Ras1 Interacts With Multiple New Signaling and Cytoskeletal Loci in Drosophila Eggshell Patterning and Morphogenesis

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ABSTRACT

Little is known about the genes that interact with Ras signaling pathways to regulate morphogenesis. The synthesis of dorsal eggshell structures in *Drosophila melanogaster* requires multiple rounds of Ras signaling followed by dramatic epithelial sheet movements. We took advantage of this process to identify genes that link patterning and morphogenesis; we screened lethal mutations on the second chromosome for those that could enhance a weak Ras1 eggshell phenotype. Of 1618 lethal *P*-element mutations tested, 13 showed significant enhancement, resulting in forked and fused dorsal appendages. Our genetic and molecular analyses together with information from the Berkeley *Drosophila* Genome Project reveal that 11 of these lines carry mutations in previously characterized genes. Three mutations disrupt the known Ras1 cell signaling components Star, Egfr, and Blistered, while one mutation disrupts Sec61β, implicated in ligand secretion. Seven lines represent cell signaling and cytoskeletal components that are new to the Ras1 pathway; these are Chickadee (Profilin), Tec29, Dreadlocks, POSH, Peanut, Smt3, and MESK2, a suppressor of dominant-negative Ksr. A twelfth insertion disrupts two genes, *Nrk*, a "neurospecific" receptor tyrosine kinase, and *Tpp*, which encodes a neuropeptidase. These results suggest that Ras1 signaling during oogenesis involves novel components that may be intimately associated with additional signaling processes and with the reorganization of the cytoskeleton. To determine whether these *Ras1 Enhancers* function upstream or downstream of the Egf receptor, four mutations were tested for their ability to suppress an activated Egfr construct (λtop) expressed in oogenesis exclusively in the follicle cells. Mutations in *Star* and *l(2)43Bb* had no significant effect upon the λtop eggshell defect whereas *smt3* and *dock* alleles significantly suppressed the λtop phenotype.

RECEPTOR tyrosine kinases (RTKs) function during development in a diverse array of species. In many instances, RTKs use the Ras signaling cascade to transduce extracellular signals into the cell nucleus. Although many of these disparate RTKs share similar downstream components, those components or mechanisms that provide developmental specificity are not always clear (Tan and Kim 1999).

In *Drosophila melanogaster*, the Ras1 cascade is used to specify cell fates in different tissues and at different times. The Ras1 cascade operates downstream of the Sevenless (Sev) RTK in eye development (reviewed by Wassarman *et al.* 1995) and the Torso (Tor) RTK in terminal differentiation of the embryo (reviewed by Duffy and Perrimon 1994). Ras1 also functions downstream of the epidermal growth factor receptor (Egfr), which is used to specify cell fates in the eye, the wing, and the embryonic ventral midline and during oogenesis to pattern the primary axes of the eggshell and embryo (reviewed by Schweitzer and Shilo 1997).

Studies of Egfr signaling during oogenesis suggest that multiple signaling events occur to correctly establish dorsoventral (D/V) and anteroposterior (A/P) polarity (reviewed by Ray and Schüpbach 1996; Nilson and Schüpbach 1999). Early in oogenesis, Gurken (Grk), the presumptive ligand for Egfr, signals from the germ line to the posterior follicle cells to establish posterior follicle cell fate. Later, these posterior cells signal back to the oocyte to initiate a reorganization of the cytoskeleton within the germ cells, mediating the localization of morphogens that determine the A/P and D/V axes of egg and embryo (González-Reyes *et al.* 1995; Roth *et al.* 1995). During this cytoskeletal reorganization, the oocyte nucleus and Grk protein are positioned at the dorsal anterior corner of the oocyte; further Grk-Egfr signaling specifies the identity of the dorsal follicle cells. This initial signaling event induces an amplification cascade involving the additional Egfr ligands Spitz, Vein, and Argos, leading to the continued patterning of the dorsal follicle cells (Wasserman and Freeman 1998; reviewed by Stevens 1998). These cells go on to secrete the dorsal structures of the eggshell, including the dorsal respiratory filaments, or dorsal appendages. Mutations in *Ras1* and Ras1 cascade components such as *Raf* and *MEK* disrupt these signaling processes and lead to dorsal appendage defects (Brand and Perrimon 1994;

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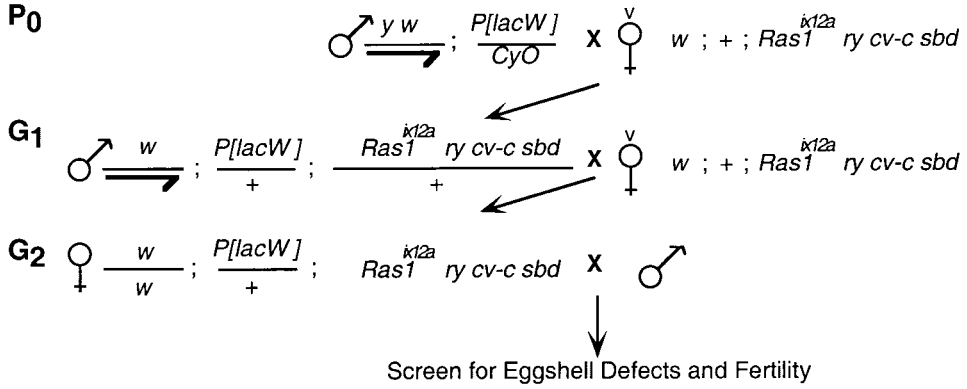

FIGURE 1.—Screen for enhancers of *Ras1*. See MATERIALS AND METHODS for details of the crosses. G_2 females were sorted into fresh vials with males and allowed to lay eggs for 2 days, and then egg morphology was screened directly in the vials under a dissecting microscope.

HSU and PERRIMON 1994; SCHNORR and BERG 1996), including forked, fused, or absent dorsal appendages. Although Ras1 cascade components are required for the specification of both the embryo and eggshell axes, mutations in genes encoding these components affect the eggshell more strongly than the embryo (HSU and PERRIMON 1994; SCHNORR and BERG 1996).

Thus, the Ras1 cascade is used in varied processes but the mechanism for evoking developmental specificity is not clear (reviewed by SIMON 2000). One possibility is that multiple signaling cascades impinge on a cell to determine its fate (reviewed by CORNELL and KIMELMAN 1994). In D/V eggshell patterning, both the TGF-α Grk and the TGF-β Dpp impinge on dorsal follicle cells to specify their identities (PERI and ROTH 2000). Alternatively, the exact level of signaling could affect kinase activity and alter effector protein function (*e.g.*, ROTH and SCHÜPBACH 1994; DYSON and GURDON 1998). Finally, the state of a cell at the time of the signaling event could alter its response. In the Drosophila eye, for example, where downstream components of the Sevenless signaling cascade have been studied intensively, the presence and activity of such proteins as Sina, Phyllopod, TTk88, Yan, and Pointed contribute to the final outcome following Ras1 activation (reviewed by RAABE 1998; DICKSON 1995). Pointed also functions in D/V axis formation, but its role may differ somewhat in oogenesis compared to R7 cell determination (MORIMOTO *et al.* 1996). These results reveal complexities that await a more thorough comparison of Ras signaling cascades throughout development.

To understand Ras signaling better, we employed a dominant modifier screen to identify components of the signaling cascade that are used during D/V patterning in oogenesis. Unlike other Egfr-dependent processes, signaling in the dorsal follicle cells leads to cell movements. We reasoned that a modifier screen would reveal novel components of the Ras1 cascade that link Egfr to morphogenesis. First we identified a weak *Ras1* allele whose activity lies on the threshold of adequate signaling during D/V axis specification. We then screened lethal, second-chromosomal mutations for dominant enhancement of a weak Ras1 eggshell phenotype. Here we describe the mutations identified as *Enhancers of Ras1*, including 11 previously characterized genes and two novel loci. Finally, we employed a gain-of-function *Egfr* construct (QUEENAN *et al.* 1997) to position a subset of these genes in the Egfr signaling pathway.

MATERIALS AND METHODS

Fly stocks and culture media: Flies were maintained on standard medium at 25°. The excision allele $Ras1^{ix12a}$ was generated by mobilizing the *P* element in the strain $Ras1^{05703}$ (SCHNORR and BERG 1996). $Egfr^{Dj}$ (CLIFFORD and SCHÜPBACH 1989), grk^{HK36} (SCHÜPBACH 1987), $okra^{RU47}$, and $chiffon^{WD18}$ (SCHÜPBACH and WIESCHAUS 1991) were provided by Trudi Schüpbach. The *P*-element collection was maintained during the screen as a collaborative effort of the Seattle area fly community. Most of these lines were generated by TÖRÖK *et al.* (1993) and are part of the Berkeley *Drosophila* Genome Project (BDGP; SPRADLING *et al.* 1995, 1999). *Df(3R)by10* (85D8-11;85E10-13), bs^{ba}, bs^{3}, *l(2)04493 (smt3)*, and $Df(2L)ast^{2}$ were obtained from the Bloomington Stock Center. $dock^{P1}$, $dock^{P2}$, and $dock^{3}$ were provided by Larry Zipursky and $chic^{07886}$ was provided by Lynn Cooley.

***Ras1* enhancer screen:** The breeding scheme used to introduce and screen the *P*-element mutations in the $Ras1^{ix12a}$ background is depicted in Figure 1. In P_0, males from the *P*-element strain to be tested were crossed with virgin females carrying the $Ras1^{ix12a}$ mutation in a *w* background. In G_1, *w*/*Y*; P[*lacW*]/+; $Ras1^{ix12a}$ *cv-c sbd*/+ males were sorted by their w^+ eyes and wild-type bristles. These males were backcrossed to the *w*; $Ras1^{ix12a}$ strain. In G_2, females of the genotype *w*; P[*lacW*]/+; $Ras1^{ix12a}$ *cv-c sbd* were selected by their w^+ eye color, stubbloid bristles, and crossveinless wing phenotypes. Five to 15 of these tester females were transferred with sibling males into fresh vials and allowed to lay eggs for 2 days. These eggs were screened under a dissecting microscope for an increase in the frequency of fused and forked dorsal appendages. Egg defects of any kind were noted, and the eggs in vials were allowed to develop to adulthood to determine the fertility of tester females. We retested 51 positive lines, repeating the crosses with more flies, examining eggs directly on agar plates, and quantifying the number of eggs that exhibited wild-type, forked, or fused dorsal appendages; 26 lines passed this secondary screen.

Genetic characterization of *Ras1 Enhancer* lines: The 26 *Ras1 Enhancer* lines were tested by us and/or the BDGP to verify that the lethality was associated with the *P*-element insertion. Nine lines interacted with *Ras1* but either contained multiple

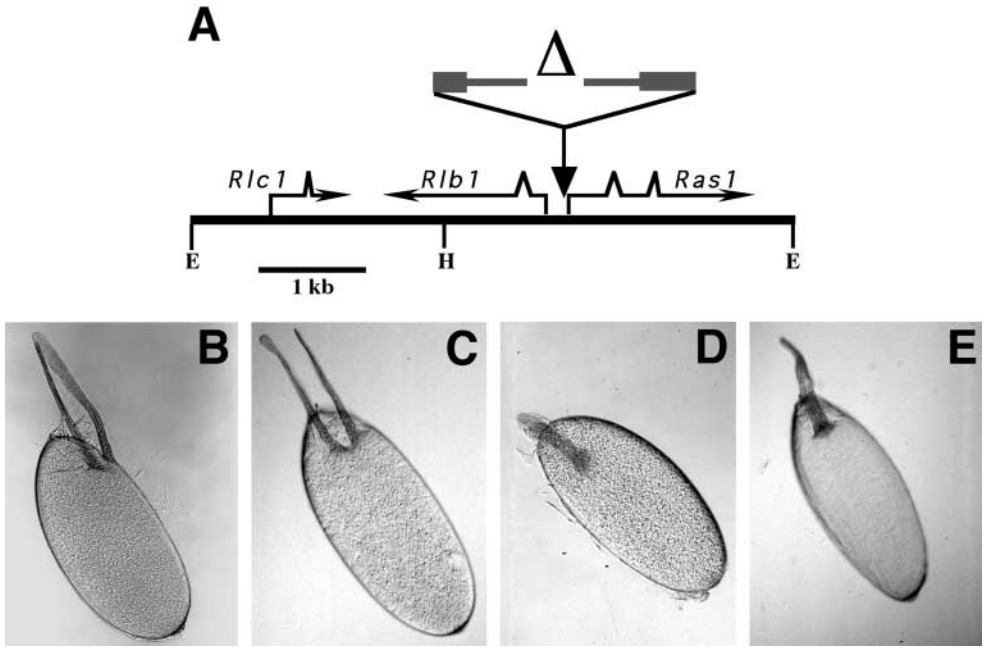

FIGURE 2.—Representative eggshell phenotypes observed in the *Ras1* enhancer screen. (A) Genomic structure of the $Ras1^{ix12a}$ excision allele. $Ras1^{ix12a}$ was created by the incomplete excision of a *PZ* element leaving 2 kb of transposon sequence just 5′ to the *Ras1* gene. We have previously shown by transformation rescue that effects on the *Rlb1* gene do not contribute to the observed phenotypes (SCHNORR and BERG 1996). (B) Wild-type egg. (C) Egg from a $Ras1^{ix12a}$ female. Any one female lays eggs that range from wild type to those exhibiting a slight expansion of the chorion at the base of the dorsal appendages. (D) Single dorsal appendage phenotype of $Ras1^{ix12a}/Df(3R)by10$, a deficiency that uncovers the *Ras1* region. (E) Egg from an $Egfr^{Df}/+$; $Ras1^{ix12a}/Ras1^{ix12a}$ female demonstrating enhancement of the $Ras1^{ix12a}$ eggshell phenotype by a member of the Ras signaling pathway.

P-element insertions or the lethality was not uncovered by a deficiency for the region. These lines and their degree of interaction were: strong interactors: *l(2)k05617*, *l(2)k05215*; moderate interactors: *l(2)k15201*, *l(2)k09403*, *l(2)k05725*; and weak interactors: *l(2)03832*, *l(2)k09610*, *l(2)k07312*, and *l(2)k14901*. In all of the remaining 17 cases presented in this article, the BDGP demonstrated that the lethality is uncovered by a deficiency for the region. In some cases, the *P* element fails to complement mutations in a known gene that maps to the region. In addition, we generated *Ras1 Enhancer* excision alleles as described previously (HOROWITZ and BERG 1995), with assistance from Jennifer Epler, Carrie Mitchell, and Matthew Quinn, for the following lines: *Star (l(2)k09312)*, *chic (l(2)k13321)*, *smt3 (l(2)k01211)*, *Tec29A (l(2)k00206)*, *bs (l(2)k07909)*, *Sec61β (l(2)k03307, l(2)k07819b, l(2)k16004)*, and *Nrk/Tpp (l(2)k014301)*. Females carrying viable, sterile excision alleles of *Star*, *smt3*, and *chic* produced eggs with a variety of ventralizing and morphogenetic eggshell defects (data not shown). Finally, we verified enhancement of the eggshell phenotype by using an independently derived allele of the following genes: *smt3*, *chic*, *Egfr*, and *DSec61β*. In the remaining lines, a formal possibility exists, albeit an unlikely one, that the genetic enhancement observed is due to a second chromosome mutation that is not associated with the *P*-element insertion.

Plasmid rescue and sequence analysis of DNA neighboring integrated transposons: To rescue DNA flanking the transposon insertion site, we followed a protocol essentially as described in ASHBURNER (1989) except that electrotransformation using a Bio-Rad (Hercules, CA) Gene Pulser was employed. To sequence flanking DNA, we conducted cycle sequencing reactions using ABI Big Dye terminators analyzed at the University of Washington biochemistry sequencing center on an ABI 377XL DNA sequencer. We used a primer recognizing the *P*-element end, 5′ GCTCTAGACGGGACCAC CTTATGT 3′.

Suppression of activated Egfr: We first tested whether each *Ras1 Enhancer* could suppress the *Egfr* allele *Ellipse*, a gain-of-function mutation that produces rough eyes when heterozygous. All the mutations were tested *in trans* to *Ellipse* (*Ellipse/Enhancer of Ras1*), but no amelioration of the rough eye phenotype was apparent under the dissecting microscope.

The activated *Egfr* (λ*top*) and Gal4 lines CU1 and T155 were provided by T. Schüpbach. The λ*top* line was generated by QUEENAN *et al.* (1997) and contains a transgene on the X chromosome encoding the λ Repressor dimerization domain fused to the *Egfr* intracellular domain and driven by the UAS yeast promoter, which requires the Gal4 protein for expression (BRAND and PERRIMON 1993). The T155 and CU1 lines express Gal4 protein in all the follicle cells beginning around stage 9 of oogenesis. Females of genotype *w* λ*top*/+; CU1/+ or *w* λ*top*/+; T155/+ produced dorsalized eggs or eggs with excess chorion blebs. We observed increasingly severe phenotypes at higher temperatures.

To test for suppression of the λtop phenotype, we crossed *w* λ*top*; *Pin/CyO* virgins to *w*/Y; *P*[*lacW*]/CyO males. Male progeny of genotype *w* λ*top*/Y; *P*[*lacW*]/*Pin* were then crossed to females carrying Gal4 enhancer lines, *w*; +/+; T155 or *w*; CU1/CyO. We then compared the phenotypes observed in females of genotype *w* λ*top*/*w*; *P*[*lacW*]/CU1 to those of genotype *w* λ*top*/*w*; *CU1/Pin*. Males from this cross did not carry the λ*top* transgene and appeared wild type.

RESULTS

$Ras1^{ix12a}$ is an excision allele derivative of a *P*-element-induced mutation ($Ras1^{05703}$) and retains 2 kb of transposon sequence 5′ of the *Ras1* gene (Figure 2A). This mutation causes a weak loss-of-function phenotype, presumably by reducing the amount of wild-type protein (SCHNORR and BERG 1996). Flies homozygous for the $Ras1^{ix12a}$ allele lay nearly wild-type eggs (Figure 2C), but

TABLE 1

Enhancers of Ras1

BDGP no.[a]	Enhancement[b]	Location[c]	Gene[d]	Comments[e]
l(2)04723	Moderate	21D3-4	*dock*	Intron 1
l(2)k09312	Strong	21E2-3	*Star*	Exon 1
l(2)k13321	Weak	26A5-6	*chic*	Intron 3: see Figure 3
l(2)k01211	Moderate	27C7-8	*smt3*	10 bp 5′ to txn start
l(2)k00206	Strong	29A1-2	*Tec29A*	Intron 5 mRNA AB009841
l(2)04614	Moderate/strong	43B1-2	*l(2)43Bb*	See text
l(2)02502	Moderate	44C1-2	*pnut*	Exon 1
l(2)k14301	Moderate/strong	49F7-8	*Nrk/TppII*	Exon 1 *Nrk*, intron 1 *TppII*
l(2)k11507	Weak	54C1-2	*POSH*	~300 bp 5′ to txn start
l(2)k05623	Weak	57E6-7	*l(2)01467*	Exon 1 CG15668 = *MESK2*
l(2)k05115	Strong	57F1-2	*Egfr*	Not determined
l(2)k07909	Moderate/strong	60C7-8	*bs*	Intron 2

[a] Berkeley *Drosophila* Genome Project strain designation.

[b] Phenotypic enhancement of a weak, homozygous *Ras1* eggshell mutant. Eggs were collected and phenotypes quantified. $Ras1^{ix12a}$ alone: >70% wild-type dorsal appendages. Weak enhancement: 41–50% wild-type dorsal appendages. Moderate enhancement: 31–40% wild-type dorsal appendages. Moderate/strong enhancement: 21–30% wild-type dorsal appendages. Strong enhancement: 0–20% wild-type dorsal appendages.

[c] Cytological location of the *P*-element insertion as reported by the BDGP; insertion site may differ slightly from gene location as reported in Gadfly.

[d] Gene affected as determined by complementation or insertion site or as reported by BDGP.

[e] Insertion site of *P* element. Note, many insertions disrupt alternatively spliced mRNAs.

when the $Ras1^{ix12a}$ allele is placed *in trans* to a deficiency for the region, females lay eggs with a single dorsal appendage (Figure 2D). Heterozygous mutations affecting other genes of the D/V pathway such as *Egfr* and *gurken* result in an increase in eggshell defects in the $Ras1^{ix12a}$ homozygous background (Figure 2E). These interactions appear specific for the Ras1 pathway since other oogenesis mutations such as $pipsqueak^{2403}$, $okra^{Ru47}$, or $chiffon^{WD18}$ modify the $Ras1^{ix12a}$ phenotype weakly or not at all (data not shown). These characteristics suggest that the $Ras1^{ix12a}$ allele is barely adequate for the correct patterning of the eggshell and is sensitive to perturbations in the level of Ras1 signaling.

Based on these observations, we conducted a genetic screen to identify heterozygous mutations that enhance the weak $Ras1^{ix12a}$ eggshell phenotype. Using the scheme in Figure 1, we screened approximately 2000 existing, second chromosome, lethal, *P*-element-induced mutations for an enhancement of the $Ras1^{ix12a}$ phenotype. In the primary screen, eggs were examined through the vial and defects were identified and roughly quantified. Of 1618 successfully tested lines, 51 (3.2%) showed some level of preliminary enhancement. Strongly enhancing mutations exhibited low fecundity and severely defective eggshells, but none possessed a phenotype as severe as that observed with $Egfr^{Df}/+$; *Ras1* or $grk^{HK36}/+$; $Ras1^{ix12a}$. Weakly enhancing mutations exhibited only slightly increased eggshell defects compared to those observed in the $Ras1^{ix12a}$ line alone. When these lines were retested by collecting eggs on agar plates and quantifying phenotypes precisely, only 26 showed consistent enhancement of the $Ras1^{ix12a}$ phenotype. Of these lines, 9 were set aside (see MATERIALS AND METHODS) due to the existence of multiple *P*-element insertions on the chromosome or to lethality not associated with the cytological location of the *P* element as determined by the BDGP. The remaining 17 lines were selected for further study.

We conducted genetic and molecular tests to determine the identity of the recovered lines. These tests were guided by the cytological map position of the *P* element as determined by the BDGP. In addition, we generated excision alleles for most lines to verify that the lethality was due to the *P* element. Finally, we employed plasmid rescue to clone the genomic DNA flanking the 5′ and 3′ ends of the *P*-element transposon from most lines. We then sequenced a portion of the cloned DNA directly flanking the *P* element and conducted a BLAST database search to identify homologous genes. The results of the genetic and molecular characterization of the *Ras1 Enhancers* appear in Tables 1 and 2.

Identity of mutant lines

Cell signaling components: Seven of the *Ras1 Enhancers* have been implicated previously in cell signaling or are genes with significant homology to previously characterized cell signaling molecules.

Egfr and Star, known signaling molecules in oogenesis: Line *l(2)k05115* strongly enhances the Ras1 eggshell phenotype and is a *P*-element allele of *Egfr* (BDGP). Likewise, line *l(2)k09312* is a strong enhancer and is affecting a previously characterized gene involved in signaling, *Star*. Complementation tests and sequence

TABLE 2

***DSec61β* alleles exhibit dominant eggshell phenotypes**

BDGP no.	Enhancement	Location[a]	Gene[b]	Comments[c]
l(2)k03307	Strong[d]	51B7-8	*DSec61β*	
l(2)k03316	Strong[d]	51B7-8	*DSec61β*	
l(2)k07819b	Strong	ND	*DSec61β*	43 bp 5′ to gene
l(2)k16004	Thin chorion[d,e]	51B7-8	*DSec61β*	Exon 1 of gene
l(2)k17010	Thin chorion[d,e]	51A 53C-E	*DSec61β*	Multiple insert line Fails to complement *l(2)k03307*

[a] Cytological location of the *P*-element insertion as reported by the BDGP.
[b] Gene affected as determined by Valcárcel *et al.* 1999 and complementation reported by BDGP.
[c] Insertion point of *P* element as determined by our sequence analysis of flanking DNA.
[d] Although these mutations demonstrate strong enhancement of a Ras1 eggshell phenotype, eggs from the starting line, *P[lacW]*/CyO, show dominant eggshell phenotypes including forked and fused appendages.
[e] Thin chorion is used to describe laid eggs that appear translucent, ruptured, or flaccid and exhibit highly variable and underdeveloped appendages. These eggs appear to have poorly developed chorions and suffer mechanical damage when laid.

analysis of the *P*-element insertion site confirmed that *l(2)k09312* is affecting the *Star* gene. *Star* is required to achieve adequate levels of signaling through *Egfr* (Heberlein *et al.* 1993; Sturtevant *et al.* 1993; Kolodkin *et al.* 1994) and encodes a single-pass transmembrane protein thought to act in processing Egfr ligands (Kolodkin *et al.* 1994; Pickup and Banerjee 1999; Bang and Kintner 2000). In addition, previous work reveals a role for *Star* in oogenesis (Ruden *et al.* 1999). We expected to recover known components of the Ras1 signaling pathway and the recovery of $Egfr^{k05115}$ and $Star^{k09312}$ confirms the efficacy of our approach and the suitability of the *Ras1* background in conducting this screen.

Neurospecific receptor kinase/tripeptidyl-peptidase II: The *l(2)k14301* mutation maps to cytological position 49F7-8. Sequence analysis of the flanking DNA demonstrated that the *P* element in *l(2)k14301* is inserted into a complex region containing overlap between two genes. Our data reveal that *l(2)k14301* is located within the 5′ end of an alternative-splice form of the *neurospecific receptor kinase* gene (*Nrk*), a putative RTK related to the *Trk* and *Ror* families of RTKs. Nrk exhibits 47% identity and 64% similarity to homologs in chicken, rat, mouse, and human. Although no mutant alleles of this gene have been characterized previously, the Nrk protein does exhibit activities expected of RTKs, including the ability to autophosphorylate *in vitro* (Oishi *et al.* 1997). Transcripts from this locus are expressed uniformly in follicle cells throughout oogenesis (C. J. Mitchell and C. Berg, unpublished data). BDGP analyses of the region suggest that a second gene, *tripeptidyl-peptidase II* (*TppII*), also produces an alternatively spliced form that may be disrupted by *l(2)k14301*. In this case, the *P* element is located in the first intron. TppII is 38% identical to human and rat proteins that degrade neuropeptides by attacking their amino termini and releasing three amino acid fragments. Purified Drosophila TppII enzyme exhibits many of the same biochemical and pharmacological properties as its distant relatives (Renn *et al.* 1998). Like *Nrk*, no mutant alleles specific for this locus are known.

blistered: The *l(2)k07909* mutation maps to 60C7-8 and fails to complement alleles of *blistered* (*bs*), the Drosophila homolog of the Serum Response Factor (SRF). *bs* encodes a MADS domain containing transcription factor involved in the regulation of tracheal development and the formation of intervein tissue in the wing (Guillemin *et al.* 1996; Montagne *et al.* 1996), processes dependent on the function of the RTKs FGF-R (*breathless*) and Egfr. Human SRF interacts with Ets domain containing proteins that in turn are activated by the mitogen-activated protein (MAP) kinase signaling pathway (reviewed by Treisman 1994).

Plenty of SH3s: The *l(2)k11507* mutation maps to 54C7-8 and fails to complement *l(2)k15815*. Sequence analysis of flanking DNA reveals that *l(2)k15815* is inserted in the first exon of *POSH*, *Plenty of SH3s* (BDGP), which encodes a putative scaffolding protein first identified by two-hybrid analysis with mouse RAC protein (Tapon *et al.* 1998). POSH contains one Zinc-C3HC4 ring-finger domain and four Src-homology-3 domains, strongly suggesting a function as an adapter protein that facilitates complex formation with multiple signaling molecules. Within the ring-finger domain, Drosophila POSH exhibits 31% identity and 47% similarity to Mus POSH while the four SH3 domains exhibit ~53% identity and ~66% similarity to this vertebrate homolog.

Tec29A: The *l(2)k00206* mutation maps to 29A1-2 and was identified by Guarnieri *et al.* (1998) and Roulier *et al.* (1998) as an allele of the *Tec29A* gene, which encodes a cytoplasmic tyrosine kinase of the Src family. *Tec29* is also called *Btk29* and was formerly called *Scr29A*. In Drosophila, mutations in *Tec29* disrupt the regulated

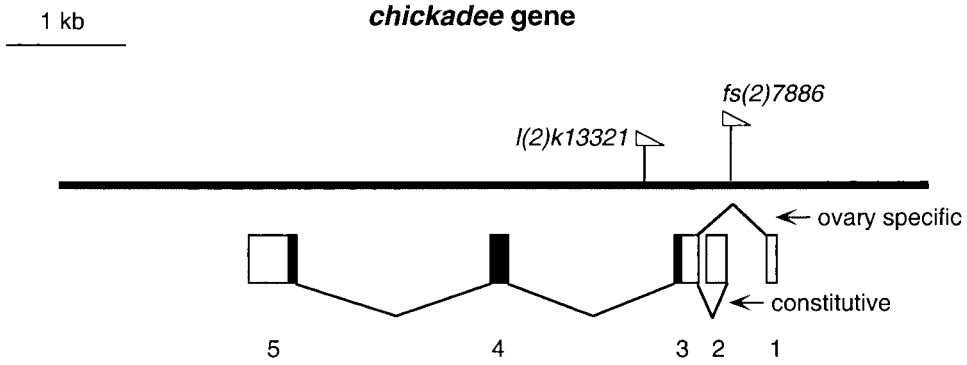

FIGURE 3.—Position of the *l(2)k13321 P* element in the *chickadee* gene. *chickadee* gene organization and position of *chic*$^{fs(2)7886}$ as determined by COOLEY *et al.* (1992). Sequence analysis of flanking DNA surrounding the *chic*$^{l(2)13321}$ insertion places the *P* element in the intron just 3′ of exon 3. Alternative 5′ untranslated regions lead to the production of an ovary-specific transcript shown above and a constitutive transcript depicted below the exons. Open boxes represent untranslated exons while solid boxes represent coding exons. Note that both transcripts use exons 3, 4, and 5. The lethal phenotype and insertion site of *l(2)k13321* are consistent with disruption of the constitutive transcript (see DISCUSSION).

growth of actin bundles in the ring canals that connect germ-line cells of the 16-cell cyst (GUARNIERI *et al.* 1998; ROULIER *et al.* 1998).

dreadlocks: The *l(2)04723* mutation maps to 21D3-4 and was identified by GARRITY *et al.* (1996) as an allele of *dreadlocks* (*dock*) in a screen for axon guidance mutants. *dock* encodes an SH2/SH3 adapter protein related to Grb2 and Nck (GARRITY *et al.* 1996); its function in axon guidance may be to modulate cytoskeletal changes within the growth cone in response to tyrosine kinase signaling (RAO and ZIPURSKY 1998).

Cytoskeletal components: In addition to *Tec29A* and *dock*, whose products link signaling pathways to the cytoskeleton, we identified two *Ras1*-interacting genes that function directly in cytoskeletal regulation.

chickadee (chic; Drosophila homolog of Profilin): Sequence analysis of flanking DNA revealed that the *l(2)k13321 P* element is inserted into the *chickadee* gene, which encodes the Drosophila Profilin homolog. The *P* element is located within an intron about 200 bp distal from exon 3 (Figure 3). The identity of the *l(2)k13321* line was independently determined by complementation tests conducted by the BDGP.

The *chic* gene contains two promoters with alternative 5′ untranslated regions that connect to a common coding region (Figure 3). Transcription driven from these promoters leads to either ovary-specific or constitutive mRNAs encoding exactly the same protein (COOLEY *et al.* 1992; VERHEYEN and COOLEY 1994). Loss of the ovary-specific transcript results in female sterility (*e.g.*, allele *chic*^{07886}) while reduction or disruption of the constitutive transcript is lethal. Given the position of the *P* element between two coding exons, we hypothesize that the lethal *chic*^{k13321} allele disrupts both the constitutive and female-specific transcript.

We asked whether the loss of Profilin protein only in the germ line would result in the enhancement of the *Ras1*^{ix12a} phenotype. We examined a female sterile allele of *chic*, *chic*^{07886}, that does not disrupt *chic* expression in the follicle cells. *chic*^{07886} in the *Ras1*^{ix12a} background produces a level of enhancement (*chic*^{07886}/+; *Ras1*^{ix12a} = 29% and +/+; *Ras1*^{ix12a} = 1.4% single dorsal appendage eggs) comparable to that observed using the *chic*^{k13321} allele (*w; chic*^{k13321}/+; *Ras1*^{ix12a} = 18% and *w;* +/+; *Ras1*^{ix12a} = 2.4%). This result suggests that a germ-line requirement for *chic* mediates D/V axis formation through the *Ras1* signaling process. This result is consistent with work by MANSEAU *et al.* (1996), who showed that female sterile alleles of *chic* produce weakly ventralized eggshells and interact with mutations in another germ-line cytoskeletal gene, *cappuccino*, to produce strongly enhanced eggshell defects.

peanut: The BDGP reported that *l(2)02502* maps to 44C1-2 and fails to complement mutant alleles of the *peanut* (*pnut*) gene, which encodes a Drosophila septin (NEUFELD and RUBIN 1994). Septins are involved in regulating the cytoskeleton during septum formation in various cell types (*e.g.*, during cytokinesis; reviewed by COOPER and KIEHART 1996; FIELD and KELLOGG 1999). In Drosophila, mutations in the *pnut* gene interact with *seven in absentia* (*sina*) during induction of the R7 cell in the eye (CARTHEW *et al.* 1994; NEUFELD and RUBIN 1994).

***l(2)k01211* is inserted 5′ of the *smt3* gene:** The genomic DNA flanking the *P*-element insertion *l(2)k01211* was rescued, sequenced, and found to be homologous to human UBL1, a ubiquitin-like protein. More thorough sequencing of the region revealed that the *P* element is ~10 bp upstream of the first exon of a gene called *smt3* (HUANG *et al.* 1998), originally cloned because of its homology to a family of ubiquitin-like (Ubl) proteins. The Ubl family represents a new class of ubiquitin-related proteins that share weak overall homology with ubiquitin (~20% identity) but have functional features in common such as a diglycine repeat at the carboxyl terminus. The Drosophila protein Smt3 shares 75 and 52% identity with the human Ubl proteins SMT3B and SMT3C (also known as SUMO-1), respectively, and 48% with Smt3p from the yeast *Saccharomyces cerevisiae*.

The BDGP reported that *l(2)k01211* failed to complement the lethality associated with *l(2)04493*. *l(2)04493* also maps to a region about 10 bp upstream of the first

exon of *smt3*. BDGP reports the other nearest transcription unit, CG8749 (*snRNP70K*), is over 3 kb upstream, suggesting that *l(2)04493* and *l(2)k01211* affect the *smt3* transcript. We tested the interaction of *l(2)04493* in a $Ras1^{ix12a}$ background and found levels of enhancement comparable to that observed with *l(2)k01211* (57% fused and forked dorsal appendages compared to 60% fused and forked dorsal appendages). Moreover, in crosses between *l(2)04493* and *l(2)k01211*, we observed rare *trans*-heterozygous escapees (0.76%; $n = 4445$) of genotype *l(2)04493/l(2)k01211*. These adult females produced few late-stage egg chambers and these rare eggs exhibited poorly developed dorsal appendages (our unpublished observations). These observations are consistent with a role of *l(2)k01211* in dorsal appendage formation.

Novel genes: Two *Ras1 Enhancer* lines, *l(2)04614* and *l(2)k05623*, affect previously uncharacterized genes. The BDGP has verified that these lines carry only one *P* element and that the lethal phenotype is closely linked to the insertion. We employed plasmid rescue to obtain flanking DNA for *l(2)k0562* but were unable to clone out flanking DNA from *l(2)04614*. Complementation analysis by BDGP, however, indicates that *l(2)04614* is an allele of *l(2)43Bb*, an essential gene identified by saturation mutagenesis of region 43A-E (Heitzler *et al.* 1993). Comparison of the exquisite fine map data from these authors and the detailed molecular map from BDGP identifies four candidate genes that may define *l(2)43Bb*. The most likely candidate encodes a protein with homology to cell adhesion molecules of the Notch and Delta family (CG11101). Alternatively, three other putative genes flank this locus and may represent *l(2)43Bb*; these genes encode proteins of no known function.

Following plasmid rescue, we sequenced flanking DNA from *l(2)k05623* and compared these data to BDGP genomic and expressed sequence tag databases. Alignment of the *P*-element insertion site, cDNA sequences, and genomic contigs revealed that *l(2)k05623* is inserted into the first exon of CG15668. The protein encoded by this gene shares homology with human and rodent proteins identified through differential expression techniques. Transcripts from these genes are upregulated during normal brain development or following Nickel induction or N-myc overexpression (Kokame *et al.* 1997; Shimono *et al.* 1999; Yamauchi *et al.* 1999). The Drosophila protein exhibits ~29% identity and 45–49% similarity to these mammalian homologs, now termed NDR1, -2, or -3 (N-myc Downstream Regulated). In a complementary study that strongly supports our findings, Huang and Rubin (2000) identified this locus in a screen for misexpression suppressors of dominant negative Ksr, a kinase with unknown substrates that acts downstream of Ras in eye development. They named the gene *MESK2*.

Dominant eggshell defects result from mutations in *DSec61β*: Not unexpectedly, we identified a number of lines that exhibit phenotypes even in the absence of the $Ras1^{ix12a}$ mutation (Table 2). These five lines show strong *Ras1* enhancement and fail to complement one another for viability. Three of these mutant lines have been mapped by the BDGP to 51B7-8 by *in situ* hybridization. Although these mutations produce significant enhancement of the Ras1 phenotype, some alleles in this group produce eggshell defects as heterozygotes in a $Ras1^{+}$ background. *l(2)k17010/CyO* and *l(2)k16004/CyO* females lay some eggs that have translucent eggshells with short or absent dorsal appendages; these females are

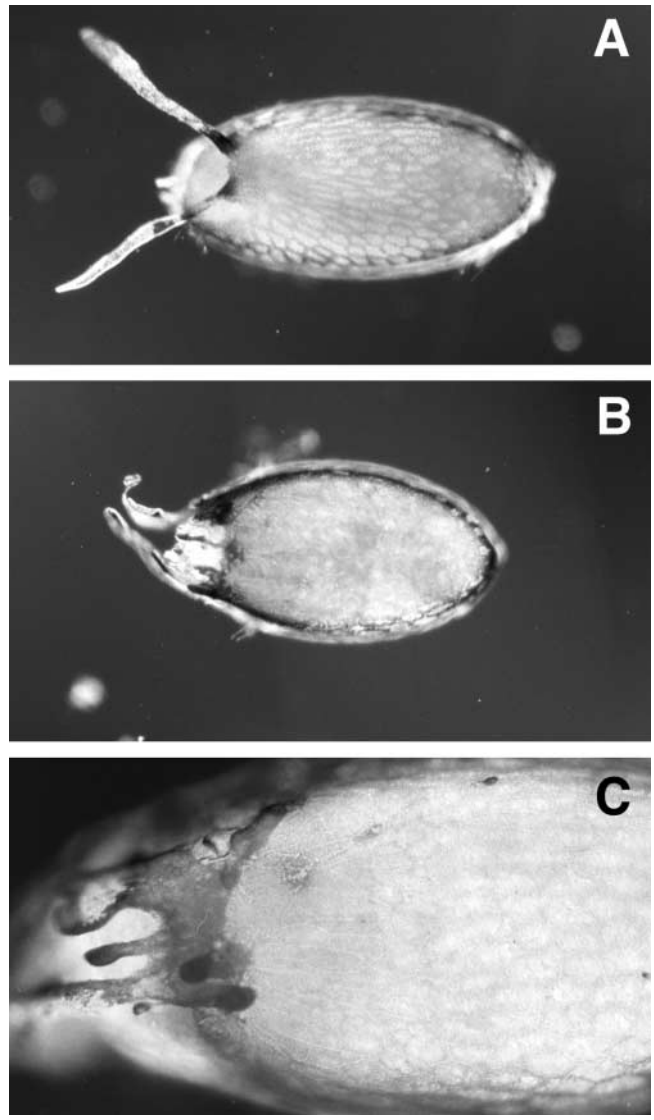

Figure 4.—Representative phenotypes observed in eggs from females carrying an activated *Egfr* (λ*top*). (A) Egg from a wild-type female. (B) Egg from a female of genotype *w UAS-*λ*top/w; CU1-GAL4/Pin* raised at 25°. We observed excess chorion material concentrated at the anterior end of the eggshell, similar to some dorsalized phenotypes. (C) A higher magnification view of egg from *w UAS-*λ*top/w; CU1-GAL4/Pin* female showing excess blebs of chorion associated with the anterior eggshell.

weakly fertile. In contrast, *l(2)k07819b* fails to complement the lethality associated with these lines, interacts with *Ras1* to produce strong dorsal appendage defects, but does not produce thin chorions.

Sequence analysis of flanking DNA from *l(2)k16004* revealed that this *P* element is inserted in the 5′ end of the *Sec61*β gene, which encodes a subunit of a putative protein translocation channel (Valcárcel *et al.* 1999). The *l(2)k07819b P* element, however, is inserted just upstream of the gene, and this more 5′ position could account for the difference in phenotype.

Suppression of an activated *Egfr*: Since our screen can identify mutations that affect either the germ line (*e.g.*, *chic*) or the follicle cells, we sought an epistasis test that could quickly distinguish whether a new component functions upstream of Egfr in the germ line or downstream of Egfr in the follicle cells. We made use of an activated *Egfr* construct that could be selectively expressed in the follicle cells of the developing eggshell using the *Gal4-UAS* system (Brand and Perrimon 1993; Queenan *et al.* 1997). Egfr (torpedo = top) is activated by replacement of its extracellular domain with the λ Repressor dimerization domain (λtop) and contains a UAS regulatory region upstream. We employed two Gal4 enhancer lines, CU1 and T155, to drive expression in a majority of the follicle cells. Previous reports using this system suggested that GAL4-driven expression is highly sensitive to temperature, and we found suitable phenotypes between the temperatures of 22° and 25° to test for suppression. At 22°, λ*top/+*; *Gal4-CU1/+* females lay some eggs that appear wild type, but others have excess chorion blebs, enlarged appendages, or other features that suggest strong dorsalization (Figure 4, B and C). At higher temperatures, fewer wild-type eggs are laid and no eggs hatch. Since we observe a decrease in the number of completely wild-type chorions over this temperature range, we reasoned that the activity level of λtop is at a threshold, and a small decrease in activity when the system is held at 25° should result in a higher frequency of wild-type eggshells.

Because of the large number of lines and difficult nature of the assay (see discussion), we focused the suppression analysis on a subset of *Ras1 Enhancer* mutations. We tested four mutations of the collection for their ability to suppress λtop (see Table 3): two new genes (*smt3* and *l(2)43Bb*) that could act in either germ cells or follicle cells and therefore lie upstream or downstream of Egfr, one moderate enhancer (*dreadlocks*) that we suspected acts downstream of Egfr, and one strong enhancer (*Star*) that we suspected acts upstream of or in parallel to Egfr. Experimental flies of the genotype λ*top/+; smt3*$^{l(2)k01211}$*/CU1* showed a higher frequency of wild-type eggshells at 25° than did the control females of genotype λ*top/+; +/CU1*. This result suggests that *smt3*$^{l(2)k01211}$ is epistatic to *Egfr* and operates downstream of the receptor in the follicle cells. We found a similar but smaller effect for *dock*^{04723}. Conversely, we found no difference in phenotype for tests conducted with the *Star*^{k09312} and *l(2)43Bb*^{04614} alleles, suggesting that these genes are not limiting in the pathway or are not required for the function of the activated λtop protein.

TABLE 3

Suppression of activated Egfr phenotype by *Enhancer of Ras1* mutations

Gene/allele	% wild-type eggshells[a]	
	*UAS:*λ*top/+; Gal4:CU1/+*	*UAS:*λ*top/+; Gal4:CU1/Mutation*
Star *l(2)k09312*	10.3 $n = 185$	3.8 $n = 212$
smt3 *l(2)k01211*	3.3 $n = 469$	25.4[b] $n = 1029$
dock *l(2)04723*	17.1 $n = 363$	33.8[b] $n = 325$
l(2)43Bb *l(2)04614*	8.2 $n = 365$	3.1 $n = 321$

[a] Represents an average of at least two experiments (except *Star*).

[b] The higher number of *UAS:*λ*top/+; Gal4:CU1/Mutation* wild-type eggshells recovered compared to *UAS:*λ*top/+; Gal4:CU1/+* suggests that *smt3* and *dock* are suppressing the eggshell phenotype associated with the λtop construct.

DISCUSSION

In this article, we present the results of a genetic screen to identify *P*-element mutations that enhance a weak Ras1 eggshell phenotype. Although our screen methodology limited our search to the second chromosome, we nevertheless identified 13 mutations that dominantly enhance the Ras1^{ix12a} eggshell phenotype. Molecular and genetic analyses in combination with the BDGP resources have allowed us to identify a majority of the genes affected by these *P*-element insertions (Figure 5). *Ras1 Enhancers* such as *Egfr* and *Star*, whose functions in Ras1 signaling have been clearly demonstrated, reveal the efficacy of our approach. Our results also make a genetic connection between a number of previously identified signaling molecules, such as Dreadlocks and Tec29, and Ras1-mediated signaling during oogenesis. Finally, several *Ras1 Enhancers* have been implicated previously in regulating cytoskeletal structure or function. This result suggests that, in addition to D/V patterning, Ras1 may also function during egg morphogenesis to link signal transduction directly to the reorganization of the cytoskeleton.

Recovery of previously identified *Ras1* signaling components: Ras1 signaling downstream of the Egfr, Torso, and Sev RTKs has been studied extensively in the fly (reviewed by Wassarman *et al.* 1995). Consequently, we expected to recover mutations in second chromosomal

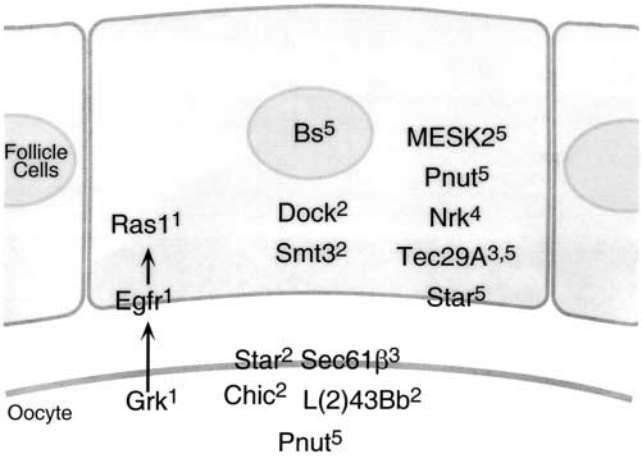

FIGURE 5.—Model depicting the possible site of function of proteins encoded by various *Ras1 Enhancers* in the egg chamber. A developing egg chamber consists of the germline-derived oocyte (below) surrounded by a layer of somatic follicle cells (above, gray shading). The Grk ligand is produced in the oocyte and activates the Egf receptor within the follicle cells. We have portrayed the site of protein activity as in either the oocyte or the follicle cells or in both tissues. These tentative positions are based on the following as indicated by superscript numbers: (1) mosaic analysis, (2) genetic epistasis tests or tissue-specific phenotypes, (3) mosaic analysis in germ-line only, (4) expression analysis of mRNA (data not shown), (5) work in other tissues. The proteins Pnut, Tec29A, Chic, Star, and Sec61β have been localized to the germ line or the follicle cell layer through genetic methods or immunostaining. In some cases (Chic, Pnut) the proteins are found in both tissues but their site of interaction with the Ras pathway is not definitively established (see DISCUSSION). The functions of the proteins Nrk, Smt3, Tec29A, POSH, MESK2, and Dock (follicle cells) have not been examined directly within this tissue but genetic and molecular evidence is consistent with these assignments (see DISCUSSION). In addition, epistasis tests with the activated Egfr construct support a function for Smt3 and Dock in the follicle cells.

genes known to function in Ras1 signal transduction, as well as genes necessary for the D/V patterning of the eggshell. Indeed, we recovered *l(2)k05115*, an allele of *Egfr*.

Another *Ras1* pathway member we identified is *Star*, a member of the *Spitz* group of genes that functions during Egfr-mediated formation of the embryonic ventral midline. *Star* mutations appear repeatedly in screens for RTK-related eye phenotypes (HEBERLEIN and RUBIN 1991; MA *et al.* 1996) and these *Star* alleles suppress gain-of-function mutations affecting *Egfr* and *sev* signaling pathways (HEBERLEIN *et al.* 1993; STURTEVANT *et al.* 1993). In addition, a dominant female sterile allele, $Star^{Kojak}$, produces phenotypes that suggest a dual function for Star in A/P and D/V patterning in oogenesis (RUDEN *et al.* 1999). Finally, *Star* encodes a single-pass transmembrane protein (KOLODKIN *et al.* 1994); recent evidence supports the hypothesis that Star is involved in processing the Egfr ligands Spitz and Gurken (GOLEMBO *et al.* 1996; RUDEN *et al.* 1999; BANG and KINTNER 2000). Our recovery of *Star* and epistasis tests with λ*top*, which place *Star* upstream of or in parallel with *Egfr* (Figure 5), support these previous findings.

Another known *Ras1* pathway member we identified is *bs*, which encodes the Drosophila homolog of the human SRF. SRF is a MADS domain-containing transcription factor that binds the serum response element, an enhancer sequence named for its presence upstream of genes that respond to growth factor stimulation (reviewed by TREISMAN 1994). Although we have not directly tested the functional requirements of *bs* in dorsal appendage formation, it is likely that SRF acts in follicle cell nuclei (Figure 5).

Genetic observations in wing and tracheal development reveal a role for SRF in processes regulated by Egfr and FGF-R signaling pathways. In Drosophila wing imaginal discs, *bs* is expressed in the future intervein tissue in a pattern complementary to that of Rhomboid, an Egfr accessory protein that facilitates presentation of ligand (FRISTROM *et al.* 1994; BANG and KINTNER 2000). Loss-of-function mutations in *bs* interact strongly with *Egfr* and other *Ras1* signaling components in the wing and suppress the effects of disruptions in that pathway (ROCH *et al.* 1998). In contrast, *bs* mutations enhance *Ras1* defects in the egg, revealing important differences in the regulation of these two processes.

In tracheal development, *bs* functions in the terminal branching process that results from activity of *breathless* (FGF-R; reviewed by METZGER and KRASNOW 1999), an RTK that can employ the Ras signaling cascade (WHITMAN and MELTON 1992; KOUHARA *et al.* 1997). Lack of *bs* or *breathless* function eliminates cellular outgrowths and terminates tracheal branching prematurely. Thus, SRF and FGF-R act in concert to regulate cellular morphogenesis and in these ways resemble SRF and Ras1 function in dorsal appendage formation.

Finally, we identified five alleles of the *DSec61*β gene, which encodes a subunit of the DSec61 protein translocation channel (VALCÁRCEL *et al.* 1999). *DSec61*β is required for embryonic development; mutants die with poorly developed, transparent cuticles due to defective cuticle secretion. *DSec61*β has also been implicated in D/V patterning of the egg. Loss of *DSec61*β from the germ line results in variable dorsal appendage phenotypes ranging from forked to fused single dorsal appendages (VALCÁRCEL *et al.* 1999). Valcárcel *et al.* propose that DSec61β is involved in Grk secretion and that the eggshell phenotypes result from secretion of lower levels of Grk ligand from the oocyte to the overlying follicle cells.

Our identification of five alleles of *DSec61*β, some with distinct phenotypes, is consistent with two potential roles for this protein during oogenesis. Four of the five alleles produced some level of dominant eggshell phenotypes characterized by translucent and flaccid eggs with shortened dorsal appendages. This phenotype was observed in a Ras^{+} background and may be due

to a defect in chorion secretion. Nevertheless, these *DSec61*β alleles also enhanced the Ras1 dorsal appendage phenotype, increasing the number of fused and forked appendages observed in *Ras1*^{ix12a} homozygotes. This enhancement may be an additive effect between two mutations affecting coupled biological processes, patterning and secretion. One *DSec61*β allele, however, *l(2)k07819b*, enhanced the Ras1 eggshell phenotype but did not produce the dominant chorion defects observed with the other four alleles. This latter observation suggests that the *DSec61*β enhancement is specific and supports the hypothesis that the DSec61 channel participates in the secretion of Grk ligand. For these reasons, we place DSec61β in the germ line (Figure 5).

Signaling molecules new to the Ras pathway: A surprising and rewarding feature of our results is the identification of signaling and cytoskeletal components that had not been linked previously to the *Ras1* pathway. Molecules such as Dock, Tec29, POSH, and potentially Nrk or TppII function in signal transduction pathways or share homology with known signaling proteins but are new to Egfr-regulated D/V patterning processes. Molecules such as Profilin (*chic*), Peanut, and Smt3 may not be directly involved in signaling, but all are associated with cytoskeletal processes in some way. These proteins may organize the signal transduction machinery or effect the reorganization of the cytoskeleton in response to signaling.

dock is the Drosophila homolog of the mammalian oncogene *Nck* and belongs to the SH2/SH3 adapter protein family. *dock* has an essential role during axon guidance in the development of the eye, functioning in the growth cones of photoreceptor axons (Garrity *et al.* 1996; Hing *et al.* 1999; Ruan *et al.* 1999). Although the mammalian Nck protein interacts with the guanine nucleotide releasing protein, Sos (Hu *et al.* 1995), no previous evidence links Drosophila *dock* to *Sos* or the Ras1 signaling cascade. Recent proposals for Dock function in the nervous system are based on its interactions with Misshapen (Ruan *et al.* 1999) and Pak (Hing *et al.* 1999), Ste20-like kinases that may regulate growth cone changes through phosphorylation of the cytoskeleton. Our epistasis tests place *dock* downstream of *Egfr*, potentially interacting with Sos and/or the Drosophila homologs of the Ste-20-like kinases (Figure 5).

Tec29 is a non-RTK molecule that was originally identified based on its homology to *Src* kinases (Simon *et al.* 1983). Mutations in *Tec29* are embryonic lethal but clonal analyses reveal a role for *Tec29* in ring canal formation during oogenesis. Egg chambers lacking Tec29 protein in the germ cells contain ring canals that are reduced in size and lack phosphoproteins as well as Tec29 itself (Guarnieri *et al.* 1998; Roulier *et al.* 1998). In addition, the nurse cells fail to transfer their cytoplasmic contents into the oocyte, producing small eggs. In spite of these defects, developing embryos deficient for maternally provided Tec29 exhibit no D/V patterning defects; indeed, 60% of embryos hatch (Roulier *et al.* 1998). This result suggests that Tec29 interacts with Ras1 through a function in the follicle cells (Figure 5).

Drosophila POSH is homologous to a mouse protein identified in a two-hybrid screen with RAC, a Ras-like GTPase that regulates cytoskeletal function. Data from cultured cells support the hypothesis that mouse POSH mediates RAC signaling by activating the JNK cascade and inducing subsequent transcriptional changes, rather than affecting cytoskeletal structure or activity directly (Tapon *et al.* 1998). Our study is the first to demonstrate a function for this gene in Drosophila. Although we have not tested POSH function through mosaic analysis, its homology to other known signaling components suggests that POSH acts in the follicle cells (Figure 5).

l(2)k14301 is inserted at 49F7 and potentially affects two genes, *Nrk* and *TppII*. *Nrk* encodes an RTK of the Ror family (Oishi *et al.* 1997). These proteins contain a cytoplasmic tyrosine kinase domain structurally related to the Trk family of receptors but differ in that their extracellular regions contain a "kringle" domain thought to mediate protein-protein interactions (Wilson *et al.* 1993). To date, the ligand for this family of receptors is unknown. One other Drosophila gene shows homology to the Ror family of RTKs (Wilson *et al.* 1993); both genes were thought to be expressed exclusively in the nervous system. Our results reveal unexpected expression of *Nrk* in the follicle cells and suggest that additional signaling events modulate follicle cell activity during eggshell morphogenesis (Figure 5). Moreover, the existence of a second RTK in this process could explain some aspects of the differential phenotypes produced by *Ras1* compared to *grk* and *Egfr* mutations (Schnorr and Berg 1996). It is possible, for example, that Nrk interacts with Ras, Tec29, POSH, and Dock to modulate cytoskeletal structure or function. Alternatively, *l(2)k14301* could be affecting TppII, a serine protease with demonstrated activity in degrading neuropeptide signals (Renn *et al.* 1998). In mammalian systems, TppII homologs can compensate for loss of ubiquitin-mediated degradation pathways (Wang *et al.* 2000). If TppII acts in oogenesis, it may interact with Smt3 (see below) in a novel pathway to modulate signaling or cytoskeletal functions in response to Ras. Thus, the recovery of a *Nrk/TppII* mutation in our screen is an exciting result that will facilitate further analyses of these patterning and morphogenetic events.

Ras signaling and the cytoskeleton: The second main class of *Ras1 Enhancers* consists of mutations that disrupt *bona fide* cytoskeletal regulatory genes. These *Enhancers* may interact in the pathway in a variety of ways, as discussed below.

chickadee (*chic*) is the Drosophila homolog of Profilin (Cooley *et al.* 1992), a conserved actin binding protein. The *chic* gene produces both constitutive and ovary-specific transcripts through alternative promoters (see

RESULTS and Figure 3). Mutations that disrupt the ovary-specific transcript result in sterile females that produce dumpless eggs; at a low frequency, these eggs exhibit fused dorsal appendages (COOLEY *et al.* 1992; VERHEYEN and COOLEY 1994; MANSEAU *et al.* 1996). In these mutants, cytoplasmic actin cables in the nurse cells are missing. As a result, the rapid transfer of cytoplasm that occurs in late-stage 10 and 11 is disrupted when the nurse cell nuclei drift into the ring canals and impede further cytoplasmic flow.

The cause of the D/V patterning defect exemplified by the fused dorsal appendages is more obscure. The female sterile alleles do not disrupt follicle cell expression of *chic*, suggesting the defect is linked to *chic* function in the germ line. *In situ* hybridization studies, however, do not reveal a significant change in the level or degree of localization of *grk* mRNA, which encodes the TGFα-like D/V morphogen (NEUMAN-SILBERBERG and SCHÜPBACH 1993). These results suggest that the defect is more subtle than a gross perturbation of the cytoskeleton (MANSEAU and SCHÜPBACH 1989; MANSEAU *et al.* 1996). Here, we have found that mutations disrupting either the ovary transcript or both the ovary and constitutive transcript of *chic* enhance a weak Ras1 eggshell phenotype. One explanation may involve the interaction of Chic, the actin cytoskeleton, and the microtubule-based cytoplasmic streaming thought to facilitate the uniform distribution of molecules in the oocyte during nurse cell cytoplasmic transfer (THEURKAUF 1994; MANSEAU *et al.* 1996). Premature streaming may inhibit the localization or function of molecules involved in translation or secretion of the Grk morphogen. Alternatively, Chic might be necessary to form an actin-based anchoring network critical for tethering Grk protein.

Although the data suggest that Chic must have a germline function (Figure 5), these data do not rule out an additional function in the follicle cells. During egg formation, follicle cells undergo shape changes and migrations while secreting the eggshell. These functions require the reorganization and directed movement of the follicle cell cytoskeleton, possibly effected in part by the Chic protein (EDWARDS *et al.* 1997; reviewed by CARPENTER 2000).

Likewise, the current evidence concerning the function of Peanut and Smt3 links these proteins to signaling and the cytoskeleton. *peanut* (*pnut*) is one of five Drosophila *septin* genes (reviewed by FIELD and KELLOGG 1999) and was originally identified as a genetic enhancer of a weak allele of *seven in absentia* (*sina*; CARTHEW *et al.* 1994). *pnut* is required for cytokinesis and is localized to the cleavage furrow of dividing cells (NEUFELD and RUBIN 1994). How does heterozygous Pnut enhance a weak Ras1 phenotype? Both Pnut and Ras1 have been implicated in cell division (NEUFELD and RUBIN 1994; PROBER and EDGAR 2000) and a reduction in these proteins may inhibit cell division early in oogenesis resulting in later effects on egg morphogenesis. This possibility seems remote, however, since we do not observe any obvious defects in the follicular layer.

Alternatively, septins may be critical for the organization of the cytoskeleton and/or the localization of signal transduction components in the follicle cells. Septins are found in the cytoplasmic bridges during spermatogenesis (HIME *et al.* 1996), but their distribution in oogenesis differs. In nurse cells, septins are not part of the ring canals or intracellular bridges but are found in the cytoplasm, while in follicle cells, septins are specifically localized to baso-lateral surfaces (FARES *et al.* 1995). Septins also localize to areas of cortical reorganization (FARES *et al.* 1995; KINOSHITA *et al.* 1997) and thus may be important for follicle cell shape changes and migrations. In yeast, septins function to localize signaling molecules to the future bud site (reviewed by LONGTINE *et al.* 1996; FIELD and KELLOGG 1999). Thus, *peanut* may be critical for the correct localization, anchoring, and stability of the Ras1 signal transduction machinery in the follicle cells (Figure 5).

Interestingly, we also identified the ubiquitin-like gene *smt3* as a *Ras1 Enhancer*. The *smt3* gene product is a member of a new family of proteins that share many functional similarities with ubiquitin. Like ubiquitin, Smt3 proteins are post-translationally conjugated to target proteins, but the functional significance of this tagging is still under investigation. In human cells, cytosolic RanGAP1 is targeted to the nuclear pore after receiving an SMT3C (SUMO-1) protein tag (MAHAJAN *et al.* 1997). SMT3C conjugation to IκBα, on the other hand, renders the inhibitory factor resistant to ubiquitination and results in the retention of NFκB in the cytoplasm (DESTERRO *et al.* 1998). In Drosophila, the NFκB homolog Dorsal and the transcriptional repressor Tramtrack69 (TTk69) are tagged by Smt3 (LEHEMBRE *et al.* 2000). Although the function of the Smt3 tag on TTk69 is not clear, Dorsal/Smt3 conjugates appear to migrate preferentially from the cytoplasm into the nucleus (BHASKAR *et al.* 2000).

These observations suggest that Ras1 signaling may be affected by Smt3 conjugation to transcription factors. The ability of the *smt3* mutant to suppress the activated Egfr eggshell phenotype suggests that Smt3 functions downstream of the Egfr receptor in the follicle cells (Figure 5). Thus, Smt3 might be involved in the modulation of transcription factors in the follicle cells. One candidate molecule is CF2, a zinc finger transcription factor negatively regulated by Egfr signaling. CF2 is retained in the cytoplasm of cells experiencing high levels of Ras1 signaling activity (MANTROVA and HSU 1998). Smt3 may function together with MAP kinase to alter the conformation of CF2 and prevent import into the nucleus. Alternatively, Smt3 may be conjugated to transcription factors to facilitate their nuclear import. For example, Smt3 is known to show punctate nuclear staining in some tissues of the fly and interact with TTk69 (LEHEMBRE *et al.* 2000). Thus, Ras1 activation may lead

to the conjugation of Smt3 to transcription factors, leading to nuclear importation and/or modification of their binding activities (Kim *et al.* 1999).

Recently, Johnson and Blobel (1999) and Takahashi *et al.* (1999) have observed the conjugation of Smt3 protein to yeast septins. Genetic studies in yeast suggest that Smt3p may be important for the disassembly of septin rings during cytokinesis (Johnson and Blobel 1999). Considering these yeast interaction data, the identification of *pnut* and *smt3* as independent modifiers of *Ras1* in our screen suggest that the *smt3/Ras1* interaction may be dependent on *pnut*. Thus, rather than modifying a transcription factor, the Smt3 protein may affect Ras1 signaling by regulating septin dynamics and the cytoskeleton.

Together, these results suggest that effective Ras1 signaling during eggshell morphogenesis depends on molecules that control the dynamic cytoskeleton. As described above, molecules such as Chic, Tec29, Pnut, Smt3, and Dock are involved in cytoskeletal reorganization. The Ras1 pathway may require a properly assembled cytoskeletal scaffold to achieve adequate signaling levels to correctly pattern the egg. Alternatively, the Ras1 signal may induce reorganization of the follicle cell cytoskeleton during the later cell migrations and subsequent secretion of the eggshell. Such large-scale reorganization may depend on a number of these *Ras1 Enhancers*.

Future directions: The completion of the Drosophila genomic DNA sequence allowed us to rapidly identify the genes in our *Ras1 Enhancer* collection. We are still working to understand the site of function of these genes during oogenesis and their relationship to *Ras1* signaling. Suppression of the λtop construct (*smt3* and *dock* mutants) strongly suggests a function for a protein in the follicle cells downstream of Egfr. Failure to suppress the λtop (*Star* and *l(2)43Bb*), however, is more difficult to interpret and suggests the gene may not be required for λtop signaling or is not limiting in the pathway (Figure 5). We have begun clonal analysis of a subset of these genes to clarify their site of action more fully.

In conclusion, we have identified 13 *Enhancers of Ras1* that define 11 known and 2 novel genes. The vast majority of these genes encode molecules that are new to D/V patterning and also new to Ras signaling, revealing the importance of analyzing apparently similar signaling processes that occur in tissues with diverse developmental outcomes. Our study provides a wealth of new genes that link signaling and the cytoskeleton, setting the stage for analysis of the molecular mechanisms that connect patterning and morphogenesis.

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