Development 120, 2593-2600 (1994)
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Down-regulation of *Drosophila Egf-r* mRNA levels following hyperactivated receptor signaling

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SUMMARY

Internalization of ligand-receptor complexes is a well-documented mechanism for limiting the duration and magnitude of a signaling event. In the case of the EGF-Receptor (EGF-R), exposure to EGF or TGF-α results in internalization of up to 95% of the surface receptor pool within 5 minutes of exposure to ligand. In this report, we show that levels of *Drosophila Egf-r* mRNA are strongly down-regulated in epidermal cells likely to have recently undergone high levels of EGF-R signaling. The cells in which *Egf-r* mRNA levels are down-regulated express the *rhomboid* gene, which is thought to locally amplify EGF-R signaling. Widespread *Egf-r* mRNA down-regulation can be induced by ubiquitous expression of *rhomboid* or by eliminating the *Gap1* gene. These results suggest that cells engaged in intense EGF-R/RAS signaling limit the duration of the signal through a combination of short-acting negative feedback mechanisms such as receptor internalization followed by a longer lasting reduction in receptor transcript levels. Control of *Egf-r* mRNA levels by altering transcription or mRNA stability is a new tier of regulation to be considered in analysis of EGF-R signaling during development.

Key words: EGF-R, down-regulation, mRNA stability, transcription, negative feedback, *rhomboid*, *Drosophila*

INTRODUCTION

Loss-of-function mutations in a small group of *Drosophila* genes known as the ventrolateral or *spitz* group genes (including *rhomboid*, *spitz* and *Star*) result in remarkably similar embryonic phenotypes suggesting that these genes function in a common pathway (Mayer and Nüsslein-Volhard, 1988; Bier et al., 1990; Rutledge et al., 1992; Kim and Crews, 1993). Defects in ventrolateral group mutants are complex, but primarily affect the formation or differentiation of epidermal structures. A variety of evidence suggests that the ventrolateral group genes are required for hyperactivation of EGF-R signaling. The most direct support for this view is that embryos carrying moderate alleles of a gene encoding a *Drosophila* homolog of the vertebrate EGF-R family (*Egf-r*) have similar phenotypes to ventrolateral group mutants (Clifford and Schüpbach, 1992; Raz and Shilo, 1992, 1993; J. O'Neill and E. Bier, unpublished data). Further evidence implicating the ventrolateral group in EGF-R signaling is that the *spitz* (*spi*) gene encodes an EGF/TGF-α-like predicted protein (Rutledge et al., 1992). Finally, genetic interactions between *rho* and genes encoding components of the EGF-R/RAS signaling pathway, as well as between *rho* and other genes of the ventrolateral group, support models in which *rho* amplifies EGF-R signaling during wing development (Sturtevant et al., 1993; Noll et al., unpublished data). Similar genetic interactions suggest that *Egf-r* works in concert with ventrolateral group genes during embryogenesis (Raz and Shilo, 1993).

The genes encoding the EGF-Receptor and putative Spi ligand are expressed broadly throughout development (Kammermeyer and Wadsworth, 1987; Zak et al., 1990; Katzen et al., 1991; Zak and Shilo, 1992; Rutledge et al., 1992), while expression of the *rhomboid* (*rho*) gene is highly localized and predominantly limited to cells requiring the activity of the ventrolateral pathway to differentiate (Bier et al., 1990; Sturtevant et al., 1993). Localized expression of *rho* is important for restricting hyperactivation of EGF-R signaling to appropriate cells since ectopic expression of *rho* leads to overproduction of cells that normally depend on the activity of the ventrolateral group genes (Sturtevant et al., 1993; Ruohola-Baker et al., 1993; J. W. O'Neill and E. Bier, unpublished data). For example, in the case of wing development, localized expression of *rho* is required for development of the normal pattern of veins. Flies homozygous for the viable rho^{ve} promoter mutation specifically lack expression of *rho* in wing vein primordia and as a consequence have truncated wing veins (Sturtevant et al., 1993). Conversely, ubiquitous expression of *rho* at any stage of vein development leads to the production of extra veins (Sturtevant et al., 1993; M. A. Sturtevant and E. Bier, unpublished data). Similar requirements for localized expression of *rho* have been observed during oogenesis (Ruohola-Baker et al., 1993) and during embryogenesis (J. W. O'Neill and E. Bier, unpublished data). These data support models in which localized expression of *rho* leads to spatially and temporally regulated hyperactivation of EGF-R signaling during development.

In this report, we show that, while the *Egf-r* gene is ubiquitously expressed during much of embryogenesis and larval imaginal disc development, *Egf-r* mRNA levels are down-regulated in cells expressing *rho*. We also show that down-regulation of *Egf-r* transcripts is dependent on *rho* function during wing development and that ubiquitous expression of *rho* leads to ectopic down-regulation of *Egf-r* mRNA levels in ectodermal cells.

MATERIALS AND METHODS

Fly stocks

All genetic markers and chromosome balancers used are described in Lindsley and Grell (1968) and Lindsley and Zimm (1992). Fly stocks were obtained from the Bloomington, Indiana *Drosophila* Stock Center and the Bowling Green, Ohio Stock Center.

Mounting fly wings

Wings from adult flies were dissected in isopropanol and mounted in Canadian Balsam mounting medium (Gary's Magic Mount) following the protocol of Lawrence et al. (in Roberts, 1986). Mounted wings were photographed under Nomarski optics with a 4× lens on a compound microscope. Alternatively, whole flies or portions of flies were photographed through a dissection microscope.

In situ hybridization to whole-mount embryos or discs

In situ hybridization to whole-mount discs and embryos was performed using digoxigenin-labeled RNA probes (Boehringer-Mannheim, 1093 657) based on the method of Tautz and Pfeiffle (1989) and modified according to Sturtevant et al. (1993) using 4 μg/ml Proteinase K instead of 40 μg/ml as required for digoxigenin-labeled DNA probes.

Antibody labeling

Antibody labeling was performed according to Bier et al. (1990). The anti-Rho antiserum was raised in rabbits against a 30 amino acid amino terminal peptide of the predicted Rho protein conjugated through a carboxyl terminal cysteine to KLH using the Pierce sulfide linkage kit. Anti-EGF-R antibody was kindly provided by Dr B. Shilo (Weizmann Institute).

Double labeling of embryos or discs

Embryos were fixed for 15 minutes in 4% formaldehyde in PBS and discs were fixed for 30 minutes in 0.1 M Pipes (pH 6.9), 2 mM $MgSO_4$, 1 mM EGTA, 1% Triton X-100 and 4% formaldehyde. After washing 5× 10 minutes in an incubation buffer containing 50 mM Tris (pH 6.8), 150 mM NaCl and 0.5% NP-40, the tissues were then blocked for 2 hours at 4°C in incubation buffer with 5 mg/ml BSA. Subsequent incubations and washes for antibody staining were done in the incubation buffer with 1 mg/ml BSA. Anti-Rho antibody (1:1,000), which was preabsorbed against fixed embryos, was incubated with preparations overnight at 4°C. After 5× 10 minutes washes, biotinylated goat anti-rabbit secondary antibodies (Vector) were added at 1:200 for 1 hour and then washed 5× for 10 minutes in PBT. At this point, the antibody-staining protocol was suspended and discs were refixed in PBS with 50 mM EGTA and 4% formaldehyde overnight to insure that the biotinylated secondary antibody remained in place during the subsequent whole-mount in situ protocol. The preparations were then washed and processed according to the standard whole-mount in situ protocol. Following development of the alkaline phosphatase reaction from the whole-mount in situ protocol, we resumed the antibody staining protocol at the Avidin-Biotinylated-HRP addition step and proceeded with the Vectastain ABC elite kit according to instructions.

Other molecular techniques

RNA probe synthesis was performed according to Boehringer-Mannheim protocols and other cloning techniques followed standard procedures, as in Maniatis et al. (1982).

RESULTS

The pattern of *Egf-r* expression during embryogenesis is complex and often nearly uniform in the epidermis (Kammermeyer and Wadsworth, 1987; Zak et al., 1990; Katzen et al., 1991; Zak and Shilo, 1992; Rutledge et al., 1992; Sturtevant et al., unpublished data); however, there are stages when *Egf-r* expression is spatially modulated. The regions of lower *Egf-r* expression are remarkably similar to the pattern of *rho* expression. *rho* is expressed in a complex pattern during embryogenesis including a ventrolateral strip of cells at the cellular blastoderm, the mesectoderm during germband extension, and lateral stretch receptor organ precursor cells and tracheal pits at full germband extension (Bier et al., 1990). Because of the intimate link between *rho* and *Egf-r* signaling, the *rho* expression pattern serves as a marker for hyperactivated EGF-R signaling. Therefore, the pattern of *Egf-r* down-regulation largely recapitulates the pattern of intense EGF-R signaling but with a slight temporal delay.

To pursue the possible connection between amplified EGF-R signaling and subsequent *Egf-r* down-regulation, we closely examined the relation of Rho protein and *Egf-r* mRNA expression during various stages of embryogenesis in double-labeling experiments (Fig. 1). The repeating theme from this double-label analysis is that *Egf-r* and Rho expression domains initially overlap (consistent with the hypothesis that cells expressing Rho undergo high levels of EGF-R signaling) and then with a short delay, *Egf-r* mRNA levels are strongly down-regulated. For example, during formation of the cellular blastoderm, *Egf-r* is initially co-expressed with Rho in a ventrolateral strip of cells (Fig. 1A; arrow). *Egf-r* expression is even somewhat elevated in these ventrolateral cells. 15 minutes later, *Egf-r* transcripts begin to fade from the most ventral territory of Rho expression (Fig. 1B; arrow). Within another 15 minutes, *Egf-r* mRNA levels are extremely reduced in all ventrolateral cells expressing Rho (Fig. 1C; arrow), leading to a virtually complementary pattern of *Egf-r* and Rho expression. Down-regulation of *Egf-r* mRNA in ventrolateral cells persists for more than an hour after *rho* mRNA (Bier et al., 1990) and Rho protein have become undetectable in these cells (Fig. 1D; arrows). Later, during germband extension *Egf-r* and Rho are co-expressed in tracheal pit precursor cells (Fig. 1E; bracket). At this higher magnification, *rho*-expressing cells can be unambiguously identified by the presence of dark punctate plaques of Rho (Fig. 1E,G,H). Within 30 minutes *Egf-r* transcripts virtually disappear from Rho-expressing cells (Fig. 1F,G; brackets) and from single isolated lateral stretch receptor organ precursor cells (Fig. 1G; arrow). Similarly, following the reappearance of *Egf-r* mRNA in all ventral cells during germband retraction, it is down-regulated in midline cells expressing Rho so that by early germ band retraction the pattern of *Egf-r* mRNA is essentially complementary to Rho (Fig. 1H).

We have also compared expression of *rho* and *Egf-r* during wing vein development. In the early pupal wing disc, *Egf-r*

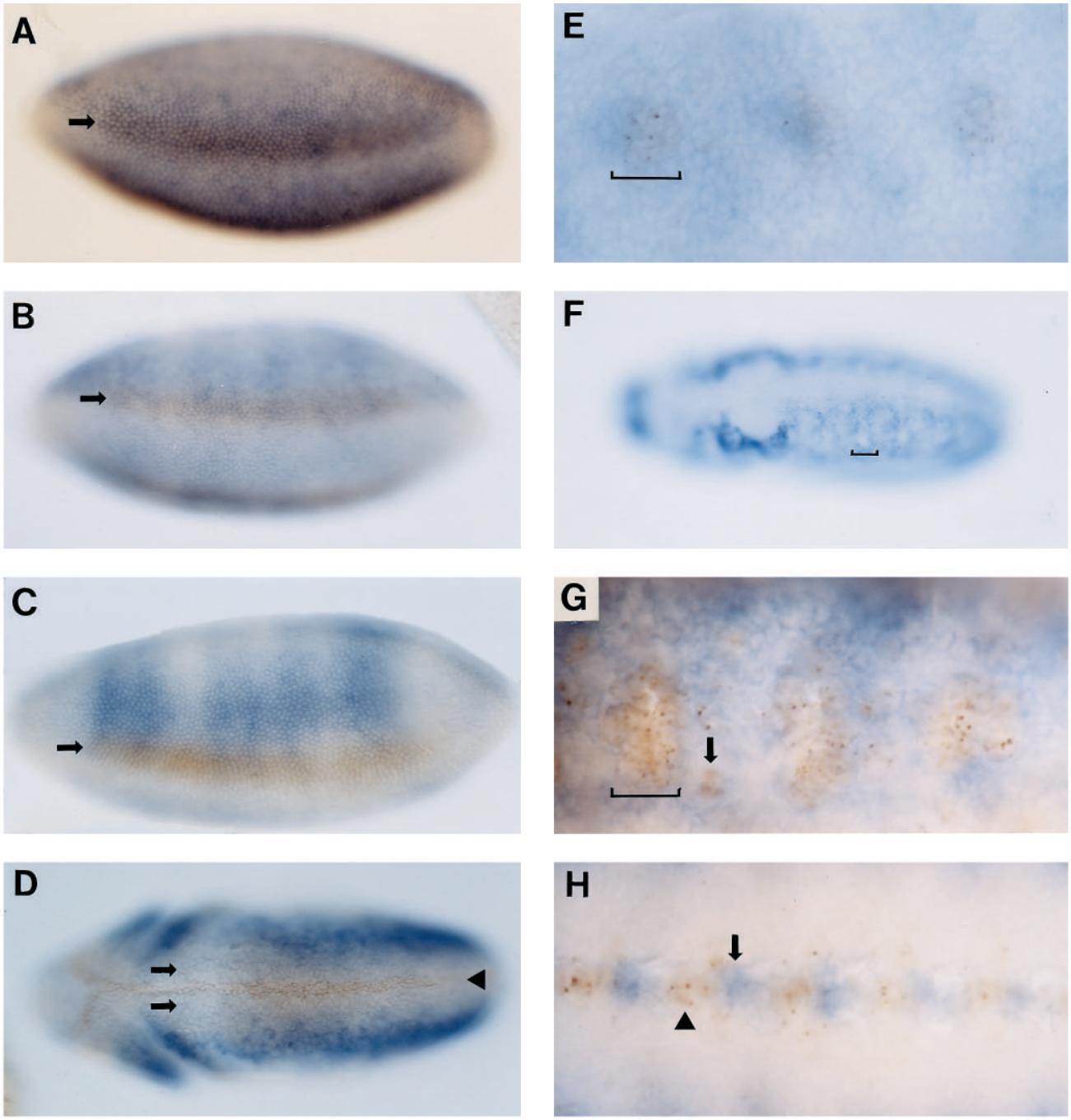

Fig. 1. Rho protein expression and *Egf-r* mRNA down-regulation during embryogenesis. Rho protein (brown punctate staining) was detected with a rabbit anti-Rho antibody (J.W. O'Neill and E. Bier, unpublished data) and visualized with a peroxidase reaction. *Egf-r* mRNA (blue staining) was detected by in situ hybridization with a digoxigenin-labeled *Egf-r* anti-sense RNA probe and visualized with an alkaline phosphatase reaction. The *Egf-r* probe was synthesized to a full-length cDNA clone (kindly provided by B. Shilo) and therefore detects the expression of all *Egf-r* transcripts. (A) *Egf-r* and Rho expression overlap during the early cellular blastoderm (arrow). (B) Within 15-20 minutes *Egf-r* expression begins to fade from Rho-expressing cells (arrow). (C) Approximately 15 minutes later the patterns of *Egf-r* and Rho (arrow) are mutually exclusive. (D) *Egf-r* down-regulation in the ventral ectoderm persists during germband extension. By this stage, Rho expression is confined to the midline (arrowhead), but *Egf-r* expression is still excluded from ventral ectodermal cells which expressed Rho during the blastoderm stage (arrows). (E) When Rho is first detected in tracheal pit precursor cells (punctate plaques above bracket), *Egf-r* expression is fairly uniform in the lateral ectoderm. High magnification view of three segments. (F) As the tracheal pits (bracket) exhibit the first signs of invagination, *Egf-r* transcripts fade from these cells. (G) Double-labeled *Egf-r* mRNA and Rho protein patterns at the same stage as in panel F. High magnification view of three segments showing essentially complementary *Egf-r* mRNA and Rho protein. Expression of *Egf-r* also appears substantially reduced in the Rho-expressing stretch receptor organ precursor cell (arrow). Note the punctate plaques of Rho. (H) The pattern of *Egf-r* mRNA-(arrow) and Rho-(arrowhead) expressing cells alternate along the midline of early germband retracted embryos. Again, note the punctate plaques of Rho.

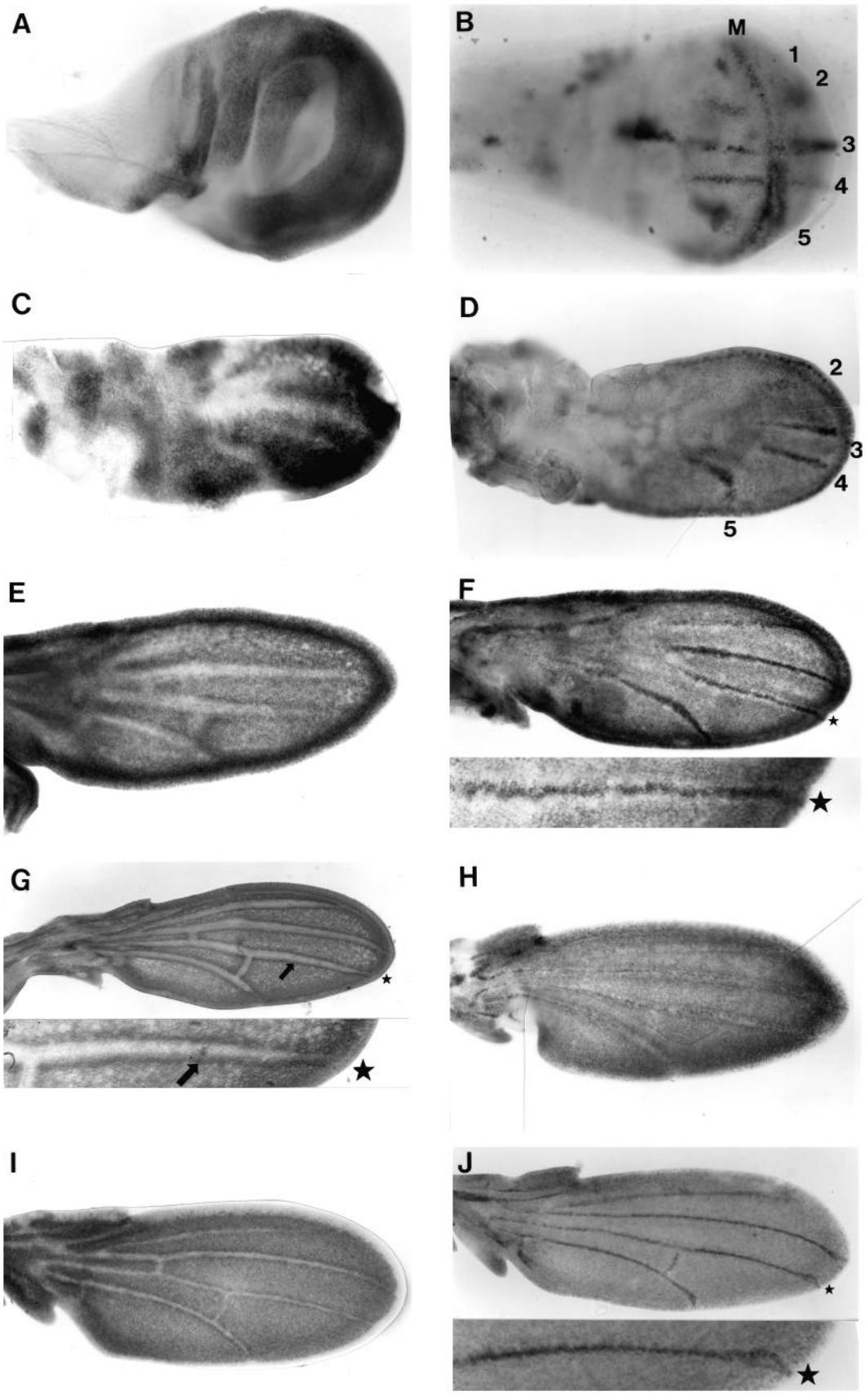

Fig. 2. *Egf-r* down-regulation during wing development. (A) *Egf-r* mRNA expression 2-4 hours AP in early everting prepupal wing discs. (B) *rho* expression 2-4 hours AP. Labels are as follows: M, future wing margin; L1-L5, longitudinal vein primordia. (C) *Egf-r* mRNA expression 6-8 hours AP. Dorsal and ventral surfaces of the wing have come together and *Egf-r* down-regulation is first apparent. (D) Double label of *Egf-r* and *rho* expression 6-8 hours AP. Note the slight gap between *Egf-r* and *rho*-expressing cells. Intersections of longitudinal veins L2-L5 with the margin are indicated. (E) *Egf-r* expression 18 hours AP. (F) Double label of *Egf-r* and *rho* mRNA expression 18 hours AP. There is a slight gap between *Egf-r* and *rho*-expressing cells. The intersection of L4 with the margin is marked with an asterisk. Inset: higher magnification view of L4 vein. (G) EGF-R protein expression at about 25 hours AP. Close examination reveals that EGF-R expression is not totally absent from these cells. A reproducible feature of *Egf-r* RNA or protein expression at this stage is that cells expressing elevated levels of EGF-R immediately flank the strip of low expressing cells (arrow). The intersection of L4 with the margin is marked with an asterisk. Inset: higher magnification view of L4 vein. (H) Double label of *Egf-r* and *rho* mRNA expression at about 25 hours AP. A slight gap is still present between *EGF-R* and *rho*-expressing cells. (I) *Egf-r* mRNA expression at about 30 hours AP. (J) Double label of *Egf-r* and *rho* mRNA expression at about 30 hours AP. The two patterns of staining are entirely complementary by this stage as judged by the absence of unstained cells. The intersection of L4 with the margin is marked with an asterisk. Inset: higher magnification view of L4 vein.

expression is virtually uniform (Fig. 2A). At this same stage, *rho* is expressed in a pattern of intersecting stripes representing the future longitudinal veins and wing margin (Fig 2B). During early pupariation after the wing disc has everted to bring the dorsal and ventral surfaces of the wing together (approximately 8 hours after pupariation (AP)), down-regulation of *Egf-r* transcripts becomes apparent (Fig. 2C). Double-label experiments reveal that domains of *Egf-r* mRNA down-regulation are slightly broader than those expressing *rho* (Fig. 2D). The pattern of *Egf-r* down-regulation becomes more sharply defined between 18 hours AP (Fig. 2E) and 25 hours AP (Fig. 2G). Double-label experiments demonstrate that *Egf-r* and *rho* expression remain nearly complementary throughout this period (Fig. 2F,H). Examination of these preparations at higher magnification (inset in Fig. 2F) suggests that cells expressing high levels of *Egf-r* mRNA are typically separated from *rho*-expressing cells by a row of one or two low-*Egf-r*-expressing cells. By 30 hours AP, *Egf-r* (Fig. 2I) and *rho* are expressed in essentially complementary patterns, as no gaps of unlabeled cells are observed in double-labeled wings (Fig. 2J). Although *Egf-r* is expressed in all intervein cells between 20 and 30 hours AP, expression is strongest in cells flanking developing veins (see inset in Fig. 2G). It is worth noting that ectopic expression of *rho* during comparable pupal stages leads to profound ectopic wing vein formation, frequently in regions flanking existing veins (M. A. Sturtevant and E. Bier, unpublished data) demonstrating that all components of the *Egf-r* signaling cascade are present in intervein cells. Other examples of localized *Egf-r* down-regulation following *rho* expression include cells behind the morphogenetic furrow in the eye (Zak et al., 1990) and cells forming adult external sensory organs (data not shown).

The clear reiterated theme during both embryogenesis and larval development is that cells initially express both *Egf-r* and *rho* and then with a brief delay, *Egf-r* mRNA levels are sharply down-regulated in *rho*-expressing cells. One possible explanation for this sequence of events is that Rho activity leads to subsequent *Egf-r* down-regulation by some negative feedback mechanism. Such negative feedback could be mediated by heightened EGF-R signaling or by a separate action of Rho. Alternatively, it is possible that control of *Egf-r* down-regulation is independent of *rho* activity. Fully resolving this important question is beyond the scope of this report; however, data described below suggest that *Egf-r* down-regulation is linked to *rho* function.

To address whether *rho* expression is coupled to *Egf-r* mRNA down-regulation, we examined the effect of ubiquitous *rho* expression during embryogenesis and wing development, as there are phenotypic consequences to ectopic *rho* expression during these stages that are opposite to phenotypes resulting from loss of *rho* function. For example, heat induction of embryos carrying a heat-shock *rho* construct (HS-*rho*) at germband extension leads to the production of supernumerary stretch receptor organ precursor cells (J.W. O'Neill and E. Bier, unpublished data), and heat inductions performed throughout wing vein development lead to the formation of ectopic veins (Sturtevant et al., 1993; M. A. Sturtevant and E. Bier, unpublished data). During the germband extended stage *Egf-r* is normally strongly expressed in the mesoderm and epidermis, but not in the neuroblast layer, leading to a railroad track staining pattern in sagittal section (Fig. 3A). Ubiquitous *rho* expression resulting from brief induction of the HS-*rho* construct followed by an hour of recovery leads to dramatic global down-regulation of *Egf-r* expression in the epidermis in greater than 90% of embryos, but does not reduce expression in the mesoderm (Fig. 3B). The lack of down-regulation in the mesoderm, perhaps due to the absence of some necessary component present in epidermal cells, provides an internal control for the in situ staining reaction at this particular stage. HS-*rho* induced *Egf-r* down-regulation in the epidermis is due to ectopic *rho* expression since uninduced embryos carrying the HS-*rho* construct (Fig. 3A) or heat-shocked wild-type embryos (Fig. 3C) have the normal railroad track pattern of epidermal and mesodermal *Egf-r* expression. To eliminate a potential role of mis-expressing *rho* during oogenesis, we examined *Egf-r* expression in embryos collected from a cross of heterozygous HS-*rho* male flies to wild-type females. As expected, approximately half of these embryos exhibit the broad epidermal down-regulation phenotype. Down-regulation of *Egf-r* mRNA levels is not a general feature of all epidermal transcripts since expression of *spitz*, which is also strongly expressed in the epidermis and mesoderm but not in the neuronal layer, is unaltered in heat-shocked HS-*rho* embryos (data not shown). Because embryos lacking zygotic *Gap1* activity exhibit a similar pattern of *Egf-r* down-regulation (Fig. 3D), *rho*-induced *Egf-r* mRNA down-regulation may be mediated by a tyrosine kinase signaling pathway.

rho is also involved in *Egf-r* mRNA down-regulation during wing development. Heat induction of the HS-*rho* stock 15-16 hours AP leads to peak formation of excess veins and blistered wings (data not shown). Wild-type or uninduced HS-*rho* wings at this stage show clear signs of *Egf-r* down-regulation in vein territories (Fig. 3E). *Egf-r* down-regulation is expanded to occupy the entire wing blade following HS-*rho* induction (Fig. 3F). Expression along the margin, which is unaffected by ectopic *rho* expression, serves as an internal staining control. Control heat-shocked wild-type flies do not exhibit this specific down-regulation, although levels of RNA are reduced somewhat in all cells (data not shown). HS-*rho* induced *Egf-r* RNA down-regulation is also obvious earlier (6-8 hours AP) during prepupal stages (data not shown). A further link between *rho* function and *Egf-r* RNA down-regulation is revealed by examining *Egf-r* expression in developing rho^{ve} mutant wings (Fig. 3I), which lack distal portions of wing veins (Fig. 3G), and in a HS-*rho* transformant line (Fig. 3J), which constitutively forms excess veins (Fig. 3H). *Egf-r* down-regulation is not observed in distal regions of developing rho^{ve} wings (Fig. 3I), while down-regulation is observed in more cells than normal in the HS-*rho* line (Fig. 3J). Thus, the pattern of *Egf-r* down-regulation in both of these cases prefigures the final vein phenotype.

DISCUSSION

The results described in this study suggest an intimate link between *rho* expression and *Egf-r* mRNA down-regulation. Down-regulation of *Egf-r* mRNA levels in developing wings requires *rho* function and ubiquitous expression of *rho* during embryogenesis and wing development leads to ectopic *Egf-r* mRNA down-regulation in epidermal cells. The short time between ectopic *rho* expression and strong *Egf-r* mRNA down-

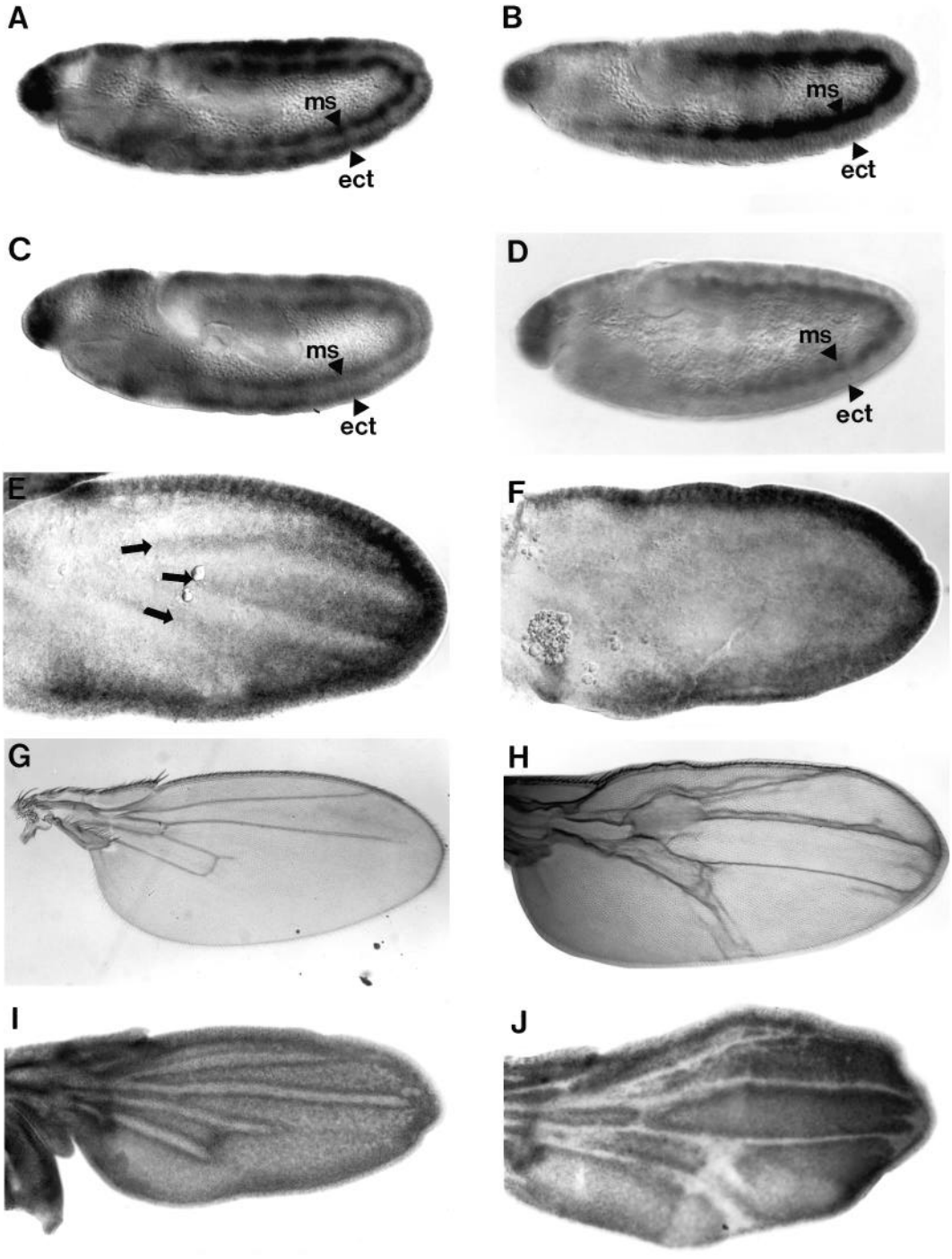

Fig. 3. *Egf-r* down-regulation in HS-*rho* embryos and in HS-*rho* and *rho*^{ve} mutant wings. (A) *Egf-r* expression in non-heat-shocked HS-*rho* embryos. *Egf-r* expression in these and non heat-shocked wild-type embryos are indistinguishable. This sagittal view gives the appearance of railroad tracks due to high levels of expression in the ectoderm (ect) and mesoderm (ms), but not in the intervening neuronal layer. (B) *Egf-r* expression in heat-induced embryos carrying the HS-*rho* construct. Embryos were heat shocked at 37°C for 30 minutes, allowed to recover for 1 hour, fixed and then hybridized with an *Egf-r* digoxigenin-labeled RNA probe according to Sturtevant et al. (1993). Expression is dramatically reduced in the ectoderm but not in the mesoderm. Mesodermal expression may be somewhat increased relative to controls, or the absence of the usual obscuring epidermal expression, which is normally heavy and out of focus in a sagittal view, may give the appearance of higher mesodermal expression. (C) *Egf-r* expression in a heat-shocked wild-type embryo. *Egf-r* mRNA levels are slightly reduced in all cells relative to uninduced controls, but the pattern of expression is indistinguishable from wild type. (D) *Egf-r* down-regulation in homozygous Df AC1 embryos which entirely lack the *Gap1* gene (Gaul et al., 1992). Df AC1 embryos can be identified based on their abnormal morphology. The *Gap1* gene is ubiquitously expressed at low levels during embryogenesis (J. W. O'Neill, unpublished results). (E) *Egf-r* expression 16 hours AP in an uninduced wing from an HS-*rho* line is indistinguishable from wild type. (F) *Egf-r* expression 16 hours AP in HS-*rho* wings following heat shock for 45 minutes at 38°C. mRNA levels in intervein regions virtually vanish while expression along the margin is relatively unaffected. Marginal cells, like embryonic mesodermal cells, may lack some component required for Rho-mediated *Egf-r* down-regulation. (G) Adult *rho*^{ve} mutant wing lacking distal portions of longitudinal wing veins. (H) An HS-*rho* transformant line, which has extra veins at room temperature due to dominant constitutive ectopic *rho* expression (Sturtevant et al., 1993). (I) *Egf-r* down-regulation in *rho*^{ve} wings does not occur distally where wing veins fail to differentiate (25 hours; compare with wild-type *Egf-r* expression in Fig. 2G). (J) Ectopic *Egf-r* mRNA down-regulation in the HS-*rho* transformant line shown in H (30 hours; compare with wild-type *Egf-r* expression in Fig. 2I).

regulation suggests that this link may be fairly direct. Other pathways in addition to that mediated by Rho, however, must also contribute to *Egf-r* down-regulation during certain developmental stages. For example, *Egf-r* transcripts are down-regulated in embryos homozygous for a null *rho* point mutant, albeit not as sharply as in wild type (data not shown). This is

not too surprising since other genes are likely to function in parallel with *rho* during embryogenesis (Bier et al., 1990) and wing vein formation (Sturtevant et al., 1993). A role for EGF-R/RAS signaling in *Egf-r* down-regulation is suggested by the ectopic down-regulation observed in heat-induced HS-*rho* embryos and in embryos lacking the *Gap1* gene. *Egf-r* function is not required for down-regulation; however, since down-regulation is initiated normally in three different strong *Egf-r* point mutants, but is not as sharply defined or as long lasting as in wild-type embryos (data not shown). As GAP proteins have been proposed to function as effectors as well as regulators of RAS (Martin et al., 1992; Medema et al., 1992; Al-Alawi et al., 1993; Declue et al., 1993; Grunicke and Maly, 1993; Marshal, 1993), it is possible that an effector function of GAP plays a role in down-regulating *Egf-r*. This possibility is consistent with the fact that *rho* interacts genetically with *Gap1* but not with *sos* or *drk* (GRB2) in wing vein formation (Sturtevant et al., 1993). Thus, Rho may induce ectopic *Egf-r* down-regulation via its role in promoting EGF-R/RAS signaling or by some other means such as a RAS-independent activity of GAP (Martin et al., 1992). It is also possible in some cases that *Egf-r* down-regulation is due to an indirect cell non-autonomous action of *rho*. For example, during much of pupal development, the strips of cells that down-regulate *Egf-r* are slightly broader than the presumptive vein cells expressing *rho* (Fig. 2C,D and E,F). Further experiments will be required to determine the role that *rho* and other genes, such as those encoding components of the EGF-R signaling pathway, play in *Egf-r* down-regulation.

Egf-r mRNA down-regulation could be due to decreased *Egf-r* transcription or decreased mRNA stability. As the probe that we used should detect all *Egf-r* transcripts, it is likely that transcripts derived from both *Egf-r* promoters are affected. EGF stimulation of various cell types can induce alterations in either mRNA stability (Le et al., 1991) or in the transcription rate of a variety of target genes (Hopkin et al., 1991), including EGF-R itself (Kesavan et al., 1990; Joh et al., 1992). Further investigation, such as constructing *Egf-r* promoter *lacZ* fusions, will be required to distinguish between these possibilities. In either case, down-regulation of *Egf-r* mRNA levels provides a novel mechanism for extending the period of low EGF-R activity following transient down-regulation via receptor internalization (Haigler et al., 1978; Lund et al., 1990; Wiley et al., 1990) and reduction of high affinity receptor activity due to protein kinase C phosphorylation of the receptor (Welsh et al., 1991).

The observation that *Egf-r* activity is decreased following receptor activation is similar to genetic observations that have been made with respect to regulation of EGF-R activity in *C. elegans.* A category of loss-of-function *let-23* (*Egf-r*) alleles cause a hyperinduced (Hin) phenotype resulting, paradoxically, in elevated EGF-R activity (Aroian and Sternberg, 1991). These Hin alleles in *trans* to *Egf-r* null alleles exhibit loss-of-function phenotypes (e.g. vulvaless) and combinations of *let-23* Hin alleles with similar alleles at two other distinct loci (*lin-2* and *lin-7*) also result in vulvaless phenotypes. It has been proposed that these mutants when individually homozygous disrupt some aspect of negative regulation of *let-23* more than they reduce signaling activity of the receptor, but that when Hin alleles are combined with stronger loss-of-function alleles signaling is also compromised leading to the vulvaless phenotype (Aroian and Sternberg, 1991; Sternberg, 1993). Although *Drosophila Egf-r* alleles corresponding to *C. elegans* Hin alleles have not been reported, mild *Egf-r* loss-of-function alleles ($Egf\text{-}r^{top}/Egf\text{-}r^{top}$) exhibit a slight gain-of-function phenotype in the wing (e.g. distal vein branching) and some combinations of HS-*rho* constructs with the gain-of-function $Egf\text{-}r^{Elp}$ allele lead to a serrated margin phenotype. Serrated wing margin phenotypes are typical of genes antagonizing *Egf-r* and *rho* activity (Sturtevant et al., 1993; Sturtevant and Bier, unpublished data). The existence of other genes behaving genetically as coactivators of EGF-R (i.e. *lin-2* and *lin-7*), which also participate in negative regulation of the receptor, is formally similar to the role played by *rho* in *Drosophila.* Whether any of the four pathways that negatively regulate *let-23* in *C. elegans* (Sternberg, 1993) involve down-regulation of EGF-R protein or mRNA levels remains to be determined.

It should be noted that the systematic down-regulation of *Egf-r* mRNA levels following heightened EGF-R signaling, which we report here, contrasts with the transient increase in *Egf-r* mRNA levels observed in several mammalian cell culture studies (Clark et al., 1985; Earp et al., 1986; Bjorge and Kudlow, 1987; Thompson and Rosner, 1989). This may reflect a fundamental difference between uncommitted embryonic cells undergoing a series of distinct cell fate choices during development and more terminally differentiated cells, which are primarily concerned with whether to proliferate in response to exogenous cues. Another potentially relevant difference is that cells in culture generally respond to soluble ligands, whereas embryonic cells are in direct contact with other cells. It is possible that direct cell-cell contact is required for the kind of regulation we have observed.

Since failure to down-regulate EGF-R activity by interfering with EGF-R internalization leads to tumor formation in mice (Masui et al., 1991) and overexpression of EGF-R or the highly related *neu* receptor are associated with aggressive forms of cancer (Neal et al., 1985; Santon et al., 1986; Slamon et al., 1987), understanding transcriptional control of *Egf-r* is relevant to oncogenesis. Down-regulation of *Egf-r* mRNA levels in specific differentiating *Drosophila* epidermal cells may play a similar role to down-regulation of FGF (Velcich et al., 1989; Moore et al., 1991) and FGF-R (Moore et al., 1991) expression in differentiating myogenic cells. Determining the mechanism of *Egf-r* transcriptional down-regulation and whether this regulation plays an important role in development are key issues to resolve in future experiments.

We thank Michael Levine, Kathryn S. Burton, Geof Rosenfeld, Gordin Gill and Richard A. Firtel for very helpful discussions and critical comments on the manuscript: Benny Shilo for the anti EGFR antibody: and Kathryn S. Burton for assembling the figures. This work was supported by NIH Grant (#RO1-NS29870-01), NSF Grant #IBN-9318242 and Research Grant #5-FY92-1175 from the March of Dimes Birth Defects Foundation. E. B. was supported by funds from the McKnight Neuroscience Foundation, Sloan Foundation and an ACS Junior Faculty Award.

REFERENCES

Aroian, R. V. and Sternberg, P. (1991). Multiple functions of *let-23*, a C. *elegans* receptor tyrosine kinase gene required for vulval induction. *Genetics* **128**, 251-256.

Al-Alawi, N., Xu, G., White, R., Clark, R., McCormick, F. and Feramisco, J. R. (1993). Differential regulation of cellular activities by GTPase-activating protein and NF1. *Mol. Cell. Biol.* **13**, 2497-2503**.**

Bier, E., Jan, L. Y. and Jan, Y. N. (1990). *rhomboid*, a gene required for dorsoventral axis establishment and peripheral nervous system development in *Drosophila melanogaster*. *Genes Dev.* **4**, 190-203.

Bjorge, J. D. and Kudlow, J. E. (1987). Epidermal growth factor receptor synthesis is stimulated by phorbol ester and epidermal growth factor. Evidence for a common mechanism. *J. Biol. Chem.* **262**, 6615-6622.

Clark, A. J., Ishii, S., Richert, N., Merlino, G. T. and Pastan, I. (1985). Epidermal growth factor regulates the expression of its own receptor. *Proc. Natl. Acad. Sci. USA* **82**, 8374-8378.

Clifford, R. J. and Schüpbach, T. (1992). The *torpedo* (DER) receptor tyrosine kinase is required at multiple times during *Drosophila* embryogenesis. *Development* **115**, 853-872.

Declue, J. E., Vass, W. C., Johnson, M. R., Stacey, D. W. and Lowy, D. R. (1993). Functional role of GTPase-activating protein in cell transformation by pp$60^{v\text{-}src}$. *Mol. Cell. Biol.* **13**, 6799-6809.

Earp, H. S., Austin, K. S., Blaisdell, J., Rubin, R. A., Nelson, K. G., Lee, L. W. and Grisham, J. W. (1986). Epidermal growth factor (EGF) stimulates EGF receptor synthesis. *J. Biol. Chem.* **261**, 4777-4780.

Gaul, U., Mardon, G. and Rubin, G. M. (1992). A putative Ras GTPase activating protein acts as a negative regulator of signaling by the Sevenless receptor tyrosine kinase. *Cell* **68**, 1007-1019.

Grunicke, H. H. and Maly, K. (1993). Role of GTPases and GTPase regulatory proteins in oncogenesis. *Crit. Rev. Onc.* **4**, 389-402.

Haigler, H., Ash, J. F., Singer, S. J. and Cohen, S. (1978). Visualization by fluorescence of the binding and internalization of epidermal growth factor inhuman carcinoma cells A-431. *Proc. Natl. Acad. Sci. USA* **75**, 3317-3321.

Hopkin, W. E., Westerhausen, Jr., D. R., Sobel, B. E. and Billadello, J. J. (1991). Transcriptional regulation of plasminogen activator inhibitor type-1 mRNA in Hep G2 cells by epidermal growth factor. *Nuc. Acids Res.* **19**, 163-168.

Joh, T., Darland, T., Samuels, M., Wu, J. X. and Adamson, E. D. (1992). Regulation of epidermal growth factor receptor gene expression in murine embryonal carcinoma cells. *Cell Growth and Diff.* **3**, 315-325.

Kammermeyer, K. L. and Wadsworth,S. C. (1987). Expression of *Drosophila* epidermal growth factor homologue in mitotic cell populations. *Development* **100**, 201-210.

Katzen, A. L., Kornberg, T. and Bishop, J. M. (1991). Expression during *Drosophila* development of DER, a gene related to erbB-1 and neu: correlations with mutant phenotypes. *Dev. Biol.* **145**, 287-301.

Kesavan, P., Das, P. and Das, M. (1990). Regulation of stability and synthesis of EGF-receptor mRNAs encoding for intact and truncated receptor forms. *Oncogene* **5**, 483-488.

Kim, S. E. and Crews, S. T. (1993). Influence of *Drosophila* ventral epidermal development by the CNS midline cells and *spitz* class genes. *Development* **118**, 893-901.

Lawrence, P. A. (1986). Basic *Drosophila* care and techniques. In *Drosophila: A Practical Approach* (ed. D. B. Roberts). pp. 1-38. Washington, DC: IRL Press.

Le, P. T., Lazorick, S., Whichard, L. P., Haynes, B. F. and Singer, K. H. (1991). Regulation of cytokine production in the human thymus: epidermal growth factor and transforming growth factor alpha regulate mRNA levels of interleukin 1 alpha (IL-1 alpha), IL-1 beta and IL-6 in human thymic epithelial cells at a post-transcriptional level. *J. Exp. Med.* **174**, 1147-1157.

Lindsley, D. L. and Grell, E. H. (1968). *Genetic Variations in Drosophila melanogaster*. Carnegie Institute of Washington, Washington, D. C.

Lindsley, D. L. and Zimm, G. G. (1992). *The Genome of Drosophila melanogaster*. San Diego, CA: Academic Press Inc.

Lund, K. A., Opresko, L. K., Starbuck, C., Walsh, B. J. and Wiley, H. S. (1990). Quantitative analysis of the endocytic system involved in hormone-induced receptor internalization. *J. Biol. Chem.* **265**, 15731-15723.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Marshall, M. S. (1993). The effector interactions of p21^{ras}. *Trends in Biochem. Sci.* **18**, 250-254.

Martin, G. A., Yatani, A., Clark, R., Conroy, L., Polakis, P., Brown, A. M. and McCormick, F. (1992). GAP domains responsible for Ras p21-dependent inhibition of muscarinic atrial K+ channel currents. *Science* **255**, 192-194.

Masui, H., Wells, A., Lazar, C. S., Rosenfeld, M. G. and Gill, G. N. (1991). Enhanced tumorigenesis of NR6 cells which express non-down-regulating epidermal growth factor receptors. *Cancer Res.* **51**, 6170-6175.

Mayer, U. and C. Nüsslein-Volhard. (1988). A group of genes required for pattern formation in the ventral ectoderm of the *Drosophila* embryo. *Genes Dev.* **2**, 1496-1511.

Medema, R. H., De Laat, W. L., Martin, G. A., McCormick, F. and Bos, J. L. (1992). GTPase activating protein SH2-SH3 domains induce gene expression in a RAS-dependent fashion. *Mol. Cell. Biol.* **12**., 3425-3430.

Moore, J. W., Dionne, C., Jaye, M. and Swain, J. L. (1991). The mRNAs encoding acidic FGF, basic FGF and FGF receptor are coordinately down regulated during myogenic differentiation. *Development* **111**, 741-748.

Neal, D. E., Marsh, C., Bennett, M. K., Abel, P. D., Hall, R. R., Sainsbury, J. R. and Harris, A. L. (1985). Epidermal-growth-factor receptors in human bladder cancer: comparison of invasive and superficial tumours. *Lancet* **1**, 366-368.

Raz, E. and Shilo, B. Z. (1992). Dissection of the *faint little ball* (FLB) phenotype-determination of the development of the *Drosophila* central nervous system by early interactions in the Ectoderm. *Development* **114**, 113-123.

Raz, E. and Shilo, B. Z. (1993). Establishment of ventral cell fates in the *Drosophila* ectoderm requires DER, the EGF receptor homolog. *Genes Dev.* **7**, 1937-1948.

Ruohola-Baker, H., Grell, E., Chou, T. B., Baker, D., Jan, L. Y. and Jan, Y. N. (1993). Spatially localized *rhomboid* is required for establishment of the dorsal-ventral axis in *Drosophila* oogenesis. *Cell* **73**, 953-965.

Rutledge, B., Zhang, K., Bier, E., Jan, Y. N. and Perrimon, N. (1992). The *Drosophila spitz* gene encodes a putative EGF-like growth factor involved in dorsal-ventral axis formation and neurogenesis. *Genes Dev.* **6**, 1503-1517.

Santon, J. B., Cronin, M. T., Macleod, C. L., Mendelsohn, J., Masui, H and Gill, G. N. (1986). Effects of epidermal growth factor receptor concentration on tumorigenicity of A431 cells in nude mice. *Cancer Res.* **46**, 4701-4705.

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ulrich, A. and McGuire, W. L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**, 177-182.

Sternberg, P. (1993). Intercellular signalling and signal transduction in C. elegans. *Annu. Rev. Genet.* **27**, 497-521.

Sturtevant, M. A., Roark, M. and Bier, E. (1993). The *Drosophila rhomboid* gene mediates the localized formation of wing veins and interacts genetically with components of the EGF-R signaling pathway. *Genes Dev.* **7**, 961-973.

Tautz, D. and Pfeiffle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**, 81-85.

Thompson, K. L. and Rosner, M. R. (1989). Regulation of epidermal growth factor receptor gene expression by retinoic acid and epidermal growth factor. *J. Biol. Chem.* **264**, 3230-3234.

Velcich, A., Delli-Bovi, P., Mansukhani, A., Ziff, E. B. and Basilico, C. (1989). Expression of the K-fgf proto-oncogene is repressed during differentiation of F9 cells. *Oncogene Res.* **5**, 31-37.

Welsh, J. B., Gill, G. N., Rosenfeld, M. G. and Wells, A. (1991). A negative feedback loop attenuates EGF-induced morphological changes. *J. Cell Biol.* **114**, 533-543.

Wiley, H. S., Herbst, J. J., Walsh, B. J., Lauffenburger, D. A., Rosenfeld, M. G. and Gill, G. N. (1990). The role of tyrosine kinase activity in endocytosis, compartmentation, and down-regulation of the epidermal growth factor receptor. *J. Biol. Chem.* **266**, 11083-11094.

Zak, N. B., Wides, R. J., Schejter, E. D., Raz, E. and Shilo, B. Z. (1990). Localization of the DER/flb protein in embryos: implications on the *faint little ball* lethal phenotype. *Development* **109**, 865-874.

Zak, N. B. and Shilo, B. Z. (1992). Localization of DER and the pattern of cell divisions in wild-type and Ellipse eye imaginal discs. *Dev. Biol.* **149**, 448-456.

(Accepted 17 May 1994)