Development 135, 829-837 (2008) doi:10.1242/dev.015206

Multiple RTK pathways downregulate Groucho-mediated repression in *Drosophila* embryogenesis

Einat Cinnamon[1], Aharon Helman[1], Rachel Ben-Haroush Schyr[2], Amir Orian[3], Gerardo Jiménez[4] and Ze'ev Paroush[1,*]

RTK pathways establish cell fates in a wide range of developmental processes. However, how the pathway effector MAPK coordinately regulates the expression of multiple target genes is not fully understood. We have previously shown that the EGFR RTK pathway causes phosphorylation and downregulation of Groucho, a global co-repressor that is widely used by many developmentally important repressors for silencing their various targets. Here, we use specific antibodies that reveal the dynamics of Groucho phosphorylation by MAPK, and show that Groucho is phosphorylated in response to several RTK pathways during *Drosophila* embryogenesis. Focusing on the regulation of terminal patterning by the Torso RTK pathway, we demonstrate that attenuation of Groucho's repressor function via phosphorylation is essential for the transcriptional output of the pathway and for terminal cell specification. Importantly, Groucho is phosphorylated by an efficient mechanism that does not alter its subcellular localisation or decrease its stability; rather, modified Groucho endures long after MAPK activation has terminated. We propose that phosphorylation of Groucho provides a widespread, long-term mechanism by which RTK signals control target gene expression.

KEY WORDS: *Drosophila*, Groucho, TLE, phosphorylation, RTK signalling, Repression

INTRODUCTION

In early *Drosophila* embryogenesis, signals mediated by different receptor tyrosine kinases (RTKs) establish cell fates in a wide range of developmental processes. All RTK pathways transduce signals via the canonical Ras/Raf/MEK/MAPK cascade, yet they clearly elicit diverse outcomes: the Torso RTK pathway defines the embryonic termini (Furriols and Casanova, 2003); two FGF receptors (FGFR) are required for the patterning of the mesoderm and trachea (Huang and Stern, 2005); and the EGF receptor (EGFR) pathway controls various processes such as the formation of the ventral neuroectoderm, the specification of muscle precursors and the invagination of tracheal branches (Shilo, 2003). These differential transcriptional and morphological responses to RTK activation are context specific, and probably depend on the strength, range and duration of the signal. Additional specificity of the response is conferred by crosstalk between RTK and other signalling pathways (Culi et al., 2001), as well as by the combinatorial activity of nuclear pathway effectors together with distinct tissue-specific factors, at the level of specific DNA enhancers (Flores et al., 2000; Simon, 2000).

The EGFR pathway induces broad changes in target gene expression in responding cells by activating, as well as inactivating, specific DNA-binding transcription factors belonging to the Ets family (Shilo, 2005). We have recently found that this pathway also modulates the function of Groucho (Gro), a pivotal global co-repressor that contains two putative, evolutionarily conserved MAPK consensus sites. Specifically, Gro is phosphorylated in response to EGFR-dependent signalling, and this modification leads to the downregulation of its repressor capacity (Hasson et al., 2005). In particular, we have shown that the activation of the EGFR pathway attenuates Gro-mediated repression in vivo, whereas mutations in either *Egfr* or *Ras* produce an opposite effect, i.e. Gro-mediated repression is strengthened. Significantly, the ubiquitously expressed Gro and its Transducin-like Enhancer-of-split (TLE) mammalian homologues interact with, and potentiate the repressor function of, a large number of transcription factors (Buscarlet and Stifani, 2007; Chen and Courey, 2000). By compromising the ability of Gro/TLE to function as a general negative transcriptional co-regulator, EGFR signalling can thus simultaneously override an entire group of repressors, affecting the spatial and temporal regulation of their target genes. In this way, relief of Gro/TLE-dependent gene silencing in response to EGFR signalling could potentially permit the coordinated derepression of a large number of genes, allowing for wide-range changes in gene expression profiles, and consequently in cell fates (Hasson and Paroush, 2006).

Here, we have generated antibodies that specifically recognise the phosphorylated form of Gro, allowing us to detect it in its modified state during the different stages of embryonic development. We use these anti-sera to explore the dynamics of Gro phosphorylation in vivo, and find that it is modified downstream of several RTK pathways. Our data suggest that Gro is phosphorylated directly by MAPK or by the MAPK kinase MEK. Importantly, a large proportion of the pool of Gro molecules per nucleus is phosphorylated, indicating that the repressor capability of Gro is attenuated by an efficient mechanism. We focus on the regulation of terminal patterning by the Torso RTK pathway, and show that Gro phosphorylation and the resulting downregulation of its repressor function is essential for the transcriptional output of this pathway and for terminal cell specification. Finally, we demonstrate that phosphorylation of Gro does not alter its subcellular localisation, nor does it bring about its degradation. Rather, nuclear Gro persists in its phosphorylated state long after MAPK/ERK activation has terminated. We propose that inactivation of Gro via phosphorylation is an essential, shared response to RTK signal transduction, and

[1]Department of Biochemistry , Faculty of Medicine, The Hebrew University, PO Box 12272, Jerusalem 91120, Israel. [2]Department of Cellular Biochemistry and Human Genetics, Faculty of Medicine, The Hebrew University, PO Box 12272, Jerusalem 91120, Israel. [3]The Rappaport Faculty of Medicine and Research Institute, Technion-Israel Institute of Technology, Haifa 31096, Israel. [4]Institut de Biologia Molecular de Barcelona-CSIC and Institució Catalana de Recerca i Estudis Avançats, Parc Científic de Barcelona, 08028-Barcelona, Spain.

*Author for correspondence (e-mail: zparoush@cc.huji.ac.il)

Accepted 12 December 2007

discuss a model whereby phosphorylation of Gro provides a transcriptional 'memory' mechanism that allows RTK cascades to confer long-lasting effects on target gene expression.

MATERIALS AND METHODS

Fly culture

Flies were cultured and crossed on standard yeast-cornmeal-molasses-malt extract-agar medium at 25°C.

Fly stocks and germ-line clones

The following mutant alleles and Gal4 drivers were used: *tsl*^{691}, *EGFR*^{f2}, *tor*^{Y9}, *upd*^{1} (FlyBase), *nos-Gal4-VP16*, *UASp-lacZ* (provided by Pernille Rørth) and *btl-Gal4;tau-GFP* (gift of Benny Shilo). The *yw* stock served as wild-type control.

Embryos lacking maternal *gro* or *DSor* activities were derived from mosaic *gro*^{E48} and *gro*^{BX22}; or *Dsor*^{LH110} (FlyBase) mutant germlines, respectively (Chou et al., 1993).

Cuticle preparation

Unhatched larvae (24-48 hours old) were dechorionated in bleach, transferred into 50% lactic acid and 50% hoyers medium, and baked at 70°C overnight.

In situ hybridisation and antibody staining

One- to 3.5-hour-old embryos were dechorionated in bleach and fixed in 4% formaldehyde/PBS/heptane for 15-20 minutes. Expression patterns of *tll*, *hkb*, *kni*, *hb* and *nos* were visualised by whole-mount in situ hybridisation using digoxigenin-UTP labelled antisense RNA probes and anti digoxigenin antibodies conjugated to alkaline phosphatase (Roche).

Fluorescent immunohistochemical detection of activated MAPK, in freshly fixed embryos (10% formaldehyde/PBS/Heptane buffer), was achieved with a monoclonal antibody against diphosphorylated Erk (dpERK) (1:100; Sigma) using the TSA biotin system (PerkinElmer Life Sciences). Secondary antibodies were conjugated to biotin (1:2000; Chemicon) and visualised by the addition of Streptavidin Cy-2 (1:500; Jackson Laboratories). Polyclonal αpGro (1:100) antibodies were generated and affinity-purified by Biosynthesis (www.biosyn.com). Rabbits were immunised with the following peptide: NH2-GCSLKTKDMEK-PGpTPGAKAR-OH. For viewing the endogenous Gro protein, monoclonal αGro antibodies were used (1:1000; Developmental Studies Hybridoma Bank). Other antibodies were: αHA monoclonal antibody (1:1000; Jackson Laboratories); αpSTAT (1:1000; Cell Signalling Technology), αEven-skipped (1:10; Hybridoma Bank), αLamin (monoclonal; 1:1000; gift of Yosef Gruenbaum) and αCic (1:1000) (Jiménez et al., 2000). For Cic detection, a preabsorbed alkaline-phosphatase-coupled secondary antibody was utilised (1:1500; Jackson Laboratories). Secondary antibodies were FITC- (1:2000), Rhodamine- (1:2000) or Cy5-conjugated (1:800) (Jackson Laboratories). Embryos were mounted using DakoCytomation medium.

Germ-line transformations

P-element-mediated transformations were performed as previously described (Goldstein et al., 2005). At least two independent insertions were analysed for each Gro variant. For maternal expression, homozygous *nanos*-Gal4-VP16 females were mated to homozygous Gro transgenic males. Virgin female offspring with one copy of the Gal4 driver and one copy of the Gro transgenic line were mated with corresponding homozygous Gro transgenic males, and their progeny collected. Maternal expression of Gro and mutant variants was confirmed by similarly driving HA-tagged Gro and staining with αHA antibodies. High uniform nuclear expression of Gro-HA was observed from stage 1 embryos up to stage 9.

Plasmids

Gro, Gro^{AA} or Gro^{DD} fragments (Hasson et al., 2005) were generated by PCR amplification and subcloned, first into *pBluescript* (Stratagene) and, once sequenced, into the *pUASp* vector (Rørth, 1998). Additional details are available on request.

In vitro kinase assay and western blot analyses

A HIS-tagged ERK2 fusion protein was expressed in *Escherichia coli*, purified on nickel beads (Qiagen) and activated using active MEK1 (Upstate). A GST-Gro fusion protein was expressed in *Escherichia coli*, bound to glutathione-agarose beads (Sigma) and incubated with or without 0.2 μg active ERK2 in a total volume of 50 μl of kinase reaction buffer (20 mM HEPES, 0.1 mM benzamidine, 25 mM β-glycerophosphate, 0.1 mM DTT, 1 mM Na_3VO_4, 10 mM $MgCl_2$ and 0.1 mM ATP) for 30 minutes at 30°C. The agarose beads were then washed in 1×PBS and the bound GST-Gro protein eluted at 95°C for 5 minutes in SDS sample buffer. Proteins were separated by SDS-PAGE and analysed by Western blotting. Dephosphorylation was performed by incubation with calf intestinal phosphatase (CIP; Roche).

Quantitative PCR

For each transgene, total RNA was prepared from 200 embryos aged 1-2 hours. QPCR was performed using the ABI Prism 7300 cycler and the Power SYBR PCR master mix (Applied Biosystems) and analysed as described (Livak and Schmittgen, 2001). The primers used were specific for the transgenic forms of *gro*: (*gro*) 5′CGATAAGAAGGCTA-CTGTCTACGA3′, (*UASp*) 5′GCAGAAATGTTTACTCTTGACCAT3′. RNA levels were normalised to the expression level of the eIF4A gene in the same samples: 5′AAGCAGGAGAACTGGAAACTG3′, 5′CGGTGA-AGTTGTGGATAGACAT3′.

RESULTS

Dynamic phosphorylation of Groucho during *Drosophila* embryogenesis

To follow the spatio-temporal dynamics of Gro modification, we raised polyclonal antibodies against a synthetic phosphopeptide containing one of the two putative MAPK consensus sites in Gro (PG*T*P; see Materials and methods). Below, we demonstrate the specificity of these affinity-purified, anti-phosphorylated Gro (αpGro) antibodies, and use them to reveal the stereotyped pattern of Gro phosphorylation in vivo, that is distinct at different developmental stages.

We have previously shown that Gro repressor activity is downregulated by the EGFR pathway in the wing disc. Consistent with this, we find strong αpGro staining in the ventral neuroectoderm of stage 10 embryos on both sides of the midline, in a region that matches the domain of EGFR activation (Fig. 1A) (Gabay et al., 1997a). Indeed, the pGro staining in this region largely overlaps with that of the doubly phosphorylated active form of MAPK (dpERK) (Fig. 1A-C), which serves as an effective readout for EGFR (and other) RTK signalling (Gabay et al., 1997a; Gabay et al., 1997b). Immunofluorescence analysis, using the available monoclonal anti-Gro antibody (αGro) (Delidakis et al., 1991), shows a reduction in staining in the ventral neuroectoderm, relative to more lateral ectodermal cells. To compare the αGro and αpGro patterns further, we performed double labelling experiments. We find that the pattern detected by the αGro antibody is largely complementary to the domain of pGro (Fig. 1D-F). The opposing αGro and αpGro staining is evident throughout embryonic development (e.g. Fig. 2; data not shown).

The above results suggest that phosphorylation of Gro reduces its detection by the monoclonal αGro antibody, perhaps because the epitope recognised by this antibody undergoes phosphorylation in response to signalling. Alternatively, phosphorylation could be inducing conformational changes in the Gro protein, or might be promoting the association of pGro-specific interacting cofactors that mask the anti-Gro epitope. To distinguish between these possibilities, we performed in vitro phosphorylation assays of Gro, followed by western blot analyses. As depicted in Fig. 1J, the largely mutually exclusive recognition by the αpGro and αGro antibodies is observed even under denaturing conditions, arguing that these antibodies are directed against the same epitope and that the phosphorylation event itself is enough to cause their differential recognition.

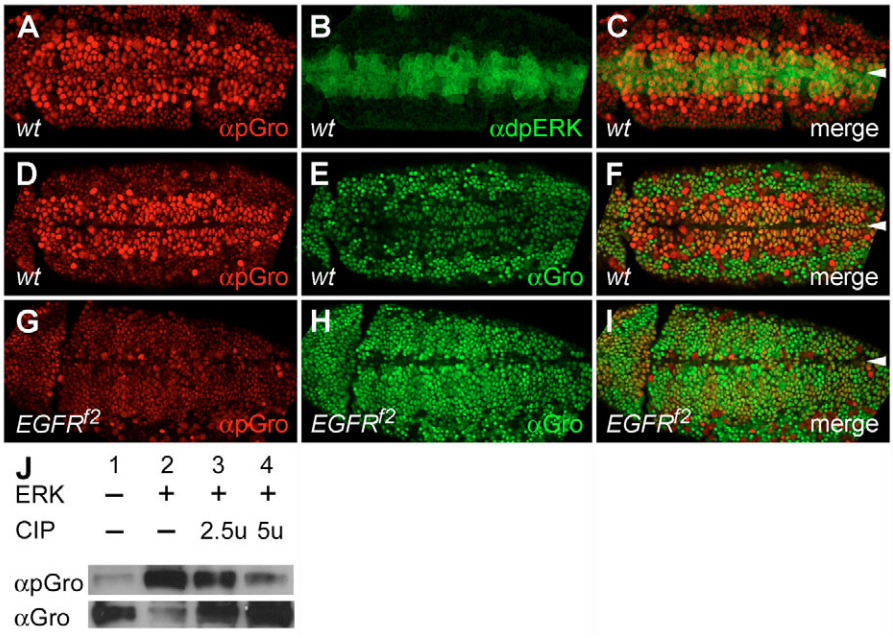

Fig. 1. Phosphorylation of Groucho in the ventral neuroectoderm depends on EGFR signalling. (A-I) Ventral views of stage 10 embryos; anterior is towards the left. (**A-C**) Wild-type embryo stained with both αpGro (A; red) and αdpERK (B; green) antibodies. (C) Merge. There is a significant overlap between the staining in cells that border the midline, whereas only pGro, but not dpERK, is detected in more lateral cells. (**D-F**) Wild-type embryo double-stained with αpGro (D; red) and αGro (E; green) antibodies. (F) Merge. The staining is largely mutually exclusive, indicating that Gro is phosphorylated in cells straddling the ventral midline, whereas in more lateral ectodermal regions it is mostly in its unphosphorylated state. (**G-I**) Homozygous *Egfr*^{f2} mutant embryos stained for pGro (G; red) and Gro (H; green). (I) Merge. pGro staining is decreased in the ventral neuroectoderm (compare with A,D), and is replaced by αGro staining (compare with E). (F,I) There is complementarity between the αpGro and αGro staining, attesting to the specificity of our αpGro antibodies (see Fig. 2D below). (C,F,I) Arrowheads indicate the ventral midline. (**J**) αpGro and αGro antibodies differentially recognise the phosphorylated and nonphosphorylated forms of Gro, respectively, in western blot analysis, using a denaturing gel. Bacterially expressed GST-Gro fusion protein is recognised mainly by αGro antibodies (lane 1). Phosphorylation of Gro by ERK2 in vitro leads to its detection primarily by αpGro, and prevents its recognition by αGro, antibodies (lane 2). Incubation of phosphorylated Gro with a nonspecific phosphatase (2.5u CIP, lane 3; 5u CIP, lane 4) reverses the recognition by the antibodies, suggesting that phosphorylation itself is enough to cause the differential recognition by the antibodies.

Based on the clear regional distinction between the ON/OFF state of Gro phosphorylation in the embryo, we conclude that during *Drosophila* embryonic development, the majority of Gro molecules are phosphorylated in cells that respond to EGFR activation.

Phosphorylation of Groucho in the neuroectoderm depends on EGFR signalling

As indicated above, phosphorylation of Gro in the ventral neuroectoderm of stage 10 embryos coincides with the region of EGFR activation. To test if this phosphorylation is indeed dependent on functional EGFR signalling, we immunostained *Egfr* mutant embryos with both αpGro and αGro antibodies. In such mutants, phosphorylation of Gro in the neuroectodermal region appears greatly reduced, with a concomitant expansion of αGro staining into that region (Fig. 1G-I). Taken together, these data suggest that Gro phosphorylation in the ventral neuroectoderm is EGFR-dependent.

Groucho is phosphorylated by the Torso RTK pathway

The pattern of pGro in stage 5 syncytial blastoderm embryos includes both poles, as well as seven transverse stripes in the central region of the embryo (Fig. 2A). Here too, the αGro staining pattern is mostly complementary to that of αpGro (Fig. 2C); at this stage, unphosphorylated Gro accumulates everywhere except for the embryonic termini and the seven stripes, which stain only weakly (Fig. 2B). Pole cells are also strongly stained by αGro, but not with αpGro, antibodies (Fig. 2C). Importantly, the αpGro staining is completely lost in embryos devoid of maternally contributed *gro* (Fig. 2D), confirming the specificity of our αpGro antibodies.

The phosphorylation of Gro at the termini coincides with the areas of Torso pathway activity, which is mandatory for the establishment of the anterior and posterior termini of the early embryo (Furriols and Casanova, 2003). Consistently, the domains of αdpERK and αpGro staining overlap at the anterior and posterior poles of blastoderm embryos (Fig. 2E-G) (Gabay et al., 1997b). Furthermore, phosphorylation of Gro is sensitive to mutations that disrupt the Torso pathway. For example, no staining is observed for pGro or dpERK at the poles of embryos laid by *torso-like*^{691} (*tsl*^{691}) mutant females, in which the Torso ligand is not processed properly (Fig. 3B and data not shown) (Casali and Casanova, 2001; Casanova et al., 1995; Stevens et al., 1990). Conversely, overactivation of the Torso pathway in *tor*^{Y9} gain-of-function mutants (Duffy and Perrimon, 1994; Sprenger and Nusslein Volhard, 1992; Sprenger et al., 1989) leads to expansion of the terminal pGro domain towards the centre of the embryo (Fig. 3C). Notably, the seven pGro trunk stripes are largely unaffected in *tsl*^{691}or *tor*^{Y9} mutants, suggesting that they are Torso independent (Fig. 3B,C) (see below).

Phosphorylation of Groucho correlates with FGFR-mediated signalling

In the *Drosophila* embryo, the FGFR pathway controls tracheal branching and morphogenesis. In stage 12 embryos, for example, localised activation of the Breathless FGFR occurs mainly in the posterior lateral migrating tip cells of the tracheal branches and, consequently, ERK is activated in these cells (see Fig. S1A in the supplementary material, arrowhead; GFP expression marks the tracheal field) (Gabay et al., 1997b). Double labelling with αpGro and αdpERK antibodies shows coincident staining (see Fig. S1C in the supplementary material), correlating phosphorylation of Gro with FGFR pathway activation.

Taken together, the above findings indicate that Gro is phosphorylated in response to multiple RTK pathways that operate at different times and places in the embryo.

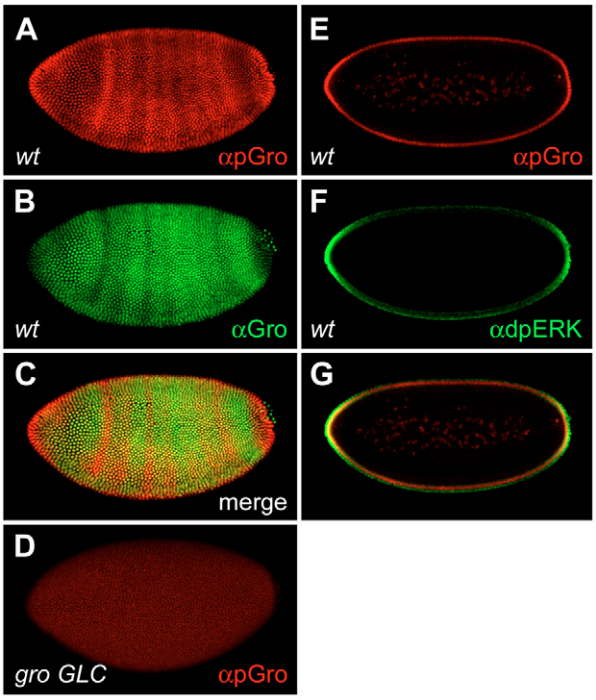

Fig. 2. Groucho is phosphorylated in cellular blastoderm embryos at the termini and in seven central transverse stripes. (**A-C**) Lateral view of a stage 5 blastoderm embryo, double-stained with αpGro (A; red) and αGro (B; green) antibodies. (C) Merge. Phosphorylated Gro is detected at both poles, and in seven stripes in the trunk region of the embryo (A). αGro staining is complementary to that of αpGro (C). (**D**) The pGro signal is significantly reduced in embryos devoid of maternally contributed Gro (germline clone; GLC), stained by αpGro antibodies. (**E-G**) Confocal optical cross-sections of a stage 4 syncytial blastoderm embryo, stained for αpGro (E; red) and αdpERK (F; green). (G) Merge. αdpERK and αpGro staining colocalises at the anterior and posterior termini. dpERK is mostly cytoplasmic, whereas pGro is predominantly nuclear. In this and subsequent figures, embryos are oriented with the anterior towards the left and dorsal side upwards.

Groucho is phosphorylated by MAPK and by other kinases

We next asked whether Gro is directly phosphorylated by MAPK, an idea consistent with Gro phosphorylation by multiple RTK pathways (Figs 1-3, see Fig. S1 in the supplementary material) and with the modification of Gro by MAPK/ERK in vitro (Fig. 1J) (Hasson et al., 2005). Unfortunately, the direct analysis of mutants devoid of maternal MAPK is technically unfeasible (Berghella and Dimitri, 1996). Instead, we monitored phosphorylation of Gro in embryos mutant for *DSor* (*Drosophila* MEK), lacking the maternal contribution of the only fly MAPK kinase (Hsu and Perrimon, 1994; Tsuda et al., 1993); these embryos do not accumulate active dpERK protein at their poles (data not shown). As shown in Fig. 3E, we find that *DSor* mutant embryos also lack detectable pGro protein at their termini. Conversely, staining of these *DSor* embryos with the αGro antibody reveals increased staining at the poles relative to wild-type embryos (Fig. 3F). These results are in agreement with Gro being a direct target for MAPK, though they do not formally rule out the possibility that it is MEK that phosphorylates Gro at the poles. Notably, the seven stripes of pGro still persist in *DSor* mutants, suggesting that some other kinase accounts for this striped pattern.

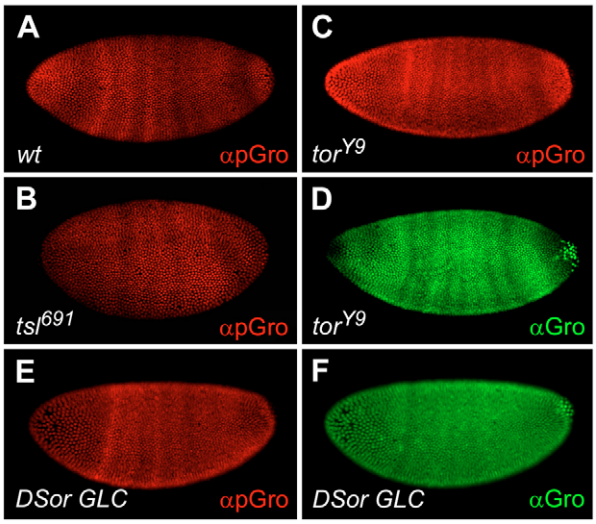

Fig. 3. Groucho is phosphorylated by the Torso pathway. Wild-type (**A**) or mutant (**B-F**) stage 5 embryos, stained with αpGro (A,B,C,E) or αGro (D,F) antibodies. The genetic inactivation of the Torso pathway, in tsl^{691} mutant embryos, leads to loss of pGro from the termini (B; compare with A). Reciprocally, pGro staining expands to more central regions in tor^{Y9} mutants, in which the Torso receptor is overactive, and a higher than normal uniform staining is observed throughout the embryo (C; compare with A). (D) A tor^{Y9} mutant embryo stained with the αGro antibody, showing a larger posterior domain devoid of staining (compare with Fig. 2B). The central pGro stripes are not dramatically affected in either tsl^{691} or tor^{Y9} mutants. (E,F) A stage 5 *DSor* mutant embryo (GLC), stained for αpGro (E; red) and αGro (F; green). pGro staining is absent from the termini (E; compare with A), and is replaced by αGro staining, which is not detected normally in this region (compare with Fig. 2B). There are seven pGro stripes in the *DSor* mutant (E).

Phosphorylation of Groucho and downregulation of its repressor function are required for terminal gene expression

The Torso pathway is one of the most studied models for RTK signalling in *Drosophila*, both in terms of identifying the molecular components of the RTK cascade as well as for studying the transcriptional regulation of pathway target genes (Duffy and Perrimon, 1994; Furriols and Casanova, 2003). We therefore chose this system to test whether phosphorylation of Gro in the embryo also results in the downregulation of its repressor activity, as has been demonstrated in the adult, and whether this modification is important for terminal patterning.

Expression of the downstream zygotic targets of the Torso pathway, *tailless* (*tll*) and *huckebein* (*hkb*), is blocked outside the termini by both Gro and the DNA-binding HMG-box repressor Capicua (Cic). At the termini, activation of the Torso pathway induces expression of *tll* and *hkb* by locally inhibiting repression exerted by Gro and Cic (Jiménez et al., 2000; Paroush et al., 1997). Phosphorylation of Cic by MAPK is one molecular mechanism employed by the Torso pathway to relieve repression in terminal regions (Astigarraga et al., 2007); once phosphorylated, Cic is targeted for degradation and is thus cleared from the poles (Jiménez et al., 2000). We therefore asked whether downregulation of Cic at the poles is the sole molecular event required for the derepression of *tll* and *hkb*, or whether phosphorylation-dependent attenuation of Gro-mediated repression is also important. To this end, we used the

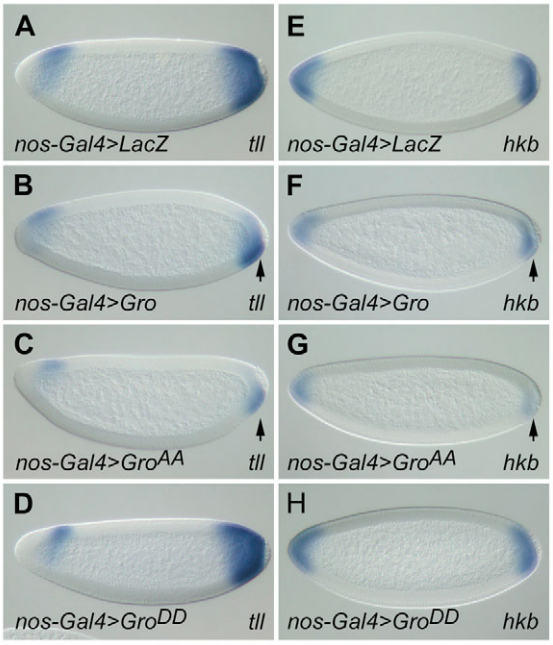

Fig. 4. Phosphorylation of Groucho and downregulation of its repressor function are required for terminal gap gene expression. Expression of *tll* (**A-D**) and *hkb* (**E-H**) in stage 4 embryos maternally expressing Gro (B,F), Gro^{AA} (C,G) or Gro^{DD} (D,H). Embryos similarly expressing *lacZ* serve as controls (A,E). Expression of Gro leads to reduction in *tll* and *hkb* transcription at both termini (B,F), whereas that of Gro^{AA} almost completely eliminates *tll* and *hkb* expression (C,G). In the posterior of the embryo, *tll* and *hkb* expression remains only in a ventral-terminal domain (arrows in B,C,F,G). Expression of Gro^{DD} has no effect, indicating that it cannot repress *tll* and *hkb* (D,H).

GAL4/UAS system to maternally express throughout the embryo (see Fig. S2 in the supplementary material) either the native form of Gro or two modified derivatives: (1) a Gro^{AA} variant, in which alanines replace the phospho-acceptor residues within the two MAPK consensus sites of Gro, rendering it unphosphorylatable; and (2) a Gro^{DD} form, in which these two amino acids are substituted by phosphomimetic aspartates (Hasson et al., 2005). If phosphorylation by Torso signalling is required to attenuate the repressor activity of endogenous Gro at the termini, then the two Gro derivatives are predicted to exert distinct effects on terminal gap gene expression: Gro^{AA} should be refractory to downregulation by the Torso pathway, and hence should cause dominant repression of pathway target genes; Gro^{DD}, however, mimics the effects of phosphorylation, and should be unable to repress *tll* and *hkb* expression.

Normally *tll* is expressed in syncytial blastoderm embryos in a posterior cap and in an anterior horseshoe-shaped stripe (Fig. 4A). At this stage *hkb* is transcribed at the most anterior tip, and in a small posterior domain that is nested within the *tll* domain (Fig. 4E) (Bronner et al., 1994; Weigel et al., 1990). As Fig. 4B,F show, the *tll* and *hkb* domains are spatially reduced at both termini by the expression of the native form of Gro, an outcome that is consistent with the role of Gro as a co-repressor of terminal gap gene expression (Paroush et al., 1997). The effects brought about by Gro^{AA}, are much stronger, however, as it causes an almost complete loss of *tll* and *hkb* expression (Fig. 4C,G). Notably, *tll* and *hkb* expression persists in a small ventral patch at the posterior pole in a significant proportion of these embryos. By contrast, expression of the pseudo-phosphorylated Gro^{DD} derivative has no effect, indicating that this form cannot repress *tll* and *hkb* (Fig. 4D,H). These results suggest that the Torso pathway is required to downregulate the co-repressor activity of Gro for correct terminal gap gene expression.

Downregulation of Groucho via phosphorylation is required for terminal patterning

Gro^{AA} and Gro^{DD} also exert differential effects on the expression of *knirps* (*kni*) and *hunchback* (*hb*), two gap genes that are regulated by Tll and Hkb. The posterior boundary of *kni* is established by direct Tll-mediated repression. Tll also indirectly activates the posterior *hb* stripe, partly by repressing *kni*, a repressor of *hb* (Moran and Jiménez, 2006). Hkb also targets *hb* expression, repressing it at the posterior tip (Margolis et al., 1995). We find that the posterior stripe of *hb* shifts posteriorly upon expression of Gro^{AA}, in accordance with the reduction in *tll* and *hkb* expression in these embryos (Fig. 5C). Notably, the small ventroposterior domain where *hkb* expression persists appears devoid of *hb* transcripts (Fig. 5C, compare with Fig. 4G). Similarly, *kni* is derepressed posteriorly in embryos expressing Gro^{AA} (see Fig. S3C in the supplementary material). By contrast, Gro^{DD} does not cause significant effects on *hb* and *kni* expression (Fig. 5D, see Fig. S3D in the supplementary material).

Maternal expression of Gro, Gro^{AA} and Gro^{DD} also leads to patterning defects that parallel their effects on terminal gene expression. Expression of native Gro leads to a low hatching rate and causes a range of segmental cuticular defects, consistent with the well-established role of Gro in segmentation (not shown) (Chen and Courey, 2000; Paroush et al., 1994). In 34.2% of the dead larvae, we also observe a loss or reduction of terminal structures, such as the head and filzkörper (see Fig. S3F in the supplementary material). Expression of Gro^{AA} also causes a low hatching rate, and in 30.5% of unhatched larvae the filzkörper is reduced even further and at times completely lost, in accordance with a strong reduction of *tll* and *hkb* expression at the posterior pole (see Fig. S3G in the supplementary material and Fig. 4B,C,F,G). Expression of Gro^{DD} also leads to early lethality; however, the effects on terminal structure morphology are minimal and are observed in only 11.1% of dead larvae, whereas the vast majority of these larvae show a fully extended filzkörper (see Fig. S3H in the supplementary material).

Collectively, these data suggest that Torso signalling downregulates Gro repressor activity via phosphorylation, and that this mode of Gro regulation is essential for accurate expression of terminal gap genes and their targets, as well as for correct specification of terminal cell fates.

Groucho may repress terminal gap genes independently of Capicua

The expression of Gro and its derivatives in the germline could potentially interfere with the early steps of anteroposterior (AP) axis specification, and hence the effects on *tll* and *hkb* gene expression may be indirect. To rule out this possibility, we confirmed that anterior and posterior determinants are correctly localised in embryos expressing Gro, Gro^{AA} and Gro^{DD}. In all three cases, we find that expression of *hb* at the anterior and that of *nanos* (*nos*) at the posterior are indistinguishable from the wild type (Fig. 5A-H), suggesting that maternal expression of Gro or its variants does not disrupt early embryonic AP axis formation.

Another possible explanation for Gro^{AA} repression of *tll* and *hkb* is that its expression leads to a failure in the clearance of Cic from the termini. However, as Fig. 5I-L shows, Cic is properly downregulated in those embryos. The ability of Gro^{AA}, and to a

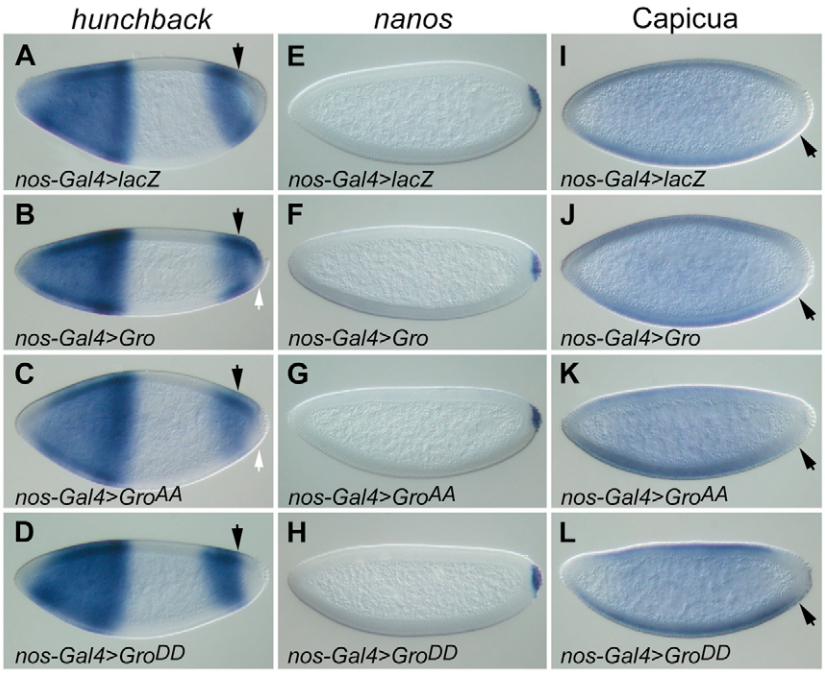

Fig. 5. Maternal expression of Groucho or its derivatives does not disrupt embryonic anteroposterior axis formation. The anterior localisation of *hb* (**A-D**) and posterior localisation of *nos* (**E-H**) RNA transcripts, as well as the degradation of the Cic protein at the termini (**I-L**) are indistinguishable in early stage 5 control embryos (A,E,I) and in embryos maternally expressing Gro (B,F,J) or its variants, Gro^{AA} (C,G,K) and Gro^{DD} (D,H,L). By contrast, the posterior zygotic *hb* stripe is derepressed posteriorly in Gro (B) and Gro^{AA} (C), but not in Gro^{DD} (D)-expressing embryos. *hb* is not derepressed in a ventroposterior domain in Gro (B) and Gro^{AA} (C) expressing embryos (white arrows), in accordance with the enduring *hkb* expression in these embryos (see Fig. 4F,G). (A-D,I-L) Black arrows indicate the same relative positions in all embryos.

lesser extent of native Gro, to repress terminal gap gene expression at the pole regions where Cic is absent suggests that Gro acts in these regions in association with some other Gro-dependent repressor(s).

Phosphorylated Groucho is a nuclear and stable protein

Our analyses of pGro distribution in vivo provide additional significant insights into the mechanism of Gro regulation via phosphorylation. First, we find that pGro is strictly nuclear at all stages (e.g. Fig. 6A,A′). Thus, phosphorylation by MAPK does not promote translocation of Gro to the cytoplasm or otherwise alters its subcellular localisation, nor does it lead to Gro degradation. This implies that phosphorylation of Gro interferes in some other manner with its ability to repress.

Second, a comparison of the dpERK and pGro patterns shows that MAPK is only transiently phosphorylated, whereas pGro perdures for longer periods of time. For example, dpERK is no longer detected in the pole regions of gastrulating embryos, yet Gro is still phosphorylated in these domains (Fig. 6B-D). Similarly, Gro, but not MAPK, remains phosphorylated at stage 9 in the ventral neuroectoderm, as a consequence of EGFR activation at stage 7 (data not shown). In addition, at stages 9 and 10, the extent of the pGro neuroectodermal domain is evidently larger than that of dpERK, probably reflecting the large domain of dpERK staining at the earlier stage (Fig. 1A). Thus, pGro is a stable protein that appears to undergo dephosphorylation at a low rate. Below, we discuss the implication of these findings to the regulation of target gene expression by RTK signalling.

DISCUSSION

Groucho is phosphorylated in response to multiple RTK pathways acting in embryogenesis

RTK pathways play key roles during development, to a large extent by eliciting changes in the expression of target genes that, in turn, induce cell proliferation and differentiation (Tan and Kim, 1999). The key RTK effector is MAPK, which, upon activation, translocates to the nucleus and directly targets substrate transcription factors. Intriguingly, a surprisingly low number of transcriptional regulators that are phosphorylated by MAPK have been identified and confirmed to date, despite their potential vital roles in normal development and in cancer.

In this context, we have recently found that the global co-repressor Gro is phosphorylated in response to EGFR signalling, and that such regulation is essential for the correct patterning of the adult wing (Hasson et al., 2005). Here, we confirm and extend these findings by showing that at least three RTK pathways – mediated by the EGFR, FGFR and Torso receptors – elicit phosphorylation of Gro in various embryonic processes. In addition, we provide several lines of evidence indicating that such phosphorylation is directly mediated by MAPK: first, MAPK/ERK2 can phosphorylate Gro in vitro (Fig. 1); second, our αpGro sera was raised against, and detects phosphorylation on, a MAPK consensus site; and third, *DSor* mutant embryos lack detectable pGro protein in the termini and neuroectoderm (Fig. 3; data not shown). Based on these findings, we conclude that phosphorylation of Gro is probably a general outcome of RTK activation in *Drosophila*, and possibly in higher organisms as well. Indeed, a recent report identified TLE proteins as possible targets of EGFR phosphorylation in mammalian cells (Olsen et al., 2006).

Notably, the αpGro antibodies also detect phosphorylated Gro in places and times where RTK pathways are not known to be active. For example, seven stripes of αpGro staining, which overlap with the Even-skipped pair-rule stripes, can be seen at the centre of early cellular blastoderm embryos (Fig. 2; data not shown). Given that this pattern is also observed in a *DSor* background (Fig. 3), we hypothesise that other MAPK family members phosphorylate Gro. In principle, several kinases that are active in the early embryo could account for this seven-striped pattern (e.g. p38, JNK and Nemo-like). We have ruled out the possibility that this phosphorylation is catalysed directly or indirectly by JAK, a tyrosine kinase that acts in segmentation (Binari and Perrimon, 1994): the stripes of pGro and of phosphorylated STAT (the target for JAK activity) do not overlap, and pGro is detected even when the JAK/STAT pathway is genetically blocked (e.g. in *unpaired* mutants; not shown) (Harrison

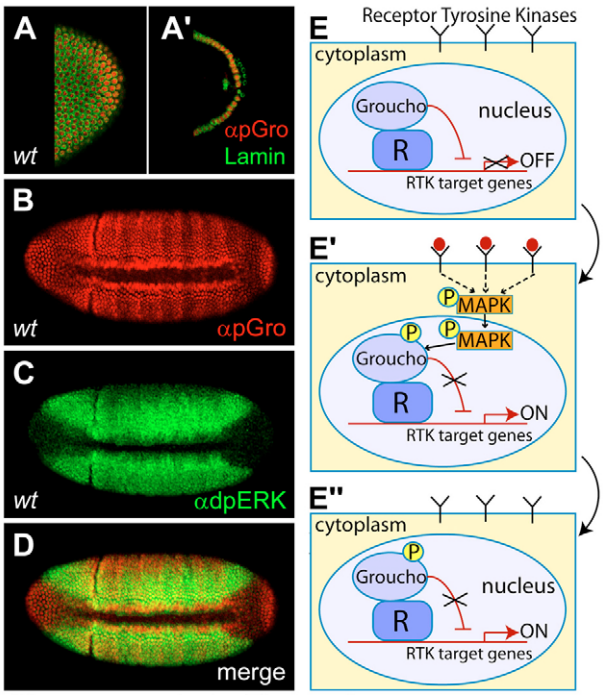

Fig. 6. Phosphorylated Groucho is a nuclear and stable protein that persists after MAPK activation has been extinguished. (**A**,**A′**) Posterior terminus of stage 5 wild-type embryo, stained with αpGro antibodies (red) and αLamin antibody (green) demarcating the nuclear membrane. Superficial (A) and transverse (A′) confocal sections show the nuclear localisation of pGro, encircled by αLamin staining. (**B-D**) Ventral view of wild-type stage 6 gastrulating embryo (note the invagination of the ventral furrow), stained for αpGro (B; red) and αdpERK (C; green). (D) Merge. pGro staining is detected at the termini even after MAPK activation has been turned off. Strong dpERK and pGro staining on both sides of the ventral furrow correlates with EGFR activation in this region. (**E-E″**) Model depicting possible implications of Gro phosphorylation to RTK target gene regulation. Prior to RTK activation (E), Gro is associated with its partner DNA-binding repressors (R), mediating repression of RTK target genes. Upon RTK pathway activation (E′), Gro is phosphorylated by MAPK. Modification of Gro downregulates its repressor activity, causing derepression of pathway target genes. MAPK is no longer active after RTK signalling has been turned off (E″), yet Gro remains stably phosphorylated and its activity attenuated, allowing for sustained RTK target gene expression.

et al., 1998). Future studies will be required to uncover those additional kinases and pathways that phosphorylate Gro, and to determine whether modification of Gro in stripes is required to downregulate its activity vis-à-vis one or more of its dependent repressors that act in the process of segmentation (e.g. Hairy and Even-skipped).

Phosphorylation of Groucho and terminal patterning

Our results suggest that the Torso pathway triggers phosphorylation of Gro, and in this way attenuates its activity. Importantly, this post-transcriptional downregulation of Gro is essential for setting up the precise domains of terminal gap gene expression and for the specification of the non-segmented embryonic poles. Thus, relief of terminal gap gene repression by the Torso pathway involves targeting of both Cic and Gro at the embryonic poles. We still do not understand how these two regulatory events are coordinated in vivo. Two possibilities are worth considering: (1) if Gro and Cic act in the same repressor complex, then phosphorylation of Gro may be part of a double-safety mechanism that ensures that even low levels of Cic at the termini are not active; (2) alternatively, Gro and Cic could be acting in distinct repression complexes, which are inactivated by the Torso pathway independently of each other. We favour the second possibility for two reasons: first, a derivative of Cic that is refractory to MAPK phosphorylation acts as a dominant repressor of terminal gap gene expression at the poles (Astigarraga et al., 2007), where Gro is phosphorylated, suggesting that Cic-mediated repression is insensitive to the phosphorylation state of Gro; second, the Gro^{AA} derivative represses terminal gap gene expression at the poles, despite the normal clearance of Cic (Fig. 4C,G, Fig. 5K). According to the latter model, Gro would have to be recruited to promoters of terminal gap genes by an as yet unidentified repressor. NTF-1 or GAGA, which can bind a cis-regulatory module in the *tll* promoter that mediates repression (Liaw et al., 1995), could correspond to this repressor. Future studies will establish whether these are Gro-dependent repressors, and whether their function is sensitive to the phosphorylation state of Gro. In any case, our findings provide evidence for a new level of regulation of terminal gene expression, that acts in parallel to the regulation of Cic by the Torso pathway and to other inputs, such as the anterior and dorsal maternal systems at the anterior pole (Pignoni et al., 1992), and the posterior maternal group at the posterior (Cinnamon et al., 2004).

Regulation of Groucho-dependent repression by phosphorylation

How does MAPK phosphorylation affect Gro activity? Hypothetically, it could influence any of the steps between the recruitment of Gro by its DNA-binding repressor partners and its interaction with other co-factors that leads to gene silencing. For example, phosphorylation of Gro by HIPK2 and CK2 impacts on its interactions with transcription factors and/or with chromatin (Choi et al., 2005; Nuthall et al., 2004). In our case, we find that MAPK phosphorylation does not affect the strength of interactions between Gro and Hairy or Odd-skipped, or with the Rpd3 histone deacetylase (HDAC) (Chen et al., 1999; Goldstein et al., 2005; Jiménez et al., 1997; Mannervik and Levine, 1999; Paroush et al., 1994), at least in vitro (A.H. and Z.P., unpublished). pGro is evidently a stable nuclear protein, excluding the possibility that, once modified, it is exported from the nucleus or degraded. It is possible that phosphorylation alters the sub-nuclear localisation of Gro in a way that precludes its ability to repress transcription. However, a more plausible explanation, insinuated by the finding that phosphorylation of Gro abrogates recognition by the αGro antibody, is that modified pGro can no longer form active complexes with HDACs and/or other co-regulatory proteins.

One of our main findings is that the phosphorylated and unphosphorylated states of Gro are largely mutually exclusive. This inference is based on the observation that the αGro antibody hardly recognises pGro, resulting in reduced or no staining where Gro is phosphorylated. This observation indicates that Gro is phosphorylated by a mechanism that is highly efficient, and supports the biological significance of Gro downregulation via phosphorylation; if only a fraction of the pool of Gro molecules in the nucleus were phosphorylated, then the remaining non-phosphorylated proteins could still be active and repression would not be relieved in response to signalling. Similarly, Cic and Yan, two repressor proteins that are also targeted by MAPK, are effectively degraded as a result of phosphorylation by RTK signals (Astigarraga

et al., 2007; Rebay and Rubin, 1995). By contrast, lower levels of phosphorylation should suffice for the upregulation of transcriptional activators and signal transducers (e.g. Pointed and MAPK, respectively).

Another aspect of Gro regulation via phosphorylation concerns its duration. A comparison between pGro and dpERK staining reveals overlapping domains at different stages of embryogenesis, suggesting that the overall dynamics of Gro phosphorylation are similar to those of RTK signalling (Figs 1, 2; see Fig. S1 in the supplementary material). A closer inspection, however, reveals that phosphorylation of MAPK precedes that of Gro, and that pGro persists after dpERK staining has faded away. For example, pGro remains at the termini until the beginning of gastrulation, when dpERK staining is no longer observed (Fig. 6B-D). Thus, pGro seems to be a stable protein, which becomes dephosphorylated at lower rates than activated MAPK. We propose that the persistence of pGro protein is an important feature of its regulation by MAPK. Thus, it is possible that prolonged phosphorylation of Gro imparts cells with long-term memory of previous RTK signalling, by enabling continuous effects on gene expression that would be necessary for cellular differentiation (Fig. 6E-E″).

Concluding remarks

Gro and its TLE mammalian homologues act as co-repressors for nuclear effectors of multiple, conserved signal transduction pathways that include Dpp/TGFβ, Notch and Wg/Wnt. Gro/TLE therefore makes an ideal focal point for crosstalk between RTK and other developmental pathways (Hasson et al., 2005; Hasson and Paroush, 2006; Orian et al., 2007). By phosphorylating and downregulating the repressor function of Gro/TLE, multiple RTK signals could impinge on the transcriptional output of other pathways, providing a synchronised regulatory mechanism of numerous target genes via a single yet efficient and persistent phosphorylation event.

We thank members of our laboratory for continued help and encouragement, especially Robert Goldstein, Rona Grossman and Orit Gozoltsany. We are grateful to Peleg Hasson, Susan Parkhurst, Benny Shilo and Talila Volk for their comments on the manuscript, and to Benny Shilo and Howard Cedar for advise and support. We thank Jordi Casanova, Mark Van Doren, Abraham Fainsod, Lilach Gilboa, Yosef Gruenbaum, David Ish-Horowicz, Norbert Perrimon, Pernille Rørth, Benny Shilo, Daniel St Johnston, Mark Wainwright, the Developmental Studies Hybridoma Bank and the Bloomington Stock Centre for antibodies, fly stocks and other reagents. This research was supported by grants from the Israel Science Foundation (501/04), the Israel Cancer Research Fund and the Król Charitable Foundation to Z.P. G.J. was supported by grants from the Spanish Ministry of Science and Education, A.O. was supported by the HFSP (CDA 0048/2004) and R.B.-H.S. was supported by a US-Israel Binational Science Foundation grant to Abraham Fainsod. Z.P. is a Braun Lecturer in Medicine, G.J. is an ICREA Investigator and A.O. is a Lymphoma and Leukemia Society and a Horev-Taub-Shalom Fellow.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/5/829/DC1

References

Astigarraga, S., Grossman, R., Diaz-Delfin, J., Caelles, C., Paroush, Z. and Jiménez, G. (2007). A MAPK docking site is critical for downregulation of Capicua by Torso and EGFR RTK signalling. *EMBO J.* **26**, 668-677.

Berghella, L. and Dimitri, P. (1996). The heterochromatic *rolled* gene of *Drosophila melanogaster* is extensively polytenized and transcriptionally active in the salivary gland chromocenter. *Genetics* **144**, 117-125.

Binari, R. and Perrimon, N. (1994). Stripe-specific regulation of pair-rule genes by *hopscotch*, a putative Jak family tyrosine kinase in *Drosophila*. *Genes Dev.* **8**, 300-312.

Bronner, G., Chu-LaGraff, Q., Doe, C. Q., Cohen, B., Weigel, D., Taubert, H. and Jackle, H. (1994). Sp1/egr-like zinc-finger protein required for endoderm specification and germ-layer formation in *Drosophila*. *Nature* **369**, 664-668.

Buscarlet, M. and Stifani, S. (2007). The 'Marx' of Groucho on development and disease. *Trends Cell Biol.* **17**, 353-361.

Casali, A. and Casanova, J. (2001). The spatial control of Torso RTK activation: a C-terminal fragment of the Trunk protein acts as a signal for Torso receptor in the *Drosophila* embryo. *Development* **128**, 1709-1715.

Casanova, J., Furriols, M., McCormick, C. A. and Struhl, G. (1995). Similarities between *trunk* and *spatzle*, putative extracellular ligands specifying body pattern in *Drosophila*. *Genes Dev.* **9**, 2539-2544.

Chen, G. and Courey, A. J. (2000). Groucho/TLE family proteins and transcriptional repression. *Gene* **249**, 1-16.

Chen, G., Fernandez, J., Mische, S. and Courey, A. J. (1999). A functional interaction between the histone deacetylase Rpd3 and the corepressor *groucho* in *Drosophila* development. *Genes Dev.* **13**, 2218-2230.

Choi, C. Y., Kim, Y. H., Kim, Y. O., Park, S. J., Kim, E. A., Riemenschneider, W., Gajewski, K., Schulz, R. A. and Kim, Y. (2005). Phosphorylation by the DHIPK2 protein kinase modulates the corepressor activity of Groucho. *J. Biol. Chem.* **280**, 21427-21436.

Chou, T. B., Noll, E. and Perrimon, N. (1993). Autosomal *P[ovoD1]* dominant female-sterile insertions in *Drosophila* and their use in generating germ-line chimeras. *Development* **119**, 1359-1369.

Cinnamon, E., Gur-Wahnon, D., Helman, A., St Johnston, D., Jiménez, G. and Paroush, Z. (2004). Capicua integrates input from two maternal systems in *Drosophila* terminal patterning. *EMBO J.* **23**, 4571-4582.

Culi, J., Martin-Blanco, E. and Modolell, J. (2001). The EGF receptor and N signalling pathways act antagonistically in *Drosophila* mesothorax bristle patterning. *Development* **128**, 299-308.

Delidakis, C., Preiss, A., Hartley, D. A. and Artavanis-Tsakonas, S. (1991). Two genetically and molecularly distinct functions involved in early neurogenesis reside within the Enhancer of split locus of *Drosophila* melanogaster. *Genetics* **129**, 803-823.

Duffy, J. B. and Perrimon, N. (1994). The *torso* pathway in *Drosophila*: lessons on receptor tyrosine kinase signalling and pattern formation. *Dev. Biol.* **166**, 380-395.

Flores, G. V., Duan, H., Yan, H., Nagaraj, R., Fu, W., Zou, Y., Noll, M. and Banerjee, U. (2000). Combinatorial signalling in the specification of unique cell fates. *Cell* **103**, 75-85.

Furriols, M. and Casanova, J. (2003). In and out of Torso RTK signalling. *EMBO J.* **22**, 1947-1952.

Gabay, L., Seger, R. and Shilo, B. Z. (1997a). In situ activation pattern of *Drosophila* EGF receptor pathway during development. *Science* **277**, 1103-1106.

Gabay, L., Seger, R. and Shilo, B. Z. (1997b). MAP kinase in situ activation atlas during *Drosophila* embryogenesis. *Development* **124**, 3535-3541.

Goldstein, R. E., Cook, O., Dinur, T., Pisante, A., Karandikar, U. C., Bidwai, A. and Paroush, Z. (2005). An eh1-like motif in *odd-skipped* mediates recruitment of Groucho and repression in vivo. *Mol. Cell. Biol.* **25**, 10711-10720.

Harrison, D. A., McCoon, P. E., Binari, R., Gilman, M. and Perrimon, N. (1998). *Drosophila unpaired* encodes a secreted protein that activates the JAK signalling pathway. *Genes Dev.* **12**, 3252-3263.

Hasson, P. and Paroush, Z. (2006). Crosstalk between the EGFR and other signalling pathways at the level of the global transcriptional corepressor Groucho/TLE. *Br. J. Cancer* **94**, 771-775.

Hasson, P., Egoz, N., Winkler, C., Volohonsky, G., Jia, S., Dinur, T., Volk, T., Courey, A. J. and Paroush, Z. (2005). EGFR signalling attenuates Groucho-dependent repression to antagonize Notch transcriptional output. *Nat. Genet.* **37**, 101-105.

Hsu, J. C. and Perrimon, N. (1994). A temperature-sensitive MEK mutation demonstrates the conservation of the signalling pathways activated by receptor tyrosine kinases. *Genes Dev.* **8**, 2176-2187.

Huang, P. and Stern, M. J. (2005). FGF signalling in flies and worms: more and more relevant to vertebrate biology. *Cytokine Growth Factor Rev.* **16**, 151-158.

Jiménez, G., Paroush, Z. and Ish-Horowicz, D. (1997). Groucho acts as a corepressor for a subset of negative regulators, including Hairy and Engrailed. *Genes Dev.* **11**, 3072-3082.

Jiménez, G., Guichet, A., Ephrussi, A. and Casanova, J. (2000). Relief of gene repression by *torso* RTK signalling: role of *capicua* in *Drosophila* terminal and dorsoventral patterning. *Genes Dev.* **14**, 224-231.

Liaw, G. J., Rudolph, K. M., Huang, J. D., Dubnicoff, T., Courey, A. J. and Lengyel, J. A. (1995). The *torso* response element binds GAGA and NTF-1/Elf-1, and regulates *tailless* by relief of repression. *Genes Dev.* **9**, 3163-3176.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$T method. *Methods* **25**, 402-408.

Mannervik, M. and Levine, M. (1999). The Rpd3 histone deacetylase is required for segmentation of the *Drosophila* embryo. *Proc. Natl. Acad. Sci. USA* **96**, 6797-6801.

Margolis, J. S., Borowsky, M. L., Steingrimsson, E., Shim, C. W., Lengyel, J. A. and Posakony, J. W. (1995). Posterior stripe expression of *hunchback* is driven from two promoters by a common enhancer element. *Development* **121**, 3067-3077.

Moran, E. and Jiménez, G. (2006). The *tailless* nuclear receptor acts as a dedicated repressor in the early *Drosophila* embryo. *Mol. Cell. Biol.* **26**, 3446-3454.
Nuthall, H. N., Joachim, K. and Stifani, S. (2004). Phosphorylation of serine 239 of Groucho/TLE1 by protein kinase CK2 is important for inhibition of neuronal differentiation. *Mol. Cell. Biol.* **24**, 8395-8407.
Olsen, J. V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P. and Mann, M. (2006). Global, in vivo, and site-specific phosphorylation dynamics in signalling networks. *Cell* **127**, 635-648.
Orian, A., Delrow, J. J., Rosales Nieves, A. E., Abed, M., Metzger, D., Paroush, Z., Eisenman, R. N. and Parkhurst, S. M. (2007). A Myc-Groucho complex integrates EGF and Notch signalling to regulate neural development. *Proc. Natl. Acad. Sci. USA* **104**, 15771-15776.
Paroush, Z., Finley, R. L., Jr, Kidd, T., Wainwright, S. M., Ingham, P. W., Brent, R. and Ish-Horowicz, D. (1994). Groucho is required for *Drosophila* neurogenesis, segmentation, and sex determination and interacts directly with *hairy*-related bHLH proteins. *Cell* **79**, 805-815.
Paroush, Z., Wainwright, S. M. and Ish-Horowicz, D. (1997). Torso signalling regulates terminal patterning in *Drosophila* by antagonising Groucho-mediated repression. *Development* **124**, 3827-3834.
Pignoni, F., Steingrimsson, E. and Lengyel, J. A. (1992). *bicoid* and the terminal system activate *tailless* expression in the early *Drosophila* embryo. *Development* **115**, 239-251.
Rebay, I. and Rubin, G. M. (1995). Yan functions as a general inhibitor of differentiation and is negatively regulated by activation of the Ras1/MAPK pathway. *Cell* **81**, 857-866.
Rørth, P. (1998). Gal4 in the *Drosophila* female germline. *Mech. Dev.* **78**, 113-118.
Shilo, B. Z. (2003). Signalling by the *Drosophila* epidermal growth factor receptor pathway during development. *Exp. Cell Res.* **284**, 140-149.
Shilo, B. Z. (2005). Regulating the dynamics of EGF receptor signalling in space and time. *Development* **132**, 4017-4027.
Simon, M. A. (2000). Receptor tyrosine kinases: specific outcomes from general signals. *Cell* **103**, 13-15.
Sprenger, F. and Nüsslein Volhard, C. (1992). Torso receptor activity is regulated by a diffusible ligand produced at the extracellular terminal regions of the *Drosophila* egg. *Cell* **71**, 987-1001.
Sprenger, F., Stevens, L. M. and Nüsslein-Volhard, C. (1989). The *Drosophila* gene *torso* encodes a putative receptor tyrosine kinase. *Nature* **338**, 478-483.
Stevens, L. M., Frohnhofer, H. G., Klingler, M. and Nüsslein Volhard, C. (1990). Localized requirement for *torso-like* expression in follicle cells for development of terminal anlagen of the *Drosophila* embryo. *Nature* **346**, 660-663.
Tan, P. B. and Kim, S. K. (1999). Signalling specificity: the RTK/RAS/MAP kinase pathway in metazoans. *Trends Genet.* **15**, 145-149.
Tsuda, L., Inoue, Y. H., Yoo, M. A., Mizuno, M., Hata, M., Lim, Y. M., Adachi-Yamada, T., Ryo, H., Masamune, Y. and Nishida, Y. (1993). A protein kinase similar to MAP kinase activator acts downstream of the *raf* kinase in *Drosophila*. *Cell* **72**, 407-414.
Weigel, D., Jurgens, G., Klingler, M. and Jackle, H. (1990). Two gap genes mediate maternal terminal pattern information in *Drosophila*. *Science* **248**, 495-498.