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The ubiquitin ligase Hyperplastic discs negatively regulates *hedgehog* and *decapentaplegic* expression by independent mechanisms

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SUMMARY

Photoreceptor differentiation in the *Drosophila* eye disc progresses from posterior to anterior in a wave driven by the Hedgehog and Decapentaplegic signals. Cells mutant for the *hyperplastic discs* gene misexpress both of these signaling molecules in anterior regions of the disc, leading to premature photoreceptor differentiation and overgrowth of surrounding tissue. The two genes are independently regulated by *hyperplastic discs*; *decapentaplegic* can still be misexpressed in cells mutant for both *hyperplastic discs* and *hedgehog*, and a repressor form of the transcription factor Cubitus interruptus can block *decapentaplegic* misexpression but not *hedgehog* misexpression. Loss of *hyperplastic discs* causes the accumulation of full-length Cubitus interruptus protein, but not of Smoothened, in both the eye and wing discs. *hyperplastic discs* encodes a HECT domain E3 ubiquitin ligase that is likely to act by targeting Cubitus interruptus and an unknown activator of *hedgehog* expression for proteolysis.

Key words: Ubiquitin, Morphogenetic furrow, hedgehog, Cubitus interruptus, slmb, Drosophila melanogaster

INTRODUCTION

Communication is essential for multicellular development. Intercellular signals regulate the timing and pattern of cellular events such as growth, division, movement, and differentiation, allowing large groups of cells to behave in a coordinated manner during events such as organogenesis. Cell-cell signaling can regulate the activity of cells at many different levels, including gene expression, cell division and motility. Within the receiving cell, signal transduction cascades transform the reception of ligand at the cell surface into the proper cellular response. This may involve regulation of protein activity by post-translational modification or by destruction or proteolytic processing. Ubiquitination, the covalent addition of a multimeric chain of the 76-amino-acid Ubiquitin (Ub) protein, is the most common intracellular signal for proteolysis. Ubiquitination is a multi-step process that begins with the activation of a Ub molecule by an E1 or Ub-activating enzyme. The activated Ub is transferred to an E2 enzyme, which is then responsible, either directly or indirectly, for attaching the Ub to a substrate protein (reviewed by Ciechanover et al., 2000). Specificity of the ubiquitination reaction is achieved at the level of the E3 ubiquitin ligase, which is thought to directly bind the substrate. Many such ligases exist and have been classified into families based on the structure of the ubiquitination domain. HECT-domain E3 ligases directly attach Ub to a substrate, while RING domain E3s direct specific substrate ubiquitination by the E2 (Jackson et al., 2000).

A small number of signaling pathways appear to direct most developmental processes. In *Drosophila*, the BMP family member Decapentaplegic (Dpp) and the founding member of the Hedgehog family (Hh) are used repeatedly throughout development. One function of Hh and Dpp is to direct the progressive differentiation of the eye imaginal disc (reviewed in Lee and Treisman, 2002). In the second instar eye disc, *hh* is expressed in a complex pattern in both the disc proper and the peripodial membrane, before being refined to a small domain centered on the dorsoventral midline of the disc's posterior margin (Cho et al., 2000). Hh signals more anterior cells to express *dpp* and *atonal (ato)*, which encodes the bHLH transcription factor required for the formation of the R8 'founder' photoreceptor in each cluster (Jarman et al., 1994). These cells then differentiate as photoreceptors and themselves express *hh*, allowing the cycle to propagate toward the anterior of the disc. *dpp* is expressed in the morphogenetic furrow, an indentation at the front of differentiation, where it is responsible for coordinating the timing of differentiation through synchronization of the cell cycle (Penton et al., 1997). Loss of either Hh or Dpp blocks the initiation of differentiation, while loss of both blocks progression (Curtiss and Mlodzik, 2000; Greenwood and Struhl, 1999). While it is known that Hh

activates *dpp* expression in the furrow (Heberlein et al., 1995; Heberlein et al., 1993; Ma et al., 1993), it is not known how *hh* expression is controlled, nor how *dpp* is turned off in cells leaving the furrow.

Much has been learned about the regulation of *hh* expression in tissues other than the eye. During embryonic development, maintenance of *hh* expression in the posterior of each segment requires the homeobox gene *engrailed* (*en*) (Tabata et al., 1992). In the head segments, however, *hh* expression is not controlled by En, but by several different homeobox and segment polarity genes (Crozatier et al., 1999; Gallitano-Mendel and Finkelstein, 1997). *hh* is also regulated by En later in development, in the posterior compartments of the leg and wing imaginal discs (Tabata et al., 1995; Zecca et al., 1995). En regulates *hh* expression indirectly, by repressing the expression of *cubitus interruptus* (*ci*) in the posterior compartment (Dominguez et al., 1996; Eaton and Kornberg, 1990; Schwartz et al., 1995). In the absence of Hh signal, the full-length Ci protein (Ci_{155}) is proteolytically processed to a 75 kDa transcriptional repressor (Ci_{75}); when the Hh signal is received this processing event is inhibited, allowing the accumulation of Ci_{155} (Aza-Blanc et al., 1997). In the anterior compartment, Ci_{75} represses *hh* expression (Dominguez et al., 1996; Methot and Basler, 1999). The Hh signal is transmitted to Ci through two transmembrane proteins, Patched (Ptc) and Smoothened (Smo) (reviewed by Ingham and McMahon, 2001). Hh binds to Ptc and inhibits its activity; in the absence of Hh, Ptc promotes internalization, dephosphorylation and degradation of Smo (Alcedo et al., 2000; Denef et al., 2000; Ingham et al., 2000).

Unlike these other tissues, in the eye the source of *hh* expression changes over time; as cells are recruited to differentiate as photoreceptors, they begin expressing *hh*. Reflecting this difference in *hh* control, *en* function is not required in the eye disc for *hh* expression (Strutt and Mlodzik, 1996). The relationship between *hh* and *dpp* expression is conserved in some tissues, but not in others. In the wing disc, as in the eye, *dpp* is transcribed in response to Hh (Basler and Struhl, 1994; Zecca et al., 1995); in the leg disc this response is limited, through the activity of Wg, to a dorsal domain in the anterior compartment (Basler and Struhl, 1994; Brook and Cohen, 1996; Jiang and Struhl, 1996). In other tissues, such as the dorsal domain of the early embryo, *dpp* expression appears to be independent of Hh signaling (St Johnston and Gelbart, 1987). Thus the mechanisms controlling the expression of these signals are likely to be at least partially context-dependent. In tissues where Hh regulates *dpp* expression, *dpp* is both activated by Ci_{155} and repressed by Ci_{75}, allowing robust control of *dpp* by Hh (Methot and Basler, 1999).

To gain further insight into the control of photoreceptor development, we have conducted mosaic screens for mutations that perturb differentiation (Benlali et al., 2000; Lee and Treisman, 2001a; Lee and Treisman, 2001b; Treisman, 2001). Many components of the *hh* and *dpp* pathways were recovered in these screens. We report here our characterization of mutations in the *hyperplastic discs* (*hyd*) locus; *hyd* encodes a HECT domain E3 ubiquitin ligase (Callaghan et al., 1998; Mansfield et al., 1994). Mutations in *hyd* cause ectopic non autonomous differentiation in the eye. This effect is due to the independent activation of *hh* and *dpp* expression in *hyd* mutant tissue, reflecting independent effects of Hyd on *hh* expression and function.

MATERIALS AND METHODS

Genetics

The mosaic screen for eye differentiation mutants on 3R has been described previously (Lee and Treisman, 2001a). Four alleles of *hyd* were recovered in this screen. Several previously isolated *hyd* alleles, including alleles in which Hyd protein is absent (Mansfield et al., 1994), gave indistinguishable clonal phenotypes in the eye disc. To make negatively labeled clones, FRT82, *hyd* or *hyd, hh* or *slmb* males were crossed to *ey*FLP1 or *hs*FLP122; FRT82, *arm-lacZ* or FRT82, Ubi-GFP females. To make clones in a *Minute* background, the females used were *ey*FLP1; FRT82, *arm-lacZ, M(3)96C*. To make positively labeled clones, FRT82, *hyd* males were crossed to UASGFP, *ey*FLP1 or *hs*FLP122; *tub*-GAL4, FRT82, *tub*-GAL80 females (Lee and Luo, 1999). The same females were used for clone-specific expression of transgenes carried by the FRT82, *hyd* males (Lee and Treisman, 2001a). Crosses in which *hs*FLP122 was used were heat-shocked at the first and second instar stage for 1 hour at 38.5°C. The alleles used were hh^{rJ413} (Heberlein et al., 1993), hh^{AC} (Lee et al., 1992), hh^{ts2} (Ma et al., 1993), $slmb^{B93}$ (Lee and Treisman, 2001a) and ato^{IH3} (J. D. L., unpublished). Transgenes used were *tub*-GAL4, *tub*-GAL80 (Lee and Luo, 1999) and UAS-ci_{76} (Aza-Blanc et al., 1997). Reporters used were *dpp-lacZ* (Blackman et al., 1991) and hh^{P30} (Lee et al., 1992). To examine the level of *hh* expression in *hyd* mutant clones, *hyd* clones carrying two copies of a *hh-lacZ* reporter in a heterozygous background were compared to wild-type clones with two copies of *hh-lacZ* in a heterozygous background. The clones shown have significantly stronger *lacZ* expression than would be expected from doubling the reporter dosage.

Immunohistochemistry

Imaginal discs were stained as described previously (Hazelett et al., 1998). Primary antibodies used were rat α-Elav (1:1) (Robinow and White, 1991), rabbit α-Atonal (1:10,000) (Jarman et al., 1994), rat α-Ci (1:1) (Motzny and Holmgren, 1995), rat anti-Smo (1:500) (Denef et al., 2000), mouse α-β-galactosidase (Promega; 1:200), rabbit α-β-galactosidase (Cappel; 1:5000), mouse anti-Neuroglian (1:1) (Hortsch et al., 1990), mouse anti-Ptc (1:150) (Capdevila et al., 1994) and rabbit α-Hh-N (1:2000) (Lee and Treisman, 2001b). Secondary antibodies were from Jackson Laboratories, conjugated to FITC, Texas Red or Cy5, used at 1:200. Fluorescent images were obtained using a Leica TCS NT confocal microscope. In situ hybridization was performed as described (Maurel-Zaffran and Treisman, 2000).

RESULTS

hyd clones cause non autonomous differentiation and overgrowth in the eye disc

To identify novel genes contributing to pattern formation in the *Drosophila* eye disc, we carried out a mosaic genetic screen in which we generated homozygous mutant clones of cells specifically in the eye disc (Benlali et al., 2000; Lee and Treisman, 2001a; Lee and Treisman, 2001b; Treisman, 2001). In this screen we recovered 4 alleles of *hyd*, which encodes a large protein containing a HECT family E3 ubiquitin ligase domain (Callaghan et al., 1998; Mansfield et al., 1994). *hyd* was initially isolated in a screen for mutations causing imaginal disc overgrowth (Martin et al., 1977). We found that adult eyes containing *hyd* mutant clones showed extensive overgrowth of the eye tissue surrounding a *hyd* clone, although the clones themselves did not persist to adulthood (Fig. 1B). In the third instar eye disc, we observed premature photoreceptor differentiation, visualized by expression of the markers Elav

and Neuroglian, in *hyd* mutant clones in a region just anterior to the morphogenetic furrow (Fig. 1E and data not shown). Very rarely, differentiation was seen in clones lying near the anterior margin (data not shown). The spatial restriction of this phenotype is consistent with work by others demonstrating the existence of a preproneural zone anterior to the furrow (Greenwood and Struhl, 1999; Heberlein et al., 1995; Ma et al., 1993; Strutt et al., 1995). Ectopic differentiation spread beyond the borders of the *hyd* mutant clones into the surrounding wild-type tissue (Fig. 1E). The proneural transcription factor Atonal (Ato) was also misexpressed within and surrounding *hyd* clones anterior to the furrow (Fig. 1H). The overgrowth of wild-type tissue in adult eyes was visible in third-instar imaginal discs as folding and distortion of the disc epithelium surrounding *hyd* clones (Fig. 1E, Fig. 3A). This effect was more widespread than the ectopic differentiation phenotype, appearing in more anterior regions of the disc.

While *hyd* clones in third instar eye discs contained Elav-expressing photoreceptors, clones in the adult eye appeared to form either scars in the eye or head cuticle at its margin (Fig. 1B). We also noticed that *hyd* clones in the posterior of the eye disc were generally smaller than wild-type clones generated in a similar cross and contained reduced numbers of photoreceptors (Fig. 1D,E). The lack of mutant ommatidia in adult eyes could reflect either a loss of *hyd* mutant clones due to competition with the surrounding tissue, or a later requirement of *hyd* for viability. To further examine this question, we made *hyd* clones in a *Minute* genetic background (Morata and Ripoll, 1975), thereby reversing the growth disadvantage of *hyd* mutant tissue (Fig. 1C). These animals survived to pharate adult stages but could not eclose; they had greatly reduced eyes that nevertheless appeared to contain properly formed *hyd* mutant ommatidia (Fig. 1C). This suggests that the lack of visible *hyd* clones in adults is due to their poor relative growth, leading to their elimination through competition with wild-type cells. We also examined third instar eye discs containing *hyd/Minute* clones, and found greatly reduced eye discs that appeared to contain full clusters of photoreceptors throughout the eye field (Fig. 1F). As cells arrest division when they differentiate, premature differentiation could explain the small size of *hyd/Minute* eye discs. Ato is normally expressed in the morphogenetic furrow and in the youngest R8 photoreceptors (Fig. 1G); in early third instar *hyd/Minute* mutant discs the only remaining Ato staining is in single (presumably R8) cells (Fig. 1I), implying that the morphogenetic furrow has already reached the anterior margin. From this we infer that differentiation in *hyd/Minute* discs still progresses from posterior to anterior, but at an accelerated rate.

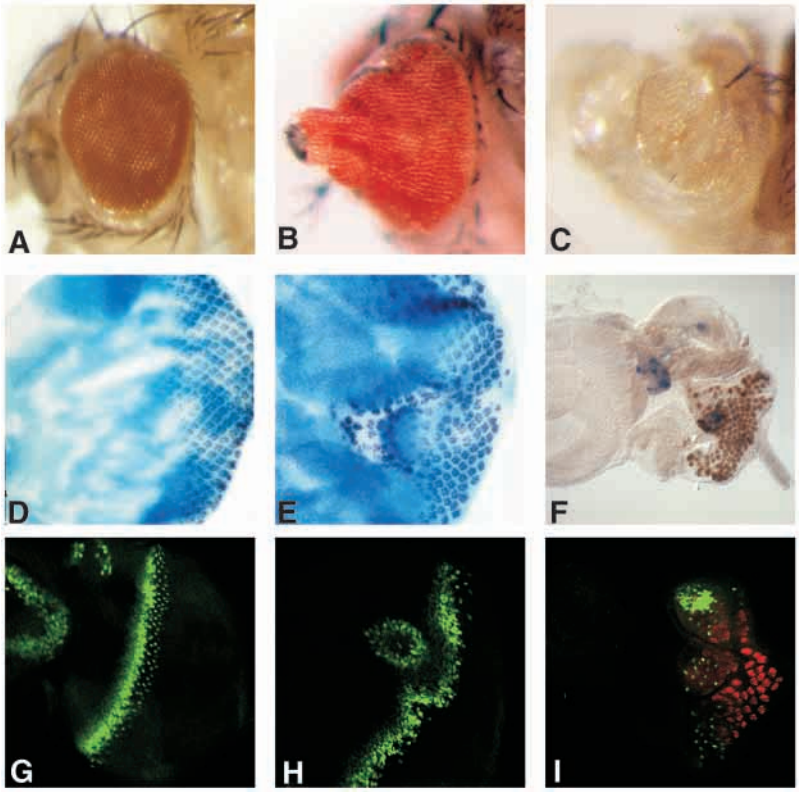

Fig. 1. Loss of *hyd* leads to ectopic differentiation and overgrowth in the eye disc. (A-C) Adult eyes; (A) wild type; (B) $hyd^{K3.5}$ clones have induced outgrowths of wild-type ($white^{+}$) tissue; (C) $hyd^{K3.5}$ mutant clones generated in a *Minute* background. All surviving photoreceptors are $white^{-}$ and therefore *hyd* mutant. (D-I) Third instar eye discs. Anterior is to the left and dorsal up in this and subsequent figures. (D-F) Anti-Elav staining in brown and X-gal staining, to show *arm-lacZ* expression, in blue. (D) Clones of wild-type tissue identified by lack of *arm-lacZ* expression. (E) Clones of $hyd^{K3.5}$ mutant cells identified by lack of *arm-lacZ* expression. Ectopic photoreceptors are visible in an anterior clone. (F) $hyd^{K3.5}$ clones generated in a *Minute* background. Remaining wild-type tissue is marked by *arm-lacZ* expression. (G,H) Anti-Ato staining in wild type (G) and in a disc containing an unmarked $hyd^{K3.5}$ clone (H), where a ring of ectopic Ato staining is visible anterior to the furrow. (I) An early third instar eye disc with $hyd^{K3.5}$ clones generated in a *Minute* background, stained with anti-Elav in red and anti-Ato in green. Ato is already restricted to single cells at the anterior of the disc.

hyd mutant cells ectopically express *hedgehog*

Growth and differentiation of cells in the eye disc both depend on Hh and Dpp secreted by more posterior cells. The non autonomous differentiation and overgrowth caused by *hyd* clones suggested that the clones might be producing one or both of these molecules; a similar phenotype is produced by ectopic expression of *hh* or activation of the *hh* pathway ahead of the furrow (Heberlein et al., 1995; Pan and Rubin, 1995; Strutt et al., 1995). We therefore looked at *hh* expression in *hyd* clones using both an enhancer trap line (Lee et al., 1992) and antibody staining. *hyd* mutant clones anterior to the furrow indeed expressed *hh-lacZ* and Hh protein earlier than surrounding wild-type cells (Fig. 2A-C and data not shown). When we made *hyd* clones in a *Minute* background, we observed *hh-lacZ* expression throughout the eye disc (Fig. 2E). The widespread *hh* expression in *hyd/Minute* clones may explain the accelerated differentiation and small size of these eye discs.

The ectopic *hh* expression we observed in *hyd* mutant clones could be a consequence, rather than a cause, of their premature differentiation. To rule out this possibility we generated *hyd*

ato double mutant clones. Clones mutant for *ato* cannot form the R8 photoreceptor (Jarman et al., 1995), which is itself required for the recruitment of photoreceptors R1-R7. Thus *ato* mutant clones do not differentiate, unless a cell at the margin of a clone is recruited by a neighboring wild-type R8. We confirmed that *ato* single mutant clones do not express *hh* (data not shown). We found that while *hyd ato* mutant clones did not differentiate, they nevertheless expressed *hh-lacZ* and were capable of directing ectopic differentiation in surrounding wild-type tissue (Fig. 2G-I). This demonstrates that loss of *hyd* function has a direct effect on *hh* expression that is not simply due to differentiation.

As an E3 ubiquitin ligase, Hyd is likely to promote the degradation of one or more proteins. Based on the ectopic expression of *hh* in *hyd* mutant clones, we hypothesized that Hyd was acting in the anterior of the third instar eye disc to prevent premature expression of *hh*. *hyd* is expressed in proliferating tissues in the embryo and larva, but its expression in the eye disc had not been described in detail (Mansfield et al., 1994). Using in situ hybridization, we found that *hyd* RNA was highly expressed in the anterior of the eye imaginal disc, especially around the dorsoventral midline (Fig. 2F). *hyd* was expressed at lower levels towards the dorsal and ventral margins but was still restricted to the anterior. This expression pattern is consistent with a role for *hyd* in preventing the premature expression of *hh*.

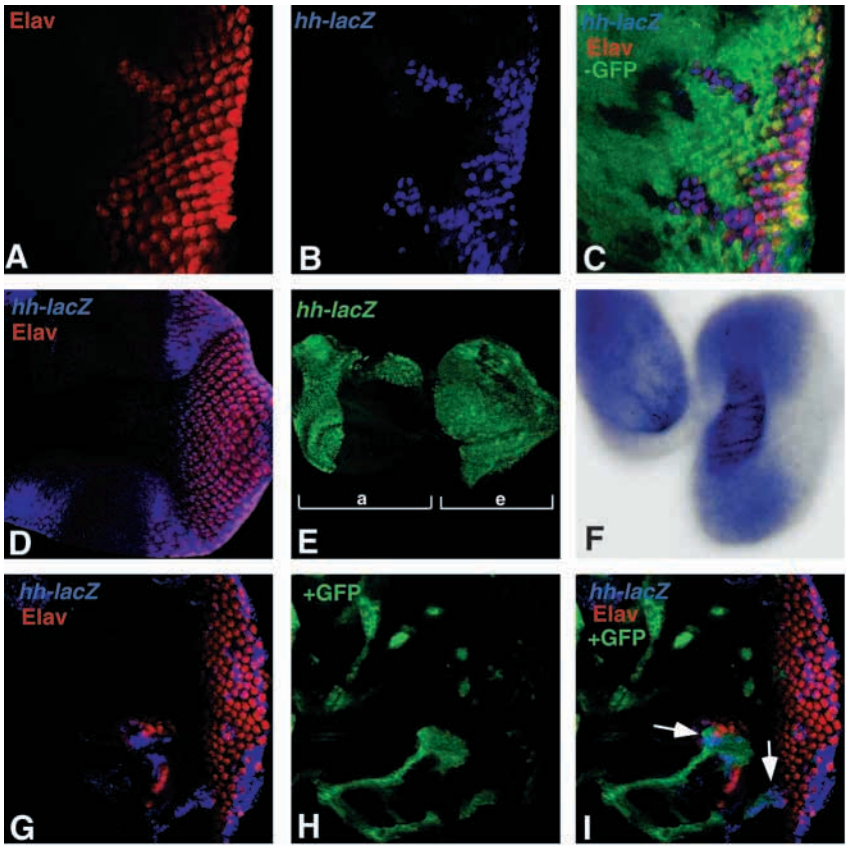

Fig. 2. Loss of *hyd* induces ectopic *hh* expression in the eye disc. All panels show third instar eye discs. (A-D) Anti-Elav staining in red, anti-β-galactosidase staining, reflecting *hh-lacZ* expression, in blue, and GFP in green. (A-C) $hyd^{K7.19}$ clones (marked by lack of GFP expression in C); (D) wild type. *hh* is misexpressed anterior to its normal domain in *hyd* mutant clones. (E) $hyd^{K7.19}$ clones made in a *Minute* background, showing *hh-lacZ* expression in green. *hh* is misexpressed throughout the eye disc. The two portions of the eye-antennal disc are indicated: e, eye disc; a, antennal disc. (F) In situ hybridization with a *hyd* antisense probe. *hyd* is predominantly expressed anterior to the furrow. The sense probe showed only very faint non-specific staining (not shown). (G-I) $hyd^{K7.19}$, ato^{IH3} clones positively marked by GFP expression in (H,I) are stained with anti-Elav in red and anti-β-galactosidase, reflecting *hh-lacZ* expression, in blue. Lack of *ato* prevents differentiation, but not *hh* expression, within the *hyd* clones (arrows); ectopic *hh* then leads to differentiation of surrounding wild-type tissue.

Loss of *hedgehog* function partially suppresses the *hyd* phenotype

If the ectopic differentiation and overgrowth associated with *hyd* clones is due to ectopic *hh* expression, it should be possible to rescue this phenotype by removing *hh* function from the clones. We therefore determined the phenotype of *hyd hh* double mutant clones. We obtained similar results with three *hh* alleles, hh^{rJ413} (Heberlein et al., 1993), hh^{ts2} (Ma et al., 1993) grown at 29°C, and the null allele hh^{AC} (Lee et al., 1992). While *hh* mutant clones appear wild type unless located on the margin of the eye disc (Dominguez and Hafen, 1997) (Fig. 3B), *hyd hh* double mutant clones showed a partial suppression of the *hyd* phenotype. Ectopic photoreceptors were no longer present in or around *hyd hh* clones (Fig. 3C), and *hyd hh* double mutant clones generated in a *Minute* background had only a few photoreceptors associated with the remaining wild-type tissue (Fig. 3D). Thus *hyd* mutant tissue requires Hh in order to differentiate. However, some *hyd hh* mutant clones were still able to stimulate proliferation of surrounding tissue, leading to overgrowth of the adult eye (Fig. 3C,E).

dpp has also been shown to stimulate proliferation in the eye disc (Pignoni and Zipursky, 1997). The remaining non autonomous overgrowth induced by *hyd hh* double mutant clones might therefore be due to ectopic expression of *dpp*. Using a *dpp-lacZ* reporter construct, we observed ectopic *dpp* expression in and around *hyd* mutant clones anterior to the furrow (Fig. 3F and data not shown). Ectopic *dpp* expression appeared more widespread than ectopic *hh* expression and occurred in more anterior regions of the disc, raising the possibility that *dpp* misexpression is not merely induced by ectopic Hh in the clones but is an independent consequence of the loss of *hyd*. Indeed, we found that *dpp* was still expressed in some *hyd hh* mutant clones (Fig. 3G,H), although its expression was limited to clones close to the morphogenetic furrow. Thus in the absence of *hyd* function, *dpp* expression is no longer strictly regulated by Hh signaling.

Hyd independently regulates *hh* and *dpp* expression

The persistence of *dpp* expression in *hyd hh* double mutant

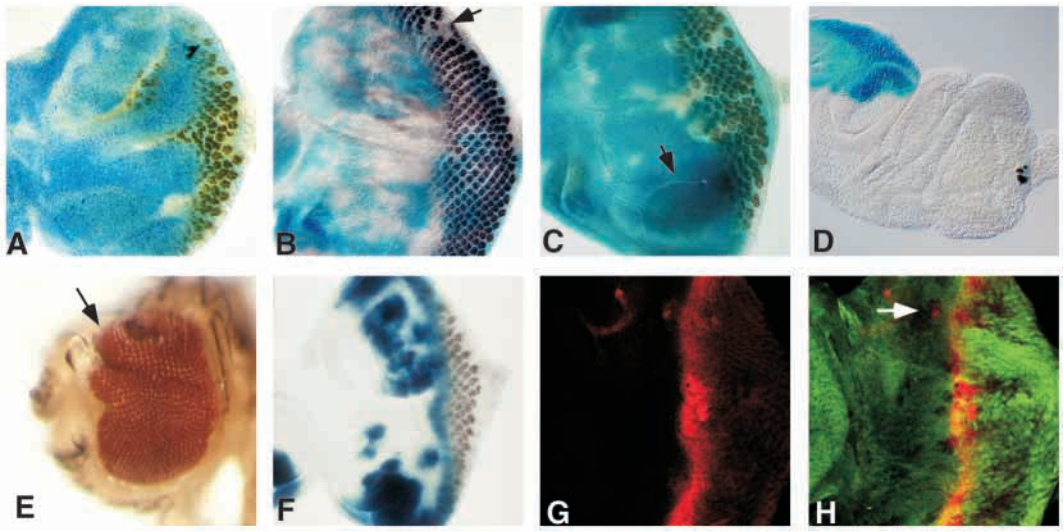

Fig. 3. Removal of *hh* function partially suppresses the *hyd* mutant phenotype. All panels show third instar eye discs. (A-D) Anti-Elav staining in brown and X-gal staining, reflecting *arm-lacZ* expression (wild-type tissue), in blue. (A) Ectopic differentiation in $hyd^{K3.5}$ mutant clones. (B) hh^{rJ413} clones; lack of photoreceptor differentiation is only apparent in clones at the posterior margin (arrow). (C) $hyd^{K3.5}$, hh^{rJ413} double mutant clones. No ectopic photoreceptors form, but some clones still induce overgrowth of wild-type tissue (arrow). (D) $hyd^{K3.5}$, hh^{rJ413} clones generated in a *Minute* background. The few photoreceptors that form are in wild-type tissue. (E) An adult eye containing $hyd^{K3.5}$, hh^{rJ413} clones and exhibiting some overgrowth (arrow). (F) Unmarked $hyd^{K3.5}$ clones stained with anti-Elav in brown and X-gal, reflecting *dpp-lacZ* expression, in blue. *dpp* is ectopically expressed anterior to the furrow. (G,H) A third instar eye disc containing $hyd^{K3.5}$, hh^{AC} mutant clones (labeled by lack of GFP expression in H). Anti-β-galactosidase (in red) is used to visualize *dpp-lacZ* expression; ectopic *dpp* is still visible in some clones (arrow in H).

clones suggested that *dpp* regulation by Hyd was at least partially independent of *hh* (Fig. 3G,H). One molecule that is known to transcriptionally regulate both *hh* and *dpp* is Ci. In the wing imaginal disc it has been shown that Hh controls *dpp* expression both by suppressing the production of Ci_{75}, which inhibits *dpp* expression, and by activating Ci_{155}, which activates *dpp* expression (Methot and Basler, 1999). In addition, Ci_{75} inhibits *hh* expression in the anterior compartment of the wing disc; *ci* transcription is repressed by *en* in the posterior compartment, allowing *hh* to be expressed there (Dominguez et al., 1996; Methot and Basler, 1999). To test whether *hyd* was acting through Ci to affect *hh* and *dpp* expression in the eye disc, we drove the expression of a truncated constitutive repressor form of Ci (Ci_{76}) (Aza-Blanc et al., 1997) specifically in *hyd* mutant clones using the MARCM system (Lee and Treisman, 2001a; Lee and Luo, 1999). *dpp-lacZ* was no longer expressed in *hyd* clones expressing UAS-ci_{76} in or anterior to the furrow (Fig. 4A,B). However, Ci_{76} did not prevent the ectopic expression of *hh* in *hyd* mutant clones (Fig. 4D-F). Continued *hh* expression in these clones sometimes led to ectopic differentiation in tissue surrounding the clone (Fig. 4D-F). Thus Ci_{76} is sufficient to block *dpp* but not *hh* expression in *hyd* clones, suggesting that Hyd regulates *hh* and *dpp* expression through at least partially independent mechanisms.

If the overgrowth phenotype induced by *hyd hh* double mutant clones is indeed due to their misexpression of *dpp*, it should be blocked by introducing Ci_{76} into the mutant cells. Indeed, when we generated *hyd hh* mutant clones expressing UAS-ci_{76}, the adult eyes no longer exhibited any overgrowth (Fig. 4C). Thus all the ectopic growth and differentiation caused by loss of *hyd* in the eye can be attributed to independent effects on *hh* expression and activation of the Hh pathway.

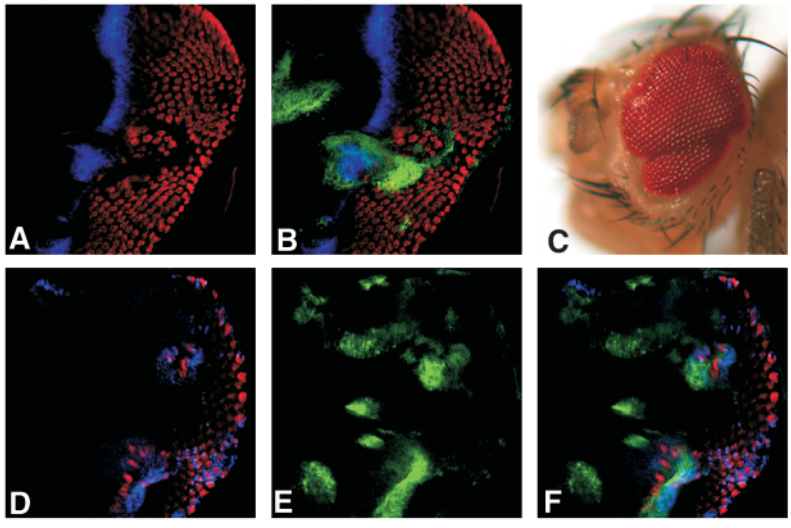

Fig. 4. *hyd* regulates *dpp* but not *hh* through Ci. (A,B,D-F) Third instar eye discs with anti-Elav staining in red. $hyd^{K7.19}$ clones expressing UAS-ci_{76} with *tub*-GAL4 are positively marked by GFP expression in B,E,F. (A,B) Anti-β-galactosidase staining reflecting *dpp-lacZ* expression in blue. (D,F) Anti-β-galactosidase staining reflecting *hh-lacZ* expression in blue. Expression of Ci_{76} in *hyd* clones blocks *dpp* expression but not *hh* expression. (C) An adult eye with $hyd^{K7.19}$, hh^{rJ413} clones expressing UAS-ci_{76}. No overgrowth is visible.

Loss of *hyd* causes accumulation of Ci but not Smoothened

If *hyd* regulates *dpp* expression by altering Ci activity, loss of *hyd* should lead to upregulation of full-length, active Ci. We indeed observed increased levels of full-length Ci in *hyd* mutant clones in the anterior of the eye disc (data not shown). However, this could be due to misexpression of *hh* in the same clones. To determine whether *hyd* has a direct effect on Ci, we examined *hyd hh* double mutant clones anterior to the morphogenetic furrow. High levels of full-length Ci accumulated in these clones (Fig. 5A-C), confirming that Hyd normally reduces Ci levels independently of Hh activity.

Hyd might act directly on Ci to promote its proteolytic cleavage or degradation. An alternative possible target for Hyd activity is Smoothened (Smo). Smo is a transmembrane protein that acts positively in Hh signaling (Alcedo et al., 1996). Smo levels are kept low by the receptor protein Patched (Ptc) in the absence of Hh, but Smo is stabilized and localized to the membrane when Hh binds to Ptc (Alcedo et al., 2000; Denef et al., 2000; Ingham et al., 2000). To test whether Hyd normally contributes to Smo degradation, we stained eye discs containing *hyd*, *hh* clones with Smo antibody. No Smo accumulation was apparent in the clones (Fig. 5D-F). Thus loss of *hyd* leads to accumulation of full-length Ci without altering the level of Smo.

hyd regulates Ci and *hh* in the wing disc

Since *hyd* is expressed in the wing disc and is required for its normal growth (Mansfield et al., 1994), we tested whether its effects on wing development might also be mediated by alterations in *hh* expression and Ci levels. In wild-type wing discs, *hh* is expressed uniformly throughout the posterior compartment of the wing pouch, while *dpp* is expressed in the anterior compartment in a stripe along the AP border (Basler and Struhl, 1994). Ci_{155} is present at high levels in a similar stripe at the AP border and at lower levels elsewhere in the anterior compartment (Motzny and Holmgren, 1995). Expression of *hh*, *dpp* and Ci_{155} in *hyd* clones remained restricted to the correct compartment. However, some *hyd* mutant clones in the posterior compartment expressed elevated levels of *hh-lacZ* (Fig. 6A,B). This misexpression of *hh* was correlated with a rounded shape and apparent overgrowth of the clones. The only known regulator of *hh* expression in the wing disc is Ci, which is restricted to the anterior compartment by En-mediated repression; Ci_{76} represses *hh* there (Dominguez et al., 1996; Methot and Basler, 1999; Schwartz et al., 1995). These results suggest that a Ci-independent activator of *hh* expression must be present in the posterior compartment and kept in check by Hyd activity.

In addition, Ci_{155} was upregulated in anterior *hyd* mutant clones (Fig. 6E,F). In contrast to the eye disc, we did not observe any *hh* misexpression in anterior *hyd* clones (Fig. 6A,B); thus Ci upregulation in *hyd* clones must be independent of *hh*. This is consistent with our findings that *hyd* regulates Ci and *hh* independently in the eye disc. Smo levels were not significantly increased in *hyd* mutant clones in the anterior compartment of the wing disc (Fig. 6C,D), suggesting that as in the eye disc, *hyd* affects Ci independently of Smo.

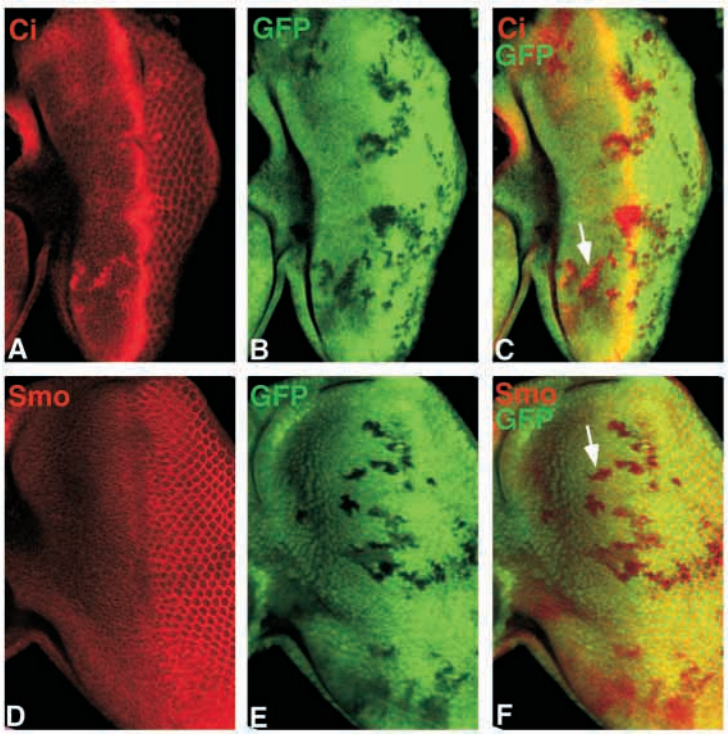

Fig. 5. Ci, but not Smo, accumulates in the absence of *hyd* and *hh*. All panels show third instar eye discs with $hyd^{K3.5}$, hh^{AC} mutant clones (marked by lack of GFP expression in B,C,E,F). (A,C) Staining with an antibody to the C-terminal region of Ci. (D,F) Staining with anti-Smo. Arrows indicate examples of double mutant clones that do not accumulate Ci (C) and do not accumulate Smo (F).

The F-box protein Slmb has been shown to promote processing of Ci to Ci_{75} as a component of an SCF ubiquitin ligase complex (Jiang and Struhl, 1998; Miletich and Limbourg-Bouchon, 2000; Noureddine et al., 2002; Theodosiou et al., 1998). We therefore compared the effects of *slmb* and *hyd* mutations on Ci levels in the wing disc. Ci_{155} was much more dramatically increased in *slmb* clones than in *hyd* clones (Fig. 6I,J). We also observed an interesting difference between *hyd* and *slmb* in the regulation of *dpp*. *dpp* expression was increased in *hyd* mutant clones close to the AP border, but was very rarely activated outside this domain (Fig. 6G,H). In contrast, *slmb* mutant clones activated *dpp* expression only when they lay outside the wing pouch (Fig. 6K,L) (Miletich and Limbourg-Bouchon, 2000), perhaps because of activation of Wg signaling, which represses *dpp* expression, within the wing pouch (Jiang and Struhl, 1998). Consistent with these third instar phenotypes, we have not observed anterior duplications like those resulting from loss of *slmb* in adult wings containing *hyd* mutant clones, although outgrowths did arise from internal regions of the wing (data not shown). Such duplications would require *dpp* to be misexpressed at a distance from its normal domain of expression (Zecca et al., 1995). Ptc expression, which requires activation of the full-length form of Ci (Methot and Basler, 1999), was not altered in either *hyd* or *slmb* mutant clones (Wang et al., 1999) (data not shown). Slmb and Hyd thus appear to have distinct effects on Ci protein accumulation and activity, suggesting that they have either different substrates or different effects on the same substrate.

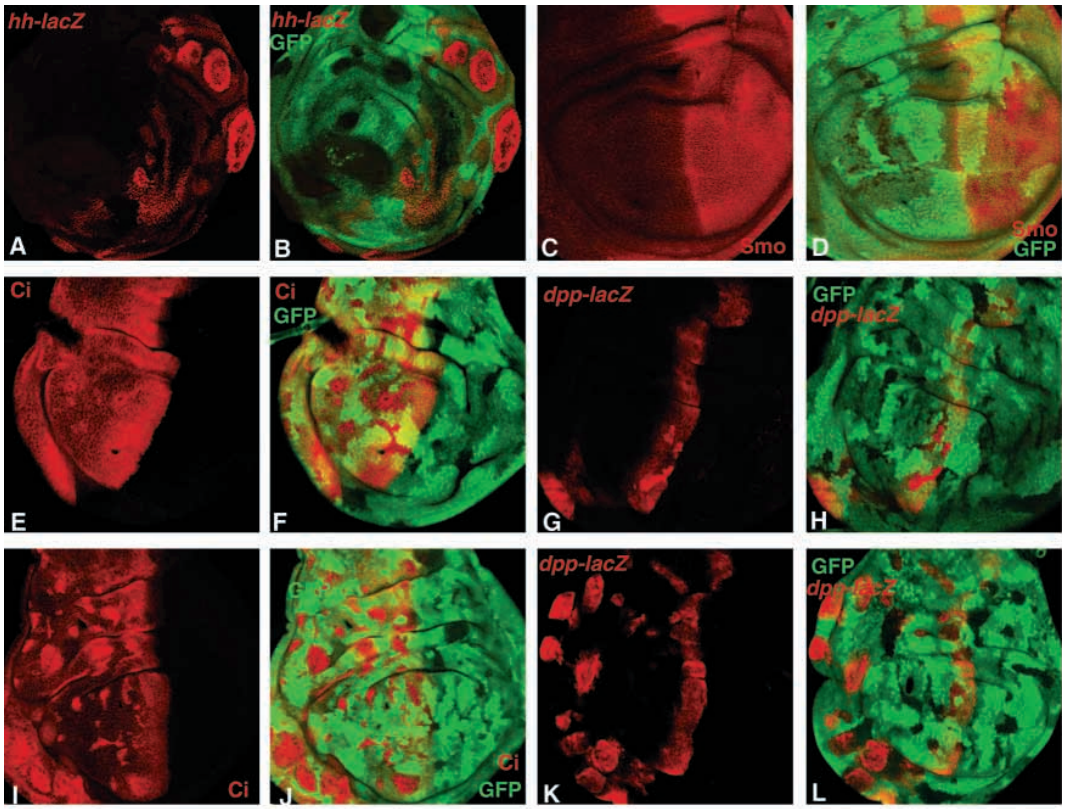

Fig. 6. *hyd* affects *hh* expression and Ci accumulation in the wing disc. All panels show third instar wing discs with anterior to the left and dorsal up. (A,B) $hyd^{K7.19}$ mutant clones (marked by lack of GFP expression in B). *hh-lacZ* is shown in red and is increased in round posterior clones. (C-H) $hyd^{K3.5}$ mutant clones (marked by lack of GFP expression in D,F,H). Staining with anti-Smo is shown in red in C and D and staining with an antibody to the C-terminal region of Ci is shown in red in E,F. Anterior *hyd* mutant clones accumulate higher levels of full-length Ci, but do not accumulate Smo protein. (G,H) *dpp-lacZ* expression (in red). *dpp* expression is increased in clones near the AP boundary, but *dpp* is not activated in more anterior clones. (I-L) $slmb^{B93}$ mutant clones (marked by lack of GFP expression in J,L). Staining with an antibody to the C-terminal region of Ci is shown in red in I,J and is stronger than in *hyd* mutant clones. (K,L) *dpp-lacZ* expression (in red). *dpp* is misexpressed only in anterior clones that lie outside the wing pouch.

DISCUSSION

hyd independently regulates *hh* and *dpp* expression

We have shown that *hyd* acts as a negative regulator of both *hh* and *dpp* expression in the anterior of the *Drosophila* eye disc. Loss of *hyd* function leads to the ectopic expression of both genes, resulting in non autonomous overgrowth of the disc and premature photoreceptor differentiation that propagates into the surrounding tissue. Our ability to suppress this overgrowth by preventing both expression of *hh* and activation of the Hh pathway indicates that the hyperplastic effects of *hyd* in the eye are entirely mediated by Hh signaling. This is probably not the case in the wing disc; in this tissue *hh* and *dpp* expression are also blocked by *hyd*, but *hyd* clones in the posterior compartment can autonomously overgrow without expressing Ci, the transcription factor required for the response to Hh (Fig. 6A-D). *hyd* may thus have an independent effect on the cell cycle in wing disc cells. The human homologue of *hyd*, EDD, is located in a chromosomal region that is disrupted in a variety of cancers (Callaghan et al., 1998). It will be of interest to determine whether loss of *hyd* is responsible for any of these syndromes, and if so, whether the tumorous growth can be attributed to misregulation of *hh* or *dpp* homologues.

Little is known about the control of *hh* expression in the eye disc. In the embryo and other imaginal discs *hh* expression is controlled by *en*, which defines the posterior compartment in a lineage-dependent manner. The eye disc has no anterior-posterior compartment boundary, and loss of *en* function in the eye has no effect (Strutt and Mlodzik, 1996). Since *hh* expression is repressed by Ci_{76} in the anterior wing disc (Dominguez et al., 1996; Methot and Basler, 1999), it seemed possible that this was also the case in the eye disc; Hh could then activate its own expression in more anterior cells by blocking Ci cleavage. However, we were not able to prevent *hh* expression by providing Ci_{76} to *hyd* mutant cells in the eye disc, although this did suffice to repress *hh* target genes such as *dpp*. In agreement with this result, *ci* mutant clones anterior to the furrow do not induce ectopic differentiation (N. Baker, personal communication), indicating that loss of the repressor form of Ci is not sufficient to allow *hh* transcription in the eye disc. *hyd* must therefore be a component of the Ci-independent mechanism that restricts *hh* expression.

Regulation of *hh* by *hyd* is also clearly independent of Ci in the wing disc, since loss of *hyd* leads to *hh* upregulation in the posterior compartment, where Ci is not present, and not in the anterior compartment. The Groucho (Gro) corepressor has been proposed to contribute to a Ci-independent mechanism of *hh* repression in cells close to the compartment boundary (Apidianakis et al., 2001). However, the effects of loss of *hyd* differ from those of loss of *gro*, which affects only the anterior compartment of the wing disc (Apidianakis et al., 2001) and promotes excessive photoreceptor differentiation only posterior to the furrow in the eye disc (Chanut et al., 2000), suggesting that a third mechanism of *hh* regulation may exist. *hh* expression may not be merely a default state resulting from the absence of the Ci repressor and Gro, but may require another activator, the levels of which are normally kept in check by Hyd.

Control of *dpp* expression by *hyd*, in contrast, appears to be mediated by Ci. Ci_{155} is upregulated in *hyd* mutant cells in the eye disc in a *hh*-independent manner, and ectopic *dpp* expression in these cells can be blocked by Ci_{76}. In the wing disc, *dpp* misexpression is limited to the *ci*-expressing anterior compartment, and is again associated with upregulation of Ci_{155}. Thus *hyd* acts on Hh signaling as well as *hh* expression, preventing full activation of the Hh pathway in anterior cells (Fig. 7).

Targets of *hyd* function

Hyd is likely to act as an E3 ubiquitin ligase; its human homologue has been shown to ubiquitinate at least one substrate in vitro (Honda et al., 2002). The substrate specificity of HECT domain E3 ubiquitin ligases appears to reside within their unique N-terminal domains (Ciechanover et al., 2000), making it difficult to predict the sequence or structure recognized by Hyd. The only potential clue is that Hyd contains a peptide-binding domain homologous to the C-terminus of poly(A)-binding protein (PABP) (Callaghan et al., 1998; Kozlov et al., 2001); the human HYD protein can interact with Paip1, a binding partner of PABP (Craig et al., 1998; Deo et al., 2001).

Hyd independently regulates *hh* and *dpp* expression, suggesting either that Hyd has multiple substrates or that its substrate has multiple functions. *dpp* expression in *hyd* clones is blocked by Ci_{76}, placing the effect of Hyd on *dpp* upstream or at the level of Ci activity. Ci_{155} but not Smo accumulates to high levels in *hyd hh* mutant cells in the eye disc and in anterior cells in the wing disc; thus Hyd may act on Ci itself, on a component of the Hh pathway between Smo and Ci, or on a nuclear cofactor that stabilizes Ci. Consistent with an effect on Ci or a cofactor, Hyd protein has been detected in both the cytoplasmic and nuclear compartments (Mansfield et al., 1994). The human Hyd homolog EDD appears to be predominantly localized in the nucleus, where it interacts with the progesterone receptor and DNA topoisomerase II-binding protein (Henderson et al., 2002; Honda et al., 2002). Ubiquitination can function to enhance the potency of transcriptional activation domains (Salghetti et al., 2001); however, the ectopic gene expression observed in *hyd* mutant clones would be difficult to explain by this mechanism.

The SCF ubiquitin ligase complex containing the F-box protein Slmb and the RING finger protein Roc1 has been implicated in the ubiquitination of Ci that mediates its processing to Ci_{75} (Jiang and Struhl, 1998; Noureddine et al., 2002; Theodosiou et al., 1998; Wang et al., 1999). There are several possible explanations for the apparent overlap between Slmb and Hyd functions. Slmb may directly ubiquitinate Ci, while Hyd acts on another substrate; the more dramatic effect of *slmb* than *hyd* clones on Ci accumulation argues for this possibility. However, it is also possible that Hyd, rather than Slmb, is the direct ubiquitin ligase for Ci. The consensus sequence for Slmb is not present in Ci, although it has been suggested that several weakly matching sequences might suffice for its recognition (Price and Kalderon, 2002; Winston et al., 1999). In addition, two groups have obtained inconsistent genetic evidence as to whether *slmb* acts upstream or downstream of *smo* and *protein kinase A* (*PKA*) (Jiang and Struhl, 1998; Theodosiou et al., 1998; Wang et al., 1999). It is unlikely that Hyd and Slmb carry out the same process in different cells, as *hyd* is expressed throughout the wing pouch (Mansfield et al., 1994), and *slmb* is clearly active in the same region (Fig. 6I) (Jiang and Struhl, 1998), although its expression has not been examined. Finally, Hyd and Slmb could both act on Ci, either additively or with different outcomes. For example, ubiquitination by Slmb promotes processing of Ci to Ci_{75} (Jiang and Struhl, 1998), while ubiquitination by Hyd might promote complete degradation of the Ci protein. Unfortunately, we were not able to obtain large enough quantities of *hyd hh* mutant tissue to test this possibility directly by western blotting. The lack of *hh* misexpression in *hyd* clones in the anterior compartment of the wing disc suggests that these cells still contain Ci_{75} as well as Ci_{155} (Methot and Basler, 1999); loss of *hyd* may therefore stabilize both forms of the Ci protein rather than altering their ratio. However, this is not a definitive test of *hyd* function, as loss of *PKA* does not lead to ectopic *hh-lacZ* expression despite its effect on Ci processing (Jiang and Struhl, 1995; Jiang and Struhl, 1998; Li et al., 1995; Pan and Rubin, 1995). Hyd-mediated degradation of both forms of Ci, or its redundancy with Slmb for Ci cleavage, would explain the limited effect on *dpp* expression in *hyd hh* double mutant clones in the eye disc and in *hyd* mutant clones in the wing disc; *dpp* misexpression in these cases is restricted to a region in which endogenous Hh may contribute to altering the ratio of the two forms of Ci.

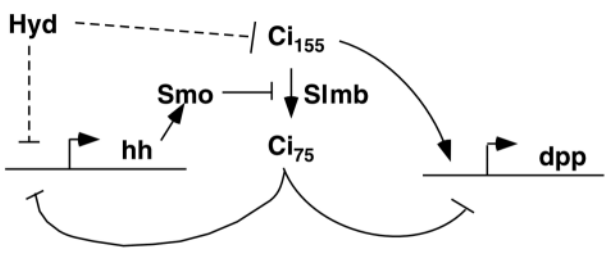

Fig. 7. Model for Hyd function. Arrows represent positive effects, and barred lines represent negative effects. Hyd represses *hh* transcription, probably indirectly. Hyd also blocks the accumulation of full-length Ci without affecting Smo. Since Slmb has been implicated in processing Ci to the repressor form Ci_{75}, we suggest that Hyd may instead act by targeting Ci for degradation.

Ubiquitination is a mechanism commonly used to regulate protein activity by targeting proteins for degradation or processing, during the cell cycle and in a number of signaling pathways (reviewed in Ciechanover et al., 2000). The Slmb-containing SCF complex is also required for the degradation of Armadillo (Arm)/β-catenin, allowing Wnt signaling (Hart et al., 1999; Jiang and Struhl, 1998; Miletich and Limbourg-Bouchon, 2000; Winston et al., 1999), as well as for the degradation of IkappaB (Spencer et al., 1999; Yaron et al., 1998). Hyd is unlikely to act on Arm in the eye disc, as Arm accumulation would prevent the ectopic photoreceptor differentiation seen in *hyd* mutant clones (Lee and Treisman, 2001a; Treisman and Rubin, 1995); this may indicate another difference in the substrate specificity of Hyd and Slmb. However, it is possible that *hyd* affects Wg signaling in the wing disc, as *hyd* mutant clones can induce ectopic expression of the Wg target gene *scute* (K. A., unpublished data). The HECT domain ligases Smurf1 and Smurf2 are important antagonists of BMP signaling, promoting downregulation of both Smads and receptors (Kavsak et al., 2000; Podos et al.,

2001; Zhu et al., 1999). Itch/Suppressor of deltex, another HECT domain ligase, ubiquitinates Notch (Cornell et al., 1999; Fostier et al., 1998; Qiu et al., 2000). In addition, nuclear Notch is degraded by Sel-10, while the ligand Delta is the target of ubiquitination by Neuralized (reviewed by Lai, 2002). Our placement of *hyd* within the Hh pathway and upstream of *hh* expression expands this growing list of cases in which signaling pathways are regulated by ubiquitination.

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