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Hedgehog restricts its expression domain in the *Drosophila* wing

Fernando Bejarano[1], *Lidia Pérez*[1], *Yiorgos Apidianakis*[2], *Christos Delidakis*[2] *& Marco Milán*[1+]

[1]ICREA and Institute for Research in Biomedicine (IRB), Parc Científic de Barcelona, Barcelona, Spain, and [2]Institute of Molecular Biology and Biotechnology, University of Crete, Heraklion, Greece

The stable subdivision of *Drosophila* limbs into anterior and posterior compartments is a consequence of asymmetrical signalling by Hedgehog (Hh), from the posterior to anterior cells. The activity of the homeodomain protein Engrailed in posterior cells helps to generate this asymmetry by inducing the expression of Hh in the posterior compartment and, at the same time, repressing the expression of the essential downstream component Cubitus interruptus (Ci). Therefore, only anterior cells that receive the Hh signal across the compartment boundary will respond by stabilizing Ci. Here, we describe a new molecular mechanism that helps to maintain the Hh-expressing and Hh-responding cells in different non-overlapping cell populations. *Master of thickveins* (*mtv*)—a target of Hh activity encoding a nuclear zinc-finger protein—is required to repress *hh* expression in anterior cells. Mtv exerts this action in a protein complex with Groucho (Gro)—the founding member of a superfamily of transcriptional corepressors that are conserved throughout eukaryotes. Therefore, Hh restricts its own expression domain in the *Drosophila* wing through the activity of Mtv and Gro.

Keywords: compartment; Mtv; Groucho; Ci

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INTRODUCTION

The limb primordia of *Drosophila* are subdivided into adjacent territories called compartments—that is, cell populations that do not mix during development (García-Bellido *et al*, 1973). Anterior and posterior cells in limb primordia acquire specific fates during embryogenesis through the activity of the homeodomain proteins Engrailed (En) and Invected in posterior cells (Tabata *et al*, 1995). Soon after the discovery that anterior and posterior cells are separated by a lineage restriction boundary (García-Bellido *et al*, 1973), it was proposed that this boundary acts as an organizing centre in the developing appendages (Crick & Lawrence, 1975). Molecular analyses have shown that specialized cells are established along the anterior–posterior boundary as a consequence of asymmetrical signalling by the diffusible protein Hedgehog (Hh), from the posterior to anterior cells (Basler & Struhl, 1994; Tabata & Kornberg, 1994). En induces expression of Hh in the posterior compartment and represses the expression of the essential downstream component of the Hh signalling pathway Cubitus interruptus (Ci). Ci can exist in two forms: a repressor form (Ci^{rep}) is generated in cells that do not receive the Hh signal; and an activator form of Ci (Ci^{act}) is generated in cells that receive the Hh signal (Fig 1I; Aza-Blanc *et al*, 1997; Ohlmeyer & Kalderon, 1998). Both forms of Ci control the expression of the secreted signalling molecule Decapentaplegic (Dpp) in a thin stripe of anterior cells along the anterior–posterior boundary (Méthot & Basler, 1999). Dpp acts as a symmetrical long-range morphogen. By this mechanism the originally asymmetrical subdivision of the limb primordium eventually leads to the establishment of a symmetrical organizing gradient of Dpp.

Asymmetrical signalling of Hh also relies on restricting the Hh-expressing cells to the posterior compartment (Fig 1I,J). Ci^{rep} has been shown to repress the expression of *hh* in anterior cells, mainly in those cells that do not receive the Hh signal (Fig 1I; Méthot & Basler, 1999). The transcriptional corepressor Groucho (Gro)—the founding member of a superfamily of corepressors that are conserved throughout eukaryotes—is also required to repress *hh* expression in anterior cells, but in cells that are close to the anterior–posterior boundary (Fig 1J; de Celis & Ruiz-Gomez, 1995; Apidianakis *et al*, 2001). Gro is recruited by several transcription factors to mediate various long-range repression mechanisms during embryonic and larval development of *Drosophila*. Here, we show that Master of thickveins (Mtv), a target of Hh activity encoding a nuclear zinc-finger protein (Funakoshi *et al*, 2001), and Gro take part in the same protein complex and mediate transcriptional repression of *hh*. Therefore, two different mechanisms are used in the *Drosophila* wing to restrict *hh* expression to the posterior compartment. Several signalling molecules have been shown to restrict their own expression or activity domains (Freeman & Gurdon, 2002); to our knowledge, this is the first time that Hh has been shown to be involved in restricting its own expression domain.

[1]ICREA and Institute for Research in Biomedicine (IRB), Parc Científic de Barcelona, Josep Samitier, 1-5, 08028 Barcelona, Spain
[2]Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology-Hellas and Department of Biology, University of Crete, 71110 Heraklion, Greece
[+]Corresponding author. Tel: +34 93 4034902; Fax: +34 93 4037109; E-mail: mmilan@pcb.ub.es

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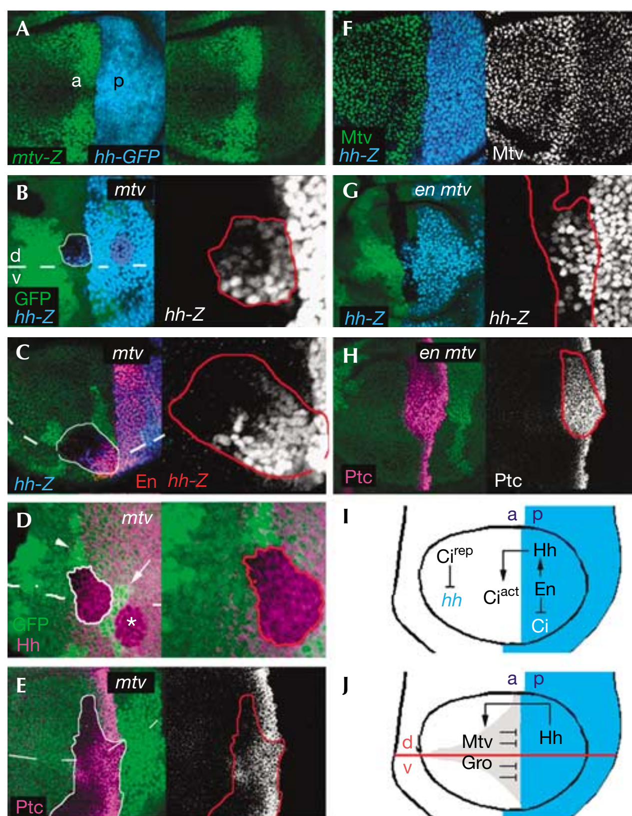

Fig 1 | Ectopic expression of *hh* in the absence of *mtv* activity. (**A**) Wing disc labelled to visualize expression of the *mtv-lacZ* reporter gene (antibody to β-Gal, green) in an *hh-GAL4;UAS-GFP* background (GFP, blue). (**F**) Wing disc labelled to visualize expression of a *hh-lacZ* reporter gene (antibody to β-gal, blue) and Mtv protein expression (green or white). (**B–E,G,H**) Clones of cells mutant for *mtv* (*mtv*^{6}; **B–E**) or for both *engrailed/invected* and *mtv* (*Df(2R)en[E] mtv*^{6}; **G,H**) and labelled by the absence of the GFP marker (green). The contour of the clone is marked by a red or white line. *hh-lacZ* (blue or white; **B,C,G**), Engrailed (En, red; **C**), Hedgehog (Hh, purple; **D**) or Patched (Ptc, purple or white; **E,H**) is ectopically expressed in a clone abutting the anterior–posterior (ap) and dorsal–ventral (dv; dashed lines) boundaries. Note in (**D**) that the marked clone with ectopic Hh protein expression was born in the anterior (a) compartment (twin clone is marked by an arrowhead) but crosses into the posterior (p) compartment. A neighbouring p clone (white asterisk) and its twin (white arrow) are shown. Violation of the ap compartment was very frequently observed in these clones (data not shown). (**I,J**) Two different mechanisms to repress *hh* expression in a cells. (**I**) En expression in p cells induces *hh* expression and represses *cubitus interruptus* (*ci*) expression. Hh activity in a cells stabilizes the transcriptional activator form of Ci (Ci^{act}). In the absence of Hh signal, Ci is transformed to a transcriptional repressor (Ci^{rep}). Ci^{rep} represses Hh expression. (**J**) Proposed model illustrating the role of Mtv and Gro in the repression of *hh* in a cells. GFP, green fluorescent protein; Gro, Groucho; Mtv, master of thickveins.

RESULTS AND DISCUSSION

The anterior–posterior asymmetrical expression of *mtv* in the developing wing primordium (Fig 1A,F) follows that of Hh activity, and is a consequence of Hh increasing *mtv* expression at the anterior–posterior boundary and En reducing *mtv* expression in posterior cells (Funakoshi *et al*, 2001). To assess the role of *mtv* in wing development, clones of cells mutant for a loss-of-function allele of *mtv*—*mtv*^{6}—were generated using the FLP/FRT technique. Clones were marked by the absence of the green fluorescent protein (GFP) marker. These mutant cells caused ectopic expression of *hh* in anterior cells (Fig 1B–D). Only those cells abutting or close to the anterior–posterior boundary were able to induce *hh*. We monitored the expression of the Hh target genes *en* and *patched* (*ptc*) in these clones. Again, loss of *mtv* caused an expansion of *en* and *ptc* expression domains in cells located in the same region (Fig 1C,E). Posterior clones did not have any effect on the expression of Hh target genes (data not shown).

In the absence of *mtv* activity, *hh* is expressed in anterior cells. The *hh* gain-of-funcion allele *Moonrat*—*hh*^{Mrt}—also resulted in derepression of *hh* in anterior cells and caused duplication of anterior structures—that is, longitudinal veins (arrow in Fig 2B)—and enlargement of the anterior compartment (de Celis & Ruiz-Gomez, 1995; Felsenfeld & Kennison, 1995). We then investigated whether reduced levels of *mtv* activity would enhance the *hh*^{Mrt} phenotype. Loss of one copy of *mtv* strongly enhanced the *hh*^{Mrt} heterozygous adult wing phenotype (compare Fig 2B and C). In *hh*^{Mrt} heterozygous wing discs, slight ectopic expression of *hh* messenger RNA, En and Araucan (Ara), another target of Hh (Gomez-Skarmeta & Modolell, 1996), was observed in anterior cells (compare Fig 2D,G with E,H). Loss of one copy of *mtv* in this *hh*^{Mrt} heterozygous background increased the number of cells ectopically expressing *hh* mRNA, En and Ara and the levels of expression (Fig 2F,I). The ectopic expression of En (arrow in Fig 2I), a target of Hh required to specify the fate of wing margin bristles at the anterior–posterior boundary (Hidalgo, 1994), is consistent with the observation that bristles along the anterior wing margin (triple row bristles, TR) were transformed into more posteriorly located sensory organs (double row bristles, DR; Fig 2C). The enlargement of the expression domain of Ara (arrowheads in Fig 2I), a target of Hh that defines the position of the third longitudinal vein (L3; Fig 2A; Gomez-Skarmeta & Modolell, 1996), explains the ectopic L3 observed in *mtv*^{6} *hh*^{Mrt} double heterozygous wings (arrow in Fig 2C). Delta protein is expressed along the presumptive longitudinal veins L3 and L4 in the wing primordium (Fig 2J). In *mtv*^{6} *hh*^{Mrt} double heterozygous wing discs, Delta is ectopically expressed in the anterior compartment (Fig 2K), presumably in those cells that will give rise to the ectopic vein L3 observed in the resulting adult wings. Note the relatively normal levels of Delta protein expression in the posterior compartment of these discs.

hh is a target of En in posterior cells; therefore, ectopic expression of *hh* might be a consequence of derepression of En in *mtv* mutant clones. However, clones lacking *mtv* and *en* activities still showed the ability to cause ectopic expression of *hh* and its target gene *ptc* (Fig 1G,H) in the anterior compartment. Anterior clones lacking *en* activity alone did not have any effect on the expression of *hh* (supplementary Fig 1 online). Together, these results indicate that Mtv is required to repress *hh* expression in anterior cells, independently of En.

Gro activity has previously been shown to be required for the repression of *hh* transcription in anterior cells (de Celis & Ruiz-Gomez, 1995; Apidianakis *et al*, 2001). Gro is ubiquitously expressed in the wing disc, but it is required only at the

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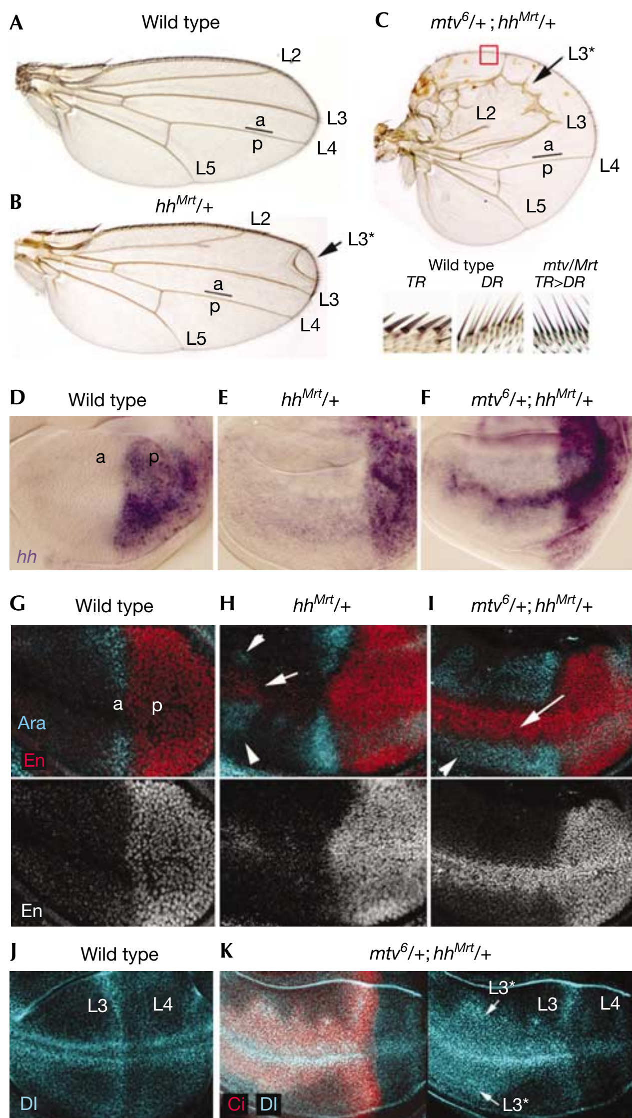

Fig 2 | Genetic interactions between *mtv* and the *hh* gain-of-function allele *Moonrat.* (**A–C**) Cuticle preparations of wild type (**A**), $hh^{Mrt}/+$ (**B**) and $mtv^{6}/+$; $hh^{Mrt}/+$ (**C**) adult wings. The adult wing is decorated by four longitudinal veins (L2–L5) and sensory organs along the margin. Note in (**B**) and (**C**) the ectopic vein L3 (L3*, marked by an arrow). In (**C**) the anterior compartment is enlarged. As shown in the magnification of the anterior wing margin (red box) at the bottom of the panel, the sensory organs located in the most anterior wing margin (Triple Row Bristles, TR) are transformed into more posteriorly located bristles (Double Row Bristles, DR). Anterior (a) and posterior (p) compartments are indicated. (**D–K**) Wild-type (**D,G,J**), $hh^{Mrt}/+$ (**E,H**) and $mtv^{6}/+$; $hh^{Mrt}/+$ (**F,I,K**) wing discs labelled to visualize expression of *hh* messenger RNA (purple; **D–F**), Araucan (Ara, blue; **G–I**), Engrailed (red in the top panels, white in the bottom panels; **G–I**), Delta (Dl, blue; **J,K**) and Ci (red; **K**). Note ectopic expression of Dl in the anterior compartment, presumably marking the presumptive ectopic vein L3 (L3*, marked by an arrow) visualized in adult flies (**C**). Hh, Hedgehog; Mtv, master of thickveins.

anterior–posterior boundary. This topological requirement seems to be similar to that of Mtv (compare Fig 1B–D with Fig 4F; note that clones were marked in this case by the presence of two copies of the GFP marker), thus indicating that Gro and Mtv might work together to repress *hh*. Interestingly, *gro* and *mtv* genetically interact *in vivo*. Transheterozygous gro^{1}/gro^{E48} adult wings showed no overt phenotype (Fig 3A), and no apparent ectopic expression of *hh* mRNA and Hh target genes Ara and En was observed in the corresponding wing imaginal discs (Fig 3B,C). Loss of one copy of *mtv* in a gro^{1}/gro^{E48} mutant background induced an enlarged anterior compartment in the adult wing (Fig 3D) and ectopic expression of *hh* mRNA, En and Ara in anterior cells (Fig 3E,F). The ectopic expression of En and Ara explains the transformation of anterior wing margin bristles into more posteriorly located sensory organs and the appearance of the ectopic L3. Genetic interactions between *gro* and *mtv* mutant alleles, and the similar clonal phenotype suggest that Mtv and Gro might function in the same complex to repress *hh* expression in anterior cells. This hypothesis was tested using a co-immuno-precipitation assay in S2 cells. Myc-epitope-tagged Mtv was immunoprecipitated with Gro protein and Gro antibody in these cells (Fig 3G). To determine whether the interaction between Gro and Mtv was direct, we carried out a two-hybrid analysis in yeast and a pull-down assay of *in vitro*-translated Gro and glutathione-*S*-transferase (GST)-epitope-tagged Mtv (supplementary Fig 2 online). In the yeast assay, Gro did not recognize Mtv, as shown by the lack of expression of the *lacZ* reporter gene. In the pull-down assay, Mtv was expressed in bacteria as a GST fusion, immobilized on glutathione-Sepharose beads and then tested for their ability to retain radiolabelled Gro protein. GST–Mtv showed little or no binding (supplementary Fig 2 online). Taken together, we can conclude that Gro and Mtv take part in the same protein complex but that they do not bind directly, and we suggest that this complex is important for the repression of *hh* transcription in anterior cells.

Anterior clones lacking *mtv* or *gro* activities induced *hh* expression only when located close to or abutting the posterior compartment (Figs 1B–D,4F; Apidianakis *et al*, 2001). This requirement indicates that ectopic *hh* expression might depend on the endogenous source of Hh coming from posterior cells. To test this hypothesis, we ubiquitously expressed a dominant-negative version of the transmembrane protein Smoothened (Smo-5A)—an essential component of the Hh signalling pathway. In this background, the activity of the Hh pathway was compromised, as shown by the reduction in Ptc protein levels (supplementary Fig 1 online), and no *hh* expression was observed in *mtv* clones (Fig 4A). High levels of Ptc are known to repress the activity of Smo but without affecting the expression of posterior *hh* (supplementary Fig 1 online). Clones lacking *mtv* and over-expressing Ptc did not cause ectopic expression of *hh* either (Fig 4B). Conversely, clones mutant for *mtv* and constitutively activating the Hh pathway—that is, mutant for *ptc*—induced *hh* expression at any distance from the anterior–posterior boundary (Fig 4C). Clones mutant for *ptc* alone did not have any effect on *hh* expression (supplementary Fig 1 online). Together, these results indicate that, in the absence of Mtv or Gro activities, anterior *hh* expression seems to be induced by the Hh signalling pathway.

High levels of Hh signalling leads to the generation of the activator form of Ci (Ci^{act}) and, consequently, to reduced levels of

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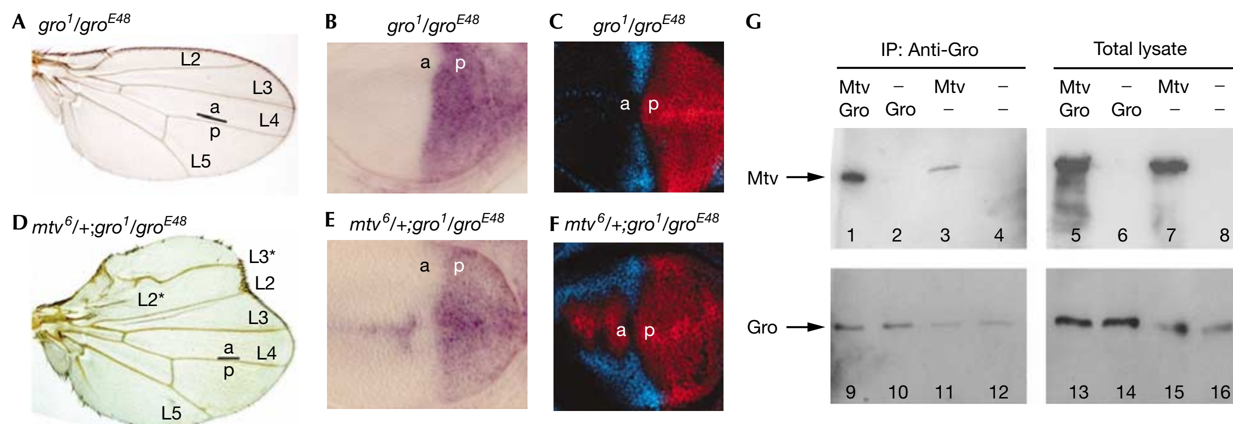

Fig 3 | Gro and Mtv interact *in vivo* and *in vitro*. (**A,D**) Cuticle preparations of gro^1/gro^{E48} (**A**) and $mtv^6/+;gro^1/gro^{E48}$ (**D**) adult wings. Note in (**D**) ectopic veins L2 and L3 (L2*, L3*), and the enlargement of the anterior compartment; compartments a and p are indicated. (**B,C,E,F**) gro^1/gro^{E48} (**B,C**) and $mtv^6/+;gro^1/gro^{E48}$ (**E,F**) wing discs labelled to visualize expression of *hh* messenger RNA (purple; **B,E**), Araucan (Ara, blue; **C,F**) and Engrailed (red; **C,F**). (**G**) Co-immunoprecipitation (IP) of Myc-tagged Mtv (Mtv–Myc) by binding to Gro. S2 cells were co-transfected to express Gro and Mtv–Myc or transfected to express Gro or Mtv–Myc alone; empty vector was co-transfected as control in the latter cases. Right panels show 1/30 of Gro and Mtv–Myc proteins in cell lysates; cell lysates were immunoprecipitated with Gro antibody (left). Mtv–Myc was co-immunopreciptitated in significant amounts in cells co-transfected with Gro and Mtv–Myc (lane 1). Mtv–Myc was also co-immunopreciptitated in lesser amount in cells transfected with Mtv–Myc alone (lane 3) as a result of the endogenous Gro present in S2 cells (lanes 11, 12, 15 and 16). In the absence of Gro antibody, Mtv–Myc was not co-immunopreciptitated (data not shown). a, anterior; Gro, Groucho; Hh, hedgehog; Mtv, master of thickveins; p, posterior.

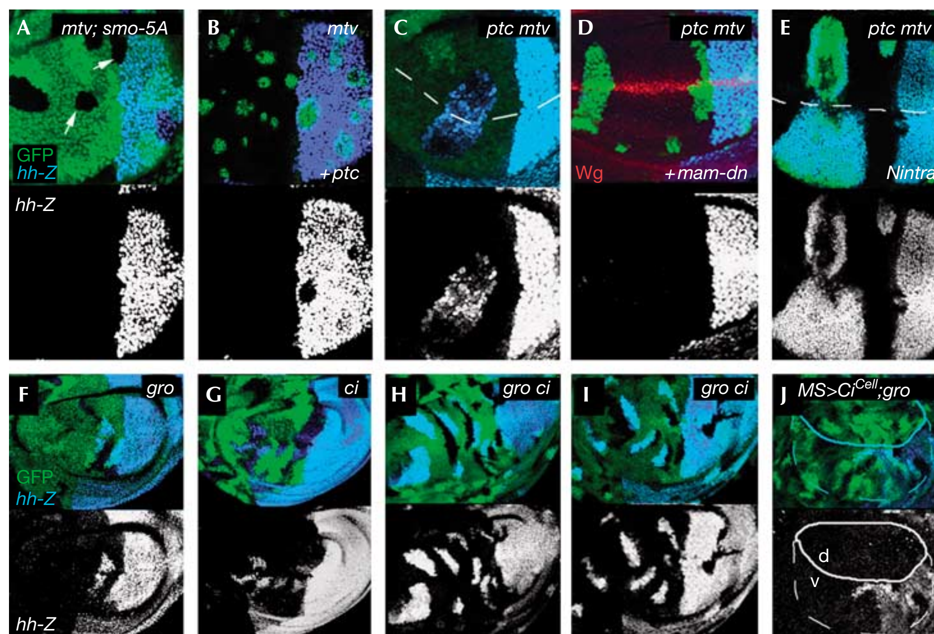

Fig 4 | Anterior *hh* expression depends on the endogenous activity of the Hh and Notch signalling pathways. Clones of cells mutant for *mtv* (mtv^6; **A,B**), *ptc* and *mtv* ($ptc^{S2}\,mtv^6$; **C–E**), *gro* (*Df(3R)Espl22*; **F,J**), *ci* (ci^{94}; **G**) or *gro* and *ci* (*Df(3R)Espl22*, ci^{94}; **H,I**) labelled by the absence (**A,C,G**), expression (**B,D,E**) or presence of two copies of the GFP marker (green; **F,H–J**). Expression of *hh-lacZ* (light blue, top; white, bottom) was monitored. The endogenous dorsal–ventral boundary is labelled by a dashed line or by Wingless (Wg, red; **D**) expression. The Hh signalling pathway was blocked by overexpression of a dominant-negative version of Smoothened in all wing cells (Smo-5A) in (**A**). Using the MARCM technique, the activity of the Hh or Notch signalling pathways was blocked or activated by means of full-length Ptc (**B**), a dominant-negative form of Mastermind (mam-dn; **D**) or by an activated form of the Notch receptor (Nintra; **E**). In (**J**), Ci^{Cell} was expressed under the control of $MS1096^{Gal4}$ control. The $MS1096^{Gal4}$ domain of expression is depicted by a white line. Gal4 expression levels are higher in the dorsal (d) wing pouch. Ci, Cubitus interruptus; GFP, green fluorescent protein; Gro, Groucho; Hh, Hedgehog; MARCM, mosaic analysis with a repressible cell marker; Mtv, Master of thickveins.

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its repressor form Ci^{rep} (Aza-Blanc *et al*, 1997; Ohlmeyer & Kalderon, 1998). Next, we investigated whether anterior *hh* expression in *mtv* or *gro* mutant clones was induced by the activity of Ci^{act} or, alternatively, by the absence of Ci^{rep}. Anterior *hh* expression is independent of Ci^{act}, as clones lacking *gro* and *ci* activities still showed the ability to cause ectopic expression of *hh* at the anterior–posterior boundary (Fig 4H,I), and the levels of *hh* expression at the anterior–posterior boundary were much higher than in *ci* single-mutant clones (Fig 4G). Therefore we conclude, that in the absence of *mtv* or *gro* activities, anterior *hh* is expressed only at the anterior–posterior boundary owing to the absence or reduced levels of Ci^{rep}. Consistently, when a truncated form of Ci that behaves as a repressor form (Ci^{Cell}; Méthot & Basler, 1999) was expressed in the dorsal compartment of the wing pouch, *hh* was not ectopically expressed in *gro* mutant clones (Fig 4J; note that Ci^{Cell} also repressed *hh* expression in posterior cells of the dorsal compartment). We observed that Ci^{Cell} was able to repress *hh* in the absence of Gro activity, indicating that Ci^{rep}-dependent repression of *hh* does not require Gro activity.

Anterior *hh* expression in the absence of Mtv and Gro activities is restricted not only to the anterior–posterior boundary, but also to the region close to or abutting the dorsal–ventral compartment boundary. The Notch signalling pathway is activated at the dorsal–ventral compartment boundary (Diaz-Benjumea & Cohen, 1995; de Celis *et al*, 1996), where it induces the expression of the long-range morphogen Wingless (Wg). We then investigated whether anterior *hh* expression depends on the activity of the Notch or Wg signalling pathways. For this purpose, we analysed the effects on *hh* expression after blocking or activating these pathways. We analysed these effects in clones mutant for both *mtv* and *ptc* because in these clones *hh* expression is induced at any distance from the anterior–posterior boundary. When the Notch signalling pathway was blocked in *ptc mtv* double-mutant clones by means of expression of a dominant-negative form of the Notch nuclear effector Mastermind (Mam-DN), no *hh* expression was observed in *ptc mtv* mutant clones (Fig 4D). When the Notch signalling pathway was constitutively activated by means of a dominant active form of Notch (Struhl & Adachi, 1998), ectopic expression of *hh* was observed at any distance from the dorsal–ventral boundary (Fig 4E). Expression of Mam-DN or Nintra alone did not have any effect on *hh* expression (supplementary Fig 1 online). Ectopic expression of Wg in *ptc mtv* double-mutant clones did not cause any effect in their ability to induce anterior *hh* expression (data not shown). These results indicate that, in the absence of *mtv* activity, the Notch and Hh signalling pathways act together to induce *hh* transcription in anterior cells.

Asymmetrical signalling by Hh from posterior to anterior cells is required for stable compartment subdivision and maintenance of a Dpp-dependent organizer in the centre of the wing primordium. This asymmetry is a consequence of having Hh-expressing and Hh-responding cells in different non-overlapping cell populations corresponding to posterior and anterior compartments, respectively. Two different and independent mechanisms are used in the *Drosophila* wing to repress *hh* expression in anterior cells (Fig 1I,J). The first acts mainly in those cells not receiving the Hh signal and is based on the repressor form of Ci (Ci^{rep}). We presented evidence that this repression does not require Gro activity. The second acts in those cells receiving the Hh signal and is based on Mtv binding Gro to mediate transcriptional repression of *hh*. We present evidence that anterior *hh* expression in the absence of Gro activity does not require Ci. Interestingly, Mtv and Gro seem to be inhibiting a new role of Notch at the dorsal–ventral boundary in driving *hh* expression in anterior cells. It is interesting to note that in this context the *hh* gain-of-function allele *Moonrat* (hh^{Mrt}) leads to derepression of *hh* in anterior cells located at the dorsal–ventral boundary (Fig 2). We speculate that Mtv and Gro might act on a *cis*-regulatory region of the *hh* gene affected in the *Moonrat* allele. Ci^{rep} seems to repress *hh* expression independently of Notch (Fig 4; see also Méthot & Basler, 1999), indicating that Ci^{rep} might act on a different *cis*-regulatory region than Mtv and Gro.

Finally, we would like to point out that many signalling molecules have been shown to restrict their own expression such as Wg or Notch (Rulifson *et al*, 1996; Herranz *et al*, 2006) or activity domains such as Hh (Chen & Struhl, 1996). This report shows that Hh is also involved in restricting its expression domain. Hh induces the expression of *mtv* in nearby anterior cells and Mtv, together with Gro, represses *hh* expression in anterior cells (Fig 1J).

METHODS

***Drosophila* strains.** mtv^6, a protein null allele of *mtv*, and mtv^{k00702} (*mtv-lacZ* in the text; Funakoshi *et al*, 2001); hh^{Mrt} (Felsenfeld & Kennison, 1995); *Df(2R)en[E]* deletes both *en* and *invected* (Gustavson *et al*, 1996); *c765-Gal4* and *UAS-Smo-5A* (Collins & Cohen, 2005); UAS-N^{intra} (Struhl & Adachi, 1998); UAS-mam-DN (Helms *et al*, 1999); ptc^{s2}, hh^{P30} (*hh-lacZ* in the text), *ci-lacZ*, *Df(3R)Espl22*, gro^1 and gro^{E48} (Flybase); ci^{94} and *UAS-*Ci^{Cell} (Méthot & Basler, 1999); *ap-Gal4* and *MS1096-Gal4* (Milan *et al*, 1998).

Antibodies. Rabbit anti-Ara (Gomez-Skarmeta & Modolell, 1996), rabbit anti-Hh (Capdevila & Guerrero, 1994), rat anti-Ci (Motzny & Holmgren, 1995), mouse anti-Mtv (Senti *et al*, 2000), mouse anti-Gro (Apidianakis *et al*, 2001); rabbit anti-Myc (Santa Cruz Biotechnology Inc., Antibodies, Santa Cruz, CA, USA) and rabbit anti-β-Gal (Cappel). Mouse anti-En (4D9), mouse anti-Wg (4D4), mouse anti-Ptc (Apa 1), mouse anti-Dl (C594.9B) and mouse anti-β-Gal (40-1A) are described in the Developmental Studies Hybridoma Bank (University of Iowa, Iowa, USA). *In situ* hybridization was carried out as described by Milan *et al* (1996).

Co-immunoprecipitation of Gro and Mtv. S2 cells were transfected two times each with 1 μg of pMT-Gal4-VP16 and UAS-Myc-Mtv and UAS-Gro or empty vector using 5 μl of CellFectin (Invitrogen, Carlsbad, CA, USA) per well. Cells were recovered for 6 h after transfection, induced for 2 days with 0.7 mM $CuSO_4$ and lysed in 500 μl of 5 mM Tris, 150 mM NaCl and 1% Triton X-100 (pH 8). A portion (1/30th) was analysed on SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Medium fractions (450 ml) were each immunoprecipitated with Gro antibody overnight at 4 °C. A 50 μl portion of protein G slurry was added for 30 min at 4 °C. The beads were washed three times with PBS and 0.1% Triton X-100, and then boiled in 50 μl of 2 × SDS–PAGE loading buffer and one-third was loaded on SDS–PAGE. All buffers were supplemented with protease inhibitors (Boehringer Ingelheim Gmbh, Ingelheim, Germany). Blots were probed with mouse anti-Myc and mouse anti-Gro.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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REFERENCES

Apidianakis Y, Grbavec D, Stifani S, Delidakis C (2001) Groucho mediates a Ci-independent mechanism of hedgehog repression in the anterior wing pouch. *Development* **128:** 4361–4370
Aza-Blanc P, Ramírez-Weber F-A, Laget M-P, Schwartz C, Kornberg TB (1997) Proteolysis that is inhibited by Hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell* **89:** 1043–1053
Basler K, Struhl G (1994) Compartment boundaries and the control of *Drosophila* limb pattern by *hedgehog* protein. *Nature* **368:** 208–214
Capdevila J, Guerrero I (1994) Targeted expression of the signalling molecule decapentaplegic induces pattern duplications and growth alterations in *Drosophila* wings. *EMBO J* **13:** 4459–4468
Chen Y, Struhl G (1996) Dual roles for patched in sequestering and transducing Hedgehog. *Cell* **87:** 553–563
Collins RT, Cohen SM (2005) A genetic screen in *Drosophila* for identifying novel components of the hedgehog signaling pathway. *Genetics* **170:** 173–184
Crick FH, Lawrence PA (1975) Compartments and polyclones in insect development. *Science* **189:** 340–347
de Celis JF, Ruiz-Gomez M (1995) *Groucho* ahd *hedgehog* regulate *engrailed* expression in the anterior compartment of the *Drosophila* wing. *Development* **121:** 3467–3476
de Celis JF, Garcia-Bellido A, Bray SJ (1996) Activation and function of *Notch* at the dorsal–ventral boundary of the wing imaginal disc. *Development* **122:** 359–369
Diaz-Benjumea FJ, Cohen SM (1995) Serrate signals through Notch to establish a wingless-dependent organizer at the dorsal–ventral compartment boundary of the *Drosophila* wing. *Development* **121:** 4215–4225
Felsenfeld AL, Kennison JA (1995) Positional signaling by hedgehog in *Drosophila* imaginal disc development. *Development* **121:** 1–10
Freeman M, Gurdon JB (2002) Regulatory principles of developmental signaling. *Annu Rev Cell Dev Biol* **18:** 515–539
Funakoshi Y, Minami M, Tabata T (2001) *mtv* shapes the activity gradient of the Dpp morphogen through regulation of thickveins. *Development* **128:** 67–74
García-Bellido A, Ripoll P, Morata G (1973) Developmental compartmentalisation of the wing disk of *Drosophila*. *Nat New Biol* **245:** 251–253
Gomez-Skarmeta JL, Modolell J (1996) araucan and caupolican provide a link between compartment subdivisions and patterning of sensory organs and veins in the *Drosophila* wing. *Genes Dev* **10:** 2935–2945
Gustavson E, Goldsborough AS, Ali Z, Kornberg TB (1996) The *Drosophila engrailed* and *invected* genes: partners in regulation, expression and function. *Genetics* **142:** 893–906
Helms W, Lee H, Ammerman M, Parks AL, Muskavitch MA, Yedvobnick B (1999) Engineered truncations in the *Drosophila* mastermind protein disrupt Notch pathway function. *Dev Biol* **215:** 358–374
Herranz H, Stamataki E, Feiguin F, Milan M (2006) Self-refinement of Notch activity through the transmembrane protein Crumbs: modulation of γ-Secretase activity. *EMBO Rep* **7:** 297–302
Hidalgo A (1994) Three distinct roles for the *engrailed* gene in *Drosophila* wing development. *Curr Biol* **4:** 1087–1098
Méthot N, Basler K (1999) Hedgehog controls limb development by regulating the activities of distinct transcriptional activator and repressor forms of Cubitus interruptus. *Cell* **96:** 819–831
Milan M, Campuzano S, Garcia-Bellído A (1996) Cell-cycling and patterned cell proliferation in the wing primordium of *Drosophila*. *Proc Natl Acad Sci USA* **93:** 640–645
Milan M, Diaz-Benjumea FJ, Cohen SM (1998) Beadex encodes an LMO protein that regulates Apterous LIM-homeodomain activity in *Drosophila* wing development: a model for LMO oncogene function. *Genes Dev* **12:** 2912–2920
Motzny CK, Holmgren RA (1995) The *Drosophila* cubitus interruptus protein and its role in *wingless* and *hedgehog* signal transduction pathways. *Mech Dev* **52:** 137–150
Ohlmeyer JT, Kalderon D (1998) Hedgehog stimulates maturation of Cubitus interruptus into a labile transcriptional activator. *Nature* **396:** 749–753
Rulifson EJ, Micchelli CA, Axelrod JD, Perrimon N, Blair SS (1996) *Wingless* refines its own expression domain on the *Drosophila* wing margin. *Nature* **384:** 72–74
Senti K, Keleman K, Eisenhaber F, Dickson BJ (2000) Brakeless is required for lamina targeting of R1–R6 axons in the *Drosophila* visual system. *Development* **127:** 2291–2301
Struhl G, Adachi A (1998) Nuclear access and action of Notch *in vivo*. *Cell* **93:** 649–660
Tabata T, Kornberg T (1994) Hedgehog is a signalling protein with a key role in patterning *Drosophila* imaginal discs. *Cell* **76:** 89–102
Tabata T, Schwartz C, Gustavson E, Ali Z, Kornberg TB (1995) Creating a *Drosophila* wing *de novo*: the role of *engrailed* and the compartment border hypothesis. *Development* **121:** 3359–3369

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