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

Jeffrey D. Lee1, Kazuhito Amanai2,*, Allen Shearn2 and Jessica E. Treisman1,†

1Skirball Institute of Biomolecular Medicine and Department of Cell Biology, NYU School of Medicine, 540 First Avenue, New York, NY 10016, USA
2Department of Biology, Johns Hopkins University, 3400 North Charles Street, Baltimore, MD 21218, USA
*Present address: Center for Developmental Biology and Department of Pharmacology, UT Southwestern Medical Center, 6000 Harry Hines Boulevard, Dallas, TX 75390, USA
†Author for correspondence (e-mail: treisman@saturn.med.nyu.edu)

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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 Smoothed, 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. Inter cellular 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 coordinate 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 (Ci155) is proteolytically processed to a 75 kDa transcriptional repressor (Ci75); when the Hh signal is received this processing event is inhibited, allowing the accumulation of Ci155 (Aza-Blanc et al., 1997). In the anterior compartment, Ci75 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 Ci155 and repressed by Ci75, 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 hh or slmb males were crossed to eyFLP1 or hsFLP122; FRT82, arm-lacZ or FRT82, Ubi-GFP females. To make clones in a Minute background, the females used were eyFLP1; FRT, arm-lacZ, M(3)96C. To make positively labeled clones, FRT82, hyd males were crossed to UASGFP, eyFLP1 or hsFLP122; 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 hsFLP122 was used were heat-shocked at the first and second instar stage for 1 hour at 38.5°C. The alleles used were hh*1141 (Heberlein et al., 1993), hh*4C (Lee et al., 1992), hh*12 (Ma et al., 1993), slmb*93 (Lee and Treisman, 2001a) and atu*102 (J. D. Lee, unpublished). Transgenes used were tub-GAL4, tub-GAL80 (Lee and Luo, 1999) and UAS-ci76 (Aza-Blanc et al., 1997). Reporters used were dpp-lacZ (Blackman et al., 1991) and hh*-lacZ (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 (Hazelet 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 (1:200) (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. 1H), 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.

**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
atoh 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.

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(K1031) (Heberlein et al., 1993), hh(h2) (Ma et al., 1993) grown at 29°C, and the null allele hh(hc) (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
Hyd represses hh and dpp expression

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 Ci75, which inhibits dpp expression, and by activating Ci155, which activates dpp expression (Methot and Basler, 1999).

In addition, Ci75 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 (Ci76) (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-ci76 in or anterior to the furrow (Fig. 4A,B). However, Ci76 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 Ci76 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 Ci76 into the mutant cells. Indeed, when we generated hyd hh mutant clones expressing UAS-ci76, 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. 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 hydK3.5 mutant clones. (B) hh^J413 clones; lack of photoreceptor differentiation is only apparent in clones at the posterior margin (arrow). (C) hydK3.5, hh^J413 double mutant clones. No ectopic photoreceptors form, but some clones still induce overgrowth of wild-type tissue (arrow). (D) hydK3.5, hh^J413 clones generated in a Murne background. The few photoreceptors that form are in wild-type tissue. (E) An adult eye containing hyd^K3.5, hh^J413 clones and exhibiting some overgrowth (arrow). (F) Unmarked hydK3.5 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^J413 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).

Fig. 4. hyd regulates dpp but not hh through Ci.
(A,B,D-F) Third instar eye discs with anti-Elav staining in red. hydK7.19 clones expressing UAS-ci76 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 Ci76 in hyd clones blocks dpp expression but not hh expression. (C) An adult eye with hydK7.19, hh^J413 clones expressing UAS-ci76. 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). Ci155 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 Ci155 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 outgrowth 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; Ci76 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, Ci155 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.

The F-box protein Slmb has been shown to promote processing of Ci to Ci155 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. Ci155 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. 5. Ci, but not Smo, accumulates in the absence of hyd and hh. All panels show third instar eye discs with hydK3.5, hhK6C 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).
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<sup>K7.19</sup> 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<sup>K5.5</sup> 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<sup>B93</sup> 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<sub>76</sub> 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<sub>76</sub> 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. Ci155 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 Ci76. In the wing disc, dpp misexpression is limited to the ci-expressing anterior compartment, and is again associated with upregulation of Ci155. 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 Ci76, placing the effect of Hyd on dpp upstream or at the level of Ci activity. Ci155 but not Smo accumulates to high levels in hh 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 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 Ci75 (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 Ci75 (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 Ci75 as well as Ci155 (Metot 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.

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.,...
REFERENCES


