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EGFR signaling attenuates Groucho-dependent repression to antagonize Notch transcriptional output

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Crosstalk between signaling pathways is crucial for the generation of complex and varied transcriptional networks. Antagonism between the EGF-receptor (EGFR) and Notch pathways in particular is well documented, although the underlying mechanism is poorly understood. The global corepressor Groucho (Gro) and its transducin-like Enhancer-of-split (TLE) mammalian homologs mediate repression by a myriad of repressors, including effectors of the Notch, Wnt (Wg) and TGF-β (Dpp) signaling cascades[1–8]. Given that there are genetic interactions between *gro* and components of the EGFR pathway[9] (ref. 9 and P.H. *et al.*, unpublished results), we tested whether Gro is at a crossroad between this and other pathways. Here we show that phosphorylation of Gro in response to MAPK activation weakens its repressor capacity, attenuating Gro-dependent transcriptional silencing by the Enhancer-of-split proteins, effectors of the Notch cascade. Thus, Gro is a new junction between signaling pathways, enabling EGFR signaling to antagonize transcriptional output by Notch and potentially other Gro-dependent pathways.

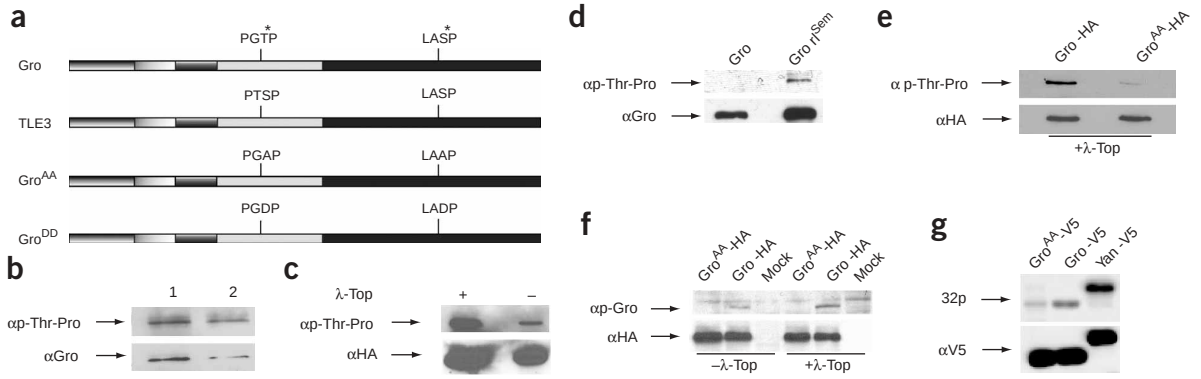

Figure 1 Gro undergoes phosphorylation in response to RTK signaling. (**a**) Schematic presentation of UAS-driven wild-type Gro, Gro^{AA} and Gro^{DD} derivatives. Putative conserved MAPK phosphorylation sites, which were mutated, are indicated by asterisks. (**b**) Extracts from 0- to 4-h-old and 0- to 12-h-old embryos were immunoprecipitated using antibodies to Gro (αGro) and western-blotted. Phosphorylation was detected with antibodies to phosphorylated threonine followed by proline (αp-Thr-Pro). Similar results were obtained using antibodies to phosphorylated serine (not shown). (**c**) Phosphorylation of immunoprecipitated HA-tagged wild-type Gro is enhanced by a factor of >4 in S2 cells coexpressing activated EGFR (λ-TOP). αHA, antibody to HA; αp-Thr-Pro, antibodies to phosphorylated threonine followed by proline. (**d**) Gro is phosphorylated in wing imaginal discs in response to MAPK activation. Wild-type Gro was expressed alone or together with rl^{Sem}, immunoprecipitated using antibodies to Gro (αGro) and western-blotted. Phosphorylation was assessed with antibodies to phosphorylated threonine followed by proline (αp-Thr-Pro; upper panel). The amount of Gro that was immunoprecipitated differed by a factor of <3 (lower panel). (**e**) Phosphorylation of immunoprecipitated HA-tagged wild-type Gro, but not of HA-Gro^{AA}, was increased in λ-TOP–stimulated S2 cells. αHA, antibody to HA; αp-Thr-Pro, antibodies to phosphorylated threonine followed by proline. (**f**) Western blot using whole-cell extracts of S2 cells stimulated with λ-TOP. Staining with antibodies to phosphorylated Gro (αp-Gro) confirmed increased phosphorylation at one of the MAPK consensus sites of Gro. αHA, antibody to HA. (**g**) V5-tagged Gro^{AA}, wild-type Gro or Yan was incubated with Erk2 in the presence of ^{32}P-ATP. Reaction products were then resolved by SDS-PAGE, followed by autoradiography (upper panel) and immunoblotting with antibody to V5 (αV5; lower panel). The relative specific activities of Gro^{AA}, wild-type Gro and Yan are 1, 3 and 12, respectively. (**c**,**f**) In nonstimulated S2 cells, phosphorylation of Gro probably stems from basal MAPK activity.

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gro is ubiquitously expressed throughout development in *Drosophila melanogaster*. Antibodies to phosphorylated threonine followed by proline recognize immunoprecipitated Gro, indicating that Gro is a phosphoprotein *in vivo* (**Fig. 1b**). To test whether Gro is phosphorylated specifically in response to receptor tyrosine kinase (RTK) signaling, we transfected hemagglutinin (HA)-tagged native (wild-type) Gro, alone or together with a constitutively activated derivative of the EGFR (λ-TOP)[10], into *D. melanogaster* Schneider (S2) cells. Staining with antibodies to phosphorylated threonine followed by proline showed that Gro phosphorylation levels are more than four times higher in cells stimulated with λ-TOP (**Fig. 1c**) and in wing imaginal discs expressing rolled^{Sem} (rl^{Sem}), a constitutively activated form of MAPK[11] (**Fig. 1d**). We mutated two potential MAPK consensus sequences, which are conserved in Gro and its vertebrate homologs, to alanine (Gro^{AA}; **Fig. 1a**). Phosphorylation of this HA-tagged Gro^{AA} variant was markedly reduced in stimulated cells compared with phosphorylation of wild-type Gro (**Fig. 1e**), suggesting that some of the modification must be occurring on these MAPK consensus sites. To confirm this possibility, we raised polyclonal antibodies directed against a synthetic phosphopeptide spanning one of these MAPK sites (PGTP). Western-blot analysis using these affinity-purified antibodies to phosphorylated Gro showed enhanced Gro phosphorylation levels in EGFR-stimulated S2 cells (**Fig. 1f**). Kinase assays showed that Gro was directly phosphorylated by MAPK (Erk2) *in vitro* (**Fig. 1g** and **Supplementary Methods** online).

To investigate whether phosphorylation affects the repressor function of Gro, we generated a construct encoding a constitutively pseudophosphorylated derivative of Gro by modifying the threonine and serine residues in the two MAPK consensus sites to aspartic acid (Gro^{DD}; **Fig. 1a**). We then misexpressed each of the constructs (wild-type Gro, Gro^{DD} and Gro^{AA}) using the *dpp-Gal4* driver and stained wing imaginal discs for expression of several genes that are normally silenced by Gro-dependent repressors. Distalless (Dll), for example, is a target of the wingless (*wg*) pathway[12], in which Gro acts as a corepressor in conjunction with Pangolin (dTCF)[3,4]. All three Gro derivatives partially repressed Dll in the *dpp* domain, restricting Dll expression to a central narrow stripe along the dorsal-ventral boundary, where the levels of *wg* signaling are highest. Notably, this Dll stripe was narrow when Gro^{AA} was expressed, and wider when Gro^{DD} was expressed, than when wild-type Gro was expressed (**Fig. 2a–c,f**). These results indicate that Gro^{AA} is a stronger corepressor than wild-type Gro, whereas Gro^{DD} is weaker. The Dpp target Spalt (Sal), which is subject to Brinker repression, was also similarly repressed to differing degrees by overexpression of the Gro derivatives (**Supplementary Fig. 1** online)[5]. Therefore, phosphorylation of Gro at sites responsive to MAPK signaling weakens its repressor capacity.

One prediction that follows from the above results is that Gro-dependent repression should be susceptible to genetic alterations in MAPK activity levels. Therefore, forced MAPK signaling should suppress the repressor function of Gro and, reciprocally, blocking this cascade should enhance Gro-mediated repression. Bric-a-brac (Bab), like Dll and Sal, was also silenced when wild-type Gro was ectopically expressed in the wing imaginal disc (**Fig. 2g**; compare with **Fig. 2d,e**). Stimulation of MAPK, using either rl^{Sem} or sSpi (a constitutively activated form of the EGFR ligand[13]), led to phosphorylation of Gro (**Fig. 1d**) and markedly reduced the ability of Gro to repress Bab and Sal (**Fig. 2h** and data not shown), supporting the notion that phosphorylation of Gro reduces its repressor potential. Consistent with these results, rl^{Sem} also attenuated silencing of a Gro-responsive target reporter in cell culture–based assays (**Supplementary Fig. 2** online). Repression by the Gro^{AA} derivative, which cannot be phosphorylated, was insensitive to these attenuating effects, as repression of Sal and Bab by Gro^{AA} was not relieved by expression of rl^{Sem} (**Fig. 2i**). Therefore, the two consensus MAPK sites mutated in Gro^{AA} must be contributing to the regulation of Gro activity by EGFR signaling.

Based on the above results, we hypothesized that blocking the MAPK pathway, and consequently decreasing phosphorylation

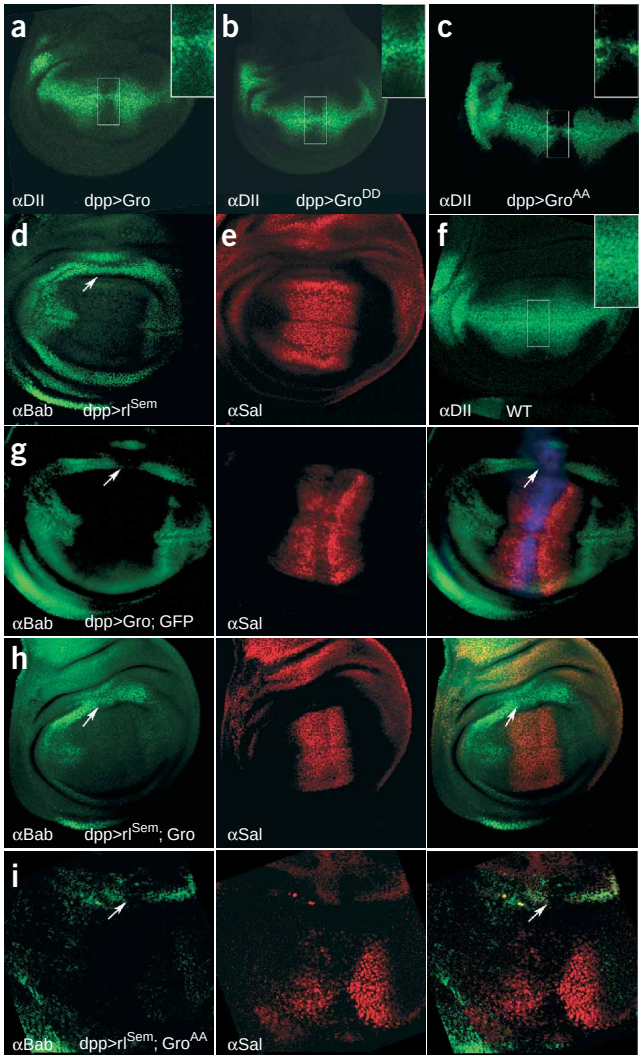

Figure 2 The repressor capacity of wild-type Gro, but not that of Gro^{AA}, is attenuated by MAPK activation. (**a–c**) Expression of Gro derivatives using *dpp-Gal4* led to differential repression of Dll. Gro^{AA} was the strongest corepressor and Gro^{DD} the weakest. (**d,e**) Expression of Bab (**d**) and Sal (**e**) is unaffected by the ectopic expression of rl^{Sem} alone. (**f**) Wild-type Dll expression. (**g**) Bab and Sal are repressed by the expression of wild-type Gro (arrow indicates overlap between *dpp* and Bab expression domains; *UAS*-GFP is shown in blue). (**h**) Repression by wild-type Gro is substantially weakened by the coexpression of rl^{Sem}. (**i**) Gro^{AA}-mediated repression is refractory to the attenuating effects of rl^{Sem}, as repression of Bab and Sal is indistinguishable from that elicited by Gro^{AA} alone (**Supplementary Fig. 1** online and data not shown). Gro^{AA} driven by *dpp* somewhat distorts the wing pouch, such that Bab expression is slightly out of the plane of focus in some areas outside the *dpp* domain. Insets in **a–c**,**f** show Dll expression in the *dpp* domain. α, antibody; WT, wild-type.

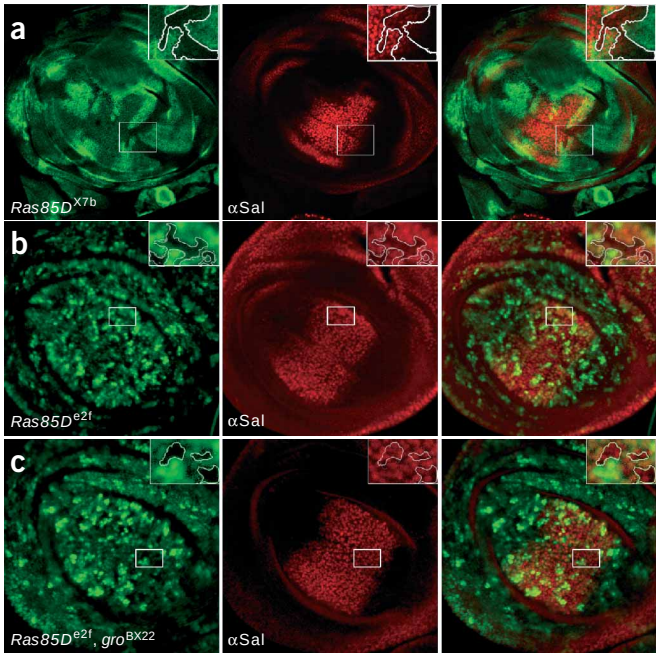

Figure 3 Ras1 activity in the wing pouch relieves repression by endogenous Gro. (**a**,**b**) Sal expression was reduced in *Ras85D* loss-of-function clones (**a**, *Ras85D*^{X7b}; **b**, *Ras85D*^{e2f}). A similar effect was observed in *Egfr* mutant clones (*Egfr*^{lK35}; not shown). (**c**) In contrast, Sal expression levels were normal in *Ras85D*^{e2f} *gro*^{BX22} double-mutant clones. Insets in **a**–**c** show representative clones. α, antibody.

of Gro, should overpotentiate the repressor activity of endogenous Gro, bringing about inappropriate repression of its target genes. In clones with homozygous mutations in either *Egfr* or *Ras85D* (also called *Ras1*), expression of Sal was autonomously decreased, but that of Gro and other wing markers was not (**Fig. 3a**,**b**; **Supplementary Fig. 3** online; and data not shown)[14]. The ectopic repression of Sal was suppressed in *Ras85D-gro* double mutant clones (**Fig. 3c**), indicating that the loss of Sal expression in *Ras85D* mutant clones was not indirectly caused by the removal of *Ras85D* but, rather, stemmed from enhanced Gro-mediated repression. These results argue that *Ras85D* signaling attenuates Gro-dependent repression, probably by promoting its phosphorylation. We suggest that downregulating Gro-dependent repression might be one of the functions of Vein, an EGFR ligand that is expressed at low levels in the developing wing pouch and is required for wing formation[15,16].

The results above support the notion that RTK signaling downregulates Gro repressor activity. Gro is a global corepressor, and Gro-dependent repressors act as downstream effectors of several signaling pathways[1–8]. Therefore, the attenuation of Gro-mediated repression could be a mechanism by which RTK pathways feed into and regulate output of other signal transduction cascades. In particular, the EGFR pathway antagonizes Notch signaling in various developmental settings[17–20]. In wing patterning, for example, the EGFR pathway promotes vein formation by overriding antivein activity mediated by the Notch pathway. In this process, both Gro and its repressor partner, the Notch effector E(spl)mβ, are antivein determinants[17,21] (**Supplementary Fig. 4** online). Could EGFR signaling be driving vein formation by phosphorylating Gro and thus downregulating E(spl)mβ- and Gro-mediated repression? If so, then the misexpression of Gro^{DD}, the presumed end product of MAPK signaling, should generate extra vein tissue, as in the ectopic activation of MAPK. Wings expressing Gro^{DD} had ectopic vein material

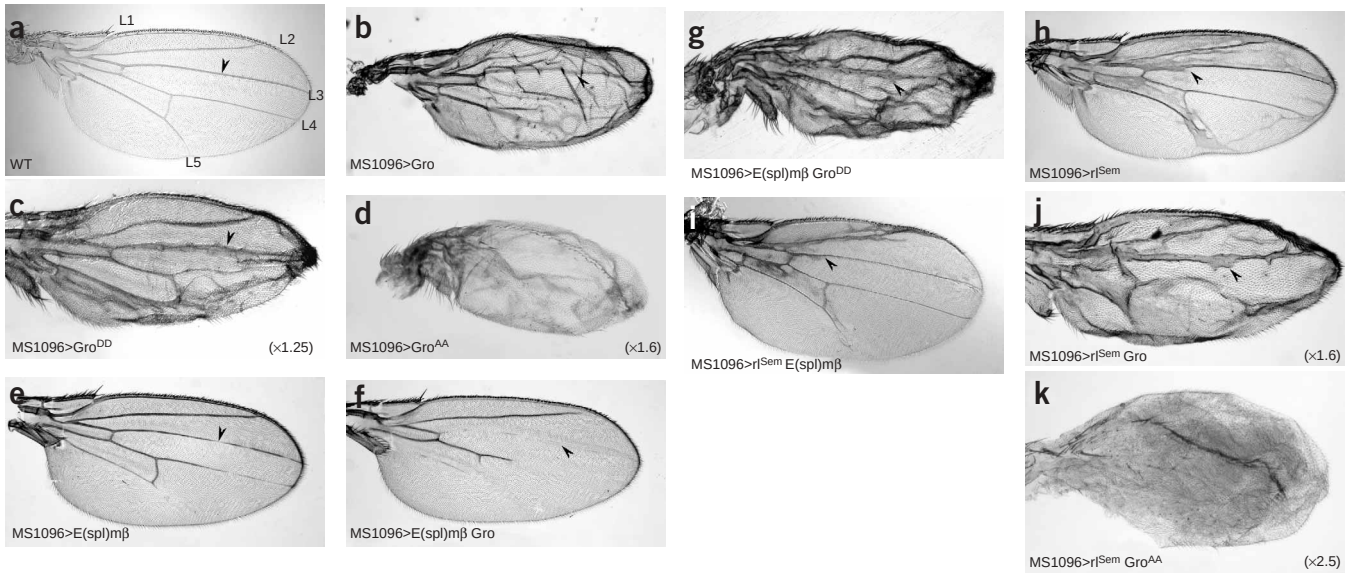

Figure 4 Gro^{DD} and Gro^{AA} have opposing effects on E(spl)mβ-induced repression of wing veins. (**a**) Wild-type wing. (**b**) Wing expressing wild-type Gro. (**c**,**d**) Expression of Gro^{DD} led to formation of extra vein tissue (**c**), whereas that of Gro^{AA} eliminated all veins (**d**). Expression of E(spl)mβ throughout the wing pouch caused a partial loss of wing veins (**e**), a phenotype that was enhanced by the coexpression of wild-type Gro (**f**). In contrast, the coexpression of E(spl)mβ and Gro^{DD} did not lead to vein loss (**g**); instead, extra vein material was evident, as observed when rl^{Sem} alone was similarly expressed (**h**). Likewise, wings expressing rl^{Sem} together with either E(spl)mβ (**i**) or wild-type Gro (**j**) had widened veins. In contrast, Gro^{AA} mediated antivein activity irrespective of rl^{Sem}, such that wings coexpressing rl^{Sem} and Gro^{AA} had little, if any, vein material and were indistinguishable from those expressing Gro^{AA} alone (**k**; compare with **d**). (**a**–**c**,**e**–**j**) L3 veins are marked by arrowheads.

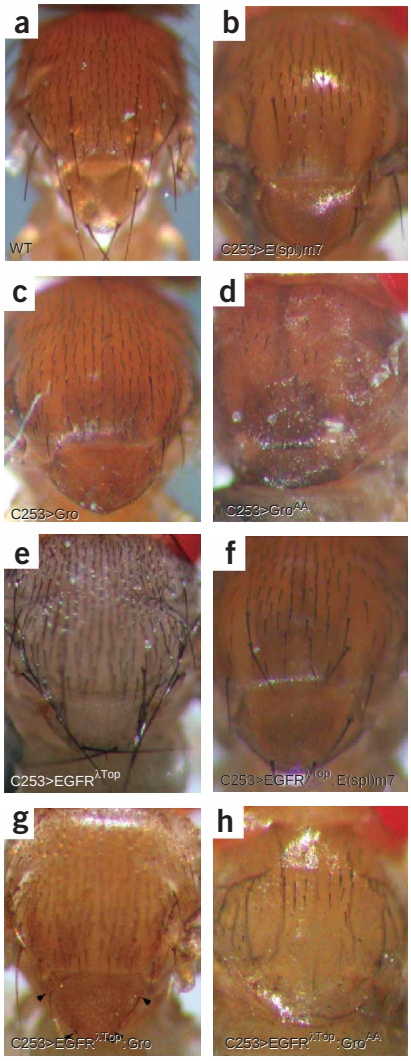

Figure 5 Gro^{AA} renders E(spl)m7-mediated repression refractory to EGFR signaling. Patterning of the notal bristles is under Notch pathway regulation[30]. (**a**) Wild-type notum. (**b**–**h**) Notum bristle phenotypes caused by the overexpression of E(spl)m7 (**b**), wild-type Gro (**c**), Gro^{AA} (**d**) or EGFR λ-TOP (**e**) alone, or by the coexpression of λ-TOP with E(spl)m7 (**f**), wild-type Gro (**g**) and Gro^{AA} (**h**), using the C253-*Gal4* driver[19]. Bristle loss is evident after Notch pathway activation, using N^{intra} (Notch intracellular domain; not shown), the E(spl)m7 repressor or its corepressor wild-type Gro (**b**,**c**), owing to the suppression of sensory organ precursor cells (data not shown)[2,19]. (**e**) In contrast, activation of EGFR signaling, by expressing Ras1^{V12}, Raf^{f179} or λ-TOP, led to ectopic bristles (data not shown)[19]. (**f**–**h**) Activated EGFR signaling suppressed the bristle-loss phenotype mediated by ectopic N^{intra}, E(spl)m7 and wild-type Gro (**f**,**g** and data not shown)[19], but not by Gro^{AA} (**h**; compare with **d**), indicating that conversion of Gro to a form that cannot be phosphorylated renders it insensitive to attenuation by activated MAPK; therefore, Notch pathway output is not compromised. Arrowheads in **g** mark bristles that are suppressed in notums expressing wild-type Gro with the C253-*Gal4* driver but rescued when λ-TOP is coexpressed.

(**Fig. 4c**), whereas those expressing Gro^{AA} lost all veins (**Fig. 4d**; compare with **Fig. 4a**,**b**). Therefore, some of the crosstalk between the EGFR and Notch pathways seems to take place at the level of Gro.

In support of this idea, we found that repression of vein formation by E(spl)mβ was enhanced by coexpression of wild-type Gro, but that E(spl)mβ was no longer able to suppress venation when coexpressed with Gro^{DD} (**Fig. 4e**–**g**). In this latter situation, we observed wider veins (particularly L3; **Fig. 4g**), suggesting that Gro^{DD} outcompetes endogenous Gro for binding to E(spl)mβ, thus compromising the antivein activity of Gro. In fact, the wing phenotypes of flies expressing both E(spl)mβ and Gro^{DD} were comparable to those of flies either expressing rl^{Sem} alone (**Fig. 4h**) or coexpressing E(spl)mβ and rl^{Sem} (**Fig. 4i**); in all these cases, we observed formation of extra vein tissue.

Along the same lines, we predicted that Gro^{AA}, which cannot be phosphorylated, would render Notch signaling refractory to antagonism by the EGFR pathway and, further, potentiate E(spl)mβ-mediated antivein activity irrespective of EGFR activation. Indeed, Gro^{AA}, but not wild-type Gro, suppressed the rl^{Sem} phenotype, such that wings of flies coexpressing both rl^{Sem} and Gro^{AA} had little if any vein material and were indistinguishable from those of flies expressing Gro^{AA} alone (**Fig. 4k**; compare with **Fig. 4d**,**j**).

Antagonism between the EGFR and Notch pathways is also evident in the patterning of the notal mesothoracic bristles, a process that involves lateral inhibition governed by Notch signaling[19]. Here too, expression of Gro^{AA} rendered E(spl)m7-mediated repression of bristle formation unresponsive to attenuation by λ-TOP (**Fig. 5**), suggesting that regulation of Gro by the EGFR pathway is a basis for antagonism of Notch signaling output also in this biological context. Thus, our data support the idea that Gro is a point of intersection between the EGFR and Notch signal transduction pathways. Crosstalk between these pathways could, of course, also take place at other levels[20,22].

Our evidence suggests that EGFR signaling leads to the attenuation of Gro-dependent repression. Although other groups have demonstrated phosphorylation of corepressors (including Gro) in cultured cells[23], these studies did not address the developmental relevance of such post-translational modifications *in vivo*. Here we show, for the first time to our knowledge in the fruit fly, how phosphorylation of a corepressor links signaling and transcriptional regulation. Downregulation of Gro, a global corepressor that acts in conjunction with many repressors[7,24], should allow RTK pathways to alter transcriptional programs involving large arrays of genes, as required for cell fate differentiation and determination[25]. Relief of Gro-mediated repression by RTK signaling could also be responsible for the transition between transcriptional silencing and activation, accounting, to some extent,

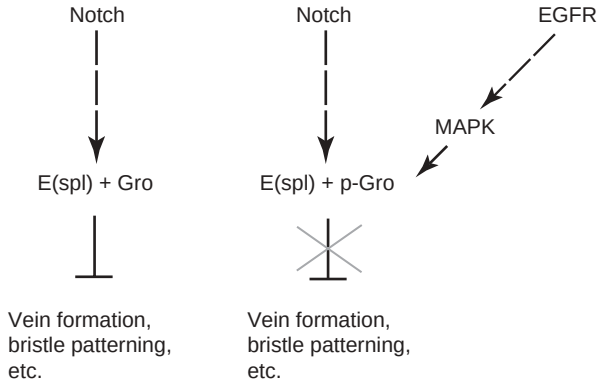

Figure 6 Model depicting crosstalk between the EGFR and Notch pathways, at the level of Gro, in vein and sensory bristle patterning.

for the bimodal nature of some Gro-dependent repressors that also function as activators. Additionally, downregulation of Gro by RTK signaling could provide the switch between Gro-dependent and Gro-independent transcriptional silencing[26,27]. Several putative or confirmed RTK pathway targets are found among genomic Gro recruitment sites[28].

Here we showed that antagonism of Notch signaling by the EGFR pathway occurs, at least in part, at the level of Gro. In response to EGFR signaling, Gro undergoes phosphorylation, a modification that compromises its repressor capability, thus decreasing the ability of the E(spl) nuclear effectors of the Notch pathway to silence their target genes (**Fig. 6**). On the other hand, in developmental settings in which Notch signaling is E(spl)-independent, the relief of Gro-dependent repression by EGFR signaling should enhance activation by Su(H)[29], allowing for synergism between the two pathways. Given that Gro also mediates repression downstream of the Wnt and TGF-β signal transduction pathways[3–6,8], and in light of the differential repression of targets of these cascades by distinct Gro derivatives (**Fig. 2**), the attenuation of Gro-mediated repression by RTK signaling might similarly modulate transcriptional outcomes of these pathways.

METHODS

Fly stocks. We used the following fly strains: *UAS*–wild-type Gro, *UAS*-Gro^{DD}, *UAS*-Gro^{AA}, *UAS*-rl^{Sem}, *UAS*-λ-TOP, *UAS*-sSpi, *UAS-Ras85D*^{V12}, *UAS*-Raf^{f179}, *UAS*-E(spl)m7, *UAS*-E(spl)mβ and *UAS*-N^{intra}. Transgenes were expressed using the Gal4/UAS binary system with the following wing drivers: *dpp-Gal4*, C253-*Gal4*, MS1096-*Gal4*, scalloped-*Gal4* and C765-*Gal4*. We generated overexpressing flip-out clones using the *act>CD2>Gal4* cassette, recombined to a *UAS*-GFP construct for the detection of the clones. We assayed at least two insertion lines independently for *UAS*–wild-type Gro, *UAS*-Gro^{DD} and *UAS*-Gro^{AA}.

Generating loss-of-function clones. We generated mutant clones of cells lacking functional EGFR (*Egfr*^{IK35}), *Ras85D*^{x7b}, *Ras85D*^{e2f}, *gro*^{BX22} or both *Ras85D*^{e2f} and *gro*^{BX22} (double mutant) using Flp-mediated mitotic recombination and identified them by the loss of the πMyc or CD2 markers and the concurrent appearance of a twin spot. We induced clones 48–72 h after egg laying by heat shock (60 min at 37 °C) and later grew larvae at 25 °C.

Immunohistochemistry. We stained wing discs in accordance with standard protocols. We used antibodies to the following proteins: Dll (1:1,000; a gift from S. Cohen, Heidelberg, Germany), Gro (1:1,000; a gift from C. Delidakis, Heraklion, Crete, Greece), Sal (1:3,000; a gift from A. Salzberg, Haifa, Israel), Bab (1:3,000; a gift from F. Laski, Los Angeles, California, USA) and CD2 (1:1,500; Serotec). We used secondary antibodies conjugated with fluorescein isothiocyanate, rhodamine red-X or Cy5 (1:400; Jackson Laboratories).

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

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