalian-activated $p21^{ras}$ protein, $p21^{v\text{-}ras}$, produced

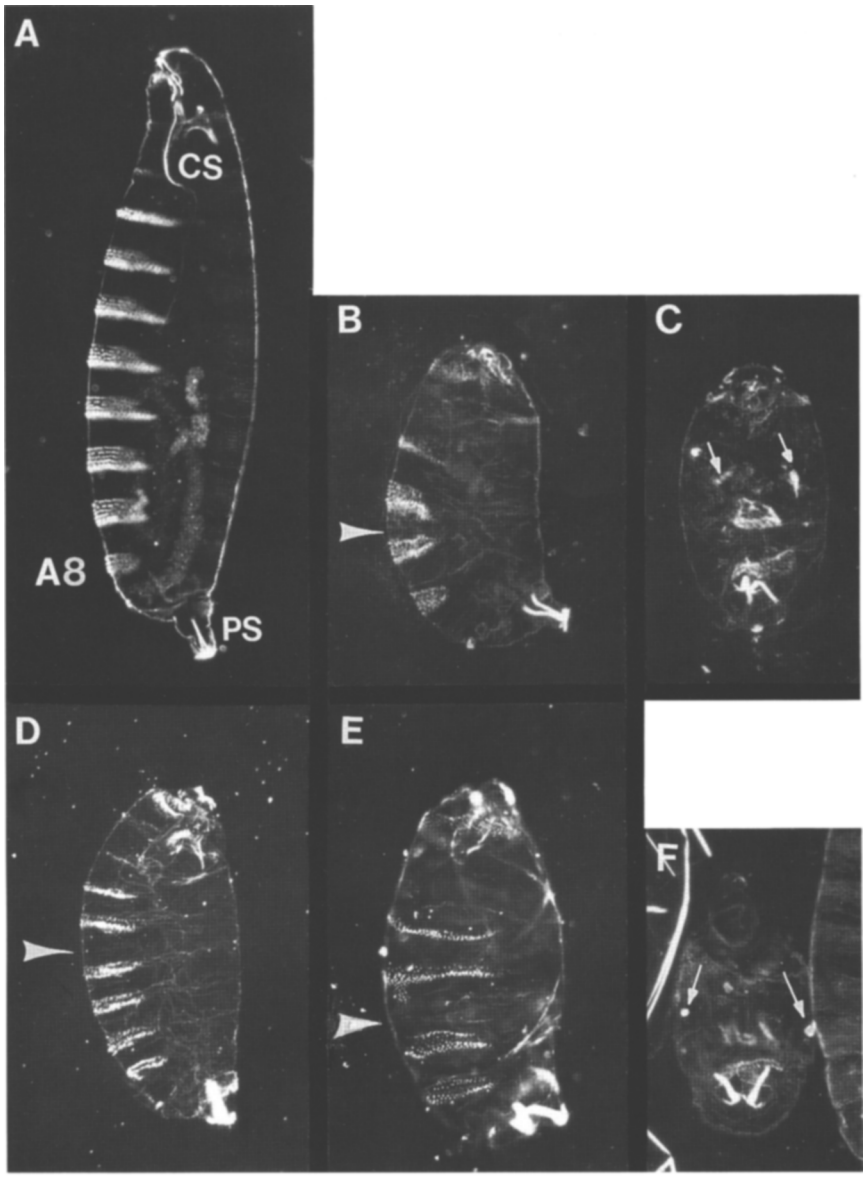

Figure 1. Expression of an activated *Ras1* gene during oogenesis generates the tor^{gof} phenotype. (*A*) A dark-field photograph of a wild-type embryo showing the well differentiated cephalopharyngeal head skeleton (CS), thoracic and abdominal segments (A8 indicates the position of abdominal segment A8), and posterior spiracles (PS). (*B,C*) Embryos derived from homozygous gain-of-function tor^{Y9} females; note the repressed abdominal segmentation pattern in *B* (arrowhead), as well as the presence of extra filzkörper material (arrows in *C*). (*D–F*) Embryos derived from females carrying *P(hs-9.4–M12)* following heat shock treatment; note the repressed abdominal segmentation (arrowheads in *D* and *E*) and extra filzkörper material (arrows in *F*).

in the baculovirus expression system, into early cleavage-stage embryos derived from homozygous tor^{XR1} females (referred to as tor^{XR1} embryos). tor^{XR1} contains a 10.5-kb deletion encompassing the *tor* gene and is both an RNA and protein null (Sprenger et al. 1989). tor^{XR1} 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 $p21^{v\text{-}ras}$ 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 $p21^{v\text{-}ras}$ into the central region of the embryos. In this assay no rescuing activity was associated with either the wild-type $p21^{c\text{-}ras}$ or the control sample lacking $p21^{ras}$ protein (see Table 1A). When $p21^{v\text{-}ras}$ was injected anteriorly (95–100% EL) in tor^{XR1} 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 $p21^{v\text{-}ras}$ into tor^{XR1} embryos is able to recover the PM cell fates, $p21^{v\text{-}ras}$ was injected into tor^{XR1} 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 tor^{XR1} embryos, posterior *1A121* staining is missing owing to the absence of the PM (Fig.

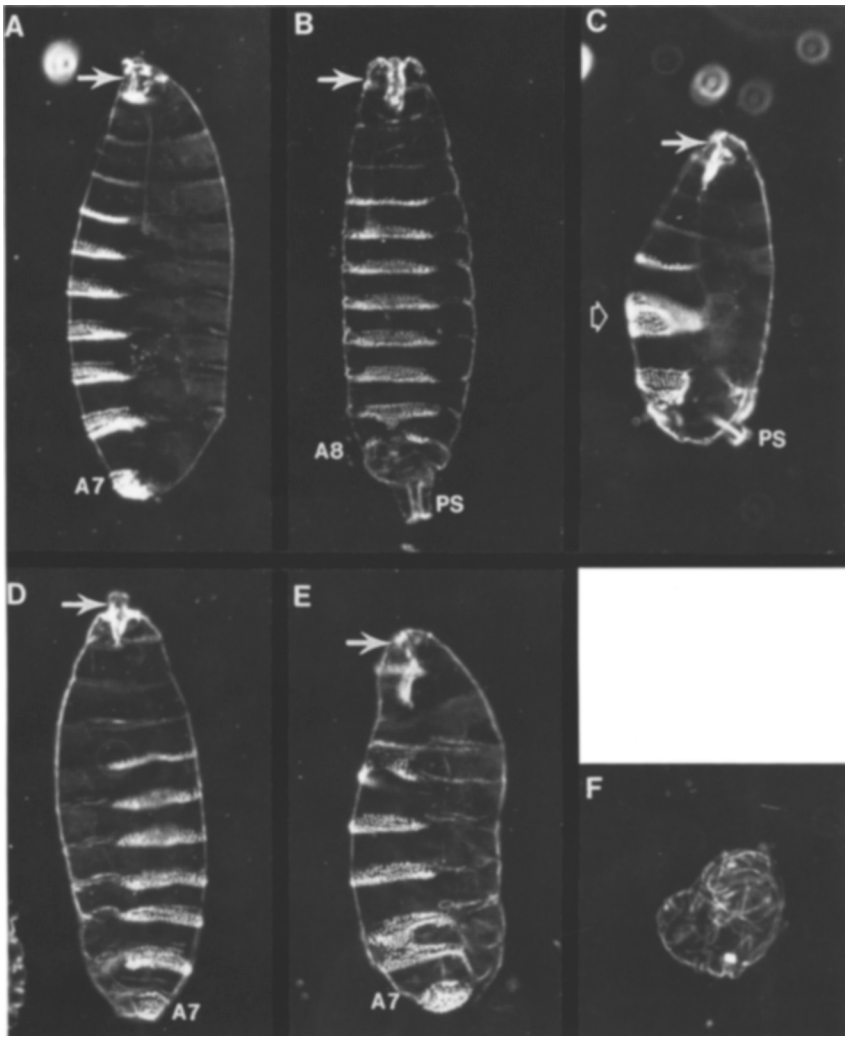

Figure 2. Injection of $p21^{v\text{-}ras}$ rescues the maternal-effect phenotype of tor^{lof} (tor^{XR1}) but not $D\text{-}raf^{lof}$ ($D\text{-}raf^{EA75}$). (*A*) A dark-field picture of the cuticular pattern elements present in a tor^{XR1} embryo. The head skeleton (indicated by a solid arrow) is truncated, and all structures posterior to abdominal segment 7 (A7) are missing. (*B,C*) Two tor^{XR1} embryos that have been injected with $p21^{v\text{-}ras}$ 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 $p21^{v\text{-}ras}$ was injected). The embryo in *C* differentiated most of the posterior terminal structures, but abdominal segmentation was severely repressed similarly to tor^{gof} 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 $D\text{-}raf^{EA75}$-rescued (*D,E*) and $D\text{-}raf^{EA75}$ null embryos (*F*) were injected with $p21^{v\text{-}ras}$. The segment fusion in *E* is also detected in some embryos injected with the control protein sample.

3C,D). Following posterior injection of $p21^{v\text{-}ras}$ 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).

$p21^{v\text{-}ras}$ 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 $p21^{v\text{-}ras}$ rescues the *tor* maternal-effect phenotype, we examined the expression of *tll*, *hkb*, and *ftz* in tor^{XR1} embryos injected with $p21^{v\text{-}ras}$ 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 $p21^{v\text{-}ras}$ 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* $p21^{v\text{-}ras}$ *to activate the terminal-class pathway*

To determine whether $p21^{v\text{-}ras}$ operates through *D-raf* to activate the terminal-class pathway, we injected $p21^{v\text{-}ras}$ 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-

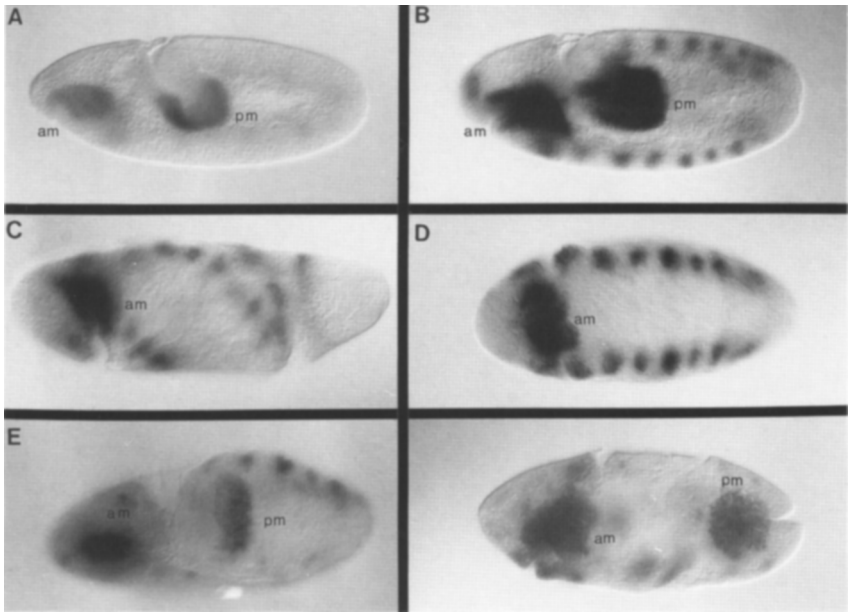

Figure 3. Injection of p21$^{v\text{-}ras}$ rescues PM formation in *tor*^{XR1} (*tor*^{lof}) embryos. The expression pattern of the enhancer trap marker *1A121* is shown at germ-band-extended stages by X-gal staining. All embryos are oriented with the anterior to the *left*. In wild-type embryos (*A*,*B*), the staining is found exclusively in both the anterior (am) and posterior (pm) midgut at stage 9 (*A*). Soon after, at stage 10, additional staining is found in the epidermis (*B*). (*C*,*D*) *tor*^{XR1}embryos with no posterior *1A121* staining past stage 9; note that *tor*^{XR1} embryos often twist during germ-band extension (*C*) and then unwind (*D*, dorsal view) at later stages. (*E*,*F*) *tor*^{XR1} embryos injected with p21$^{v\text{-}ras}$ posteriorly; note the recovery of posterior staining indicative of the differentiation of the PM primodia. The PM rescued in *tor*^{XR1} embryos were malformed (*E*,*F*); possibly owing to abnormal posterior invagination during germ-band extension. Nevertheless, the PM fate is specified by p21$^{v\text{-}ras}$ in these *tor*^{XR1} embryos.

spond to embryos that do not have maternal *D-raf* activity but have received a wild-type *D-raf* gene paternally. In both classes of *D-raf* embryos, *tll* and *hkb* are not expressed at the posterior terminus (Melnick et al. 1993). When p21$^{v\text{-}ras}$ was injected into *D-raf* embryos posteriorly, no rescue of any telson structures was detectable in either the *D-raf*-rescued embryos (Fig. 2D,E) or the *D-raf* null embryos (Fig. 2F; Table 1A). Consistent with this result, posterior expression of either *tll* or *hkb* was never detected in injected *D-raf* embryos (data not shown). Occasionally, the *D-raf*-rescued embryos showed abdominal segmentation defects (Fig. 2E). However, these defects were also detected in some wild-type embryos injected with the control sample. These results show that the p21$^{v\text{-}ras}$-rescuing activity requires the presence of D-raf protein, indicating that p21^{ras} acts upstream and through the D-raf serine/threonine kinase.

Injection of p21$^{v\text{-}ras}$ can rescue the csw *maternal-effect phenotype*

tor signaling is partially blocked in embryos derived from females completely lacking *csw* activity in their germ line (referred to as *csw* null embryos), as indicated by the reduced expression of *tll* posteriorly (Perkins et al. 1992). This is in contrast to *tor*^{lof} and *D-raf* embryos in which posterior *tll* expression is eliminated completely. At the posterior terminus, *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 *hkb* (Weigel et al. 1990), which is not expressed at the posterior terminus of *csw* null embryos (Fig. 5A; L. Perkins and N. Perrimon, in prep.). To examine the relationship of *csw* with respect to *p21^{ras}*, p21$^{v\text{-}ras}$ was injected into *csw* null embryos posteriorly. In these injected *csw* null embryos, posterior *hkb* expression was recovered (Fig. 5B), as well as the PM primodium as visualized by the *1A121* enhancer trap marker (Table 1B). These results indicate that p21$^{v\text{-}ras}$ rescues the *csw* maternal-effect phenotype, further supporting the notion that p21^{ras} mediates *tor* signaling.

Injection of dominant-negative p21^{rasN17} protein reduces tor *signaling*

The experiments described above indicate that activated p21^{ras} can turn on the terminal-class pathway through the D-raf kinase in the absence of tor RTK. However, these results do not demonstrate whether p21^{ras} is a normal component of this pathway. To address this question, we injected a dominant-negative form of p21^{ras}, p21^{rasN17}, into the posterior poles of wild-type embryos. p21^{rasN17} antagonizes wild-type p21^{ras} by competitive binding to guanine nucleotide-releasing factor (GRF; Farnsworth and Feig 1991). If p21^{ras} is necessary for *tor* signaling, injection of p21^{rasN17} should block, at least partially, *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 *ftz* (Hiromi and Gehring 1987). In wild-type embryos, *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 p21^{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 *csw* was ob-

Table 1. *Injection of $p21^{v\text{-}ras}$ and $p21^{rasN17}$ into early* Drosophila *embryos*

Injection sample	Recipient	Number of developed embryos	Number with filzkörper	Number with PM	Number with seventh *ftz* stripe shifted	Number without PM	Percent rescue	Percent affected
			A. Rescue of the filzkörper structures					
Uninjected	tor^{XR1}	400	0				0	
Control sample	tor^{XR1}	25	0				0	
$p21^{v\text{-}ras}$	tor^{XR1}	106	54				51	
$p21^{c\text{-}ras}$	tor^{XR1}	40	0				0	
Uninjected	$Draf^{EA75}$	200	0				0	
$p21^{v\text{-}ras}$	$Draf^{EA75}$	145	0				0	
			B. Rescue of the PM					
Uninjected	tor^{XR1}	100		0			0	
$p21^{v\text{-}ras}$	tor^{XR1}	13		5			38	
Uninjected	csw^{KN27}	100		0			0	
$p21^{v\text{-}ras}$	csw^{KN27}	13		8			61	
			C. $p21^{rasN17}$ reduces tor *signaling*					
Uninjected	*4304*	100			0			0
BSA	*4304*	20			0			0
$p21^{rasN17}$	*4304*	50			45			90
Uninjected	*1A121*	100				0		0
$p21^{rasN17}$	*1A121*	11				9		81

Injected into early cleavage-stage embryos were 1–3% egg volume of $p21^{v\text{-}ras}$ (0.05–0.1 mg/ml), $p21^{rasN17}$ (2–3 mg/ml), $p21^{c\text{-}ras}$ (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 tor^{XR1} females (tor^{XR1} embryos) were injected with $p21^{v\text{-}ras}$, $p21^{c\text{-}ras}$, 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 ($D\text{-}raf^{EA75}$ embryos) were injected with $p21^{v\text{-}ras}$ 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 materials. Uninjected embryos were collected overnight from the same batch of females used in the injection experiments. Of $p21^{v\text{-}ras}$-injected tor^{XR1} embryos, 51% developed either complete telson structures or just the filzkörper material, whereas $p21^{v\text{-}ras}$-injected $D\text{-}raf^{EA75}$ embryos did not develop any structures posterior to A7. $p21^{c\text{-}ras}$ and the control sample did not show any rescuing activity. The level of the rescuing activity correlates with the amount of $p21^{v\text{-}ras}$ protein present in the sample (for details, see Materials and methods).

(*B*) Homozygous tor^{XR1} females and females carrying germ-line clones deprived of *csw* activity (csw^{KN27}) were crossed to males carrying the *1A121* marker, which labels the PM primodium. Embryos derived from these crosses were injected with $p21^{v\text{-}ras}$ 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. csw^{KN27} and tor^{XR1} embryos do not develop the PM (Weigel et al. 1990; L. Perkins and N. Perrimon, unpubl.). However, following $p21^{v\text{-}ras}$ injection, 61% of csw^{KN27} embryos and 38% of tor^{XR1} embryos developed the PM. The rescued PM in csw^{KN27} embryos had normal morphology. However, the PMs rescued in tor^{XR1} embryos were malformed (Fig. 3E,F). This may reflect the fact that a higher amount of $p21^{v\text{-}ras}$ protein is required in tor^{XR1} embryos to completely recover all terminal structures. Nevertheless, the results indicate that $p21^{v\text{-}ras}$ is sufficient to specify PM fate.

(*C*) Wild-type embryos derived from flies carrying the *ftz–lacZ* (*4304*) and the *1A121* marker were injected with $p21^{rasN17}$ 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 anti-β-galactosidase antibody. Of the $p21^{rasN17}$ 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 tor^{lof} 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 $p21^{rasN17}$ 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 $p21^{rasN17}$ was injected at 25–35% EL. Injection of an unrelated protein such as BSA at a similar concentration as $p21^{rasN17}$ does not affect *ftz* expression pattern (data not shown). The association of this specific terminal-class phenotype with the injection of $p21^{rasN17}$ into wild-type embryos indicates that $p21^{ras}$/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

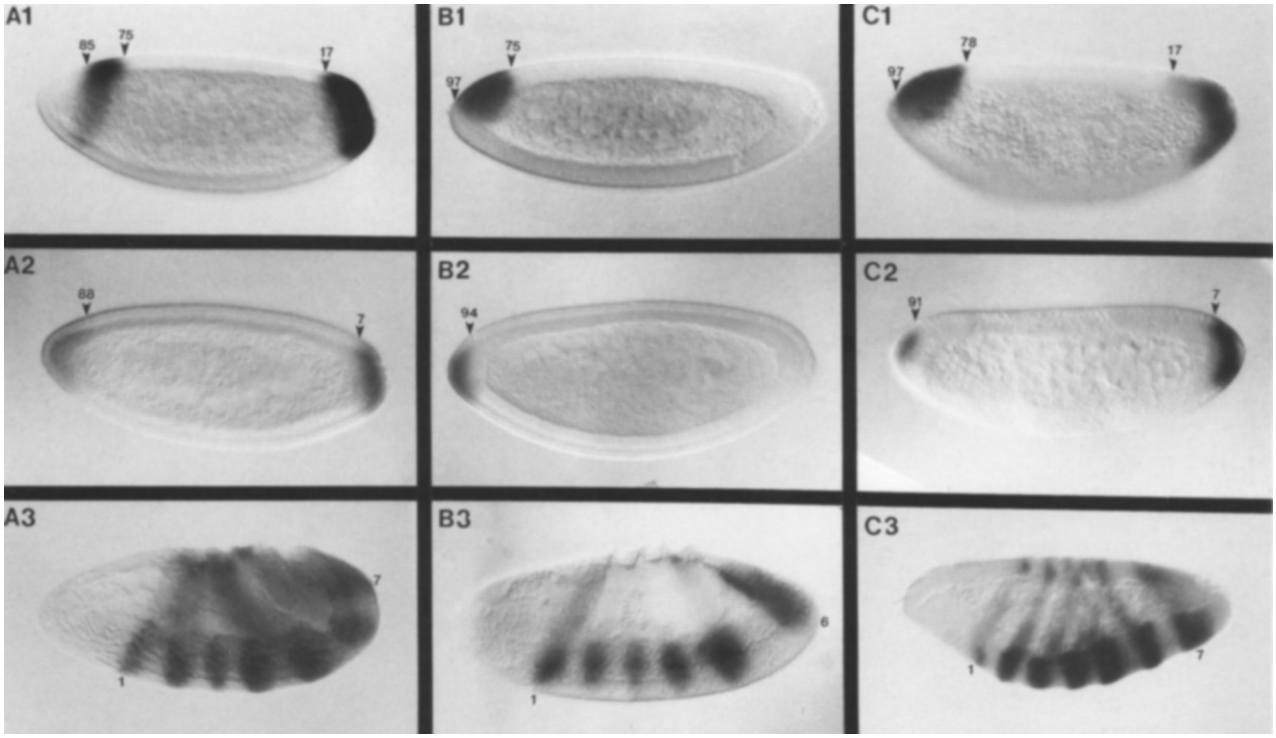

Figure 4. p21[v-ras] activates the *tor* signaling pathway. The in situ hybridization patterns of *tll* (*A1*, *B1*, *C1*), *hkb* (*A2*, *B2*, *C2*), and X-gal staining of *ftz–lacZ* (*A3*, *B3*, *C3*) in wild-type (*A*), *tor*^{XR1} (*B*), and p21[v-ras] injected *tor*^{XR1} (*C*) embryos are shown. All embryos are oriented with the anterior to the *left* and dorsal *up*. The domains of *tll* and *hkb* expression are indicated as percent EL by numbers located above the arrowheads.

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*^{x122}, a loss-of-function allele, produced two phenotypic classes of embryos when crossed to *Sos*^{x122}/+ males. (1) The *Sos*-rescued embryos (genotype *Sos*^{x122}/+) 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*^{x122}/*Sos*^{x122}) 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 *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.

Discussion

The role of p21[ras]/Ras1 in 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 *tor* gain-of-function mutations. Similar experiments using an activated Ras2 protein do not lead to the same defects. Ras1 and Ras2 belong to p21[ras] and R-ras families of ras proteins, respectively. Although no specific function has been ascribed to *Ras2*, *Ras1* is involved in *sevenless*, and possibly *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 *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 p21[ras], p21[v-ras] protein, into the posterior pole of *tor*^{XR1} 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 p21[rasN17], a dominant-negative form of p21[ras]. p21[rasN17] antagonizes wild-type p21[ras] by competitive binding to GRF. This protein has been shown to selec-

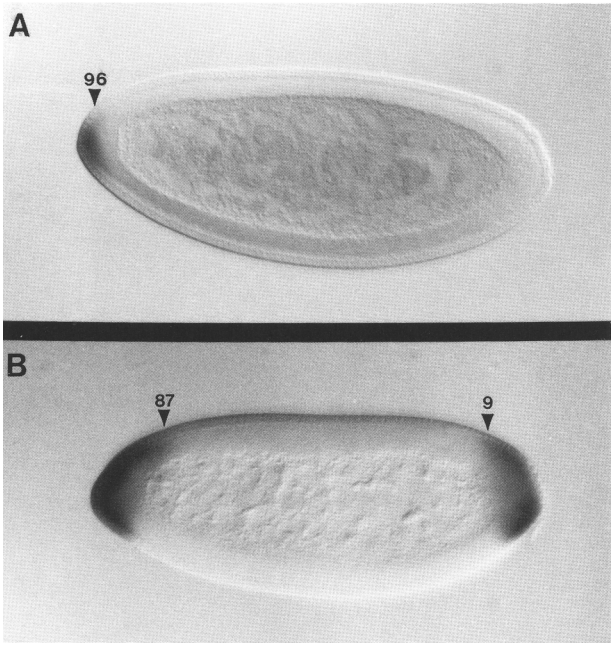

Figure 5. p21$^{v\text{-}ras}$ rescues *hkb* expression in *csw* null embryos. The expression pattern of *hkb* is shown in a *csw*^{KN27} embryo (*A*) and a p21$^{v\text{-}ras}$ injected *csw*^{KN27} embryo (*B*). *hkb* is only expressed in a reduced domain anteriorly in *A* (cf. Fig. 5A with Fig. 4A2). Following the injection of p21$^{v\text{-}ras}$ 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 p21$^{v\text{-}ras}$-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.).

tively block p21^{ras}-mediated signaling in both mammalian and *Xenopus* systems (Whitman and Melton 1992). Several lines of evidence strongly suggest that the terminal-class phenotype associated with the injection of p21^{rasN17} in wild-type embryos is caused by the reduction of p21^{ras} activity. First, the phenotype generated by injection of p21^{rasN17} is specific. Injected p21^{rasN17} affects the expression of the seventh *ftz* stripe and the determination of PM fate, both of which are regulated by the *tor* signaling pathway. Second, these specific terminal class phenotypes can be produced by injection of p21^{rasN17} into wild-type embryos at 25–35% EL, which is outside of the prospective terminal anlagen (0–20% EL). The fifth and sixth *ftz* stripes located around the site of injection are not disrupted, indicating that the terminal-class phenotypes associated with p21^{rasN17} injections are not caused by nonspecific disruption of inherent cytoplasmic structures present in the terminal region of the eggs. Third, we have shown that embryos derived from mosaic females lacking normal levels of *Sos*$^{+}$ (GRF) activity in their germ line have a terminal-class phenotype. These results indicate that p21^{ras}/Ras1 is an intrinsic component of *tor* signal transduction.

Furthermore, we have shown that all the rescuing activity of p21$^{v\text{-}ras}$ as assayed by both the recovery of cuticular elements posterior to abdominal segment seven and posterior *tll* and *hkb* expression in *tor*^{XR1} embryos is absolutely dependent on the presence of *D-raf* activity. Studies in mammalian cells have shown that the activity of p21^{ras} 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 p21^{ras}/Ras1 in the *tor* pathway is completely mediated through the D-raf kinase, providing the most convincing evidence that p21^{ras} 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 p21^{ras} 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 p21^{ras}/Ras1 is a component of the *tor* signaling pathway where it operates upstream of *D-raf* and is both neces-

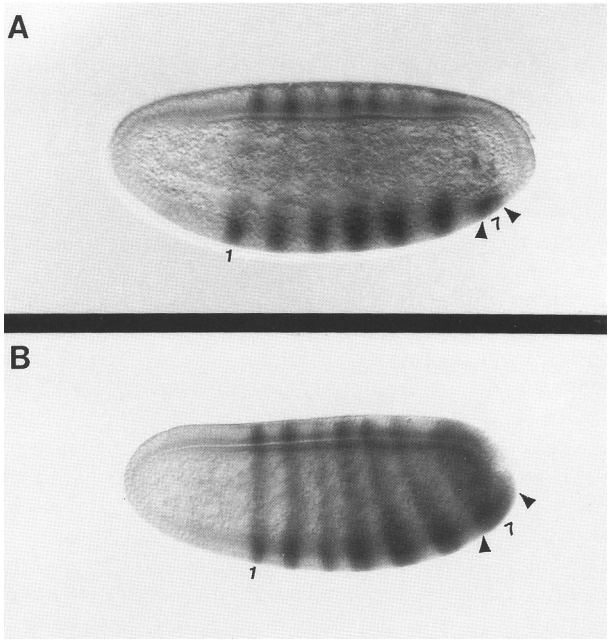

Figure 6. p21^{ras} is an intrinsic component of the *tor* signaling pathway. Embryos derived from the *4304* stock were stained using an anti-β-gal antibody at late cellular blastoderm stages to visualize the *ftz–lacZ* expression pattern. *A* and *B* are oriented with the anterior to the *left* and dorsal *up*. (*A*) The wild-type *ftz* pattern; (*B*) the altered *ftz* pattern following injection of p21^{rasN17} at 25% EL. Note that the seventh *ftz* stripe in *B* is expanded posteriorly.

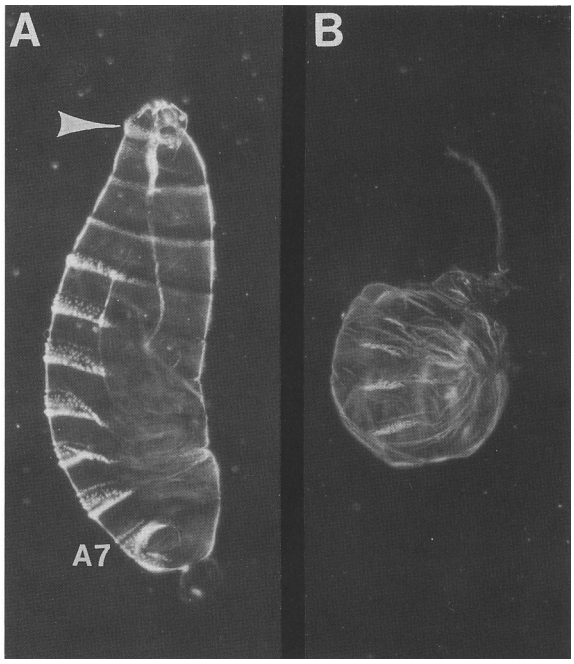

Figure 7. The maternal-effect phenotypes of the *Sos* gene. Two phenotypic classes of embryos are obtained from females carrying homozygous germ-line clones of Sos^{x122}. The embryo in *A* is a *Sos*-rescued embryo (genotype $Sos^{x122}/+$). This class of embryos has phenotypes similar to that of tor^{lof} 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^{x122}/Sos^{x122}), which shows little cuticle differentiation.

sary and sufficient to specify posterior terminal cell fates. In contrast to the clear rescue of posterior defects in tor^{XR1} embryos, injection of $p21^{v\text{-}ras}$ at the anterior of the embryos does not lead to the recovery of normal head cuticular structures although it affects the spatial expression of terminal gap genes. Because head formation requires the inputs from both the *tor* pathway and the *bicoid* patterning system (Finkelstein and Perrimon 1990; Pignoni et al. 1992), it is likely that the failure of $p21^{v\text{-}ras}$ to rescue proper head differentiation reflects the underlying balanced cross-regulation of both patterning systems. Hyperactivation of the *tor* signaling pathway following $p21^{v\text{-}ras}$ injection in embryos will result in malformation of the head skeletal structures. This is similar to the effect of tor^{gof} mutations that lead to a spliced embryonic phenotype where head structures are very abnormal (Klingler et al. 1988; Strecker et al. 1989).

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-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, $p21^{v\text{-}ras}$ 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^{lof} phenotype among wild-type embryos injected with $p21^{rasN17}$, which blocks $p21^{ras}$ activation by binding to GRF(*Sos*) competitively. This observation is consistent with the fact that many *Sos*-rescued embryos (genotype $Sos^{x122}/+$) have a phenotype weaker than that of tor^{XR1} embryos. There are a number of possibilities to explain these results. First, the *Sos* mutation used in our germ-line mosaic analyses, Sos^{x122}, 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 $p21^{ras}$/Ras1 or more downstream components remains open (Williams et al. 1992).

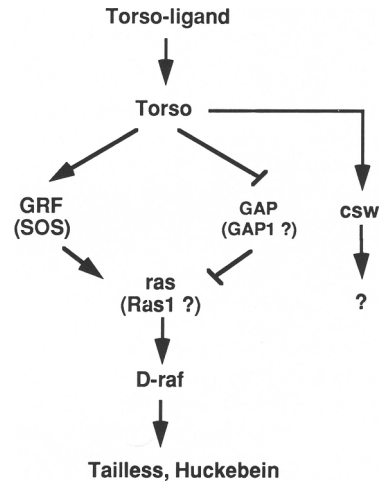

Figure 8. The updated terminal-class model. (→) Activation effects; (—|) negative regulatory effects. It is unclear whether a ras–GAP activity is involved in the terminal-class pathway (for details, see discussion). (Torso) Torso receptor tyrosine kinase; (csw) corkscrew protein tyrosine phosphatase; (GRF) guanine nucleotide-releasing factor; (Sos) son of sevenless; (GAP) GTPase-activating protein; [ras (Ras1)] $p21^{ras}$ 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 $p21^{v\text{-}ras}$ was injected anteriorly or in the central region of tor^{XR1} 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 $p21^{ras}$/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., filzkörper materials) following injection of $p21^{v\text{-}ras}$. The differential instructive effects of tor^{gof} mRNA and activated $p21^{v\text{-}ras}$ 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 *C(1)DX, y f/Y; Flp^{38}/Flp^{38}* females crossed to *w ovo^{D1} v^{24} FRT^{101}/Y; Flp^{38}/Flp^{38}* males (Chou and Perrimon 1992). Flp^{38} is the yeast site-specific recombinase under a heat shock promoter, and FRT^{101} is the Flp^{38} recognition site located at 14A. *tor* stocks are *b cn tor^{Y9} bw/CyO* and *b pr cn tor^{XR1}/CyO* (Klingler et al. 1988; Sprenger et al. 1989). A *D-raf* null allele, *D-raf^{EA75}* (Melnick et al. 1993; Perrimon et al. 1985), and a loss-of-function *Sos* allele, Sos^{x122} (Rogge et al. 1991), were used to generate *D-raf* and *Sos* embryos, respectively. *4304* expresses *lacZ* under the control of the *ftz* promoter elements. *1A121* expresses *lacZ* in both the AM and PM primordia (Perrimon et al. 1991). Two *P(hs–$Ras2^{V12}$)* transgenic lines that gave similar results are X-linked *44/22/88* and autosomal *42PG1* (Bishop and Corces 1988). Flies were raised on standard *Drosophila* media at 25°C. Chromosomes and mutations that are not described in the text can be found in Lindsley and Zimm (1992).

hs–Ras1 *constructs*

hs–Ras1 The 1.4-kb *Ras1* cDNA (EF1 in pGM) (Neuman-Silberberg et al. 1984) was cut out by *Eco*RI 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-m5.2*, and *hs–EF-f12*.

hs–$Ras1^{Q13}$ 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–$Ras1^{Q13}$)* 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 these 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–$Ras1^{Q13}$)* and *P(hs–$Ras2^{V12}$)* 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-raf^{EA75}* using the FLP-DFS technique (Chou and Perrimon 1992). Germ-line clones of Sos^{x122} were generated by X-ray treatment (Perrimon et al.1984) using a DFS mutation, *P[ovo^{D1}]^{13}*, carried by a P-element transposon insertion on 2L (T.B. Chou and N. Perrimon, in prep.). Briefly, progeny from a cross between *Sos^{x122}/SM6* virgin females and *ovo^{S1} v^{24}/Y; P[ovo^{D1}]^{13}/CyO* males were irradiated during the first-instar larval stage with 1000 rads (Torrex 120 X-ray machine; 100 kV, 5 mA, 3-mm aluminum filter) to induce germ-line clones. Under these conditions, ~2% of females with germ-line clones are recovered. *ovo^{S1} v^{24}/+; Sos^{x122}/P[ovo^{D1}]^{13}* females carrying Sos^{x122} homozygous germ-line clones were crossed to either *+/Y; Sos^{x122}/CyO* or *y^{w}/Y; +/+* males and raised at 25°C. The presence of the *ovo^{S1}* mutation on the X chromosome is to ensure complete sterility of the *ovo^{S1}, v^{24}/+; +/P[ovo^{D1}]^{13}* females (T.B. Chou and N. Perrimon, in prep.).

Injection of $p21^{v\text{-}ras}$, $p21^{c\text{-}ras}$, and $p21^{rasN17}$ proteins

The $p21^{v\text{-}ras}$ and $p21^{c\text{-}ras}$ proteins were made in *Spodoptera frugiperda* (Sf9) cells (3×10^6) infected with recombinant baculovirus encoding *$p21^{v\text{-}ras}$* or *$p21^{c\text{-}ras}$* (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 $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% NP-40) supplemented with phenylmethylsulfonyl fluoride (1 mM), aprotinin (0.15 U/ml) and leupeptin (20 μM). The lysate was centrifuged at 10,000*g* for 10 min to remove insoluble material. The cell lysates contain ~0.05–0.1 mg/ml of $p21^{v\text{-}ras}$ or $p21^{c\text{-}ras}$ proteins by Western immunoblotting. The control sample was made as lysed Sf9 cells without viral infection and contains no detectable amount of $p21^{ras}$ when assayed by Western Blotting. The $p21^{rasN17}$ 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-

cording to Campos-Ortega and Hartenstein 1985). At a concentration of 0.05–0.1 mg/ml of p21$^{v\text{-}ras}$ protein, a complete rescue of telson structures was observed (Fig. 2B,C). The amount of injected p21$^{v\text{-}ras}$ protein correlated with the rescuing activity observed. Reduction of p21$^{v\text{-}ras}$ protein present in the lysate by two- to threefold resulted in partial rescue of the *tor^{lof}* phenotype because only the dorsal straight spinules and rudimentary spiracle structures were recovered in injected *tor^{XR1}* embryos (data not shown).

X-gal antibody staining and in situ hybridization

Whole-mount in situ hybridization with *tll* 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 *ftz–lacZ* or *1A121* enhancer trap markers, virgin homozygous *tor^{XR1}* 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 p21^{ras} 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 Mannhein) 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 Axiophot microscope.

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