Dominant Enhancers of *Egfr* in *Drosophila melanogaster*: Genetic Links Between the *Notch* and *Egfr* Signaling Pathways

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ABSTRACT

The Drosophila epidermal growth factor receptor (EGFR) is a key component of a complex signaling pathway that participates in multiple developmental processes. We have performed an F_1 screen for mutations that cause dominant enhancement of wing vein phenotypes associated with mutations in *Egfr*. With this screen, we have recovered mutations in *Hairless* (*H*), *vein*, *groucho* (*gro*), and three apparently novel loci. All of the *E(Egfr)*s we have identified show dominant interactions in transheterozygous combinations with each other and with alleles of *N* or *Su(H)*, suggesting that they are involved in cross-talk between the N and EGFR signaling pathways. Further examination of the phenotypic interactions between *Egfr*, *H*, and *gro* revealed that reductions in *Egfr* activity enhanced both the bristle loss associated with *H* mutations, and the bristle hyperplasia and ocellar hypertrophy associated with *gro* mutations. Double mutant combinations of *Egfr* and *gro* hypomorphic alleles led to the formation of ectopic compound eyes in a dosage sensitive manner. Our findings suggest that these *E(Egfr)*s represent links between the *Egfr* and *Notch* signaling pathways, and that *Egfr* activity can either promote or suppress *Notch* signaling, depending on its developmental context.

GENETIC interaction screens provide a useful approach for the identification of factors that interact with a gene of interest. In recent years such screens have identified numerous components of receptor tyrosine kinase (RTK) signal transduction pathways in *Drosophila melanogaster* and *Caenorhabditis elegans* (HAN and STERNBERG 1990; ROGGE *et al.* 1991; SIMON *et al.* 1991; DOYLE and BISHOP 1993; BRUNNER *et al.* 1994; LU *et al.* 1994; KORNFELD *et al.* 1995; SINGH and HAN 1995; KARIM *et al.* 1996). Many of the components identified in these screens act as part of a "RAS cassette" that is used in multiple signal transduction pathways in different developmental contexts (reviewed in PERRIMON and DESPLAN 1994; DOWNWARD 1995; KAYNE and STERNBERG 1995). Since the components of the RAS cassette participate in the transduction of many different developmental signals, the specificity of the developmental response must be conferred by other factors, such as (1) the ligand-receptor pairs that initiate a developmental signal (RUTLEDGE *et al.* 1992; NEUMAN-SILBERBERG and SCHÜPBACH 1993; SCHWEITZER *et al.* 1995b; SCHNEPP *et al.* 1996), (2) additional factors or cofactors that modulate the signals in developmentally specific contexts (RUOHOLA-BAKER *et al.* 1993; STURTEVANT *et al.* 1994; SCHWEITZER *et al.* 1995a; GOLEMBO *et al.* 1996), (3) inputs from other signal transduction pathways (PAROUSH *et al.* 1994; TUCK and GREENWALD 1995; DAUB *et al.* 1996; DE CELIS 1997), or (4) the specific downstream effectors that implement the developmental response (DICKSON and HAFEN 1993; DUFFY and PERRIMON 1994). Thus, it is useful to conduct similar screens in different developmental contexts.

In Drosophila, the developmental roles of three RTKs, Sevenless (SEV), Torso (TOR), and the Epidermal Growth Factor Receptor (EGFR), have been particularly well studied. SEV and TOR each regulate a single developmental process: SEV is exclusively required for the specification of the R7 photoreceptor during the development of the compound eye (RUBIN 1991; DICKSON and HAFEN 1993) and TOR is required for specification of the embryonic termini (PERRIMON 1993; SPRENGER and NÜSSLEIN-VOLHARD 1993). In contrast, EGFR is involved in many developmental processes including oogenesis (PRICE *et al.* 1989; ROTH *et al.* 1995), neural precluster determination in the developing retina (BAKER and RUBIN 1989, 1992), growth of imaginal disks, specification of wing vein and bristle patterns (CLIFFORD and SCHÜPBACH 1989), and multiple processes during embryogenesis (CLIFFORD and SCHÜPACH 1992; RAZ and SHILO 1993). Three different ligands, Gurken (Grk; NEUMAN-SILBERBERG and SCHUPBACH 1993), Spitz (Spi; RUTLEDGE *et al.* 1992; FREEMAN 1994; TIO *et al.* 1994; SCHWEITZER *et al.* 1995b), and Vein (Vn; SCHNEPP *et al.* 1996; SIMCOX 1997) are involved in the modulation of EGFR signaling in different developmental contexts. However, the mechanisms by which the EGFR signal generates unique outcomes in response to activation by these ligands is unclear.

Flies homozygous, hemizygous, or transheterozygous

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for weak hypomorphic alleles of *Egfr* survive to adulthood, but display a variety of developmental defects in the wing veins, bristles, eyes, and ocelli (CLIFFORD and SCHÜPBACH 1989). The severity of these defects is correlated with the degree of reduction in EGFR signaling activity associated with any particular genotype (CLIFFORD and SCHÜPBACH 1989; and see Figure 1, a–c). Here we describe an F_1 genetic screen for mutations that act as dominant enhancers of *Egfr* wing vein defects. We describe interactions between *Egfr* and six other loci including vein (*vn*), *Hairless* (*H*), and *groucho* (*gro*). Our results are discussed with respect to cross-talk between the EGFR and Notch signaling pathways during development.

MATERIALS AND METHODS

Stocks: The mutations and balancer chromosomes used in this study are described in LINDSLEY and ZIMM (1992). In most respects we have adhered to the nomenclature presented in LINDSLEY and ZIMM. However, for purposes of clarity, we will refer to *E(spl)*^{2} as *groucho* (*gro*) to distinguish it from the other genes of the *Enhancer of split complex* (*E(spl)-C*). *Df(2R)Egfr18* is a small deficiency that removes most or all of the *Egfr* transcription unit, behaves as a genetic null of *Egfr*, and is not known to remove any other complementation groups (PRICE *et al.* 1989). *Df(3R)E(spl)BX22* is a 14-kbp deletion that removes several transcripts from the *E(spl)-C* (PREISS *et al.* 1988). *Df(3R)E(spl)BX22* homozygotes are nonviable, but can be rescued by a *gro*$^+$ transgene, indicating that *gro* is the only essential gene removed by this deletion.

Deficiency strains listed in Table 2 and the *gro*^{1} stock used in this study were obtained from the Bloomington Drosophila Stock Center. *Df(3R)E(spl)BX22* and *Df(3R)E(spl)BX36* were provided by M. MUSKAVITCH. Other *E(spl)* strains were provided by E. VERHEYEN. Alleles of *vn* were provided by A. GARCIA-BELLIDO. Stocks were normally maintained at 18°, and crosses were brooded and reared at 25° unless otherwise noted. Cultures were maintained on corn meal agar medium (CLINE 1978).

Screens: Several screens were performed to detect dominant mutations that enhance the visible wing vein defects observed in adult flies homozygous for *Egfr*^{t1}. These screens followed two protocols. In the first protocol, *b pr cn Egfr*^{t1} *bw* males were exposed to EMS as described in CLIFFORD and SCHÜPBACH (1989). Groups of 80–100 mutagenized males were placed in culture bottles with 100 *Egfr*^{t1} *bw/CyO, l(2) DTS-100* virgin females and brooded for 4 days at 18°. Flies were then transferred to fresh bottles to produce a second brood. At 8 days post mutagenesis the males were removed and the females were transferred to fresh bottles to produce a third brood. After the adults were removed from the culture bottles, the bottles were transferred to 29° until the F_1 emerged as adults. The wings of 12,372 *Egfr*^{t1} *bw/b pr cn Egfr*^{t1} *bw* F_1 males were examined for enhanced wing vein defects similar to those observed in *Egfr*^{t1} hemizygotes (Figure 1c).

The protocol described above allowed detection of *E(Egfr)* mutations on the autosomes. A second screening protocol was employed to allow recovery of mutations on the *X* chromosome, as well as the autosomes. This protocol was essentially identical to the autosome protocol, except that virgin females of genotype *b pr cn Egfr*^{t1} *bw/CyO, l(2) DTS-100* were exposed to EMS and mated to *Egfr*^{t1} *bw/Egfr*^{t1} *bw* males, and all adults were discarded at 8 days post mutagenesis. F_1 males (11,008) were screened by this protocol (Table 1).

Phenotypic analysis: Wings and legs were manually dissected, washed briefly in isopropanol, mounted in "Gary's Magic mountant" (LAWRENCE *et al.* 1986) and photographed on an Olympus Vanox photomicroscope. Bristle counts were conducted at 25× magnification on a dissecting microscope. A minimum of 20 flies of each genotype were examined for wing vein and bristle morphology. A total of 46 macrochaetae were scored on the head, thorax, and sternoplurum of each fly. For scanning electron micrographs, live specimens were mounted directly on specimen holders with silver paste and photographed on a Cambridge 250-T scanning electron microscope at 20 kV.

TABLE 1

Screen results: dominant *Enhancers of Egfr*

	Screening protocol		
	1	2	Total
F_1 males examined	12,372	11,008	23,380
Primary candidates (rescreen)	110	25	135
Secondary candidates	23	5	28
Second chromosome			
Compound alleles of *Egfr*	15	4	19
Second site enhancers	1	0	1
Third chromosome	7	1	8
X chromosome	—	0	0

RESULTS

Mutagenesis screen: Adult flies homozygous or transheterozygous for hypomorphic alleles of *Egfr* display defects in the eyes and ocelli, the macrochaetae, and the wing veins (CLIFFORD and SCHÜPBACH 1989; PRICE *et al.* 1989). These defects are sensitive to the level of *Egfr* activity present in the fly. For example, the wings of *Egfr*^{t1} homozygotes are often indistinguishable from wild-type wings (Figure 1a) and the most severe phenotype observed is the deletion of the anterior crossveins (acv; compare Figure 1, a and b). In contrast, the wings of flies that are hemizygous for *Egfr*^{t1} display a more severe defect: they are typically missing a section of the fourth longitudinal (L4) wing vein (compare Figure 1, a and c).

EMS mutagenesis screens were performed to identify dominant mutations that enhance the severity of the wing vein phenotypes observed in *Egfr*^{t1} homozygotes. EMS-treated *b pr cn Egfr*^{t1} *bw/b pr cn Egfr*^{t1} *bw* or *b pr cn Egfr*^{t1} *bw/CyO* adults were mated to individuals from an *Egfr*^{t1} *bw/CyO* tester strain (see MATERIALS AND METHODS). The wings of 23,380 *Egfr*^{t1} homozygous F_1 offspring were examined for enhanced wing vein phenotypes (Table 1 and Figure 1). F_1 individuals (135) with enhanced wing vein defects were rescreened to determine if the observed defects were heritable and dependent on an *Egfr*^{t1}/*Egfr*^{t1} genetic background. After rescreening, 28 independent mutant lines were established and retained as putative *Enhancers of Egfr* (*E(Egfr)*s).

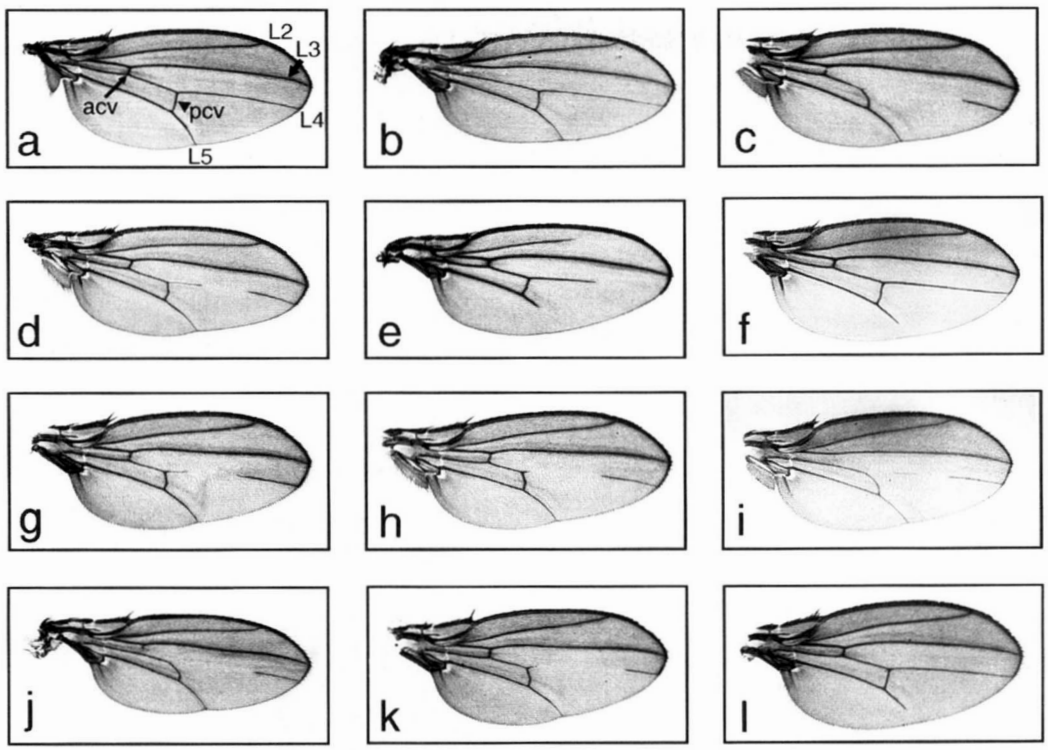

FIGURE 1.—Effects of *E(Egfr)s* on the wing vein pattern of $Egfr^{f1}$ homozygotes. All of the *E(Egfr)* mutations isolated in this screen cause dominant disruption of the L4 wing vein in an $Egfr^{f1}/Egfr^{f1}$ background (d and g–k) . *E(Egfr)B56* and H^{C21} each have a dominant phenotype in a wild-type background (f and l, respectively). (a) Wild-type wing; L2, L3, L4 and L5 indicate the second, third, fourth and fifth longitudinal veins, respectively. The anterior (acv) and posterior (pcv) crossveins are indicated. (b) $Egfr^{f1}/Egfr^{f1}$ wing, note that the acv is missing. (c) $Egfr^{f1}/Df(2R)Egfr18$. (d) *E(Egfr)B56*, $Egfr^{f1}/+$, $Egfr^{f1}$. (e) *E(Egfr)B56*, $Egfr^{f1}$/ *E(Egfr)B56*, $Egfr^{f1}$. (f) *E(Egfr)B56*/+. (g) $Egfr^{f1}/Egfr^{f1}$; $vn^{C221}/+$. (h) $Egfr^{f1}/Egfr^{f1}$; *E(Egfr)C12*/+. (i) $Egfr^{f1}/Egfr^{f1}$; $gro^{C105}/+$. (j) $Egfr^{f1}/Egfr^{f1}$; *E(Egfr)C22*/+. (k) $Egfr^{f1}/Egfr^{f1}$; $H^{C21}/+$. (l) $H^{C21}/+$.

Genetic mapping: Of the 28 mutations recovered, 20 segregated with the second chromosome and eight segregated with the third chromosome. Recombination and deficiency mapping were used to assign the putative *E(Egfr)*s to specific locations in the *D. melanogaster* genetic and cytogenetic maps (Table 2). In some cases, complementation tests allowed us to correlate *E(Egfr)* mutations with previously identified genes (Table 2B). Details of mapping and complementation tests are provided below.

Mutations on the second chromosome: The protocols used for our screens required that mutagenesis be conducted on flies that were homozygous or heterozygous for $Egfr^{f1}$, a weak allele of *Egfr*. New mutations in *Egfr*, superimposed on $Egfr^{f1}$, would be expected to cause a further decrease in *Egfr* activity, and an increase in the severity of the associated phenotypes. Thus we expected to recover compound mutations in *Egfr* as byproducts of our screen. Complementation tests and recombination mapping experiments indicated that 19 of the 20 putative second chromosome *E(Egfr)* mutations were most likely compound alleles of *Egfr*. These mutations were not examined further in this study. The one remaining *E(Egfr)* mutation on the second chromosome, *E(Egfr)B56*, was mapped by recombination to position 2-69 and subsequently assigned to cytogenetic interval 49D1; 49D4, as defined by the right and left hand breakpoints of *Df(2R)vg-135* and *Df(2R)vg-B*, respectively (Table 2).

Mutations on the third chromosome: Four of the eight *E(Egfr)* mutations on the third chromosome were found to be alleles of *Hairless* (*H*), as judged by the following criteria. Mutations *C21, C57,* and *C73* caused dominant shortening of the fifth longitudinal (L5) wing veins (Figure 1l) and loss of bristles and duplication of socket cells (Figure 2, c and h). *C21, C57,* and *C73* were lethal in homozygotes, and in transheterozygous combinations with H^1, H^2, or each other. Mutation *C24* was viable *in trans* to H^1, H^2, *C21, C57,* and *C73*, but the machrochaetae and microchaetae were almost completely eliminated from the heads and thoraxes of *C24/H* transheterozygotes (Figure 2, f and k), and the wings of *C24/H* transheterozygotes displayed shortened L4 and L5 wing veins. No recombination was detected between *C24* and H^{21C} among 893 recombinant chromosomes scored. *C24/C24* homozygotes were viable and wild type in appearance, except that ~13% were missing one or (rarely) both postvertical bristles from the head. Hereafter we shall refer to these new alleles of *H* as H^{C21}, H^{C24}, H^{C57} and H^{C73}.

The remaining *E(Egfr)* mutations (*E(Egfr)C105, E(Egfr)C221, E(Egfr)C22,* and *E(Egfr)C12*) were mapped by meiotic recombination, and complementation tests to deficiencies (Table 2). *E(Egfr)C105* was found to

TABLE 2

Meiotic and cytogenetic map locations of *E(Egfr)*s

	Deficiency breakpoints	*B56*	*C12*	*C22*
A. Results of complementation tests to deficiencies				
E(Egfr)B56				
Df(2R)vg135	48C-48D; 49D	+		
Df(2R)vg-C	49B2-3; 49E7-49F1	L2, L4, L5		
Df(2R)CX1	49C1-49C4; 50C23-50D2	L2, L4, L5		
Df(2R)vg-D	49C1-2; 49E4-5	L2, L4, L5		
Df(2R)vg-B	49D3-4; 49F15-50A3	+		
E(Egfr)C12				
Df(3R)GB104	85D11-13; 85E10		+	
Df(3R)M-Kx1	86C1; 87B5		+	
Df(3R)ry85	87B15-C1; 87F15-88A1		+	
Df(3R)red31	87F12-14; 88C1-3		+	
Df(3R)red1	88B1; 88D3-4		+	
Df(3R)sbd105	88F9-89A1; 89B9-10		+	
Df(3R)sbd26	89B9-10; 89C7-89D1		+	
Df(3R)P9	89D9-89E1; 89E4-5		+	
E(Egfr)C22				
Df(3R)e-N19	93B; 94			−
Df(3R)e-F1	93B6-7; 93E1-2			+
Df(3R)e-BS2	93C3-6; 93F14-94A1			−

	Approximate meiotic position	Cytogenetic position	Identity
B. Summary of *E(Egfr)* map locations and identities			
E(Egfr)B56	2-69	49D1-4	ND
E(Egfr)C12	3-54.5	ND	ND
E(Egfr)C22	3-77	93E1-94A1	ND
E(Egfr)C105	3-87	96F10-11	*gro*
E(Egfr)C221	3-19.5	64C13-65A	*vein*
E(Egfr)C21	ND	ND	*H*
E(Egfr)C24	ND	ND	*H*
E(Egfr)C57	ND	ND	*H*
E(Egfr)C73	ND	ND	*H*

+, deficiency complements *E(Egfr)*; −, deficiency is lethal *in trans* to *E(Egfr)*; L2, L4, L5, deficiency produces defects in these wing veins *in trans* to *E(Egfr)*; ND, not determined.

be an allele of *groucho* (*gro*), a gene in the *Enhancer of split* complex (*E(spl)-C*). It failed to complement *Df(3R)E(spl)BX22*, gro^1, and seven *E(spl)* alleles that behave as *gro* point mutations: $E(spl)^{r17}$, $E(spl)^{r18}$, $E(spl)^{r19}$, $E(spl)^{r20}$, $E(spl)^{r21}$, $E(spl)^{r22}$, and $E(spl)^{r26}$ (PREISS *et al.* 1988). Most mutations in *gro* are recessive lethal, but gro^1 is homozygous viable and causes an increase in the size and number of bristles on the head and humerus, and enlargement and/or fusion of the ocelli (LINDSLEY and ZIMM 1992; and see Figure 4b). *E(Egfr)C105*/gro^1 transheterozygotes displayed enlarged and fused ocelli, and an increased number of frontorbital microchaetae, but the supernumerary bristles were of normal size (Figure 4c). *E(Egfr)C105*/$E(spl)^{r17}$ transheterozygotes were semi-viable: ~30% survived to adulthood but died within 24 hr after eclosion. These escapers had rough eyes and thickened L5 wing veins; phenotypes previously described in association with *E(spl)* and *gro* mutations (DIAZ-BENJUMEA and GARCIA-BELLIDO 1990a; FISCHER-VIZE *et al.* 1992; LINDSLEY and ZIMM 1992; DE CELIS and RUIZ-GOMEZ 1995; HEITZLER *et al.* 1996). Transheterozygous combinations of *E(Egfr)C105* with the other six *E(spl)* alleles tested were inviable. Hereafter we will refer to this mutation as gro^{C105}.

E(Egfr)C221 was found to be an allele of *vn*. It failed to complement *Df(3L)ZN47*, vn^1, $vn^{ddd\text{-}1}$, $vn^{ddd\text{-}3}$, $vn^{ddd\text{-}13}$, and vn^{M2}. *E(Egfr)C221*/vn^1 transheterozygotes were viable and displayed deletions of the acv and a portion of the L4 proximal to the pcv; a phenotype similar to that observed in vn^1 homozygotes (DIAZ-BENJUMEA and GARCIA-BELLIDO 1990a). *E(Egfr)C221*/$vn^{ddd\text{-}13}$ transheterozygotes were semi-viable at 25°. Escapers survived for up to 3 days, and were of normal appearance except for held-out wings and the absence of acv's. Hereafter we will refer to this mutation as vn^{C221}.

E(Egfr)C22 was mapped to meiotic map position 3-77 and subsequently placed in cytogenetic interval 93E1; 94A1, based on its failure to complement *Df(3R)e-N19* and *Df(3R)e-BS2*, and its ability to complement *Df(3R)e-F1* (Table 2).

E(Egfr)C12 was mapped to meiotic map position 3-54.5. Deficiencies that removed sequences in cytogenetic interval 85D11 to 89E4 were crossed to *E(Egfr)C12* (Table 2). All were able to complement *E(Egfr)C12* lethality, so we could not assign the gene to a cytogenetic interval. There were several gaps in the deficiency set used for this region of the chromosome (see Table 2). The gene may be located in one of these gaps.

Enhancement of *Egfr* wing vein phenotypes: The dominant effects of six *E(Egfr)* mutations on the wings of $Egfr^{t1}$ homozygotes are shown in Figure 1 (d and g–k). The wing vein phenotypes produced by each of these mutations were almost identical to the effects of a direct reduction in *Egfr* activity (compare to Figure 1c). In addition to enhancing the L4 wing vein defect, all but two of the *E(Egfr)*s examined increased the frequency of acv deletion above the baseline frequency observed in $Egfr^{t1}$ homozygotes (Table 3). *E(Egfr)B56*, $Egfr^{t1}$ double homozygotes showed a more striking phenotype: the second, fourth and fifth longitudinal wing veins (L2, L4 and L5, respectively) were all shortened (Figure 1e). *E(Egfr)B56*, $Egfr^+$/*E(Egfr)B56*, $Egfr^+$ homozygotes also displayed short L2, L4 and L5 wing veins, but the phenotype was less severe in an $Egfr^+$ genetic background. *E(Egfr)B56*/+ flies sometimes displayed a dominant short L5 phenotype (Figure 1f).

Dominant interactions in transheterozygous combinations of *E(Egfr)* mutations with *Df(2R)Egfr18*: *E(Egfr)*s were isolated on the basis of their enhancement of $Egfr^{t1}$ mutant phenotypes. To distinguish between allele specific interactions with $Egfr^{t1}$ and interactions sensitive to a general reduction in *Egfr* activity, each *E(Egfr)* was crossed to *Df(2R)Egfr18*, and transheterozygotes were examined for wing defects. In a wild-type genetic back-

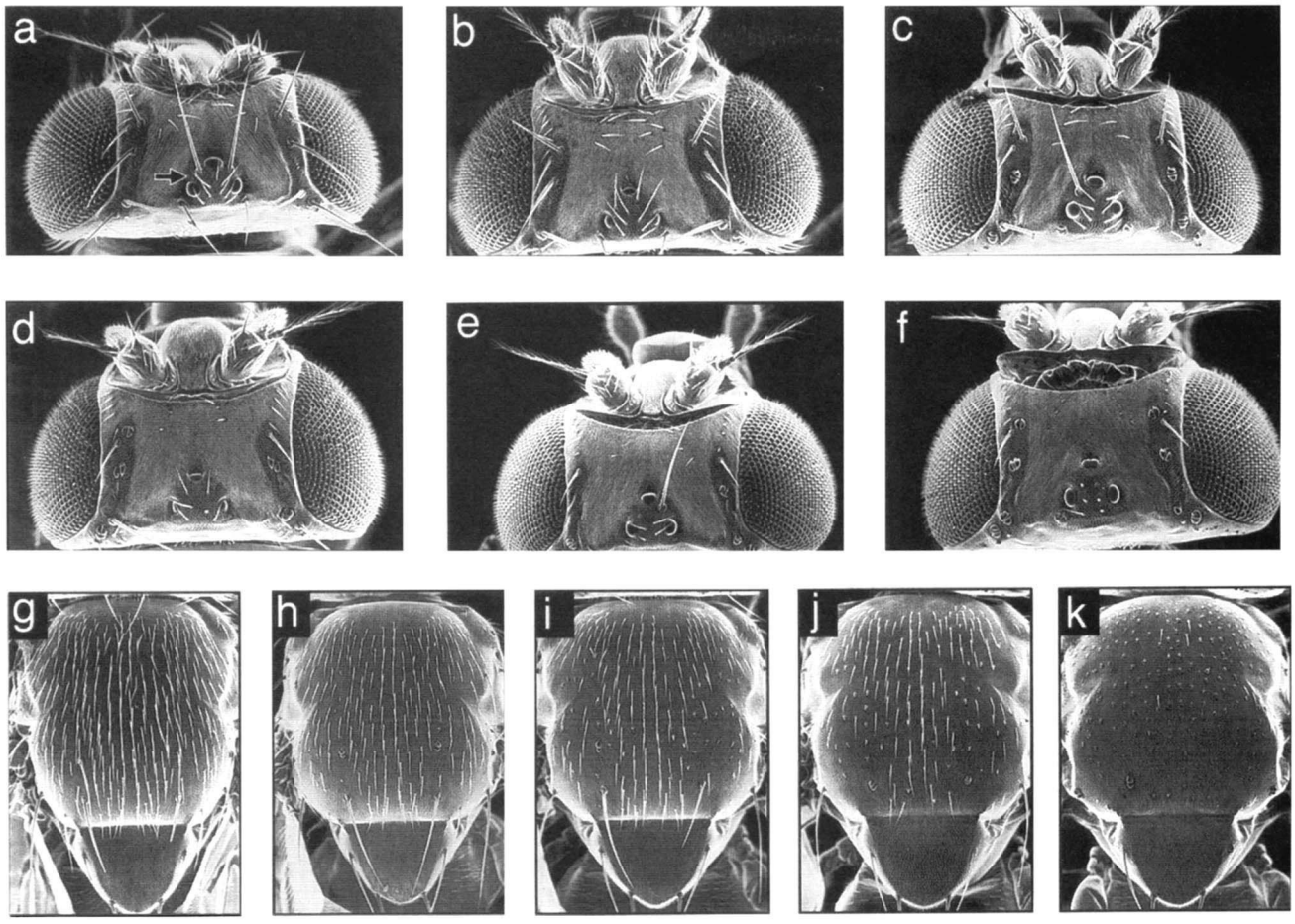

FIGURE 2.—Mutations in *Egfr* and *gro* enhance bristle loss in *H*/+ flies. Scanning electron micrographs of the bristle patterns on the heads and thoraxes of wild type (a), $Egfr^{f1}/Egfr^{f1}$ (b and g), H^{C21}/+ (c and h), $Egfr^{f1}/Egfr^{f1}$; H^{C21}/+ (d and i), H^{C21}/gro^{C105} (e and j) and H^{C21}/H^{C24} (f and k). Anterior is up in all panels. Arrow in a points to the base of the left ocellar bristle. The ocellar bristles are usually absent in $Egfr^{f1}$ homozygotes (b and d). Note the decreased number of macrochaetae and microchaetae in parts d–f and i–k.

ground *Df(2R)Egfr18/*$Egfr^{+}$ hemizygotes have wild-type wing venation. Each *E(Egfr)/+; Df(2R)Egfr18/+* transheterozygous combination caused thinning or gaps in the L4, although in some cases the penetrance was very low (Figure 3, c and d; Table 4). Thus, the interactions we have observed do not appear to be specific for $Egfr^{f1}$;

TABLE 3

Phenotypic manifestations of *E(Egfr)*s

Genotype	Disruption of L4 wing vein (frequency)	Frequency of acv disruption	*E(Egfr)* homozygous phenotype[a]
$Egfr^{f}/Egfr^{f}$	No	0.01	n/a
Df(2R)Egfr18/$Egfr^{f}$	Yes (0.78)	0.20	n/a
E(Egfr)B56, $Egfr^{f}$/+, $Egfr^{f}$	Yes (0.14)	0.00	Viable, L2, L4, L5
$Egfr^{f}/Egfr^{f}$; *E(Egfr)C12/+*	Yes (0.92)	0.29	LL
$Egfr^{f}/Egfr^{f}$; H^{C21}/+	Yes (1.00)	0.09	LL
$Egfr^{f}/Egfr^{f}$; *E(Egfr)C22/+*	Yes (0.11)	0.25	PL
$Egfr^{f}/Egfr^{f}$; H^{C24}/+	None[b]	0.00	Viable, PVB
$Egfr^{f}/Egfr^{f}$; H^{C57}/+	Yes (ND)	ND	LL
$Egfr^{f}/Egfr^{f}$; H^{C73}/+	Yes (ND)	ND	LL
$Egfr^{f}/Egfr^{f}$; gro^{C105}/+	Yes (1.00)	0.14	LL
$Egfr^{f}/Egfr^{f}$; vn^{C221}/+	Yes (0.24)	0.57	LL

L2, L4, L5, deletion of the second, fourth and fifth longitudinal veins, respectively; LL, larval lethal; PL, pupal lethal; PVB, occasional deletion of postvertical bristles; n/a, not applicable; ND, not determined.

[a] These phenotypes were determined in an $Egfr^{+}/Egfr^{+}$ genetic background.

[b] Dominant expression of this phenotype has faded, but it is still expressed in H^{C24} homozygotes.

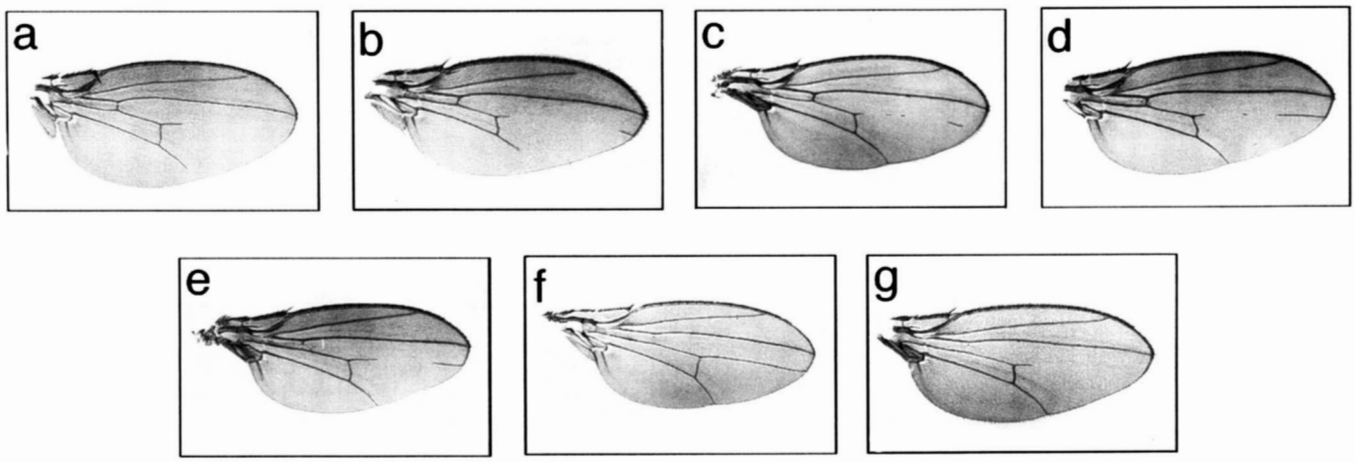

FIGURE 3.—Dominant interactions between *E(Egfr)* mutations in the wings. Wings were taken from transheterozygotes of the following genotypes: (a) vn^{C221}/H^{C21}. (b) *E(Egfr)B56/+*; $vn^{C221}/+$. (c) *E(Egfr)B56/Df(2R)Egfr18*. (d) *Df(2R)Egfr18/+*; $vn^{C221}/+$. (e) gro^{C105}/vn^{C221}. (f) gro^{C105}/*E(Egfr)C22*. (g) *E(Egfr)C12*/vn^{C221}. Wings from *E(Egfr)B56/+* and $H^{C21}/+$ single heterozygotes are shown in Figure 1f and 1l, respectively. Wings from all other single heterozygotes are wild type in appearance.

they can be produced by a general reduction in *Egfr* activity.

Dominant interactions in transheterozygous combinations of *E(Egfr)* mutations with each other: Two alleles of *H* (H^{C21} and H^{C24}) and each of the other *E(Egfr)*s were crossed *inter se*, in an $Egfr^{+}$ genetic background. With one exception (H^{C21}/gro^{C105}; see below), transheterozygotes for each combination had deletions or gaps in the acv and/or L4 wing veins (Figure 3 and Tables 4 and 5). Deficiencies *Df(2R)vgC*, *Df(3R)BX22*, *Df(3L)ZN47*, and *Df(3R)e-BS2*, which fail to complement *E(Egfr)B56*, gro^{C105}, vn^{C221} and *E(Egfr)C22*, respectively, produced dominant interactions similar to those generated by the corresponding point mutants (data not shown). This suggests that the interactions associated with *E(Egfr)B56*, gro^{C105}, vn^{C221} and *E(Egfr)C22* are due to loss of the normal wild-type functions of these genes.

E(Egfr)B56 and H^{C21} produced very similar wing phenotypes. Both caused dominant shortening of L5, both produced defects in L4 and L5 when placed in transheterozygous combinations with other *E(Egfr)* mutations, and both caused shortening of the L2 in some circumstances. However, *E(Egfr)B56*/gro^{C105} transheterozygotes displayed L4 wing vein defects while H^{C21}/gro^{C105} transheterozygotes did not.

gro^{C105} produced a marked enhancement of H^{C21} bristle loss (compare Figure 2c to 2e and Figure 2h to 2j): gro^{C105}/H^{C21} transheterozygotes had an average of 14.7 macrochaetae per fly, as compared to an average of 29.5 macrochaetae present on $H^{C21}/+$ heterozygotes. gro^{C105} also enhanced the H^{C21}-associated loss of microchaetae (Figure 2). Similar enhancement of *H* phenotypes was observed in *Df(3R)BX22*/H^{C21} transheterozygotes, consistent with the interpretation that the interactions we observed were due to reduction of *gro* activity in gro^{C105}/H^{C21} transheterozygotes. We did not detect any comparable bristle defects in *E(Egfr)B56*/gro^{C105} transheterozygotes.

***E(Egfr)* mutations act as dominant modifiers of *Notch* and *Suppressor of Hairless*:** *E(Egfr)* mutations were crossed *inter se* to alleles of *Notch* (*N*) and *Suppressor of Hairless* (*Su(H)*). Heterozygotes for N^{55e11} or $Su(H)^{S5}$ exhibit dominant notched wing phenotypes (LINDSLEY and ZIMM 1992; FORTINI and ARTAVANIS-TSAKONAS 1994). This wing-notching was partially or completely suppressed in all transheterozygous combinations of N^{55e11} or $Su(H)^{S5}$ with any of the *E(Egfr)*s except $N^{55e11}/+$; $gro^{C105}/+$ (Table 6). $N^{55e11}/+$ flies also display deltas at the distal ends of the wing veins, and these too were suppressed in transheterozygotes. $Su(H)^{AR9}$ is known to promote wing vein loss when transheterozygous with N gain-of-function (gof) alleles (FORTINI and ARTAVANIS-TSAKONAS 1994). Transheterozygous combinations of $Su(H)^{AR9}$ with *E(Egfr)B56*, gro^{C105}, or vn^{C221} also resulted in deletion of wing veins (Table 6). In summary, *E(Egfr)*s acts as dominant modifiers of mutations in several elements of the *N* pathway.

TABLE 4

Dominant interactions between *Df(2R)Egfr18*, *E(Egfr)B56*, and third chromosome *E(Egfr)*s: phenotypes observed in transheterozygotes

	Df(2R)Egfr18	*E(Egfr)B56*
vn^{C221}	L4 (1.00)	L2, L4 (1.00), L5
gro^{C105}	L4 (0.20)	L4 (0.21)
H^{C24}	L4 (0.06)	L4 (0.74), L5
E(Egfr)C22	L4 (0.04)	L4 (0.54), L5
H^{C21}	L4 (0.03), L5, B	L4 (1.00), L5
E(Egfr)C12	L4 (0.33)	L2, L4 (0.94), L5
E(Egfr)B56	L4 (0.47)	L2, L4 (1.00), L5

L2, L4, L5, deletion of the second, fourth and fifth longitudinal wing veins, respectively; B, promotion of bristle loss. Numbers in parentheses indicate penetrance of L4 defects. Crosses involving H^{C21} were performed at 18°, all other crosses were done at 29°. (H^{C21} displays a temperature-sensitive L4 defect which is nonpenetrent at 18°).

TABLE 5

Dominant interactions in transheterozygous combinations of third chromosome *E(Egfr)*s

	E(Egfr)C12	H^{C21}	*E(Egfr)C22*	H^{C24}	gro^{C105}	vn^{C221}
vn^{C221}	L4 (100%)	L4 (100%), L5	L4 (100%), acv	L4 (80%)	L4 (95%), acv	LL
gro^{C105}	acv	B	acv	acv	LL	
H^{C24}	L4 (100%), L5	L4 (100%), L5, B	acv	PVB		
E(Egfr)C22	acv	L4 (~17%), L5	PL			
H^{C21}	L4 (100%), L5	LL				
E(Egfr)C12	LL					

Crosses involving H^{C21} were performed at 18°; all other crosses were done at 29°. LL, larval lethal; PL, pupal lethal; B, enhanced loss of head and thoracic bristles; PVB, loss of postvertical bristles. Numbers in parentheses indicate penetrence of L4 defects.

Egfr mutations enhance *H* bristle loss: $Egfr^{t1}/Egfr^{t1}$; H^{C21}/+ flies had a reduced number of machrochaetae (average = 24.5) as compared to H^{C21}/+ (average = 29.5; Figure 2). Bristle loss was most noticeable on the head. This effect was partly due to the elimination of the ocellar bristles, which are often deleted in $Egfr^{t1}$ homozygotes (CLIFFORD and SCHÜPBACH 1989), but are often present in *H*/+ heterozygotes (BANG *et al.* 1991). However additional bristles, such as the anterior orbital bristles (which are usually present in either of the single mutants), were often deleted from $Egfr^{t1}/Egfr^{t1}$; H^{C21}/+ flies. $Egfr^{t1}/Egfr^{t1}$; H^{C21}/+ flies also had a reduced number of microchaetae on the head and thorax, as compared to H^{C21}/+. *Df(2R)Egfr18*/+; H^{C21}/+ and *Df(2R)Egfr3*/+; H^{C21}/+ transheterozygotes also displayed reduced numbers of macrochaetae (average = 23.7 and 22.5, respectively). This enhancement of H^{C21}/+ bristle loss by *Egfr* mutations was similar to that observed in gro^{C105}/H^{C21} or H^{C21}/H^{C24} transheterozygotes, but less pronounced. Similar interactions were observed between $Egfr^{t1}$ and other strong alleles of *H*.

***Egfr* mutations enhance multiple *gro* phenotypes:** To confirm that the *E(Egfr)* phenotype observed in association with gro^{C105} was due to a reduction in *gro* activity, we constructed $Egfr^{t1}/Egfr^{t1}$; *Df(3R)BX22*/+ flies. Flies of this genotype displayed gaps in the L4 wing veins, similar to those observed in the wings of $Egfr^{t1}/Egfr^{t1}$; gro^{C105}/+ flies.

$Egfr^{t1}/Egfr^{t1}$; gro^{C105}/+ did not display any *groucho* phenotypes. To determine if $Egfr^{t1}$ caused enhancement of *groucho* phenotypes, we constructed a balanced stock of genotype $Egfr^{t1}$/*CyO*; gro^{1}/gro^{1}. The original gro^{1}/gro^{1} stock, and out-crossed derivatives, showed incomplete penetrance for the *groucho* phenotype (as judged by bristle hyperplasia and/or enlarged ocelli; Table 7). In these gro^{1} and outcrossed gro^{1} stocks we observed a range of 3–71% penetrance, depending on genotype (see also ZIEMER *et al.* 1988; LINDSLEY and ZIMM 1992). In $Egfr^{t1}$/*CyO*; gro^{1}/gro^{1} flies the *groucho* phenotype was 100% penetrant (Table 7). Penetrance was increased in both male and female flies. Furthermore, $Egfr^{t1}$/*CyO*; gro^{1}/gro^{1} males often displayed strongly enhanced *groucho* phenotypes that were seldom observed in females of the same genotype (Table 7). In the affected males, large patches of bristles were present over each eye, the head cuticle was distorted into lumps and pits,

TABLE 6

***E(Egfr)*s interact with mutations in *N* pathway genes**

	Wing notching		Deltas	L4 wing vein defects			
	N^{55e11}	$Su(H)^{S5}$	N^{55e11}	$Su(H)^{AR9}$	gro^{C105a}	H^{C21a}	H^{C24a}
E(Egfr)B56	−	−−	−−−	++	++	+++[b]	+++
E(Egfr)C12	−−	−−−	−−	0	0	+++[b]	+++
E(Egfr)C22	−	−	−	0	0	++[b]	0
gro^{C105}	0	−−	−	+	NA	0	0
H^{C21}	−−−	−−−	−−−	ND	0[b]	NA	+++[b]
vn^{C221}	−−−	−−−	−−−	+++	+++	+++[b]	+++

Transheterozygous combinations of *E(Egfr)*s and mutations in *N* pathway genes were constructed. *E(Egfr)*s were scored for their influence on dominant N^{55e11} of $Su(H)^{S5}$ wing notching phenotypes, N^{55e11} wing vein deltas, and the L4 wing veins of $Su(H)^{AR9}$, gro^{C105}, H^{C21}, and H^{C24} in transheterozygotes. 0, no effect; −, weak suppression; −−, moderate suppression; −−−, complete suppression; +, weak enhancement; ++, moderate enhancement; +++, strong enhancement; NA, not applicable (gro^{C105} and H^{C21} homozygotes are inviable); ND, not determined.

[a] Data in these columns are derived from Tables 4 and 5 and are included here for comparison purposes.

[b] All genotypes were raised at 25° except those where L4 wing vein deletions were examined in transheterozygous combinations with H^{C21} (see Table 4 legend).

TABLE 7

Reduction of *Egfr* activity leads to an increase in *gro*^{1}/*gro*^{1} penetrance and increased expressivity of *gro*^{1}/*gro*^{1} in males

	Males			Females		
	Nonexpressing	Expressing	Strong expression	Nonexpressing	Expressing	Strong expression
gro^1/*gro*^{1} (outcrossed line)	15 (26)	32 (55)	11 (19)	17 (35)	32 (65)	0
Egfr^{t1}/*CyO; gro*^{1}/*gro*^{1}	0	2 (4)	50 (96)	0	58 (100)	0

Nonexpressing indicates *gro* phenotypes (bristle hyperplasia and/or ocellar hypertrophy) were not detected at 40× magnification on a dissecting microscope. Expressing indicates *gro* phenotypes were detected at 40× magnification on a dissecting microscope, but were not easily scored at 10× magnification. Strong expression indicates *gro* phenotypes could be scored at 10× magnification. Values in parentheses are percentages.

and the ocelli were often greatly enlarged to produce a tumorous mass in the center of the frons (Figure 4e). These ocellar tumors frequently contained small patches of cells that contained bright red pigments. Bristle and ocellar defects of this severity were never observed in *gro*^{1}/*gro*^{1} adults from the original stock, *gro*^{1} homozygotes derived from outcrossing the *gro*^{1} stock to wild-type stocks, or *gro*^{1}/*Df(3R)BX22* hemizygotes, so we believe that the observed enhancement was not due to removal of modifiers from the original *gro*^{1} stock during construction of the double mutant flies.

Further reduction of *Egfr* activity produced a somewhat paradoxical effect, in that some aspects of the *groucho* phenotype were less severe. The ocelli and bristle patterns of most *Egfr*^{t1}/*Egfr*^{t1}; *gro*^{1}/*gro*^{1} flies appeared normal or near normal. Since *Egfr* mutations cause bristle loss and the reduction or deletion of ocelli (CLIFFORD and SCHÜPBACH 1989; and see Figures 2b and 4d), and *gro*^{1} causes the opposite effects, mutations in the two genes may neutralize each others' effects on the bristles and ocelli of double homozygotes. The apparent neutralization of ocellar and bristle defects was not complete: bristle hyperplasia and ocellar enlargement and/or fusion were still observed sporadically in *Egfr*^{t1}/*Egfr*^{t1}; *gro*^{1}/*gro*^{1} individuals. At the same time, other phenotypes associated with mutations in *gro* were expressed. *Egfr*^{t1}/*Egfr*^{t1}; *gro*^{1}/*gro*^{1} males displayed wing defects, including ectopic vein material, notching of the wing margin, ectopic wing hairs, anterior outgrowths of the wing blade, bifurcation of legs, and longitudinal fusion of the tibia and femur (Figure 4, g–l; Table 8). These more severe phenotypes were sporadic and were almost exclusively restricted to males.

A mutation in *rolled* also causes enhancement of *gro* phenotypes: *rolled* encodes a Drosophila mitogen-activated protein kinase (BIGGS *et al.* 1994) that participates in EGFR signal transduction (BRUNNER *et al.* 1994; DIAZ-BENJUMEA and HAFEN 1994). To determine whether other elements in the *Egfr* signaling pathway also enhanced *gro* defects, we constructed *rl*^{1}/*rl*^{1}; *gro*^{1}/*gro*^{1} double mutant flies. These flies displayed a spectrum of *gro* enhancement comparable to that observed in *Egfr*^{t1}/*Egfr*^{t1}; *gro*^{1}/*gro*^{1} double mutants, except that anterior wing outgrowths were not observed (Table 8). The phenotypic expression of *rl*^{1} was also enhanced in double mutants: the eyes were smaller and rougher, and the wings were more strongly rolled (data not shown).

Simultaneous reduction of *Egfr* and *gro* activities leads to the formation of ectopic eyes: *Egfr*^{t1} and *gro*^{1} are both weak hypomorphic alleles. Efforts were made to construct more severe *Egfr, gro* double mutant combinations that would lead to increased penetrance or more severe phenotypic interactions. Stocks heterozygous for moderate or severe alleles of *Egfr* and homozygous for *gro*^{1} were constructed. Flies of genotypes *Egfr*^{f3}/*CyO; gro*^{1}/*gro*^{1}, *Egfr*^{f1}/*CyO; gro*^{1}/*gro*^{1}, and *Df(2R)Egfr3/CyO; gro*^{1}/*gro*^{1} displayed an array of enhanced *gro* defects similar to those observed in *Egfr*^{t1}/*CyO; gro*^{1}/*gro*^{1} or *Egfr*^{t1}/*Egfr*^{t1}; *gro*^{1}/*gro*^{1} (see above). In addition, these genotypes also produced ectopic compound eyes (Figure 5). Like the endogenous compound eyes, the ectopic eyes were organized into arrays of ommatidia. They were frequently adjacent to the endogenous eye, but were separated from it by a discrete border, and often showed more pronounced defects in ommatidial packing and the distribution of the interommatidial bristles. As described above, the more extreme *groucho* phenotypes (*i.e.*, ectopic eyes, and fused or bifurcated legs) were almost exclusively restricted to males.

Egfr^{t1}/*Df(2R)Egfr18; gro*^{1}/*gro*^{1} flies displayed a combination of *Egfr* and *gro* phenotypes. *Egfr*^{t1}/*Df(2R)Egfr18* flies rarely have ocellar bristles, and their ocelli are reduced to tiny rudiments (CLIFFORD and SCHÜPBACH 1989; and see Figure 4d). The L4 wing vein is usually deleted in flies of this genotype (see above; Figure 1c; Table 3). In *Egfr*^{t1}/*Df(2R)Egfr18; gro*^{1}/*gro*^{1} double mutants the L4 wing veins were restored, and the ocellar bristles were present but disarrayed (Figure 5, g and h). In many cases supernumerary ocellar bristles and/or interocellar bristles were present, producing a patch of bristles where the ocelli would normally have been. The ocelli were either reduced or eliminated, but most males of this genotype displayed one or more ectopic eye structures dorso-medial to the normal eyes (Figure 5, g and h). These ectopic eyes were much smaller than those described above (compare Figure 5, a, c and e to g). Some of these structures contained discrete omma-

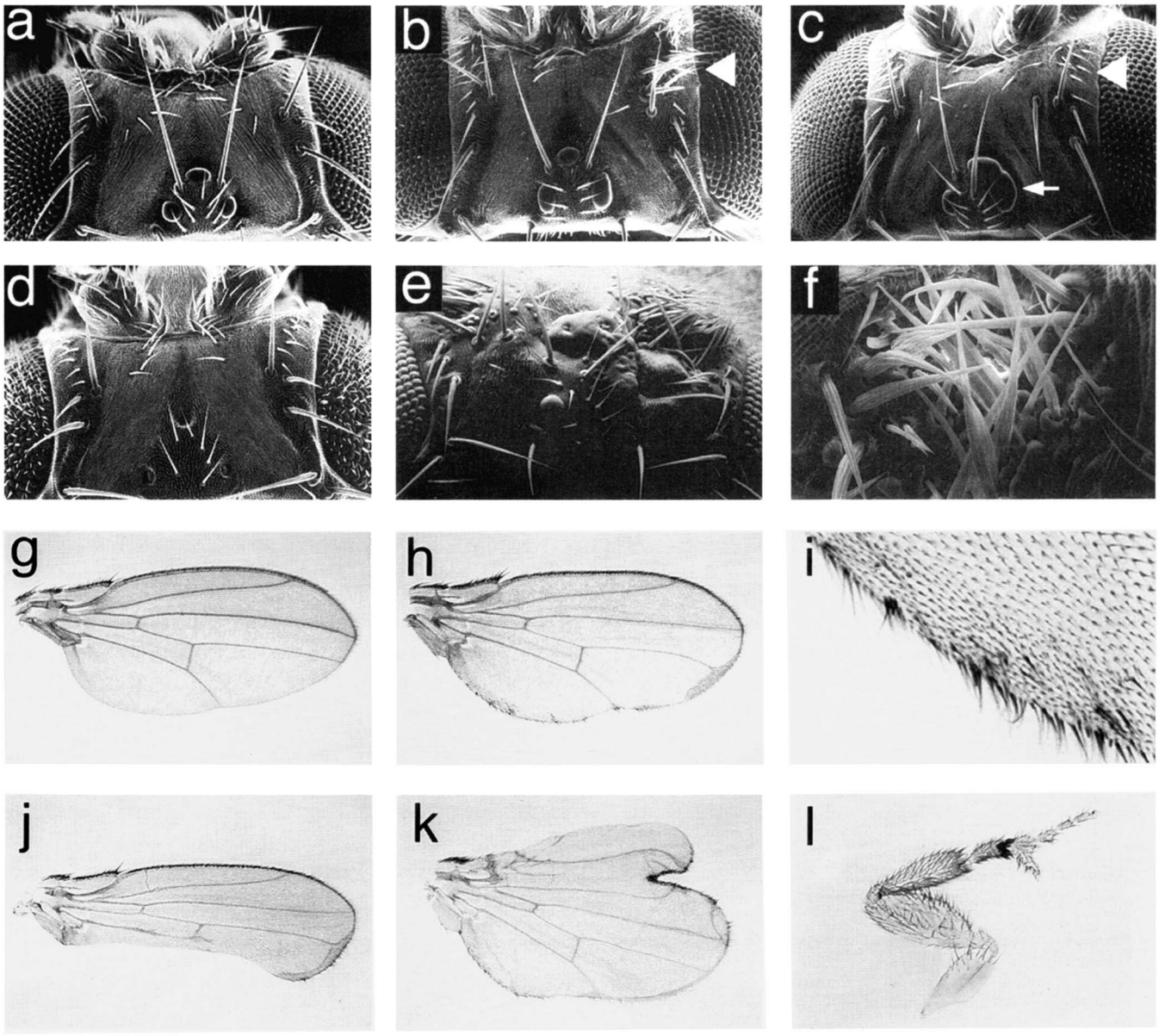

FIGURE 4.—Mutations in *Egfr* enhance the bristle, ocellar, wing, and limb phenotypes of gro^1/gro^1 flies. (a–f) Scanning electron micrographs of the dorsal aspect of the head from flies of the following genotypes: (a) wild type, (b) gro^1/gro^1, (c) gro^1/gro^{C105}, (d) $Egfr^{f1}/Df(2R)Egfr18$, (e) $Egfr^{f1}/CyO; gro^1/gro^1$, (f) $Egfrf13/CyO; gro^1/gro^1$. Anterior is up in all panels. Supernumerary frontorbital bristles are present to varying degrees in b, c and e (arrowheads in b and c). A closeup of supernumerary frontorbital bristles is shown in f. The ocelli are somewhat enlarged in gro^1/gro^{C105} (c, arrow), greatly reduced in $Egfr^{f1}/Df(2R)Egfr18$ (d), and greatly enlarged in $Egfr^{f1}/CyO; gro^1/gro^1$ (e). The lump in the top center of e is a hypertrophic ocellus. (g–l) Light micrographs of wings and legs. Wings from gro^1/gro^1 mutants are usually wild type in appearance (g). $Egfr^{f1}/Egfr^{f1}; gro^1/gro^1$ double mutants display a variety of wing defects including ectopic vein material along the distal margins (h and j), ectopic hairs (h and i), recession or notching of the posterior wing margin (j), and mirror image outgrowths from the anterior wing margin (k). They also display bifurcation of limbs (l). l, the first leg of a male, displays a bifurcated sex-comb. Defects like those shown in e and f and h–l are almost exclusively found in males (Table 7 and see text).

tidia, but they did not contain interommatidial bristles. In most cases the lens material of these small ectopic eyes was fused into a uniform glaze, and they were recessed into the cuticle of the head. These small, fused eyes still contained red pigmentation like the normal compound eyes, as opposed to the amber coloring of ocelli. Repeated efforts to generate more severe combinations of *gro* alleles in an $Egfr^{f1}/Egfr^{f1}$ background (*e.g.*, $Egfr^{f1}/Egfr^{f1}; gro^1/Df(3R)BX22$) were unsuccessful. These observations may indicate that more severe combinations of *gro* are inviable in an $Egfr^{f1}$ mutant background.

DISCUSSION

Components of the EGFR signaling pathway in wing vein development: Signaling via EGFR and its downstream effectors is a major determinant of wing vein development. The genes *argos, rolled* (*rl*; *MAP kinase*),

TABLE 8

Mutations in *Egfr* and *rl* act as enhancers of *groucho*

Genotype	Bristle pattern and ocellar morphology	Wing morphology	Leg morphology
gro^1/gro^1 (out-crossed)	Bristle hyperplasia, ocelli enlarged and/or fused	Wings normal	Legs normal
$Egfr^{t1}/Egfr^{t1}$; gro^1/gro^1	Enhanced bristle hyperplasia, enhanced ocellar enlargement, enhanced fusion of ocelli	Wings notched, hairy wing margins, ectopic vein material, anterior wing outgrowths	Legs fused, bifurcated
rl^1/rl^1; gro^1/gro^1	Enhanced bristle hyperplasia, enhanced ocellar enlargement, enhanced fusion of ocelli	Wings notched, hairy wing margins, ectopic vein material	Legs fused, bifurcated

Dsor1 (*Mek*), *l(1)ph* (*Raf*), *Star/asteroid*, and *rhomboid/veinlet* all encode known or suspected components of the EGFR signal transduction pathway (reviewed in SCHWEITZER and SHILO 1997), and mutations in each of these can cause loss of the L4 and other wing veins (DIAZ-BENJUMEA and GARCIA-BELLIDO 1990a; LINDSLEY and ZIMM 1992; STURTEVANT *et al.* 1993; TSUDA *et al.* 1993; BRUNNER *et al.* 1994; DIAZ-BENJUMEA and HAFEN 1994; HSU and PERRIMON 1994; SAWAMOTO *et al.* 1994; SCHWEITZER *et al.* 1995a; STURTEVANT and BIER 1995). SCHNEPP *et al.* (1996) have recently shown that *vn* encodes a neuregulin-like secreted peptide that is strongly implicated as a ligand for EGFR. Our identification of a mutation in *vn* verifies that our screening protocol is capable of identifying factors that participate in EGFR signaling.

Genetic interactions between the EGFR and N signaling pathways: *H* and *gro*, two of the genes identified as *E(Egfr)*s in this study, encode known components of the N signaling pathway. Activation of the N receptor causes translocation of Su(H) from the cytoplasm to the nucleus (FORTINI and ARTAVANIS-TSAKONAS 1994), where Su(H) stimulates transcription of genes in the *E(spl)-C* (BAILEY and POSAKONY 1995; LECOURTIS and SCHWEISGUTH 1995). H acts as an antagonist of Su(H) (BROU *et al.* 1994) and thereby inhibits N signaling (BANG *et al.* 1995) and *E(spl)-C* transcription (BAILEY and POSAKONY 1995; LECOURTIS and SCHWEISGUTH 1995). Gro has been proposed to act as a transcriptional corepressor that acts in concert with the activity of the Hairy-related basic helix-loop-helix (bHLH) gene products of the *E(spl)-C* (PAROUSH *et al.* 1994) to help propagate the N signal (PREISS *et al.* 1988; SCHRONS *et al.* 1992; HEITZLER *et al.* 1996). Consistent with their opposing roles in the *N* pathway, mutations in *H* and *gro* have opposite influences on the decisions of cells to follow alternative developmental fates, and this influence is reflected in their opposing phenotypes in the wing vein and bristle patterns of the fly.

Genetic interactions between the N and EGFR signaling pathways have been reported previously. DIAZ-BENJUMEA and GARCIA-BELLIDO (1990b), who also examined wing vein patterns, observed mutual enhancement between N^{Ax} (gof) and *Egfr* loss-of-function (lof) mutations, and mutual suppression between lof alleles of *Delta* (*Dl*; encodes a N ligand) and *Egfr*. BAKER and RUBIN (1992) observed that *Egfr* gof alleles ($Egfr^E$; a.k.a. *Ellipse*) enhanced N^{spl} in the eyes and *Dl* lof alleles in the wings of double mutant flies. VERHEYEN *et al.* (1996) recovered mutations in *Egfr* and two other *Egfr* pathway components (*Son of sevenless*, and *pointed*) as enhancers of N signaling in the eye. All of the above interactions are consistent with the view that the *Egfr* and *N* signaling pathways oppose each other in the wing and eye. Combinations of lof mutations in the two pathways tend to cancel each others' phenotypes (mutual suppression), while combination of a gof mutation in one pathway and a lof mutation in the other pathway tend to cause mutual enhancement of phenotypes. Our data indicate that mutations in *E(Egfr)B56*, *E(Egfr)C12* and *E(Egfr)C22* and *vn* suppress *N* lof phenotypes, and enhance *H* lof in a manner consistent with the above pattern.

Based on the genetic interactions we have observed, *E(Egfr)B56*, *E(Egfr)C12* and *E(Egfr)C22* may encode positive transducers of the EGFR pathway or negative regulators of the N pathway. *E(Egfr)C12* and *E(Egfr)C22* primarily cause defects in the acv and the L4 wing vein. In this respect their phenotypes are most similar to those associated with alleles of *Egfr*, *rl*, or *vn*, and may reflect a primary role in the EGFR pathway. Previously described interactions between *Egfr*, *rho*, *rl* and *vn* (DIAZ-BENJUMEA and GARCIA-BELLIDO 1990b; STURTEVANT *et al.* 1993; BRUNNER *et al.* 1994) are consistent with this interpretation. *E(Egfr)B56* causes defects in the L2, L4 and L5 wing veins, phenotypes that are very similar to those caused by *H* or N^{Ax} mutations. This similarity may indicate a primary role for *E(Egfr)B56* in the N pathway.

Interactions between *Egfr*, *vn*, *H* and *gro*: Mutations in both *H* and *gro* enhance the L4 defects associated with mutations in *Egfr* or *vn*. Even an extremely weak allele of *H* (H^{C24}) was able to enhance *Egfr* and *vn* wing defects in double heterozygotes, suggesting that *Egfr* and *vn* are remarkably sensitive to reductions in *H* activity. In turn, *Egfr* mutations enhanced the *H* associated loss of macrochaetae and microchaetae from the head and thorax, so EGFR and H appear to cooperate in at least two developmental processes. Mutations in *vn* did

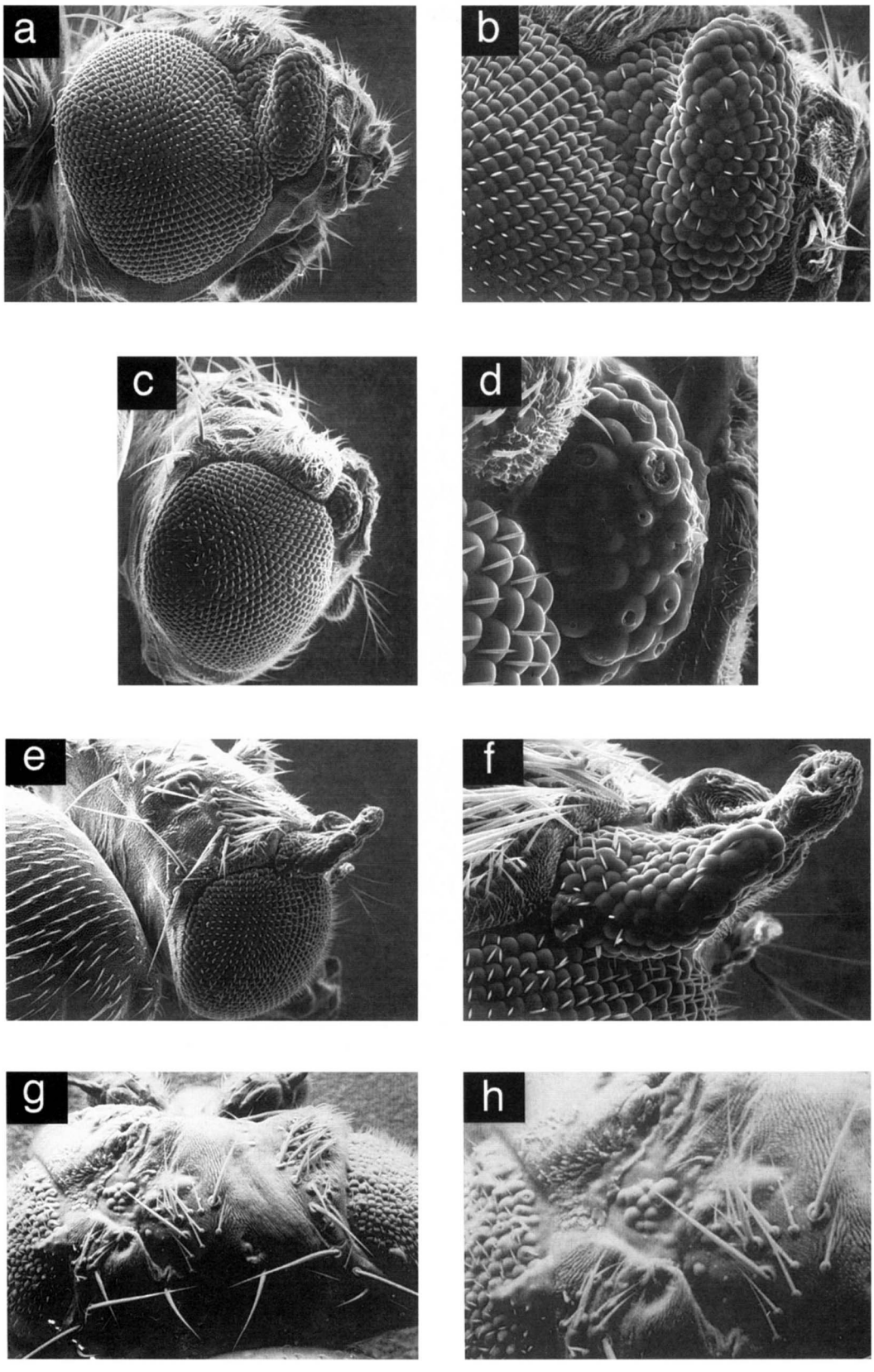

FIGURE 5.—Simultaneous reduction of *Egfr* and *gro* activities leads to the formation of ectopic eyes. (a and b) $Egfr^{f13}/CyO; gro^{1}/gro^{1}$. (c and d) $Egfr^{f1}/CyO; gro^{1}/gro^{1}$. (e and f) *Df(2R)-Egfr3/CyO;* gro^{1}/gro^{1}. (g and h) *Df(2R)Egfr18/* $Egfr^{f1}; gro^{1}/gro^{1}$. a, c, e and g are enlarged in b, d, f and h, respectively. All ectopic eyes contained the red pigmentation typical of the compound eye, but the interommatidial bristles were partially (a, b, e and f) or completely (c, d, g and h) absent. The ectopic eye shown in e and f is supported by a projection of cuticle, visible in the top right corner of f. The ectopic eye in g and h is quite degenerate, but is larger and better formed than most ectopic eyes from this genotype. Defects like those shown in all panels are almost exclusively found in males.

not appear to influence the severity of *H* associated bristle loss: the interactions between *vn* and *H* appear to be confined to the wing. This implies that Vn does not participate in *Egfr* signaling during the differentiation of sensory organ precursors. However we cannot exclude the possibility that reduced levels of *vn* activity are sufficient for bristle differentiation. All of the interactions between *H* and *Egfr* or *H* and *vn* can be interpreted as additive, in that lof mutations in all three genes have a tendency to promote wing vein loss and

mutations in *H* and *Egfr* have a tendency to delete bristles, but their combined effects are synergistic.

Since reductions in *gro* activity are associated with thickened veins and tend to promote vein development, it was surprising to find that gro^{C105} and *Df(3R)BX22* promote the loss of L4 wing vein when transheterozygous with mutations in *Egfr* or *vn*. Homozygosity for gro^{1} had the opposite effect: it suppressed the L4 wing vein defects normally observed in $Egfr^{t1}$/*Df(2R)Egfr18* flies. Reduction of *Egfr* activity led to the differentiation of broad bands of ectopic vein material along the posterior wing margins of gro^{1} homozygotes. It appears that mutations in *Egfr* and *gro* can either suppress or enhance each other, depending on the relative levels of *Egfr* and *gro* activities (see below).

Dosage sensitive interactions between *gro* and *Egfr* were also apparent in other developmental contexts. *Egfr*; gro^{1} double mutants displayed hyperplasia of macrochaetae, enlarged ocelli, notched wings, ectopic wing hairs, leg fusions and bifurcations, outgrowths of the anterior wing margin, and ectopic eyes. Except for hyperplasia of the macrochaetae and enlarged ocelli, most of these defects are not normally observed in gro^{1} homozygotes, but all have been observed in flies that are transheterozygous for gro^{1} and more severe *gro* alleles (DE CELIS and RUIZ-GOMEZ 1995), or in clones of cells that are homozygous for severe *gro* alleles (HEITZLER *et al.* 1996).

Many of these defects are consistent with a requirement for Gro in N signaling (SCHELLENBARGER and MOHLER 1978; HARTENSTEIN and POSAKONEY 1990; COUSO and MARTINEZ ARIAS 1994). However, Gro is also required in additional contexts that appear to be distinct from its function in N signaling (PAROUSH *et al.* 1994; DE CELIS and RUIZ-GOMEZ 1995; DE CELIS *et al.* 1996; HEITZLER 1996). The ectopic wing hairs we observe on the wings of *Egfr*, gro^{1} and rl^{1}; gro^{1} double mutants are similar to defects seen in *hairy* (*h*) mutant flies (INGHAM *et al.* 1985) or *gro*/*h* transheterozygotes (HEITZLER *et al.* 1996), and may indicate that *Egfr* mutations reduce Gro's activity as a corepressor with Hairy. Anterior wing margin outgrowths in *gro* mutant tissues are thought to reflect a disruption of Gro function with an unidentified partner or partners (DE CELIS and RUIZ-GOMEZ 1995; HEITZLER 1996). Our observations that reduction of *Egfr* activity can generate ectopic wing hairs and anterior wing outgrowths in *Egfr*; gro^{1} double mutants suggest that *Egfr* influences aspects of *gro* function that are distinct from its roles in the N pathway.

The spectrum of defects enhanced in *Egfr*; gro^{1} or rl^{1}; gro^{1} double mutants appears to reflect a reduction in most or all aspects of *gro* activity. The simplest interpretation of these observations is that EGFR and Rl (MAP-K) promote the activity of Gro. Gro and its mammalian homologue, TLE1, are phosphorylated on serine residues (HUSAIN *et al.* 1996) and thus may be downstream targets of an EGFR-regulated phosphorylation cascade.

The contributions of *Egfr* and *gro* to the formation of ectopic eyes: The formation of ectopic eyes is consistent with several previous observations with respect to the function of *Egfr*. $Egfr^{E}$ (activated *Egfr*; also known as *Ellipse* or *Elp*) inhibits the formation of ommatidial preclusters in the eye imaginal disk (BAKER and RUBIN 1989). This observation suggests that the normal function of *Egfr* is to suppress the formation of ommatidia in inappropriate locations (BAKER and RUBIN 1989, 1992). Subsequent studies led to the conclusion that reduced *Egfr* levels promote the formation of ommatidial preclusters in neighboring cells (XU and RUBIN 1993). The ectopic eyes we observed may result from the combined influences of reduced *gro* and *Egfr* activity in two different steps of eye development. First, reduction of *gro* activity promotes neurogenesis (SCHRONS *et al.* 1992) and permits an increased number of cells to pursue proneural fates. This process may be enhanced by reduction of *Egfr* activity (see below). Then, in a second step, *Egfr* activity levels influence the commitment of ectopic proneural cells to alternative developmental programs. Normal levels of *Egfr* activity would favor a developmental program that leads to bristle development (the most prominent aspect of the gro^{1} phenotype). Moderate reduction of *Egfr* activity would still permit proneural cells in the head ectoderm to pursue bristle fates, but would also favor differentiation to ocellar fates. Further reduction of *Egfr* activity would favor differentiation of ectopic ommatidial preclusters and ultimately ectopic eyes. The small patches of red-pigmented cells in observed in the ocellar tumors of $Egfr^{t1}$/*CyO*; gro^{1}/gro^{1} flies may be the first indication of transformation to ommatidial fates. *Egfr* activity is also required for many steps in the development of the compound eye (FREEMAN 1994, 1996, 1997; TIO *et al.* 1994; TIO and MOSES 1997). The reduced size and degenerate morphology of the ectopic eyes observed in $Egfr^{t1}$/*Df(2R)Egfr18*; gro^{1}/gro^{1} flies may indicate that *Egfr* activity levels are marginally adequate to support ectopic eye development in this genotype.

A model for communication between the N and EGFR signal transduction pathways: Our results suggest a model wherein EGFR has two independent links with the N pathway, a positive link through *gro* and an inhibitory link through some other mechanism (Figure 6). DE CELIS (1997) has presented evidence for feedback loops between both the *Egfr* and *N* pathways and the activity of the *decapentaplegic*/*thick veins* (*dpp*/*tkv*) signaling pathway. The *dpp*/*tkv* pathway may represent this inhibitory link. The *canoe* (*cno*) gene product has also been suggested to provide a link between the *Egfr* and *N* pathways. Phenotypes associated with mutations in *cno* are similar to those of mutations in *N*, and *cno* interacts genetically with *N* and *scabrous* (MIYAMOTO *et al.* 1995), while binding studies indicate that Cno associates with activated Ras (KURIYAMA *et al.* 1996). Thus *cno* has been independently linked to both pathways, but its possible influence on *Egfr* signaling is unclear. The mutual enhancement of *H* and *Egfr* mutations, and the

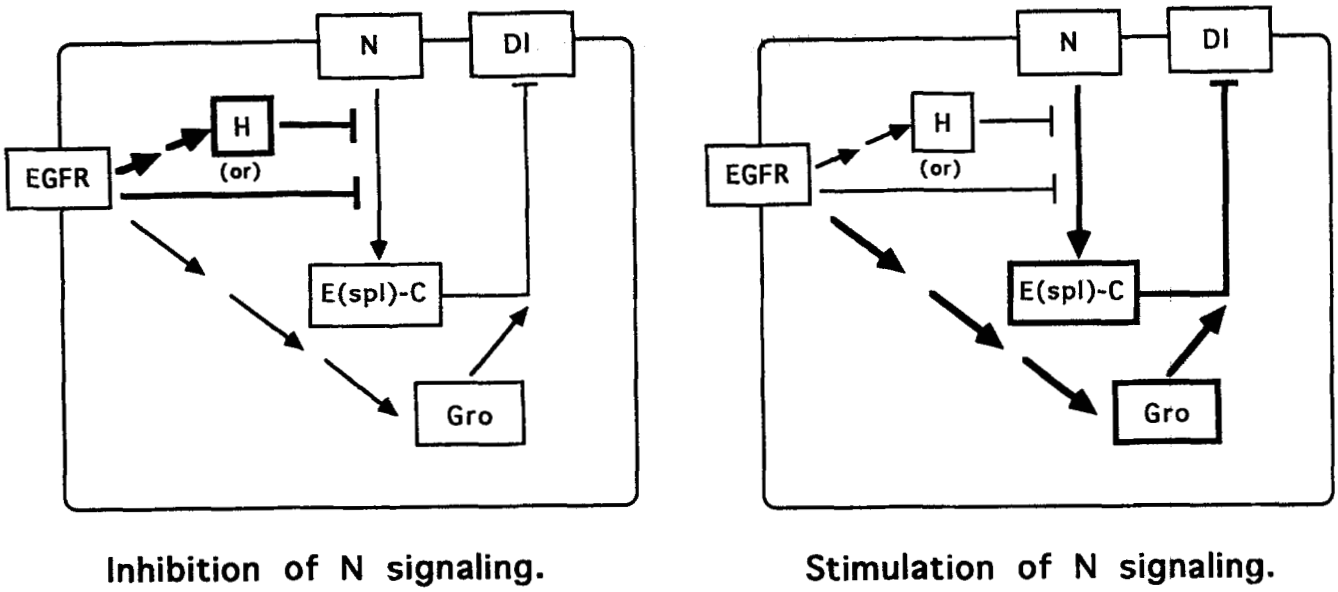

FIGURE 6.—A model for the action of Egfr in N signaling. We propose that EGFR influences N signaling by two independent routes. First, based on genetic interactions described in this paper and elsewhere (see text), we suggest that EGFR acts as an inhibitor of *N* signaling. This inhibition may occur through *H*, and/or through other intermediates. Second, we suggest that EGFR stimulates the activity of *gro* and thereby promotes N signal transduction by acting in concert with E(spl) bHLH proteins (PAROUSH *et al.* 1994; HEITZLER *et al.* 1996). Thus, EGFR can either promote or inhibit the activity of the N pathway, depending on the relative strengths of the two alternative inputs.

sensitivity of these interactions (see above) may indicate the involvement of *H* in the proposed second link between the two pathways. If so, it may it may participate in cooperation with *dpp/tkv* and/or *cno*, or by some other mechanism.

According to our model, the dosage sensitive response of gro^1 to reductions in *Egfr* activity can be interpreted as follows. *Egfr* influences bristle development by two routes. In wild-type flies, one route functions to suppress bristle formation via *Egfr*'s effect on *gro*. In gro^1 homozygotes this route of *Egfr* input into cell fates is sensitized and responds to small reductions in *Egfr* activity ($Egfr^{l1}/+$), which then leads to the enhanced *groucho* phenotype (more bristles). A second route of *Egfr* input promotes bristle determination via *Egfr*'s inhibitory link to the *N* pathway. In gro^1 homozygotes, this second input route is not sensitized, so a greater reduction in *Egfr* activity (*e.g.*, $Egfr^{l1}/Egfr^{l1}$) is required to alter this response. This simultaneous reduction in *Egfr* inputs through both routes leads to mutual suppression of the gro^1-associated overgrowth and the $Egfr^{l1}$-associated inhibition of bristles. Our observations suggest similar dosage-sensitive interactions between *gro* and *Egfr* in wing vein and ocellar development.

Possible application of our model to N and EGFR in other developmental contexts: Our observations indicate links between the *Egfr* and *N* pathways in the processes of wing vein specification, bristle determination, and eye development. However the two pathways also seem to impinge upon each other in oogenesis, where both pathways are required for proper follicle cell function in egg chamber formation, and the establishment of the anterior/posterior axis (SCHÜPBACH 1987; PRICE *et al.* 1989; RUOHOLA-BAKER *et al.* 1991; GOODE *et al.* 1992; XU *et al.* 1992; GONZALEZ-REYES *et al.* 1995; ROTH *et al.* 1995). During early oogenesis, EGFR signaling is required for the establishment of posterior fates in a specific population of follicle cells (GONZALEZ-REYES *et al.* 1995; ROTH *et al.* 1995). This process is initiated by a signal from the oocyte. N and Dl are also required in the posterior follicle cells for the establishment or maintenance of posterior follicle cell fates (RUOHOLA-BAKER *et al.* 1991; BENDER *et al.* 1993). In the context of our model, the EGFR signal may influence N signaling in the posterior follicle cells to set the expression state of the Dl ligand, which would then be maintained by lateral inhibition as described by HEITZLER *et al.* (1996).

The Lin-12 and Let-23 signal transduction pathways of *C. elegans* are analogous to the N and EGFR pathways of Drosophila. TUCK and GREENWALD (1995) have described a transcription factor, encoded by the *lin-25* gene of *C. elegans*, that appears to function downstream of Ras (Let-60) to influence signaling via Lin-12, a *C. elegans* N homologue. KENYON (1995) has described a model whereby Lin-25 acts downstream of Let-60 to regulate the expression of a ligand for Lin-12. Thus it appears that Lin-25 and Gro may play similar roles in linking the analogous signal transduction pathways in the two organisms.

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