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Groucho mediates a Ci-independent mechanism of *hedgehog* repression in the anterior wing pouch

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SUMMARY

Groucho (Gro) is the founding member of a family of transcriptional co-repressors that are recruited by a number of different transcription factors. *Drosophila* has a single *gro* gene, whose loss of function affects processes ranging from sex determination to embryonic patterning and neuroblast specification. We have characterized a function of Gro in imaginal development, namely the repression of *hedgehog* (*hh*) in anterior wing pouch cells. *hh* encodes a secreted morphogen with potent patterning activities. In *Drosophila* thoracic appendages (legs, wings, halteres), *hh* is expressed in posterior compartments and induces the anteroposterior (AP) pattern organizer in the cells across the AP boundary. *hh* is repressed in anterior compartments at least partly via Ci[rep], a form of the multifunctional transcription factor Cubitus interruptus (Ci). We show that cells in the wing primordium close to the AP boundary need *gro* activity to maintain repression of *hh* transcription, whereas in more anterior cells *gro* is dispensable. This repressive function of Gro does not appear to be mediated by Ci[rep]. Analysis of mutant *gro* transgenes has revealed that the Q and WD40 domains are both necessary for *hh* repression. Yet, deletion of the WD40 repeats does not always abolish Gro activity. Our findings provide new insights both into the mechanisms of AP patterning of the wing and into the function of Gro.

Key words: *groucho*, *hedgehog*, *cubitus interruptus*, *Drosophila*, Wing patterning, Repression, Co-repressor

INTRODUCTION

The secreted proteins of the Hedgehog (Hh) family play a central role in the patterning of many structures in insects and vertebrates (Hammerschmidt et al., 1997). In *Drosophila*, Hh is used for, among other things, the induction of the anteroposterior (AP) boundary organizer of wing, haltere and leg imaginal disks, thus linking the processes of compartmentalization established by the selector protein Engrailed (En) with growth and patterning orchestrated by morphogens emanating from the AP organizer (Lawrence et al., 1996; Neumann and Cohen, 1997). Thoracic imaginal disk primordia arise in the embryo at positions spanning parasegment boundaries. As a consequence they consist of two groups of founder cells that either do (posterior, P) or do not (anterior, A) express the *en* gene. The two states of *en* expression are inherited by the progeny of the founder cells via chromatin-mediated mechanisms, thus dividing the imaginal disk in two compartments of distinct lineage. A sharp boundary exists where the two compartments contact each other and forbids cell mixing between A and P. *hh* is expressed in all posterior compartment cells and signals to A cells across the AP boundary. This signalling has a quadruple role: (1) it maintains the straightness of the boundary, presumably by regulating selective cell adhesion (Rodriguez and Basler, 1997; Blair and Ralston, 1997; Dahmann and Basler, 1999); (2) it induces the expression of *decapentaplegic* (*dpp*) or *wingless* (*wg*) (depending on the particular disk) in the A cells closest to the AP boundary (Tabata and Kornberg, 1994; Basler and Struhl, 1994) – Dpp and Wg act as long range morphogens that pattern both A and P compartments of the disks (Neumann and Cohen, 1997); (3) in the wing at least, it is directly responsible for a part of the AP pattern, that of a stripe of A cells near the AP boundary, the same cells that express *dpp* (Strigini and Cohen, 1997; Vervoort et al., 1999). Hh achieves all these effects by diffusing away from the P compartment and forming a sharp concentration gradient to which A cells can respond in a graded fashion. Indeed the fourth role of Hh is to shape its own concentration gradient by inducing the expression of its receptor, Patched (Ptc), which sequesters extracellular Hh, thus limiting its diffusion (Chen and Struhl, 1996).

The ability of A cells to respond to Hh depends on the exclusive A expression of the gene *cubitus interruptus* (*ci*) (Aza-Blanc and Kornberg, 1999). *ci* is uniformly expressed in the A compartment and encodes two forms of a Zn-finger transcription factor of the Gli family. The full-length Ci[act] form is needed to turn on the transcription of *dpp*, *ptc* and other transcriptional targets of Hh. The alternative form of Ci is generated by regulated proteolysis: most cells of the A

compartment (those that are away from the source of Hh) process the *ci* product into an N-terminal 75 kDa fragment, which retains its DNA-binding activity, but lacks a transcriptional activation domain and acts instead as a repressor (Aza-Blanc et al., 1997). Anterior cells that receive the Hh signal respond by blocking this proteolysis and activating the full-length 155 kDa Ci post-translationally, e.g. by stimulating its nuclear import (Chen et al., 1999; Méthot and Basler, 2000). One of the outcomes of this complex regulation of Ci is that in the wing disk the Hh target gene *dpp* is actively repressed by Ci[rep] in A cells away from the AP boundary (Ci[rep] high, Ci[act] absent), but is strongly activated in cells close to the boundary (Ci[rep] low or absent, Ci[act] high) (Méthot and Basler, 1999). Posterior cells do not respond to Hh because they lack *ci* expression. This is achieved via the repression of *ci* by En (Schwartz et al., 1995).

Hh and its vertebrate homologues exert such a potent influence on nearby cells that their mis-expression produces gross patterning abnormalities, often leading to lethality. It is therefore of utmost importance to control rigorously where and when *hh* genes will be expressed. Several regulatory inputs are known in the control of vertebrate *hh* genes (Epstein et al., 2000); however, relatively little is known about *hh* transcriptional regulation in *Drosophila* appendages. One level of control of *hh* expression is mediated by Ci: *hh* is only expressed in P cells because Ci[rep] represses it in A cells (Méthot and Basler, 1999). Although this appears to be sufficient for repressing *hh* in the Hh non-responsive cells of the A compartment, it raises the question of how *hh* expression is kept off in the Hh responsive cells, which should lack Ci[rep]. As ci^{-} mosaic clones cause an equally mild derepression of *hh* in all cells of the A compartment (irrespective of proximity to the AP boundary) (Méthot and Basler, 1999; Dahmann and Basler, 2000), it has been proposed that low levels of Ci[rep] are present in the Hh responsive cells and these are sufficient to keep *hh* transcription off.

Gro is a widely used co-repressor and null alleles are embryonic lethal. However, some viable hypomorphic combinations were shown to produce an expansion of the wing's AP pattern, as a result of ectopic *hh* and *en* expression at the anterior dorsoventral (DV) boundary (de Celis and Ruiz-Gomez, 1995). Mosaic clones of stronger (lethal) *gro* alleles displayed similar adult phenotypes. It was not clear from this study whether the primary defect in these genotypes was derepression of *en*, which can cause ectopic *hh* expression (Tabata et al., 1995), or derepression of *hh*, which in turn can cause ectopic *en* expression (Blair and Ralston, 1997; Strigini and Cohen, 1997). To address these points, we examined the expression of *hh* and various target genes in the background of *gro* loss-of-function clones in the wing disk. We further asked whether Ci might play a role as a DNA tether for Gro in this setting. Our results show that *hh*, rather than *en*, is the primary target of Gro mediated repression. Gro is necessary to keep *hh* off in a subdomain of the A compartment comprising the cells near both AP and DV boundaries, but this repression seems to be independent of Ci[rep]. Analysis of mutated *gro* transgenes in this setting has revealed that both Q and WD40 domains of the protein are necessary for *hh* repression.

MATERIALS AND METHODS

Fly strains

Mutant alleles

gro^{E48}, *Df(3R)Espl22* (*gro* null allele) and $ci^{Ce\text{-}2}$ are all described in FlyBase (http://flybase.bio.indiana.edu). hh^{E23} is described in Basler and Struhl (Basler and Struhl, 1994).

lacZ reporters

ptc-lacZ was a gift from Steve Cohen (EMBL). *dpp-lacZ* stands for *P[BS3.0]H1-1*; *en-lacZ* stands for $P[en\text{-}lacZ(Xho)]en^{Xho25}$; *hh-lacZ* for $P[PZ]hh^{P30}$ and *ci-lacZ* for $P[lacW]ci^{Dplac}$, all described in FlyBase.

Gal4 lines

omb-Gal4 stands for $P[GAL4]bi^{omb\text{-}Gal4}$ (FlyBase). *UAS-gro* and mutant versions were generated by us using P-element transformation in a yw^{67c23} background.

Plasmid construction

The last 315 amino acids of Groucho (GroΔWD40) were deleted by digesting the *gro* cDNA with *Acc*I, followed by intramolecular ligation (this deletes the WD40 domain and adds a tail of 21 novel amino acids in place of the last 5 residues of Gro). Residues 116-438 (GroΔGCS mutant) were deleted by digestion with *Bst*EII, followed by in-frame intramolecular ligation. This deletes a small part of the Q domain, the entire GP, CcN and SP domains, and the first 40 amino acids of the WD40 domain. Amino acids 17-153 (GroΔQ mutant) were deleted by digestion with *Aoc*I and *Nco*I, followed by filling-in with Klenow DNA polymerase and in-frame ligation.

Point mutations were generated using two rounds of PCR amplification (information on conditions and primers used is available upon request). In GroNLS^{-} two Lys to Glu mutations were introduced in the putative nuclear localization sequence (amino acids 216 and 219). In Grocdc2^{-}, Ser 247, a potential p34cdc2 phosphorylation site, was replaced by Leu. The presence of the introduced mutations was confirmed by DNA sequencing.

UAS-gro was made by ligating a *Bam*HI(5′)-XhoI(3′) (vector polylinker sites) fragment of a Bluescript full-length *gro* cDNA clone into *Bgl*II-*Xho*I sites of pUAST. All *UAS-gro* mutants were subcloned from sev-CaSpeR constructs, as *Cla*I(5′)-*Not*I(3′) (vector polylinker sites) fragments into *Eco*RI-*Not*I sites of pUAST.

Other plasmids were $pUAST\text{-}ci^{wt}$ Méthot et al. (Méthot et al., 1999), *pGEX-gro* (Dubnicoff et al., 1997), *pJG4-5-ci* (Monnier et al., 1998), *pEG202-gro* (Alifragis et al., 1997) and *mt-GAL4* (Eastman et al., 1997).

Mosaic induction

Mitotic clones (Xu and Rubin, 1993) were induced using a *hsFLP* insert on the X, the *FRT82B* (on the base of 3R) and were marked by either *hs-πmyc*, *hs-GFP* or *Ubi-GFP* transgenes. Flies were raised at 25°C, unless otherwise noted. *hsFLP* was induced by heat-shocking 1st to 2nd instar larvae of the following genotypes for 1 hour at 38°C:

(a) y w hsFLP/w; dpp-lacZ/+; FRT82B gro^{E48}/FRT82B πmyc (or hs-GFP)

(b) y w hsFLP/w; ptc-lacZ/+; FRT82B gro^{E48}/FRT82B πmyc

(c) y w hsFLP/w; en-lacZ/+; FRT82B gro^{E48}/FRT82B πmyc (or hs-GFP)

(d) y w hsFLP/w; dpp-lacZ/+; FRT82B hh^{E23} gro^{E48}/FRT82B πmyc (or hs-GFP)

(e) y w hsFLP/w; ptc-lacZ/+; FRT82B hh^{E23} gro^{E48}/FRT82B πmyc

(f) y w hsFLP/w; en-lacZ/+; FRT82B hh^{E23} gro^{E48}/FRT82B πmyc

(g) y w hsFLP/w; FRT82B Ubi-GFP hh-lacZ Df(3R)Espl22/FRT82B πmyc

(h) y w hsFLP/y w; FRT82B Ubi-GFP hh-lacZ Df(3R)Espl22/FRT82B πmyc; ci^{Ce2}/+

(i) y w hsFLP/ w omb-Gal4; UAS-gro/+; FRT82B Ubi-GFP hh-lacZ Df(3R)Espl22/ FRT82B πmyc

(a) and (c) were repeated using *FRT82B Df(3R)Espl22*. (h) was raised at 29°C, where the effects of ci^{Ce2} are stronger; (g) was repeated at 29°C for comparison, and no differences were noted between the two temperatures. (i) was repeated using *UAS-groΔQ*, *UAS-groΔWD40* and *UAS-grocdc2*$^{-}$ transgenes.

Immunohistochemistry

For antibody staining, dissected larvae were fixed for 20 minutes at room temperature in PEM and 4% formaldehyde. Where necessary, 1-1.5 hour of heat shock at 38°C and 1-1.5 hour recovery was carried out before dissection, to induce the *hs-πmyc* or *hs-GFP* clone markers. Antibodies used were mouse anti-Gro (Delidakis et al., 1991) 1:5; rat pan-TLE (Stifani et al., 1992) 1:5; mouse anti-Myc (Developmental Studies Hybridoma Bank (developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City)) 1:3000; and rabbit anti-β-galactosidase (Cappel) 1:10000. Fluorescent secondary antibodies were from Jackson Immunochemicals or Molecular Probes. They were preadsorbed and used at a final dilution of 1:200 to 1:1000. Antibody incubations were done at 4°C for 4 hours to overnight.

For antibody staining of S2 cells, cells were spotted on depression slides coated with poly-D-lysine. After settling, they were fixed for 15 minutes in 2% formaldehyde; this and all subsequent incubations were done on the slide. Primary and secondary antibody dilutions were as above, but all incubations were performed at room temperature for 1 hour. DNA was counterstained using TOPROIII (Molecular Probes) at 1 μM for 5 minutes.

Fluorescent samples were observed using a Leica SP confocal microscope. Transmitted light images were obtained on a Leica Diaplan microscope.

Transfections

For all transfection assays a standard calcium phosphate method was used. UAS expression constructs were co-transfected with mt-GAL4 plasmid. 16 hours post-transfection, CuSO4 was added to a final concentration of 0.7 mM, in order to induce the mt-GAL4, which in turn induces the UAS constructs. For each transfection of 1 ml of S2 cells (0.5×10^6 cells/ml) 2 μg total plasmid DNA was used. Cells were collected and processed for immunochemistry 40-48 hours post-transfection.

RESULTS

gro is necessary to repress *hh* expression in specific sites of the anterior wing pouch

As *gro* hypomorphic backgrounds are known to expand the anteroposterior (AP) axis in the wing (de Celis and Ruiz-Gomez, 1995), we tested the expression of various AP patterning genes, visualized by *lacZ* reporters, in more severe *gro* loss-of-function backgrounds. To achieve this, we studied mitotic clones homozygous for gro^{E48}, a strong hypomorphic lethal, or *Df(3R)Espl22*, a null allele – the two behaved identically in our assays. *hh-lacZ* exhibited autonomous derepression in many anterior *gro*$^{-}$ clones (posterior clones had no effect) – derepression depended on the location of the clone; 14 disks were scored that bore a total of 51 large and numerous small clones. Clones abutting or close to the AP boundary showed the strongest derepression of *hh-lacZ*; expression levels were comparable with those in the posterior compartment (we operationally define these clones as type I; Fig. 1A). Clones arising a little further from the AP boundary fell into two categories: those that were close to the DV boundary expressed moderate to high levels of *hh-lacZ* (type II), while those far from it only weakly derepressed *hh-lacZ* (type III). Larger type II clones showed this effect more clearly with high levels close to the DV boundary, which gradually decreased to type III levels as one moves out away from that boundary (Fig. 1B). Finally, clones far from the AP boundary had no *hh-lacZ* expression (type IV). In general, larger clones displayed higher levels of *hh-lacZ* compared with small clones at similar positions in the wing pouch, suggesting some degree of positive autoregulation of *hh* expression. We conclude that Gro probably mediates repression of *hh* directly and, in fact, is necessary for preventing *hh* expression in anterior cells close to the AP and DV boundaries in the wing pouch. The requirement for Gro fades away in a graded manner in regions away from the AP and DV boundaries. *hh-lacZ* derepression was also variably observed in *gro*$^{-}$ clones at the wing hinge and notum, but these were not analysed systematically.

When *en-lacZ* was analysed in *gro*$^{-}$ backgrounds, derepression was much less frequently observed. Whereas *hh-lacZ* was expressed throughout the *gro*$^{-}$ territory (at varying levels, as described above), *en-lacZ* was expressed only patchily within the clone (Fig. 1D,E). It is therefore unlikely that *en* is a direct target of Gro, rather this sporadic derepression is probably a secondary effect of ectopic *hh* expression, as high levels of Hh can induce *en* expression anteriorly (Blair and Ralston, 1997; Strigini and Cohen, 1997). We confirmed this by generating mitotic clones mutant for both *gro* and *hh*: in no case did we observe ectopic expression of *en-lacZ* (not shown).

Other Hh targets behaved similarly. *dpp-lacZ* and *ptc-lacZ* were ectopically expressed in a subset of anterior *gro*$^{-}$ clones, most frequently those close to the DV boundary. Fig. 2A shows this for *dpp-lacZ*, where it is clear that derepression was non-autonomous, as surrounding wild-type cells also turned on *lacZ* expression. This suggests that *dpp* is not a direct target of Gro repression, it is rather turned on in response to ectopic Hh signalling from the clone. Consistent with this interpretation, *hh*$^{-}$ *gro*$^{-}$ clones did not derepress either *dpp-lacZ* (Fig. 2C) or *ptc-lacZ* (not shown).

The effect of clones abutting the AP boundary could not be assessed on *dpp-lacZ* and *ptc-lacZ*, as those are normally highly expressed there (unlike *hh-lacZ*). However, *dpp* expression retracts from the AP boundary in the late third instar larva, as a narrow stripe of anterior cells abutting the boundary express *en* at that time as a response to Hh. This anteriorly expressed En partially represses *dpp*. *gro*$^{-}$ clones within this anterior Engrailed domain showed autonomous derepression of *dpp-lacZ* (Fig. 2A,B). We assume this means that En needs Gro as a co-repressor in this region, as it does on other occasions (Jiménez et al., 1997). Indeed, derepression of *dpp-lacZ* in this region was independent of ectopic Hh signalling, as it took place in *hh*$^{-}$ *gro*$^{-}$ clones (Fig. 2C). In contrast to *dpp-lacZ*, we never observed derepression of *ci-lacZ* in posterior *gro*$^{-}$ clones (not shown), suggesting that En does not require Gro to repress *ci*.

Gro-mediated repression of *hh* is independent of Ci

The posteriorly restricted expression pattern of *hh* has been shown to depend on repression by Ci[rep] (Méthot and Basler, 1999). Ci[rep] is generated by the default proteolytic

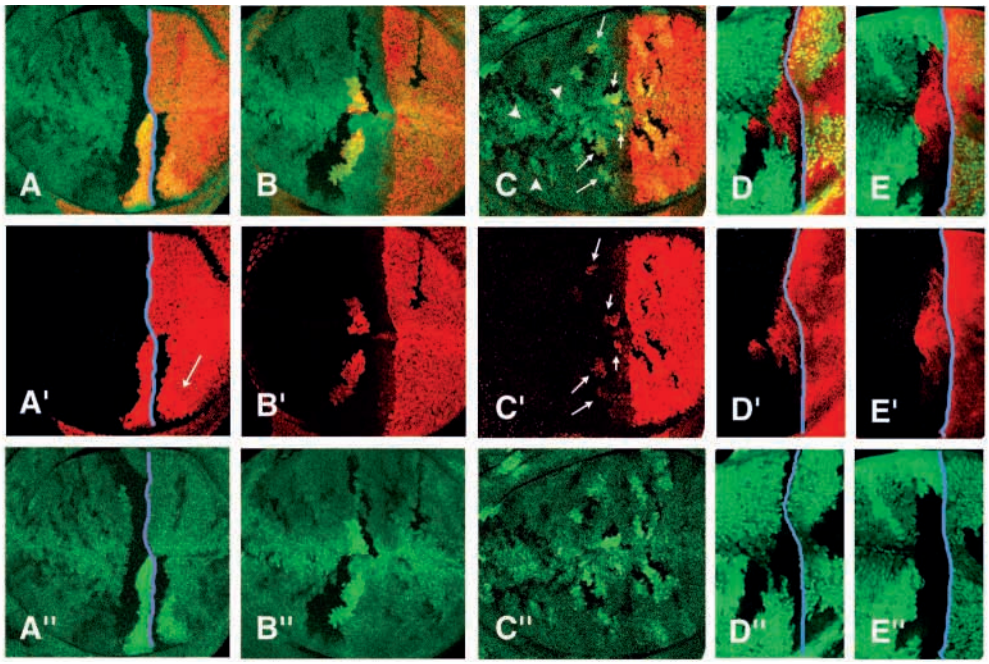

Fig. 1. Response of *hh-lacZ* (A-C) and *en-lacZ* (D-E) to *gro* loss of function in the wing. *Df(3R)Espl22* clones are shown, marked by increased GFP expression (bright green) in A-C and by absence of GFP expression (absence of green) in D-E. The $gro^{+/+}$ twin spots (clones arising from the sister cell bearing the reciprocal recombination product) lack GFP and *hh-lacZ* (A-C, see Materials and Methods) or have twice the level of GFP (D-E). Anti-β-galactosidase staining is shown in red. (A) A large anterior clone abutting the boundary (blue line) expresses *hh-lacZ* strongly (type I) – note comparable expression levels in a posterior clone, where *hh-lacZ* copy number is also 2 (arrow). (B) Large anterior clones further away from the AP boundary also express *hh-lacZ* strongly, but the intensity drops away from the DV boundary (type II/III). (C) Examples of small type II (short arrows) and III clones (long arrows) that express *hh-lacZ* only when located within an anterior domain close to the AP boundary. Several non-expressing clones (type IV-arrowheads) are seen in C, away from the AP boundary. (D,E) These large type I/II clones shown should express *hh* strongly throughout the clone (compare with A,B); however, *en-lacZ* derepression is more restricted. (A′-E′) Red channel only. (A″-E″) Green channel only. The blue line in A,D E marks the AP boundary. Anterior is towards the left and dorsal is upwards.

processing of the *ci* product in cells that do not receive Hh input (namely anterior cells away from the AP boundary). By contrast, anterior cells that receive Hh do not process the *ci* product to the repressor form, but rather convert it into the Ci[act] transcriptional activator (Aza-Blanc et al., 1997; Chen et al., 1999; Méthot and Basler, 1999). Therefore, it is not clear how *hh* transcription is blocked just anterior to the AP boundary, although it has been proposed that minute amounts of Ci[rep] present there may suffice for *hh* repression (Méthot and Basler, 1999). According to this model, the derepression of *hh-lacZ* by *gro* loss of function could be taken as an indication of Gro recruitment by Ci[rep]. If Gro were an obligate cofactor for Ci[rep], we would expect clones mutant for either to have an identical effect on Ci[rep] target genes. This is definitely not so: *dpp-lacZ* is consistently derepressed in ci^{-} clones, but it is only derepressed in a fraction of gro^{-} clones – in fact our data argue that Gro is not directly involved in *dpp* repression anterior to its normal stripe, in contrast to the well documented direct effect of Ci[rep] (Müller and Basler, 2000). Alternatively, Ci[rep] might require Gro co-operation only in some cases, e.g. in the regulation of *hh*. This is not true either: whereas *ci* loss of function clones derepress *hh-lacZ* in all anterior compartment cells (Méthot and Basler, 1999), we have shown that *gro* is only required in cells close to the AP/DV boundaries, suggesting that Ci[rep] does not need Gro to repress *hh* away from the boundaries. Further evidence against the co-operation between Ci and Gro is the quantitative aspect of *hh-lacZ* derepression: whereas ci^{-} clones express low levels of *hh-lacZ* regardless of their position in the anterior wing pouch (Méthot and Basler, 1999; Dahmann and Basler, 2000), gro^{-} clones can express anything from high to null levels of *hh-lacZ*, depending on the position and size of the

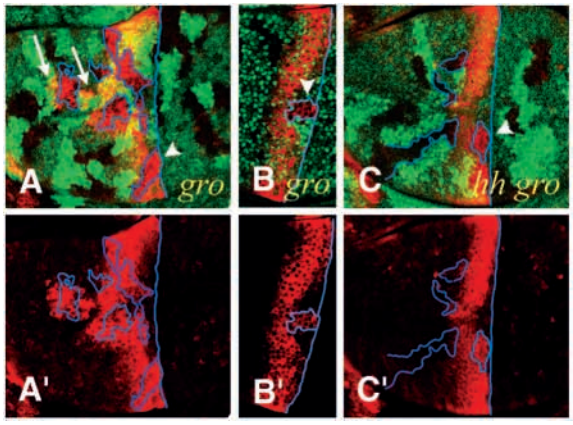

Fig. 2. Response of *dpp-lacZ* to *gro* loss of function in the wing. gro^{E48} clones are marked by loss of GFP (green A,C) or Myc epitope (green, B) and β-galactosidase is shown in red (red channel shown separately in A′-C′). A subset of gro^{-} clones in A (some clones are outlined in blue) express *dpp* ectopically. This effect is non-autonomous, as neighbouring wild-type cells are also induced to express *dpp* (arrows). Ectopic *dpp-lacZ* is never seen anterior to its normal stripe in hh^{E23} gro^{E48} clones (C), suggesting that its ectopic expression is mediated by *hh* activity. The few β-galactosidase-positive cells in the anterior clones in C are within the normal *dpp* expression stripe. *dpp-lacZ* is autonomously derepressed in anterior clones posterior to the normal expression stripe (B), where it would be normally downregulated by En. This can happen in gro^{-} (A,B) as well as in hh^{-} gro^{-} (C) clones – marked by arrowheads. All disks are oriented with anterior towards the left and dorsal upwards. The straight blue line indicates the position of the AP boundary.

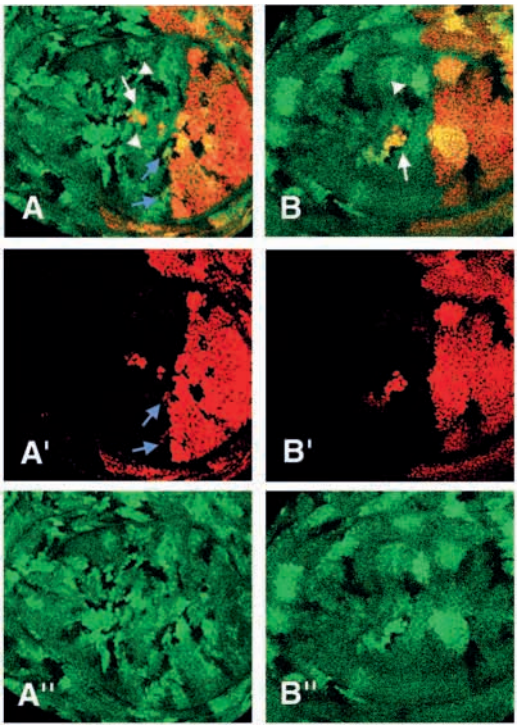

Fig. 3. Response of *hh-lacZ* to *gro* loss of function in a ci^{Ce2}/+ background. *Df(3R)Espl22* clones are marked by increased GFP, as in Fig. 1A-C. (A′,B′) Red channel only (*hh-lacZ*). (A″,B″) Green channel only (GFP). Only clones near the DV boundary show good *hh-lacZ* derepression (white arrows). Type I clones (blue arrows), which would normally express high levels of *hh-lacZ*, show weak or no expression, whereas type III clones (arrowheads) are fully repressed.

clones. We, therefore, favour an alternative explanation, namely that Ci[rep] and Gro act independently of each other.

In its simplest form, our hypothesis states that *hh* transcription is kept off by Ci[rep] in cells away from the AP boundary, whereas in cells close to the AP boundary it is kept off by a Gro-containing complex. To test this, we decided to study the response of *hh-lacZ* to loss of *gro* in a genetic background, where excess Ci[rep] activity was provided in cells close to the AP boundary. ci^{Ce2} is an allele that places a frameshift in the *ci*-coding region, causing premature termination and inability to produce full-length Ci[act] (Méthot and Basler, 1999). However, the truncated ci^{Ce2} product can provide Ci[rep] activity – as this is produced due to a premature stop codon and not via proteolysis, it is independent of Hh signalling input. Although ci^{Ce2} is homozygous lethal, it survives as a heterozygote over ci^{+} with part of the wing pattern missing, that between veins L3 and L4, precisely the region corresponding to the anterior compartment cells adjacent to the AP boundary, where Hh input is needed maximally and consequently Ci[rep] is normally kept at low amounts. If Ci and Gro act independently of each other to repress *hh*, it is conceivable that the increased levels of Ci[rep] in a ci^{Ce2}/+ background would be sufficient to repress *hh* and, thus, would diminish the importance of the Gro contribution in cells near the AP boundary. gro^{-} clones in a ci^{Ce2}/+ background confirmed this prediction: we indeed observed a diminished ability of cells close to the AP boundary

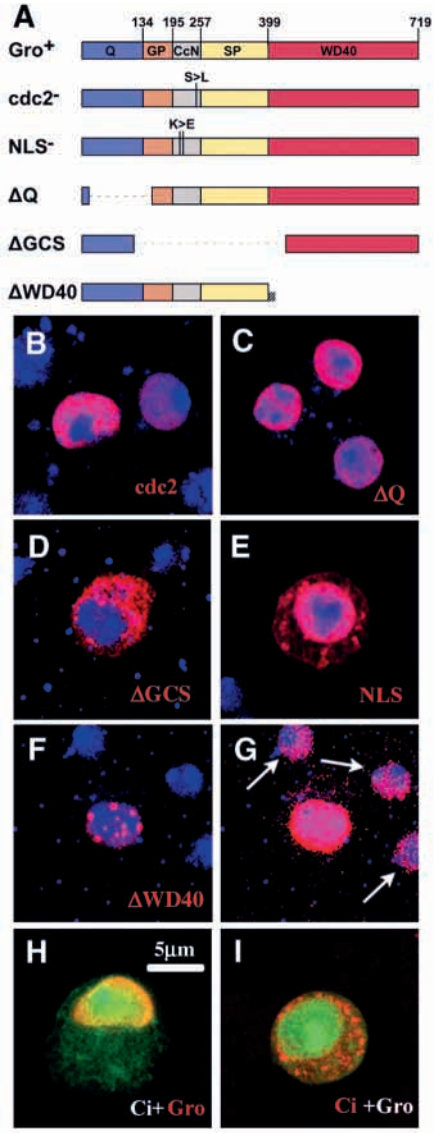

Fig. 4. (A) Schematic of the different mutant versions of *gro* transgenes. The hatched box in GroΔWD40 corresponds to 21 foreign amino acids added as a consequence of the construction strategy. (B-G) S2 cells transfected with different gro mutant expression vectors and stained with the anti-Gro (red – all panels except D) or pan-TLE monoclonal antibody (red – D) and TOPROIII (blue – overlap with Gro immunostaining seen as magenta) as a DNA counterstain – co-transfection with GFP was used to identify the transfected cells (not shown). (B) $Grocdc2^{-}$, (C) GroΔQ, (D) GroΔGCS, (E) $GroNLS^{-}$ and (F,G) GroΔWD40. Note that transfected Gro is overexpressed with respect to endogenous; G is the same panel as F, except that the detection sensitivity is increased for red and endogenous Gro immunoreactivity is detectable in the three untransfected cells (arrows). As exogenous Gro is in excess, the localization observed is not influenced by interaction with endogenous Gro. Wild-type Gro also accumulates in the nucleus: in H, $UAS\text{-}gro^{+}$ (red) was co-transfected with HA-tagged full length *ci* and counterstained with GFP (green), which localizes predominantly in the nucleus, but also in the cytoplasm. I shows another cell from the same transfection stained with an anti-HA antibody to detect Ci (red), which is exclusively cytoplasmic, thus providing no evidence for Ci-Gro interaction.

to derepress *hh-lacZ*. Even clones abutting the boundary (small type I clones) expressed barely detectable levels of *hh-lacZ* (Fig. 3A, blue arrows), whereas nearby clones that would be expected to weakly derepress *hh* (type III), showed no expression (Fig. 3A,B arrowheads). We conclude that in this genetic background, Gro-mediated repression of hh is less important, although not completely dispensable. An unexpected aspect of *gro*$^{-}$ clones in the *ci*^{Ce2}/+ background was the behaviour of type II clones (Fig. 3A,B, white arrows): unlike type I or type III clones, their *hh-lacZ* expression did not seem to be affected by *ci*^{Ce2} (i.e. they displayed moderately high levels of *hh-lacZ* (see Discussion)).

The mosaic analysis data presented above strongly argue against the participation of Ci[rep] and Gro in the same repression complex. Nonetheless, we addressed the possibility of interactions between Ci and Gro proteins using a number of different assays; the results were always negative. Bacterially produced GST-Gro protein was unable to pull down either Ci[75] (a C-terminally truncated form similar to Ci[rep]) or Ci[155] (full-length Ci) (not shown). Yeast two hybrid assays showed a very weak interaction of Gro with either form of Ci, which allowed slow growth in the absence of the selectable marker (leucine), but was insufficient to turn on a more stringent *lacZ*-based reporter (not shown). Finally, co-transfection of *gro* with *ci* was performed to detect potential co-localization, as full-length Ci accumulates in the cytoplasm, whereas Gro is nuclear. Co-expression of these two proteins did not alter their subcellular localization, thus providing no evidence for a potential interaction (Fig. 4H,I).

Rescue of *gro* loss of function by mutant *gro* transgenes

To gain further insight into the ability of Gro to repress anterior *hh* expression, we undertook an analysis of in vitro mutagenized Gro transgenes. We asked whether anterior cells containing Gro exclusively provided by the transgene were able to repress *hh-lacZ*. For this reason we generated null *gro* clones in a background of targeted *UAS-gro(mutant)* expression. Overexpression of *gro* often leads to lethality (not shown). For this experiment, we used *omb-Gal4*, which drives expression in a wide central wing pouch domain, encompassing the region of interest and allows survival to the pharate adult stage. We confirmed that these animals do not have any defects in *hh-lacZ* expression in the posterior wing compartment (not shown).

We generated six *UAS-gro* transgenes: the wild type and five mutants (Fig. 4A). Point mutations targeted a conserved putative p34cdc2 phosphorylation site (*grocdc2*$^{-}$) or the nuclear localization signal (*groNLS*$^{-}$). Deletions removed the following domains of the protein: the N-terminal Q domain (*groΔQ*), the central GP/CcN/SP domains (*groΔGCS*) and the C-terminal WD40 repeats (*groΔWD40*) – short regions of adjacent domains were also affected (see Materials and Methods). We initially used each construct in transient transfections of Schneider S2 cells to determine their subcellular localization (Fig. 4B-H). Full-length Gro as well as GroΔQ, Grocdc2^{-} and GroΔWD40 were all nuclear. All except GroΔWD40 showed the same subnuclear localization, accumulating predominantly at the nuclear periphery and avoiding the more strongly TOPROIII-stained, presumably heterochromatic, regions. GroΔWD40 showed a striking punctate pattern accumulating in particles located near the nuclear periphery. The same pattern was seen when this construct was expressed in imaginal disks (not shown). GroNLS^{-} was still predominantly nuclear, although there was substantial cytoplasmic staining as well. GroΔGCS was exclusively cytoplasmic, suggesting that regions within the GP/CcN/SP domains, in addition to the NLS, are important for nuclear localization. We decided to test the exclusively nuclear forms of Gro for their ability to repress *hh*.

When *UAS-gro* was expressed in wing disks in the background of *gro*$^{-}$ clones, *hh* repression was fully restored: no anterior *hh-lacZ* expression was obtained even in type I/II clones; the same was true for *UAS-grocdc2*$^{-}$ (Fig. 5A). By contrast, when *UAS-groΔQ* or *UAS-groΔWD40* was the sole source of Gro, there was strong anterior derepression of *hh-lacZ*, similar to control *gro*$^{-}$ clones (Fig. 5B-D). We confirmed that all transgenes were expressed at roughly the same levels by anti-Gro antibody staining (Fig. 5, blue channels). Furthermore, some displayed a dominant phenotype when overexpressed using *omb-Gal4*: the wild-type, *grocdc2*$^{-}$ and *groΔWD40* transgenes all resulted in pharate adult lethality with severe shortening of the proximodistal axis of the leg (not shown). We conclude that both the WD40 region and the Q domain of Gro are necessary for anterior *hh* repression, whereas the cdc2 phosphorylation site is dispensable. Furthermore, GroΔQ seems to be completely inactive, whereas GroΔWD40, though incapable of repressing *hh*, behaves like wild-type Gro in disrupting leg development upon overexpression.

DISCUSSION

A role of Gro in *hh* regulation

We have characterized a role of the co-repressor Gro in *hh* repression in the anterior wing compartment near the AP boundary. Although Ci[rep]-mediated repression can account for the lack of *hh* expression away from the AP boundary (Méthot and Basler, 1999), it has not been firmly established that it is operational close to the AP boundary. These cells receive high Hh signal and as a result not only do they not process Ci to Ci[rep], but also they activate full-length Ci into a strong activator, Ci[act], by post-translational modification (Aza-Blanc et al., 1997; Chen et al., 1999; Méthot and Basler, 1999). There is indirect evidence that Hh-receiving cells do not contain sufficient Ci[rep] levels to repress *hh*: In posterior cells, *ci* is repressed by En; other than this, the cellular mechanism for Hh signal transduction is present (Ramirez-Weber et al., 2000). When full-length *ci* is provided by ectopic expression in the posterior compartment, *hh-lacZ* is not repressed (Méthot and Basler, 1999). This suggests that these cells cannot produce appreciable amounts of Ci[rep], consistent with their responding to Hh signalling. That this is indeed the case was shown by the fact that ectopic expression of *ci* does repress posterior *hh-lacZ* in *smo* loss-of-function clones, where the Hh signal transduction has been disrupted (Méthot and Basler, 1999). If anterior cells that are exposed to Hh behave similarly, then the lack of *hh* expression there cannot be attributed to Ci[rep]. We propose that a Gro-dependent repression complex supplies this function, as *gro*$^{-}$ clones exhibit strong derepression of *hh-lacZ* near the AP boundary. The Gro

complex is not required in anterior cells far from the boundary, as those receive no Hh signal and thus contain sufficient Ci[rep] to repress *hh*. Accordingly, by supplying increased levels of Ci[rep] near the AP boundary via the ci^{Ce2} allele, we were able to largely abolish the need for Gro-mediated *hh* repression (Fig. 3), with the exception of the DV boundary (see below). As Gro is a ubiquitous co-repressor, one has to postulate the existence of a DNA-tethering factor, which will be referred to as 'X' for the purpose of this discussion, and some process of spatial regulation of the X-Gro complex activity. We have tested the possibility that X is a form of Ci itself and the answer was negative: using three different assays – GST pulldowns, yeast two-hybrid and transfection colocalization – we were unable to show any interaction between Gro and either form of Ci. Most importantly, the fact that Ci[rep] does not require Gro to repress *hh* in anterior cells away from the boundary supports a model where Ci and Gro repress *hh* independently of each other.

The quantitative aspect of *hh* derepression in gro^- clones was intriguing: clones abutting the AP boundary (type I) expressed the highest *hh-lacZ* levels, which dropped gradually as clones arose further from the P compartment. This is best seen in Fig. 1C and might reflect the fact that Ci[rep]-dependent repression gradually increases away from the boundary, and this is independent of *gro*. This interpretation assumes that basal (unrepressed) *hh* transcription in the A compartment would be high and subject to the dual repressors (Ci and X-Gro). Alternatively, basal *hh* transcription could be low, but, in addition to the repression control, *hh* could display a positive response to Hh signalling at the AP boundary. The latter model is consistent with the fact that in ci^- cells, basal *hh* expression appears to be low (Méthot and Basler, 1999). It also agrees with the behaviour of large type I gro^- clones in the present study. In these clones, high levels of *hh-lacZ* could be observed throughout the clone, even at a distance from the AP boundary (see, for example, Fig. 5C). This could be accounted for by Hh signalling, which, having risen over some threshold owing to *hh* derepression, further stimulates *hh* transcription to a high level. This effect would spread to the edge of the clone, beyond which activation of the X-Gro repressor would silence *hh* transcription. The putative inducer of *hh* by Hh signalling may be Ci[act], as with all other direct Hh target genes; alternatively it may be another factor induced by Ci[act]. The hypothesis that Ci[act] itself can activate *hh* transcription is not unreasonable, as *hh* should contain a regulatory region(s) that bind(s) Ci[rep]. Ci[act] and Ci[rep] contain the same DNA-binding domain and recent work has shown that the two forms of Ci bind the same target sites (Müller and Basler, 2000), although some enhancers may be configured in such a way as to respond preferentially to either the activator or repressor form.

A schematic representation of the above model is shown in Fig. 6. For the sake of simplicity we postulate the existence of a low level ubiquitous activator of *hh* (basal levels) with a stronger activator located in P cells to account for the high levels of *hh* expression there. In A cells that do not receive the Hh signal, the basal activity of *hh* is repressed by Ci[rep] and *gro* is not required. In A cells close to the Hh source, the basal transcription of *hh* would be enhanced by positive autoregulation; however, the presence of the repressive X-Gro complex does not allow this activation to take place. Implicit in this model is that X is itself activated by Hh (e.g. transcriptionally induced via Ci[act]), so that it only functions in Hh-receiving cells. In addition X production/activity should be spatially limited to the A compartment (e.g. repressed by En), as ectopic expression of full-length *ci* in the posterior cannot induce X-Gro activity to repress endogenous *hh* (Méthot and Basler, 1999). According to this model, ci^- clones close to the AP boundary express basal *hh* levels, as they lack both the X-Gro repressor (no activation of X in the absence of Ci[act]) and the activator of *hh* transcription (Ci[act] itself or a downstream target) (Fig. 6B). By contrast, gro^- clones in the same region only lack the repressive X-Gro complex and thus actively transcribe *hh* in response to Ci[act] (Fig. 6C); the high levels of *hh* produced are sufficient to initiate Hh signalling, which can propagate this effect of *hh* derepression throughout the clone.

gro^- clones near the DV boundary behaved somewhat aberrantly. *hh-lacZ* derepression there was more efficient, observable in further anteriorly arising clones (type II), compared with equivalent clones away from the DV boundary (type III) – it even occurred in the presence of increased Ci[rep] (Fig. 3). Although the mechanism remains to be discovered, one way to account for this special behaviour, without invoking additional regulators, is that Ci[rep] is less active near the DV boundary and/or Ci[act] is more active, and this modulation of Ci activity in favour of the activator form allows high level *hh* expression at a greater distance from the Hh source and even in the $ci^{Ce2}/+$ background. Interestingly, ci^- clones show little or no *hh-lacZ* derepression at the DV boundary (Méthot and Basler, 1999), consistent with Gro, rather than Ci[rep], being the major *hh* repressor there.

The model we have put forward is perhaps the simplest, but by no means the only one that fits the existing data. For example, Gro might interact with Ci[act] itself, switching it from an activator into a repressor, given the right enhancer context, much like the effect Gro has on other activators, such as Dorsal (Dubnicoff et al., 1997; Valentine et al., 1998). This interaction may be weak and/or require additional factors, accounting for our inability to detect it. To resolve the mechanism of *hh* repression at the AP boundary will necessitate detailed molecular dissection of the *hh* regulatory regions and characterization of relevant *trans* acting factors. Whatever the mechanism, it appears that a Gro-containing complex is deployed in the wing to block the spread of *hh* expression anteriorly from the AP compartment boundary. This should ensure a spatially fixed organizer (*dpp* expression stripe), in contrast to a moving one, as found in the fly retina (Heberlein and Moses, 1995).

Functions of Gro domains

Gro is the founder of a family of transcriptional co-repressors encountered in invertebrates and vertebrates (Chen and Courey, 2000). Gro proteins are multipurpose co-repressors, as they can interact with a good number of DNA-binding repressors. Once recruited to a target gene, they can interact with histones and histone deacetylase Rpd3/HDAC1. They also have the ability to homo-tetramerize, which has led investigators to propose a model for repression whereby Gro is recruited to a regulatory region via a DNA-bound repressor and subsequently binds more Gro molecules via Gro-Gro and Gro-chromatin interactions (Palaparti et al., 1997; Chen et al., 1998; Chen et

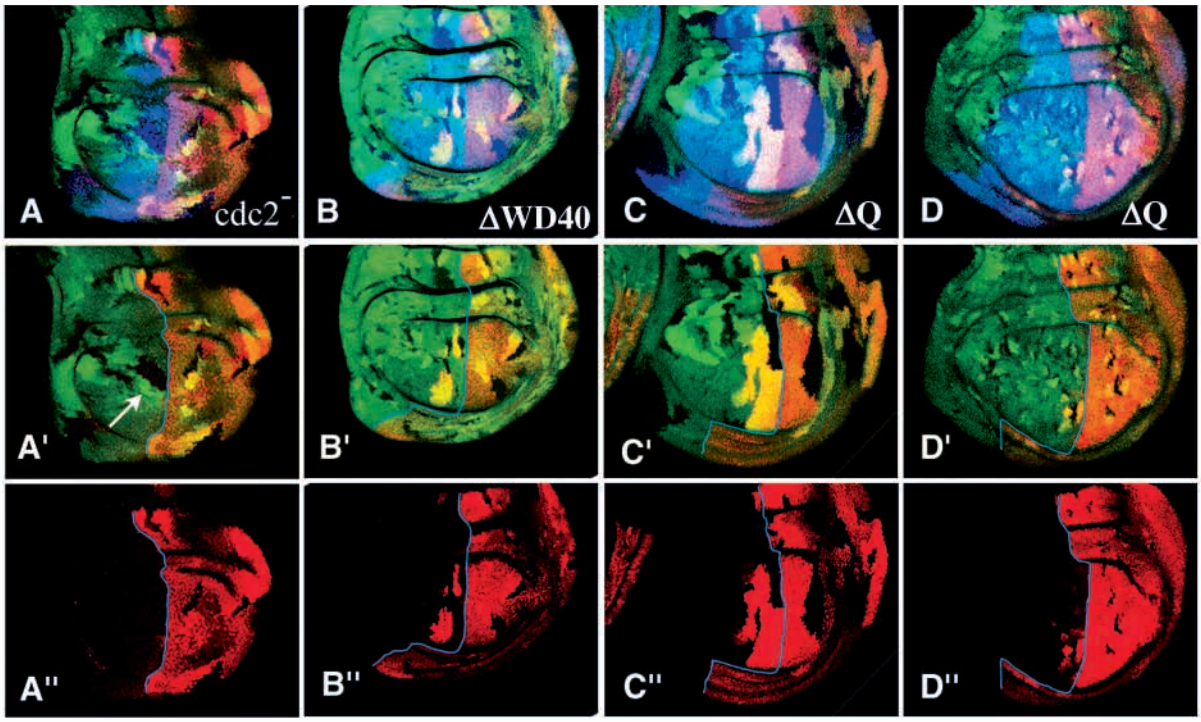

Fig. 5. (A-D) *Df(3R)Espl22* clones marked with increased GFP (bright green) and stained for *hh-lacZ* (red) in the background of *UAS-gro(mutant)* transgene expression using *omb-Gal4*; overexpressed Gro is visualized in the blue channel. (A′-D′) green (GFP) and red (*hh-lacZ*) channels only. (A″-D″) red channel only (*hh-lacZ*). (A) *UAS-grocdc2*$^{-}$ rescues the *gro*$^{-}$ defect, as it does not allow *hh-lacZ* derepression in anterior clones (arrow). (B) *UAS-groΔWD40* and C, D: *UAS-groΔQ* do not restore *hh* repression. The position of the AP boundary is indicated by a blue line. Anterior is towards the left and dorsal upwards.

al., 1999). In this way, the original recruitment may nucleate a repressive chromatin configuration that spreads a distance away from the original binding site. In accordance with such an 'active' mechanism for transcriptional repression, it has been shown that Gro can cause long-range repression (also termed gene silencing), referring to its ability to repress transcription when recruited to DNA sites away from activator-binding sites (Zhang and Levine, 1999). It is thus not surprising that Gro has a multidomain structure to implement its different functions and to contact its various partners.

Where the interaction of Gro with DNA-binding repressors has been molecularly mapped, the WD40 repeats feature as the most common interacting domain (Jiménez et al., 1997; Tolkunova et al., 1998). However, this is by no means always so. For example, insect and vertebrate TCF appears to interact with the Q domain (Brantjes et al., 2001) and repressors binding via the WD40 repeats may also contact the SP domain (Paroush et al., 1994; Jiménez et al., 1997; McLarren et al., 2001). It has been proposed that efficient Gro recruitment might necessitate multiple protein-protein contacts with one or more DNA tethered factors (Valentine et al., 1998; Eberhard et al., 2000), something that could easily be achieved if Gro uses different domains to simultaneously interact with different partners. In order to effect repression, Gro uses multiple contacts after its recruitment by DNA-bound factors: The Q domain has been shown to mediate tetramerization of Gro proteins (Chen et al., 1998), a process that appears to be a prerequisite for transcriptional repression. A Gro fragment containing the Q-GP-CcN-SP domains has been shown to be necessary and sufficient for histone binding, with a strong preference for deacetylated histones (Palaparti et al., 1997; Flores-Saaib and Courey, 2000). The GP domain is needed for interaction with the histone deacetylase Rpd3 (Chen et al., 1999). The CcN domain contains a canonical NLS and nearby sequences conforming to consensus phosphorylation sites for casein kinase (CKII) and p34cdc2.

We tested a number of Gro mutants both for subcellular localization (Fig. 4) and for activity in vivo (Fig. 5). Grocdc2^{-} and GroΔQ showed the same nuclear accumulation as wild-type Gro. GroΔWD40 was also nuclear, but it showed a striking departure from the rather uniform wild-type pattern, as it localized predominantly to a small number of subnuclear particles. GroNLS^{-} was both nuclear and cytoplasmic, whereas GroΔGCS was exclusively cytoplasmic. This suggests that the GP, CcN and SP domains contain at least two different regions needed for efficient nuclear accumulation, one of which is the canonical NLS. We can speculate that such other regions might be those necessary for association with histones or with DNA-bound repressors, which might promote nuclear accumulation of Gro even in the absence of the NLS.

In vivo activity was tested by assaying the ability of mutant Gro proteins to repress anterior *hh-lacZ* expression. GroΔQ and GroΔWD40 proteins were inactive in this assay, although both transgenes were expressed at high levels (Fig. 5). In contrast, Grocdc2^{-} was as active as wild-type Gro. The inability of GroΔQ to function as a co-repressor is expected, as the Q domain is the strongest repression domain and is needed both for tetramerization as well as for histone

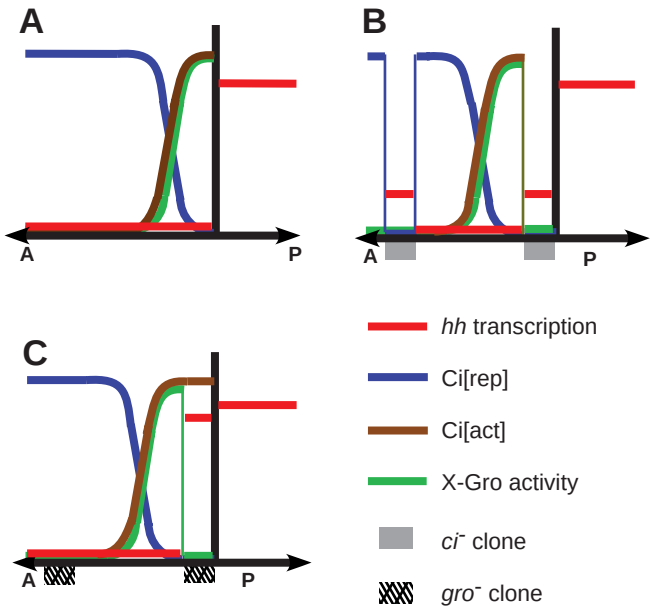

Fig. 6. Transcriptional inputs proposed to regulate *hh* in the A compartment. (A) The wild-type situation. The levels of Ci[rep] (blue) and Ci[act] (brown) are complementary, as these forms are reciprocally controlled by Hh signalling at the AP boundary (black vertical line). Ci[act] induces the expression or the activity of repressor X, which together with Gro forms a repressive complex (green) – thus, the green line follows the brown, except in cases of *gro* loss of function (C). As there is some repressor complex (blue or green) present at any given position in the A compartment, *hh* expression (red) is nil. B shows two *ci*⁻ clones (grey bars on the AP axis), where both forms of Ci (blue and brown) are eliminated. They both contain low basal levels of *hh* transcription. This is because of the absence of both repressor and activator complexes within the clones. Note that the brown and blue curves have been shifted anteriorly (to the left) – this is because the loss of *ci* near the boundary disables the induction of *ptc* and therefore posterior Hh can diffuse further (Chen and Struhl, 1996) anteriorly to generate Ci[act] and suppress Ci[rep]. C shows two *gro*⁻ clones (cross-hatched bars on the AP axis). Those affect only the X-gro repressor activity (drop in green line). As a result of high levels of Ci[act] activator (brown) and lack of repressor (green or blue) within the more posterior (rightmost) clone, *hh* is expressed at high levels. No effect is expected in the more anterior clone. Again, note that the brown and blue curves have been shifted anteriorwards as a result of high ectopic Hh activity that influences the relative amounts of Ci[act] and Ci[rep]. In B, we assume that the low basal *hh* levels within the clones are not sufficient to produce a similar effect.

interaction. The inactivity of the GroΔWD40 mutant might be accounted by its inability to interact with the X-factor tether. Or one could suggest an alternative explanation based on the localization data: that GroΔWD40 is retained in subnuclear particles and as a result cannot gain access to target genetic loci. Whether the aberrant subnuclear localization of GroΔWD40 is a cause or a consequence of its inactivity is a matter for further study. Despite its aberrant localization, GroΔWD40 was as active as wild-type Gro and Grocdc2⁻ when overexpressed by *omb-Gal4*: all three transgenes resulted in abnormal leg development. Gro-ΔQ, -NLS⁻ and -ΔGCS did not have such an effect. This shows that GroΔWD40 retains some activity, although in the absence of data regarding the cause of defects in leg patterning, we cannot conclude on the function of the mutant protein. 'Short' Gro family proteins that lack WD40 repeats exist in vertebrates. These, human AES and mouse Grg5, contain only Q and GP domains, thus they are not entirely equivalent with the ΔWD40 mutant. It has been shown that these proteins are cytoplasmic, although they are readily transported to the nucleus upon interaction with a Tcf partner (Roose et al., 1998). Their role in transcription seems to be context dependent, as they can act as co-repressors in some cases (Tetsuka et al., 2000), whereas in others they might counter repression by 'long' Gro proteins (Roose et al., 1998). One study suggests that this anti-repression effect is not necessarily due to the absence of the CcN/SP/WD40 domains, but rather due to the inability of the GP domain of the 'short' proteins to interact with HDAC1 (Brantjes et al., 2001). In this study, GroΔWD40 was active in one assay and inactive in another. It will be interesting to determine its activity in additional biological contexts where Gro is required.

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