Deciphering synergistic and redundant roles of Hedgehog, Decapentaplegic and Delta that drive the wave of differentiation in *Drosophila* eye development

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Summary

In *Drosophila*, a wave of differentiation progresses across the retinal field in response to signals from posterior cells. Hedgehog (Hh), Decapentaplegic (Dpp) and Notch (N) signaling all contribute. Clones of cells mutated for receptors and nuclear effectors of one, two or all three pathways were studied to define systematically the necessary and sufficient roles of each signal. Hh signaling alone was sufficient for progressive differentiation, acting through both the transcriptional activator Ci155 and the Ci75 repressor. In the absence of Ci, Dpp and Notch signaling together provided normal differentiation. Dpp alone sufficed for some differentiation, but Notch was not sufficient alone and acted only to enhance the effect of Dpp. Notch acted in part through downregulation of Hairy; Hh signaling downregulated Hairy independently of Notch. One feature of this signaling network is to limit Dpp signaling spatially to a range coincident with Hh.

Key words: *Drosophila* eye, Morphogenetic furrow, Hedgehog, Decapentaplegic, Notch, Delta

Introduction

The adult eye of *Drosophila* is remarkable for a wave of differentiation that spreads from posterior to anterior across the retina field of the eye imaginal disc. Eight hundred or so ommatidia begin differentiation in about 30 columns. Adjacent columns begin differentiation approximately 90 minutes apart, so that it takes about two days before the entire retinal field is differentiating. The advancing anterior boundary of the differentiating region is morphologically recognizable from an indentation known as the morphogenetic furrow, and is associated with changes in cell cycle, adhesion and gene expression (Wolff and Ready, 1993; Heberlein and Moses, 1995; Lee and Treisman, 2002). A potentially similar wave of differentiation also occurs in zebrafish retina (Neumann and Nusslein-Volhard, 2000; Stenkamp et al., 2000).

Progression of the morphogenetic furrow depends primarily on Hedgehog, which is secreted by differentiating photoreceptor neurons. Hedgehog is necessary for furrow progression (Ma et al., 1993). Ectopic Hh expression is sufficient to initiate ectopic furrows in the anterior undifferentiated region (Heberlein et al., 1995). Despite the primary role of Hh, cells unable to receive Hh signals are still able to differentiate because Hh triggers the secretion of secondary signals, and cells unable to respond to Hh still respond to these other signals (Strutt and Mlodzik, 1997). Dpp is one important secondary signal, and acts redundantly with Hh. Cells must be able to respond to either Hh or Dpp to begin differentiating (Heberlein et al., 1993; Greenwood and Struhl, 1999; Curtiss and Mlodzik, 2000). It has been proposed that Notch signaling also contributes (Baker and Yu, 1997; Li and Baker, 2001). Ectopic Notch and Dpp together can initiate differentiation as effectively as ectopic Hh does (Baonza and Freeman, 2001).

In this paper we sought to define the individual roles and interactions of each signal through the study of loss-of-function mutations affecting response to Hh, Dpp or N signals, both alone and in combination. We also sought to determine the basis of the redundancy between Hh and Dpp, and how the two signals replace each other, and we investigated whether there is redundancy between Hh and N, and for what events , if any, each signal is individually sufficient in the absence of the others.

Although redundant functions can be studied through ectopic expression experiments, a loss-of-function approach that removes components from the redundant pathways has the advantage of addressing gene function at the normal time and place, and at normal expression levels. As each of Hh, Dpp and N signals are important many times during *Drosophila* development from embryogenesis onwards, it was necessary to use a conditional genetic approach. We employed mosaic analysis using the FLP/FRT system to obtain clones of retinal cells lacking function of Hh, Dpp and N pathway components. Each of these genes also plays roles in the initiation of the morphogenetic furrow at the posterior margin of the eye field, so that furrow initiation fails when eye margin cells are mutated, leaving the region to the anterior undifferentiated (Lee and Treisman, 2002). The present study is therefore restricted to the progressive onset of differentiation by cells within the retinal field.

Initially, we found that removing Smo did not prevent accumulation of the Ci activator Ci155. However, we found

that differentiation of Smo-mutant cells depended not on Ci, but on Dpp and N signaling. Perhaps because Dpp and Hh would otherwise act over different ranges, the pace of furrow progression is constrained by inhibitors, such as Hairy, which are themselves regulated by Dpp, Hh and N.

Materials and methods

Mosaic induction

Clones of cells mutant for relevant genes were obtained by FLP-mediated mitotic recombination (Golic, 1991; Xu and Rubin, 1993). Homozygous mutant cells were identified through lack of Ci155 antibody staining, or the absence of the transgene-encoded markers armβ-gal or hsGFP (Vincent et al., 1994; Motzny and Holmgren, 1995; Methot and Basler, 1999).

smo

Clones were obtained in:
eyF; *smo*^{3} FRT40/[*armlacZ*] FRT40,
hsF; *smo*^{3} FRT40/[*armlacZ*] FRT40,
hsF; *smo*^{D16} FRT40/[*armlacZ*] FRT40, and
y hsF; *smo*^{1} FRT42 *en* /*smo*^{3} FRT42 [*smo*$^{+}$ *hs:Gfp*] [*ci*$^{+}$]; *ci*^{94} /Dp(1;4)y^{+} *spa* larvae with equivalent results. *smo*^{3} and *smo*^{D16} are both null alleles (Chen and Struhl, 1998).

smo tkv

Clones were obtained in:
hsF; *smo*^{3} *tkv*^{strII} FRT40/ M [*armlacZ*] FRT40 larvae. *tkv*^{strII} is a null allele (Greenwood and Struhl, 1999).

tkv

Clones were obtained in:
hsF; *tkv*^{a12} FRT40/ M [*armlacZ*] FRT40 larvae as described (Burke and Basler, 1996). *tkv*^{a12} is a null allele (Penton et al., 1994).

smo Mad

Clones were obtained in:
hsF; *smo*^{3} *Mad*$^{1-2}$ FRT40/[*armlacZ*] FRT40 larvae as described (Curtiss and Mlodzik, 2000). *Mad*$^{1-2}$ is an insertion in *Mad* regulatory sequences that prevents most Dpp signaling with little effect on growth (Wiersdorff et al., 1996).

ci

Clones were obtained in:
y hsF; FRT42 [*ci*$^{+}$]/FRT42; *ci*^{94} larvae as described (Methot and Basler, 1999). *ci*^{94} is a null allele from which the promoter region and first exon have been deleted, including start sites for transcription and translation (Slusarski et al., 1995; Methot and Basler, 1999).

smo ci

Clones were obtained in:
y hsF; *smo*^{1} FRT42 *en* /*smo*^{3} FRT42 [*smo*$^{+}$ *hs:Gfp*] [*ci*$^{+}$]; *ci*^{94} larvae as described (Methot and Basler, 1999). Control *smo* clones were obtained in parallel from phenotypically *y*$^{+}$ larvae:
y hsF; *smo*^{1} FRT42 *en* /*smo*^{3} FRT42 [*smo*$^{+}$ *hs:Gfp*] [*ci*$^{+}$]; *ci*^{94} /Dp(1;4)y^{+} *spa*.

Mad ci

Clones were obtained in:
y hsF; *Mad*$^{1-2}$ FRT40/[*ci*$^{+}$] FRT40; *ci*^{94} larvae. This [*ci*$^{+}$] transgene was provided by R. Holmgren.

tkv ci

Clones were obtained in:
y hsF; *tkv*^{a12} FRT40/[*ci*$^{+}$] FRT40; *ci*^{94}, and
y hsF; *tkv*^{a12} FRT40/M21 [*ci*$^{+}$] FRT40; *ci*^{94} larvae.

Su(H) ci

Clones were obtained in:
y hsF; *Su(H)*$^{\Delta 47}$ FRT40 [*w*$^{+}$ *l(2)35Bg*$^{+}$]/[*ci*$^{+}$] FRT40; *ci*^{94} larvae. *Su(H)*$^{\Delta 47}$ is a 1.9 kb deletion that removes the *Su(H) l(2)35Bg* intergenic region, including the transcriptional start site and ATG codon of both genes, and is a null allele (Morel and Schweisguth, 2000).

Su(H)

Clones were obtained in:
y hsF; *Su(H)*$^{\Delta 47}$ FRT40 [*w*$^{+}$ *l(2)35Bg*$^{+}$]/[*armlacZ*] FRT40 larvae.

Mad Su(H) ci

Clones were obtained in:
y hsF; *Mad*$^{1-2}$ *Su(H)*$^{\Delta 47}$ FRT40 [*w*$^{+}$ *l(2)35Bg*$^{+}$]/[*ci*$^{+}$] FRT40; *ci*^{94} larvae.

Ci misexpression

Fng:Gal4 (gift of M. Mlodzik) was used to misexpress ci derivatives from UAS transgenes. We also used the Gal4 line *hairy*^{H10} to misexpress UAS:ci^{Cell} anterior to the morphogenetic furrow (Ellis et al., 1994).

Antibody labelling

Eye discs were labelled for Atonal as described (Lee et al., 1996). Other labelling was performed either in 0.1 M sodium phosphate (pH 7.2), 1% normal goat serum, 0.1% saponin, following a 45 minute fixation in cold PLP (Tomlinson and Ready, 1987), or in 0.1 M sodium phosphate (pH 7.2), 0.3% sodium deoxycholate, 0.3% Triton X-100, after fixation at room temperature in 3.7% formaldehyde, 100 mM PIPES (pH 6.95), 1 mM EGTA, 2 mM $MgSO_4$. Preparations were examined using BioRad MRC600 and Radiance 2000 confocal microscopes, and digital images were manipulated using Adobe PhotoShop 4.0 and NIH Image 1.62 software. Antibodies against β-galactosidase were obtained from Cappel and from the Developmental Studies Hybridoma Bank (mAb40-1), polyclonal rabbit anti-GFP was obtained from Molecular Probes, and other antibodies were obtained from their developers: anti-ato (Jarman et al., 1994); anti-Ci155 (mAb2A1) (Motzny and Holmgren, 1995); anti-senseless (Nolo et al., 2000); and anti-hairy antibodies (Brown et al., 1995). Preparations were obtained over several years. Consequently, variations in procedures, antibody batches, and confocal hardware and software make comparing signal intensity between preparations unreliable, except where specifically noted in the text.

Results

The crucial step initiating retinal differentiation is specification of a founder R8 photoreceptor cell within each ommatidium. Once specified, R8 cells initiate the recruitment of other retinal cells in response to the receptor tyrosine kinase Egf receptor and Sevenless, which are activated by signals emanating from the R8 cells (Tomlinson and Ready, 1987; Freeman, 1997; Nagaraj et al., 2002). R8 cells are specified by the proneural gene *atonal* (*ato*) (Jarman et al., 1994). Cell specification after R8 does not depend directly on Atonal, Hh or Dpp function, and can occur in cells genetically null for *atonal*, ahead of the morphogenetic furrow, or in cells unable to respond to Hh and Dpp, so long as R8 cells are present nearby (Jarman et al., 1994; Dominguez et al., 1998; Curtiss and Mlodzik, 2000).

R8 specification in the morphogenetic furrow is illustrated in Fig. 1. *senseless* and other target genes are expressed in response to rising Atonal activity (Baker, 2002). Atonal expression is transient, whereas Senseless is maintained in differentiating R8 cells throughout the eye disc (Nolo et al.,

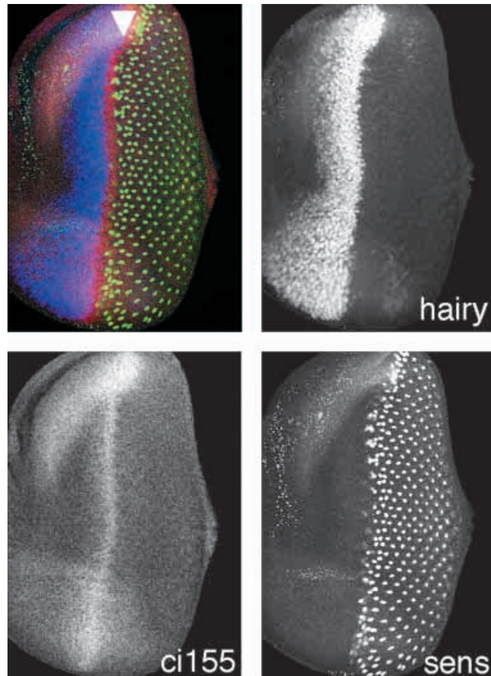


Fig. 1. Progressive eye differentiation. A wild-type eye imaginal disc labeled for gene products that reveal pattern and differentiation is shown with anterior to the left. Arrowhead indicates the center of the morphogenetic furrow that separates anterior undifferentiated and posterior differentiating portions of the eye disc. Ahead of the morphogenetic furrow the Hairy protein accumulates in a rising gradient (blue). Hairy is downregulated just anterior to the morphogenetic furrow, just as levels of Ci155 peak (red). Ci155 protein is stabilized in response to Hh signaling. As R8 specification occurs within the morphogenetic furrow, Atonal activity is reported by expression of the target gene *senseless* (green). Sens expression is also maintained in the differentiating R8 cells posterior to the furrow.

2000) (Fig. 1). *hairy* encodes a negative regulator of Atonal function, and is expressed ahead of the morphogenetic furrow and downregulated as differentiation begins (Fig. 1) (Brown et al., 1995). Notch activation downregulates Hairy (Baonza and Freeman, 2001). These events coincide with peak Hh signal at the anterior of the morphogenetic furrow, as revealed by accumulation of the Ci155 protein (Motzny and Holmgren, 1995; Strutt and Mlodzik, 1996) (Fig. 1).

Ectopic expression has identified roles of Dpp and N downstream and parallel to Hh (Pignoni and Zipursky, 1997; Baonza and Freeman, 2001). Mutations of Dpp receptors or N pathway genes do not affect the progression of differentiation (Burke and Basler, 1996; Baker and Yu, 1997). To ascertain the necessary and sufficient roles of Hh, Dpp and N signals at their normal time and place of action, and at endogenous expression levels, we have determined the effects on differentiation of mutations of *ci*, *Mad* and *Su(H)*, the essential transcription factor targets of Hh, Dpp and N, respectively, in all single-, double- and triple-mutant combinations. We also assessed the affects of some other genotypes. The complete results are summarized in Table 1. Overlap and redundancy between signals makes parallel comparison of all these results necessary in order to obtain a full picture (Table 1).

Table 1. Gene expression in mosaic clones of mutant cells

Clone	R8 differentiation	Hairy downregulation
smo	Absent at first Later recovery	Delayed in clone center
tkv smo	None	Delayed in clone center
smo Mad	None	Delayed in clone center
tkv	Normal	Normal
Mad	Normal	Normal
ci	Normal	Normal
smo ci	Normal	Not examined
Mad ci	Delayed	Delayed in clone center
tkv ci	None	Delayed in clone center
Mad Su(H) ci	None	No downregulation
Su(H) ci	Delayed (weakly neurogenic)	No downregulation
Su(H)	Normal or accelerated (strongly neurogenic)	Brief and partial delay
Mad Su(H)	Normal (strongly neurogenic)	Delayed

Dpp contributes to differentiation and Ci155 accumulation

Ato expression at first fails in clones mutant for the Hh transduction protein Smo. Ato is absent cell autonomously, consistent with Hh being the cell-cell signal that normally regulates the onset of Ato expression at this time. Several hours later, *smo*-mutant cells differentiate following the appearance of weak, delayed Ato expression (Strutt and Mlodzik, 1997; Dominguez, 1999) (Fig. 2A).

To test whether the *smo* mutations completely abolished Hh signaling, Ci155 accumulation was examined. As well as stimulating Ci155 transcriptional activator activity, Hh causes Ci155 accumulation by preventing SCF^{Slimb} from processing Ci155 to the repressor form Ci75 (Ingham and McMahon, 2001). Ci155 is specifically detected by monoclonal antibody 2A1, which does not recognize Ci75 (Motzny and Holmgren, 1995; Aza-Blanc et al., 1997). In the *smo*-mutant cells, Ci155 stabilization was not abolished but only delayed (Dominguez, 1999) (Fig. 2B). As the *smo* mutations have since been shown to be null (Chen and Struhl, 1998), a slower-acting, *smo*-independent mechanism of Ci155 stabilization was implied. Ci155 was later lost from the most posterior margins of *smo*-mutant clones (Fig. 2B).

Since *smo*-mutant cells differentiate in response to Dpp (Greenwood and Struhl, 1999; Curtiss and Mlodzik, 2000), we tested whether Ci155 was being stabilized by Dpp signaling. Ci155 levels were examined in clones of cells mutant for both *smo* and *tkv*, or for both *smo* and *Mad*. *tkv* encodes the Type I Dpp receptor, *Mad* encodes the essential transcription factor target. Ci155 did not accumulate when both Hh and Dpp signal reception was inactivated, indicating that Dpp signal reception was required to accumulate Ci155 proteins in the absence of Hh signal reception (Fig. 2C,D). A modest but reproducible reduction in Ci155 levels was seen in cells mutant for *tkv* alone, indicating that Dpp signaling through Tkv contributes to the level of Ci155, even in the presence of the Hh pathway (Fig. 2E).

Ci is dispensable

Dpp signaling might promote furrow progression and differentiation by regulating Ci155 processing to Ci75, as Hh does. If so, *ci*-mutant cells should resemble cells that are unable to respond to either Hh or Dpp, and thus would be unable to differentiate. An alternative model was that Dpp

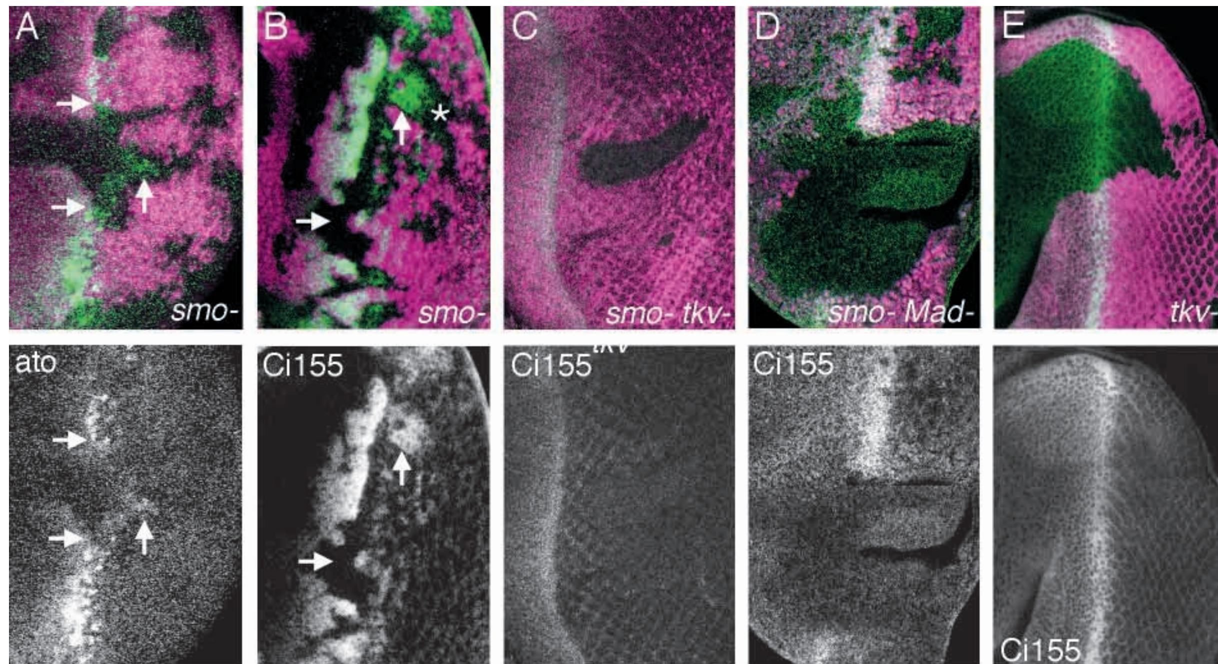


Fig. 2. Genetics of Ci155 accumulation. In A-E, clones of cells of the indicated mutant genotypes are identified by the absence of *lacZ* marker gene expression (magenta). The green channel shows Atonal protein in panel A and Ci155 for panels B-E. Absolute levels of Ci155 should not be compared between panels, as these preparations were recorded on widely different occasions. (A) Cells lacking *smo* fail to turn on *atonal* at the normal time anterior to the morphogenetic furrow (green; horizontal arrows). Lower Atonal levels that still suffice for R8 specification appear more posteriorly (vertical arrow). (B) Cells lacking *smo* fail to accumulate Ci155 ahead of the morphogenetic furrow (horizontal arrows). Ci155 does accumulate more posteriorly in the *smo*-mutant cells (vertical arrow), before disappearing as differentiation begins (e.g. asterix). (C) By contrast, Ci155 does not accumulate in cells mutant for both *smo* and *tkv*. (D) Ci155 does not accumulate in cells mutant for both *smo* and *Mad.* (E) There is a subtle but reproducible reduction in Ci155 levels in clones mutant for *tkv*, which is associated with little or no change in the pattern or timing of differentiation.

acted independently of its effects on Ci. If so, *ci*-mutant clones should exhibit delayed differentiation as do clones mutant for *smo*. Cells homozygous for a deletion of the *ci* gene were examined to distinguish between these two models.

Unexpectedly, clones of *ci*-mutant cells differentiated normally in all respects (Fig. 3A-C). The temporal and spatial pattern of *ato* expression was completely normal in *ci*-mutant cells, unlike in cells unable to respond to Hh or in cells unable to respond to Hh or Dpp (Fig. 3A). Normal neural differentiation occurred in *ci*-mutant cells without any delay (Fig. 3B and data not shown). Cells lacking *ci* were morphologically normal in adults, both externally and on sectioning to reveal differentiated cellular morphology (Fig. 3C). There were no defects in ommatidial chirality or planar polarity. The genotype of the *ci*-mutant cells was unequivocally confirmed using antibodies against Ci to detect *ci*-mutant cells directly (Fig. 3A). While this paper was under review, Pappu et al. also reported normal eye development by large clones lacking *ci* (Pappu et al., 2003).

Ci75 plays an important role

One possibility suggested by these results is a novel pathway of Hh signal transduction that is dependent on *smo* but not on *ci*. A second possibility is that Hh signaling is essential only to prevent formation of the repressor protein Ci75. If Ci155 was unimportant, deletion of the entire *ci* gene could mimic Hh signaling, by eliminating Ci75.

If *smo* signals independently of *ci* in the eye, *smo ci* clones should show delayed differentiation like *smo* clones (e.g. Fig. 3D). If *smo* is only essential to eliminate Ci75, *smo ci* clones should develop normally without any delay, like *ci* clones. Fig. 3E shows that *smo ci* clones developed normally like *ci*, and were not delayed like *smo* clones of the same size (Fig. 3D). Therefore, *smo* appeared to be essential only to prevent processing of Ci to the Ci75 repressor protein.

We targeted ectopic expression of different forms of Ci protein to the developing eye. The Gal4 line $hairy^{H10}$ was used to target UAS:ci^{Cell} expression anterior to the morphogenetic furrow (Ellis et al., 1994). ci^{Cell} encodes a Ci protein truncated at amino acid 975 and mimics Ci75 (Methot and Basler, 1999). Atonal expression was reduced, although eye patterning occurred normally (data not shown). Fng:Gal4 (a gift of M. Mlodzik) was used to misexpress UAS:ci^{Cell} ventrally (Fig. 3F). When expressed under the control of Fng:Gal4, ci^{Cell} prevented furrow progression and differentiation in ventral cells, but permitted furrow progression across the dorsal region of the eye disc (Fig. 3G). Fng:G4 and h^{H10} were also used to drive expression of UAS:ci^{U}, which encodes a Ci protein with a deletion of amino acids 611-760, which is defective in processing to Ci75 and which behaves as a Hh-dependent activator protein (Methot and Basler, 1999), and UAS:ZnAD and UAS:ZnRD transgenes, in which the DNA binding domain of Ci was coupled to transcriptional activator and repressor domains, respectively (Hepker et al., 1997). These were

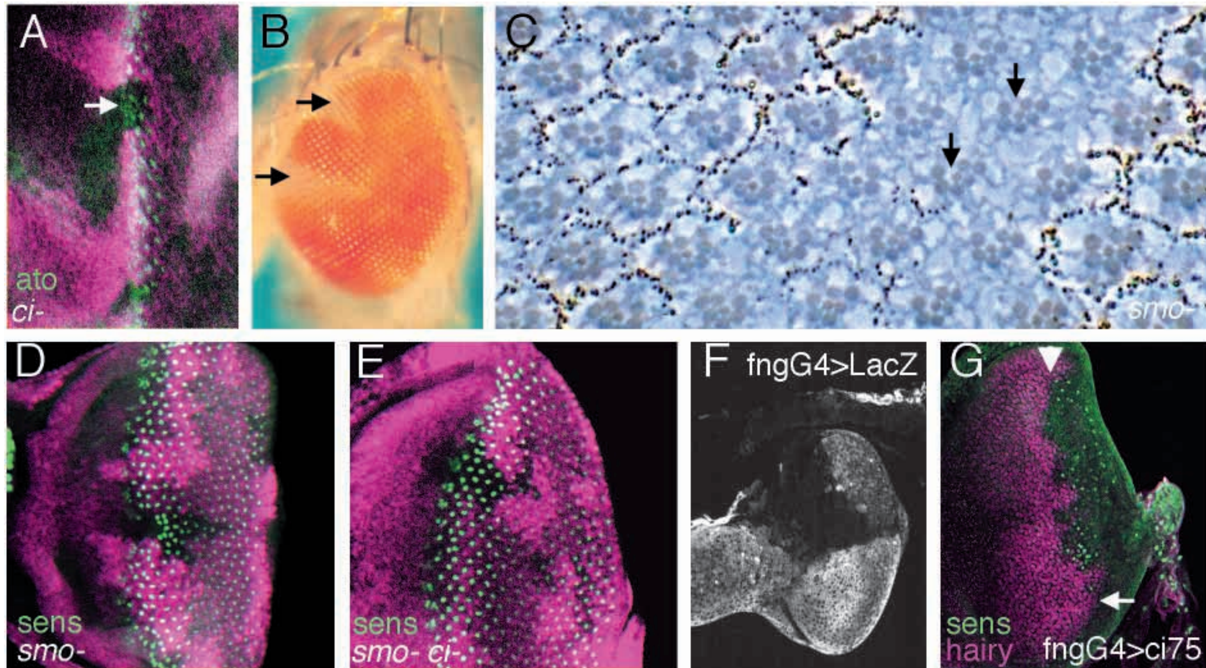


Fig. 3. Ci is dispensable for the patterning and progression of eye differentiation. (A) Ci155 in magenta; atonal in green. Initiation and progression of Atonal expression occurs normally in cells deleted for the *ci* gene. (B) Clones of cells lacking both eye pigmentation and Ci function contribute to normal adult eye structures (arrows). (C) Normal ommatidia are seen in sections through *ci*-null mutant clones marked by unpigmented pigment cells. A basal plane of section is shown so that normal R8 differentiation is apparent (arrows). The equator runs through the *ci*-mutant region. (D) Morphogenetic furrow progression is retarded through *smo*-mutant cells. The product of the Atonal target gene *sens* reveals both Atonal activity and subsequent differentiation of R8 cells (green). Cells mutant for *smo* lack the clone marker (magenta) and are also mutant for *engrailed* (*en*), a gene that is not required during eye development (Strutt and Mlodzik, 1996), see panel E. (E) Senseless expression and progression (green) occur completely normally in *smo ci*-mutant cells (also mutant for *en*). The clone is similar in size to the *smo* clone in panel E, induced simultaneously in a sibling larva (see Materials and methods for details). (F) Fng:Gal4 drives UAS-*lacZ* reporter gene expression in the ventral eye disc anterior to the morphogenetic furrow. Fng:Gal4 also drives expression more weakly in the posterior dorsal eye disc, away from the equator, expanding to reach the equator and morphogenetic furrow in the late third instar (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998) (M. Mlodzik, personal communication). (G) Ventral Ci75 expression retards furrow progression (arrowhead shows position of furrow dorsally, arrow indicates ventral delay).

without detectable effect (data not shown). Taken together, these findings suggested that differentiation might depend on blocking production of Ci75 in response to Hh or Dpp.

Ci-independent furrow progression in response to Dpp and N

There also had to be a *ci*-independent signal required to induce differentiation even in the absence of Ci. If differentiation depended entirely on eliminating Ci75, deletion of the *ci* gene would remove the barrier to differentiation anywhere in the eye field, but instead *ci*-mutant cells initiated differentiation in the same temporal progression as wild-type cells (Fig. 3A). *ci*-independent differentiation could not depend on Hh, because *smo ci* cells also initiated differentiation with precisely normal timing (Fig. 3E).

The second signal might be Dpp, acting independently of Ci. If this was correct, we would expect that *Mad ci* mutant cells would fail to differentiate. *Mad ci* mutant cells were examined and were found to differentiate, but such differentiation was delayed (Fig. 4A,B). This result confirmed that Dpp signaling through *Mad* contributed to differentiation in the absence of *ci*, but that some signal from posterior cells was still able to progress slowly through *Mad ci* mutant cells.

One possibility is that mutation of *Mad* did not completely abolish Dpp signaling. The *Mad*$^{1-2}$ allele, although similar to null alleles of *tkv* in effects on patterning, permits more normal growth than do *tkv*-null alleles and so retains a minimal response to Dpp. Perhaps this limited activity becomes significant in the absence of *ci*. Another possibility is that N signaling might contribute to differentiation in *Mad ci* cells. To distinguish whether N or residual Dpp signaling was responsible for the differentiation of *Mad ci*-mutant cells, we examined *tkv ci*-mutant cells, as well as cells triply mutant for *Su(H)*, *Mad* and *ci*.

Differentiation was not observed in *tkv ci*-mutant clones. Clones of cells mutant for both *tkv* and *ci* grew poorly, and only small clones were recovered (Fig. 4C,D). Such clones were rarely recovered in the posterior region of the eye disc, where they seemed to sort out. Large clones of *tkv ci*-mutant cells were obtained using the Minute Technique. These large clones always had round shapes, indicating that there was reduced mixing with wild-type cells throughout the eye disc (data not shown). No retinal differentiation was detected. These findings indicate that some Dpp signaling occurred in *Mad ci*-mutant cells, which contributed to the slow differentiation of *Mad ci* cells. Clones of *Mad Su(H) ci*-mutant cells also failed to

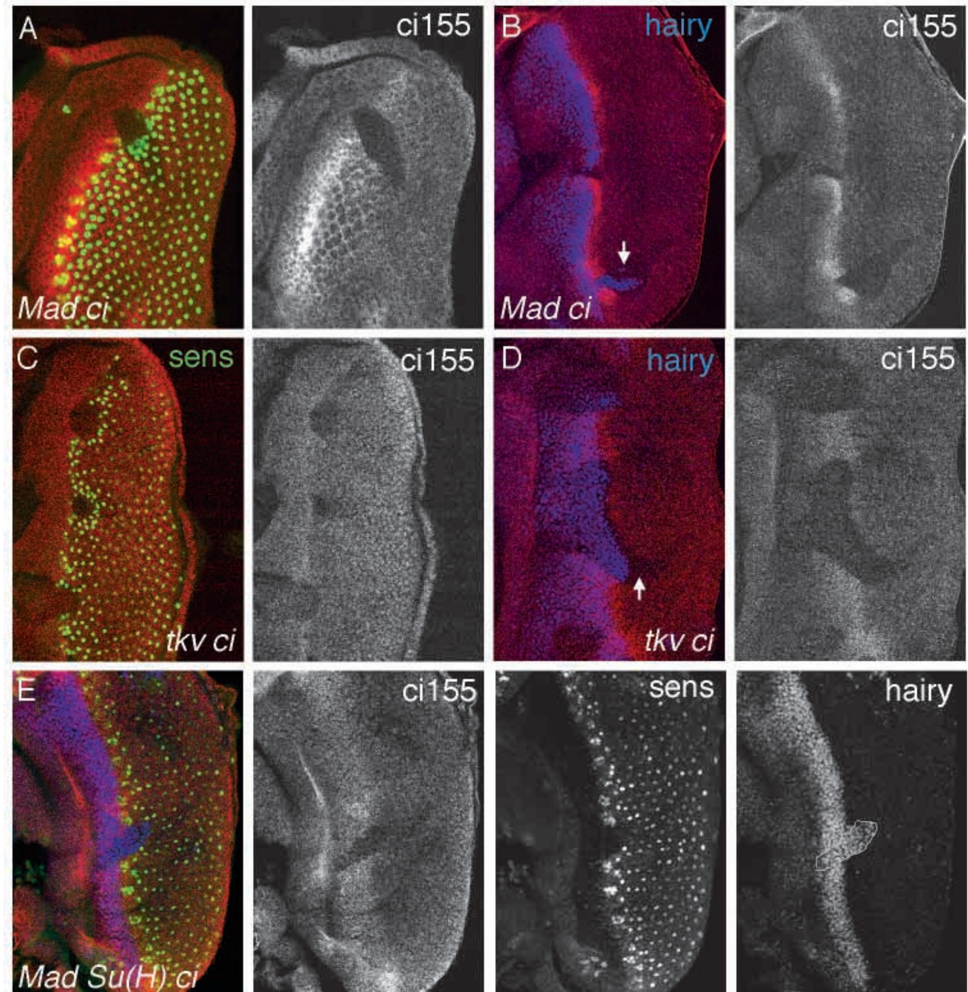


Fig. 4. Genetics of differentiation and Hairy downregulation. Panels A-H show Senseless expression (green) and Hairy expression (blue) in various genotypes. Clones of mutant cells are revealed by the absence of Ci155 (red; panels A-F), or absence of the *lacZ* marker gene (red; panels G,H). In E-H, outlines of clone boundaries have been overlaid on the Hairy channel to help assess autonomy. Because of variations in reagents and procedures over time (see Materials and methods), labeling intensities can only rigorously be compared between cells of different genotypes within the same preparation. (A) Differentiation is delayed in *Mad ci*-mutant cells. (B) *Mad ci*-mutant cells maintain Hairy expression longer, except where they are close to wild-type cells. If wild-type cells are nearby, Hairy expression is lost close to the normal time (e.g. arrow). (C) *tkv ci*-mutant cells do not differentiate. (D) *tkv ci*-mutant cells maintain Hairy expression longer, except where they are close to wild-type cells. If wild-type cells are nearby, Hairy is lost close to the normal time (e.g. arrow). In addition, Hairy levels are reproducibly lower in mutant cells than in wild type. (E) *Mad Su(H) ci*-mutant cells do not differentiate. Mutant cells maintain Hairy expression indefinitely, even where they are neighboring wild-type cells. (F) Differentiation of *Su(H) ci*-mutant cells is slightly delayed. There is an excess of Senseless-expressing R8 cells, though not so extreme as in *Su(H)*-mutant clones (compare with panel G). *Su(H) ci*-mutant cells maintain Hairy expression indefinitely, even where they are neighboring wild-type cells. Note the overlap between Senseless-expressing and Hairy-expressing regions. (G) *Su(H)*-mutant cells differentiate at or before the normal time and are strongly neurogenic (Li and Baker, 2001). Weak Hairy expression is maintained after the normal time but it is soon lost. There is limited overlap between Senseless-expressing and Hairy-expressing regions. (H) *Mad Su(H)*-mutant cells differentiate at or before the normal time and are strongly neurogenic. Hairy expression is maintained after the normal time but it is soon lost. There is limited overlap between Senseless-expressing and Hairy-expressing regions.

differentiate, indicating a role for N signaling in the delayed differentiation of *Mad ci*-mutant cells (Fig. 4E).

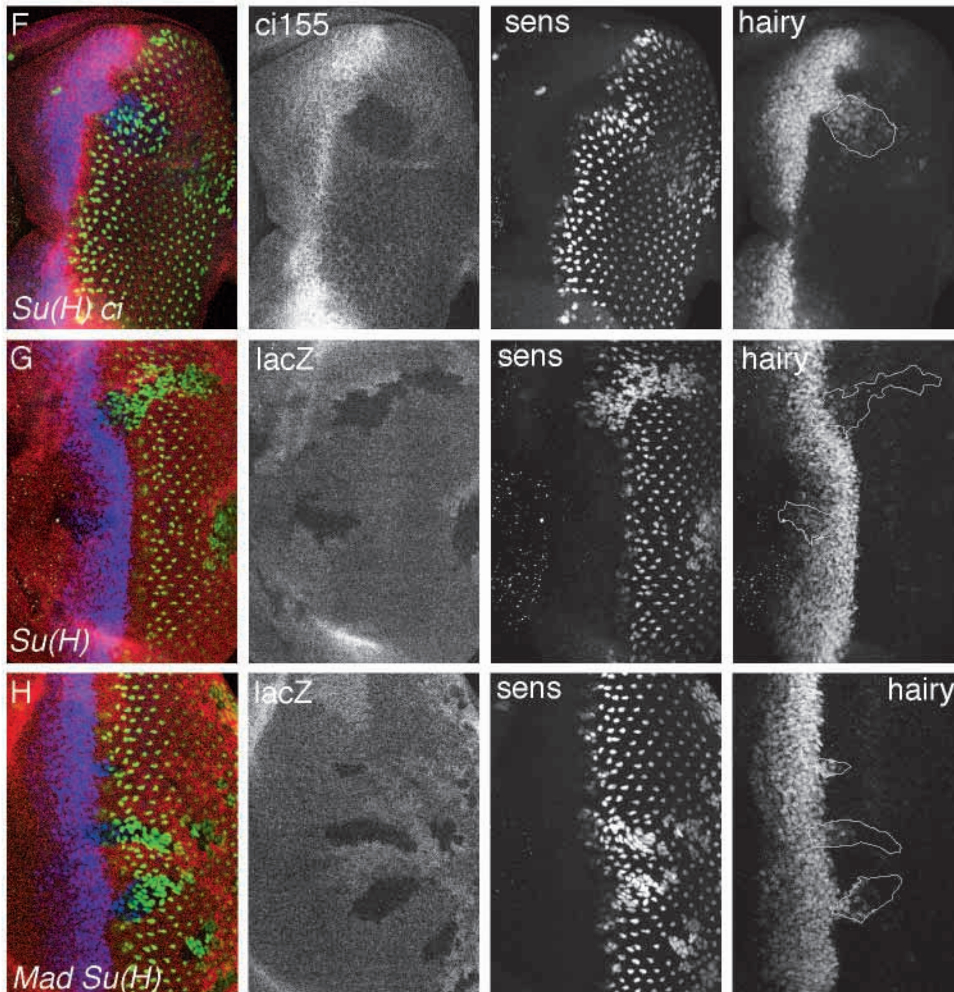
N augments Dpp signaling

Two interpretations of the role of N could be considered. One was that N signaling and Dpp signaling were each required for differentiation in the absence of *ci*. The alternative was that N activity enhanced sensitivity to Dpp so that the limited Dpp signaling that occurs in *Mad*-mutant cells became sufficient for delayed differentiation.

If N signaling was required for differentiation in the absence of *ci*, we would predict that *Su(H) ci*-mutant cells would be unable to differentiate. However, *Su(H) ci*-mutant cells did initiate differentiation, but such differentiation was delayed (Fig. 4F). Thus N signaling was not absolutely required for differentiation. Delayed differentiation in the absence of *Su(H)* and *ci* must depend on Dpp signaling, as it did not occur in *Mad Su(H) ci*-mutant cells. *Su(H)* mutations must reduce the effectiveness of Dpp, as the timing of differentiation was normal in *ci*-mutant cells but delayed in *Su(H) ci*-mutant cells (differentiation in *ci*-mutant clones must be due to Dpp signaling, because *tkv ci*-mutant cells did not differentiate). These results indicate that N signaling made Dpp signaling more effective, at least in the absence of Ci.

Ci155 augments Dpp signaling

The differentiation of *Su(H) ci*-mutant cells differed from that of *Su(H)*-mutant cells. *Su(H)*-mutant cells show a profound neurogenic phenotype in which the majority of mutant cells take an R8 fate (Ligoxygakis et al., 1998) (Fig. 4G). Morphogenetic furrow progression can accelerate so that differentiation begins earlier at the anterior of large *Su(H)*-null clones than it does in nearby wild-type regions (Li and Baker, 2001) (Fig. 4G). By contrast, differentiation of *Su(H) ci*-mutant cells was delayed, and R8 differentiation was increased only moderately compared with wild type (Fig. 4F). These data show that differentiation without *Su(H)* was more effective in the presence of *ci*. This was the first data to indicate a role of



Ci155 that could not be replaced by deleting the *ci* gene to eliminate Ci75. More evidence is reported below.

Hh-dependent differentiation requiring Ci155

One model for the importance of Ci75 was that Ci75 antagonized Dpp and N function. In this view the only normal role of Hh would be to help downregulate Ci75 at the furrow so that Dpp and N could act more promptly. Delayed differentiation in *smo* clones would be due to ci75 antagonizing the Dpp/N function that we have found is sufficient for normal furrow progression in the absence of *ci*. This model does not predict delayed differentiation in *Su(H) ci*-mutant clones (Fig. 4F; see above).

To isolate the role of Hh we examined cells mutant for *Mad* and *Su(H)*. If the only role of Hh was to derepress Dpp and N signaling, then *Mad Su(H)*-mutant cells would not differentiate because of missing Dpp and N signals. By contrast, *Mad Su(H)* cells differentiated at the normal rate (Fig. 4H). A neurogenic phenotype reflected dependence of lateral inhibition on *Su(H)* (Ligoxygakis et al., 1998). Initiation of Atonal expression has also been reported in *Dl Medea*-mutant cells, which should resemble *Mad Su(H)* cells in lacking N and Dpp signaling (Baonza and Freeman, 2001). These results show that Dpp and N were dispensable for differentiation if Hh signaling was intact. As *Mad Su(H) ci* cells did not differentiate, differentiation of *Mad Su(H)* depended on a positive role of Ci that was not mimicked by deleting the *ci* gene to remove Ci75. Thus Hh, through Ci155, could drive differentiation in the absence of Dpp and N signaling.

N and Hh each turn off Hairy

Notch signaling contributes to differentiation by downregulating Hairy and Extramacrochaetae expression at the morphogenetic furrow (Baonza and Freeman, 2001). Hairy is a transcriptional repressor protein that antagonizes Atonal (Ohsako et al., 1994; Brown et al., 1995). We monitored Hairy expression to evaluate the contributions of Hh, Dpp and N signaling to turning off Hairy, and to correlate this with differentiation. The results are summarized in Table 1. Hh, Dpp and N signals do not appear essential to turn Hairy on, although Dpp does contribute because Hairy levels appear lower in clones mutant for *tkv*, *Mad* and their combinations with other mutations (Greenwood and Struhl, 1999) (Fig. 4, and data not shown).

One, or more, of Dpp, Hh or N signaling is required to turn Hairy off at the morphogenetic furrow, because Hairy expression was maintained cell autonomously in *Mad Su(H) ci* clones (Fig. 4E). Hairy was turned off in *Mad ci* clones and *tkv ci* clones, although downregulation was delayed in the center of the clone (Fig. 4B,D). This implies that N signaling is sufficient to downregulate Hairy in response to a signal from more posterior cells outside the clone.

N signaling was required, and was sufficient, for Hairy downregulation, because Hairy was not shut off in *Su(H) ci* clones (Fig. 4F). If N signaling was essential to shut off Hairy under all circumstances then we would expect Hairy expression to be maintained autonomously in *Su(H)* clones. By contrast, there was only a brief delay to shutting off Hairy in *Su(H)* clones (Fig. 4G). Hairy was also shut off in *Mad Su(H)* clones, although after a delay (Fig. 4H). These results show that either N or Hh signals from posterior cells is sufficient to shut off Hairy expression, but that Hairy expression is maintained indefinitely in *Mad Su(H) ci* and *Su(H) ci* cells unable to respond to either pathway. Downregulation of Hairy in response to Hh as well as N explains why N is not required for differentiation in response to Hh, even though it is required for differentiation in response to Dpp.

Hairy retards differentiation

Downregulating Hairy was neither necessary nor sufficient for differentiation. Whereas *Mad Su(H) ci*-mutant cells both failed

to differentiate and maintained Hairy expression (Fig. 4E), *tkv ci* cells did not differentiate even though Hairy expression was shut off (Fig. 4C,D). By contrast, Hairy expression was maintained in *Su(H) ci*-mutant clones, even though *Su(H) ci* cells could differentiate. However, Hairy downregulation may contribute to prompt differentiation because differentiation is delayed in *Su(H) ci* compared with *ci*, and is less neurogenic in *Su(H) ci* than in *Su(H)* (Fig. 4F,G).

As Hairy was shut off by either Hh or N signaling, Hairy maintenance away from the boundaries of *Mad ci*, *tkv ci* and *Mad Su(H)* clones shows that these genotypes were defective for Hh and Dl secretion. Hh is normally secreted by differentiating photoreceptor cells (Ma et al., 1993). Atonal and then Egf receptor activity promotes expression of Dl, the activating ligand for Notch (Baker and Yu, 1998; Tsuda et al., 2002). Both Hh and Dl expression should be absent or delayed in *Mad ci*, *tkv ci* and *Mad Su(H)* clones.

Discussion

Redundant, overlapping roles of Hh, N and Dpp

Table 1 summarizes all of our data. Initially our results suggested that Dpp and Hh might be redundant through common regulation of Ci. When Ci was found to be dispensable, the simplest interpretation was that the repressor Ci75 was more important than Ci155. Others recently arrived at a similar conclusion (Pappu et al., 2003). However, further work revealed roles for Ci155, and redundancies between Hh, Dpp and N, as the explanation for normal progression of differentiation in the absence of any one of the important components *ci*, *Mad* or *Su(H)*. There might be other signals awaiting discovery, as Hh, Dpp and N cannot explain the expression of *hairy* or of genes such as *eyes absent* during furrow progression (Curtiss and Mlodzik, 2000) (Fig. 4E and N.E.B., unpublished).

The pathways implied by our results shown in Fig. 5. Fig. 5A shows how Hh, Dpp and N signaling pathways act within each cell. Fig. 5B illustrates the spatial and temporal relationships of the extracellular signals during morphogenetic furrow progression.

The development of *Mad Su(H) ci*-mutant cells is a helpful starting point, as they may reflect a ‘ground state’ of eye development that requires extracellular signals to differentiate. *Mad Su(H) ci* cells fail to express the *atonal* or *senseless* genes that initiate R8 differentiation, and, consequently, fail to support retinal differentiation. This shows that the absence of Ci75 is not sufficient for differentiation. Dpp alone can induce Ato (e.g. in *Su(H) ci* clones), but N and Dpp signaling together are required to activate Atonal with normal kinetics, as occurs in *ci*-mutant cells. N signaling alone (in *tkv ci* clones) is insufficient. In the presence of Ci, prompt differentiation required Hh to downregulate Ci75, and differentiation was delayed in *Smo* clones that lacked this input. The normal role of Hh is not just to remove Ci75 thus permitting Dpp and N to work, because Atonal is turned on normally in *Mad Su(H)* clones that do not respond to Dpp or N signals. Such differentiation depends exclusively on Hh yet progresses normally, except that a neurogenic phenotype reflects dependence of lateral inhibition on *Su(H)*. Hh depends positively on *ci* to drive differentiation in *Mad Su(H)* cells and, therefore, requires Ci155. The positive role of *ci* can also be

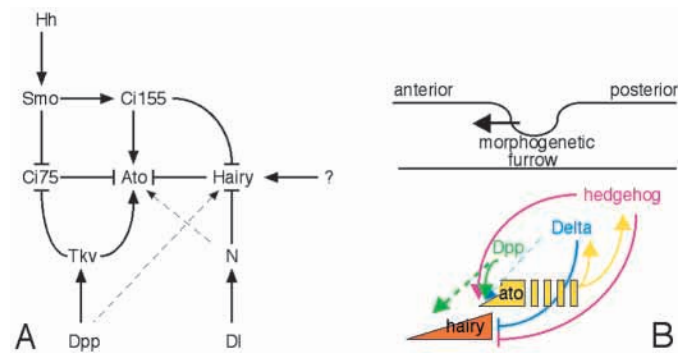


Fig. 5. Positional signals and regulatory gene expression. (A) Cell autonomous responses of Atonal and Hairy expression to Dpp, Hh and N signal reception as inferred from our results, illustrating the roles of Ci155 and Ci75. Positive and negative interactions do not imply that direct molecular interactions between proteins or between proteins and genes have been demonstrated, only that interactions occur within the same cell without further intercellular signals. Ci75 must repress Ato since mutation of *smo* imposes a delay on differentiation that is released by deleting *ci*. Ci155 must activate Ato since *Mad Su(H)* cells differentiate but *Mad Su(H) ci* cells do not. Tkv must activate Ato independently of Ci because *ci* cells differentiate normally but *tkv ci* and *Mad ci* cells do not. Tkv is shown inhibiting Ci75, because Tkv promotes Ci155 accumulation in *smo*-mutant cells; this Ci155 is presumed to be inactive. Tkv also promotes Ci155 accumulation in cells not mutant for *smo*. Hairy has been shown previously to repress Ato function (Brown et al., 1995). Ci155 must repress Hairy as Hairy is maintained cell autonomously by *Mad Su(H) ci*-mutant cells but can be downregulated by *Mad Su(H)* cells. N must repress Hairy as Hairy is maintained cell autonomously by *Mad Su(H) ci*-mutant cells but can be downregulated by *Mad ci* cells. N may activate Atonal independently of Hairy as well, because the furrow progresses faster through *Su(H)*-mutant clones where Hairy is still expressed (Li and Baker, 2001). Hairy expression must be initiated in part by another signal, although Dpp has an input, as revealed by the quantitative reduction of Hairy levels in cells mutant for *tkv* (Greenwood and Struhl, 1999) and *Mad*, and all their combinations with *Su(H)* and *ci*. The network accounts for all the mutant phenotypes. In the absence of Ci, normal differentiation occurs in response to Dpp and N. In the absence of *smo*, differentiation is delayed because activation pathways through Ci155 are lost but repression by Ci75 retained. In the absence of both Dpp and N, differentiation occurs in response to Hh. Absence of either *tkv* or *N* alone has little effect, reflecting either the dominant role of Hh, or perhaps that both positive and negative inputs are lost in each case. (B) Extracellular signaling to Atonal and Hairy. Hh, Dl and Dpp are shown at the locations of their expression. The signal acting most anteriorly to the morphogenetic furrow is Dpp. Dpp is expressed at the anterior of the morphogenetic furrow in response to Hh. Dpp promotes Hairy expression. More posteriorly, Dpp synergizes with the relatively indiffusible Dl signal to induce Atonal. Dl is expressed in response to Atonal, and later in response to activation of Egfr by ligands produced by Atonal-dependent R8 cells. Hh also induces Atonal through the Ci155/Ci75 ratio. Hh is secreted by photoreceptor cells specified by Egfr activation by ligands produced by Atonal-dependent R8 cells. Both N and Hh downregulate Hairy. Despite intrinsically different ranges of Dpp and Hh signals, their activation of Ato coincides because Dpp also elevates Hairy, which must be downregulated by Hh or Dl.

inferred from the delayed differentiation of *Su(H) ci* clones in comparison with *Su(H)* clones.

We find that Hairy is downregulated redundantly by Hh and

N signaling. Prolonged Hairy expression is not sufficient to block differentiation completely but it does antagonize it (e.g. in *Su(H) ci* clones). Downregulation of Hairy in response to Hh as well as N explains why both *ci* and *Su(H)* mutant clones can differentiate promptly, and why N enhances differentiation in response to Dpp but is not required for differentiation in response to Hh.

Hh or Dpp is sufficient for differentiation

Comparison between *Mad Su(H) ci* cells that do not differentiate and *Mad Su(H)* cells that do shows that Hh signaling is sufficient to initiate eye differentiation. This is consistent with previous studies of ectopic Hh activation (Heberlein and Moses, 1995; Li et al., 1995; Ma and Moses, 1995; Pan and Rubin, 1995; Strutt et al., 1995). Our experiments confirm this conclusion at the normal time and place of Hh signaling at the anterior of the morphogenetic furrow, and confirm directly that Dpp and N signaling are not necessary for Hh signaling to be sufficient.

Comparison between *Mad Su(H) ci* cells and *Su(H) ci* cells shows that Dpp signaling is sufficient to initiate eye differentiation in its normal location in the absence of Hh or N signals, but such differentiation is delayed. The normal timing of differentiation is restored by combined Dpp and N signals (in *ci* clones). This is the basis for the ectopic differentiation on co-expression of Dpp and Dl ahead of the furrow (Baonza and Freeman, 2001).

Superficially, our results differ from previous ectopic expression studies that concluded that Dpp signaling alone was not sufficient to induce ectopic differentiation in all locations (Pignoni and Zipursky, 1997; Greenwood and Struhl, 1999; Baonza and Freeman, 2001). This discrepancy is probably explained by the baseline repressor activity of Su(H) protein (Hsieh and Hayward, 1995; Morel and Schweisguth, 2000). Our previous work shows that without N signaling, repressor activity of Su(H) protein retards differentiation (Li and Baker, 2001). Dpp signaling is sufficient for differentiation in our experiments where the *Su(H)* gene has been deleted. In the presence of the *Su(H)* gene, Dpp may be most effective at locations where there is little Su(H) repressor activity, such as close to the morphogenetic furrow where N signaling is active.

Comparison between *Mad Su(H) ci* cells, which do not differentiate, and *Mad ci* or *tkv ci* cells, which differentiate slowly or not at all, shows that Notch signaling alone is insufficient for differentiation. Premature differentiation reported when N is activated ectopically ahead of the furrow must reflect endogenous Dpp signaling at such locations (Baonza and Freeman, 2001; Li and Baker, 2001).

Mechanisms of redundancy

Our experiments reveal an outline of the mechanisms of Hh, Dpp and N redundancy (Fig. 5A). First, our results show that Mad and Ci independently reinforce differentiation, presumably through the transcription of target genes because Mad is sufficient for differentiation in the absence of Ci, and vice versa. Our results show unequivocally that the transcriptional activator Ci155 activates differentiation in addition to Ci75 antagonizing differentiation.

It was surprising to find that Dpp stabilizes Ci155 in the absence of Smo, which suggested Dpp input into Hh signal transduction. Although the requirement for *smo*-dependent input through *fused* makes it unlikely that Ci155 is functional in *smo* clones (Ohlmeyer and Kalderon, 1997; Methot and Basler, 1999), Ci155 accumulation might be associated with reduced Ci75 levels. Ci75 is shown to repress differentiation in *smo* clones because *smo ci* clones differentiation normally. Ci155 stabilization cannot be due to an indirect effect of Dpp signaling on Hh, Ptc or Smo expression levels because the effect is detected in the absence of *smo*, and, therefore, reflects an effect on Hh signal transduction components downstream of Smo. One idea is that Dpp signaling (or Dpp-induced differentiation) may replace SCF^{Slimb} processing of Ci (which cleaves Ci155 to Ci75) with Cullin3-mediated Ci degradation, just as normally occurs posterior to the morphogenetic furrow (Ou et al., 2002). In a *smo* clone, Ci155 would accumulate because Smo is required for Cullin3 to degrade Ci (Ou et al., 2002) (N.E.B., unpublished). However, the SCF^{Slimb}-to-Cullin3 switch may not be the only effect of Dpp on Ci processing, because Tkv slightly enhanced Ci155 accumulation even when *smo* is present (Fig. 2E).

Finally, downregulation of Hairy by N requires the *Su(H)* gene. N also overcomes baseline repressor activity of Su(H) protein to promote progression of differentiation (Li and Baker, 2001). This role of N must be independent of Hairy.

Signal combinations control the rate of differentiation

Dl, Hh and Dpp are generally thought to signal over very different distances. How can signals of such different range substitute for one another to permit normal eye development? Fig. 5B shows signal sources and targets in the eye disc. Dpp is transcribed in response to Hh signaling and is produced where Ci155 levels are highest (Heberlein et al., 1993; Strutt and Mlodzik, 1996). Dl is regulated by Hh indirectly through Ato and Ato-dependent Egfr activity in differentiating cells (Baker and Yu, 1998; Tsuda et al., 2002). Hh is expressed most posteriorly of the three, in differentiating photoreceptors (Ma et al., 1993).

Eye differentiation uses Hh to progress through cells unable to respond to Dpp (*tkv*, *Mad*) or N (*Su(H)*). The range of Hh diffusion depends in part on the shape of the morphogenetic furrow cells (Benlali et al., 2000). The Dpp that drives differentiation through *ci*-mutant cells unable to respond to Hh must diffuse from outside the *ci* clones because Dpp synthesis is Hh dependent (Heberlein et al., 1993; Methot and Basler, 1999). Large *ci* clones develop normally so Dpp diffusion cannot be limiting (*dpp*-mutant clones offer no information about the range of Dpp because they express and differentiate in response to Hh). Instead the rate of progression in response to Dpp is controlled by Dl. Dl signals over, at most, one or two cell diameters at the morphogenetic furrow (Baker and Yu, 1997).

The previous view of eye patterning was influenced by the morphogen function of Hh and Dpp in other discs (Nellen et al., 1996; Strigini and Cohen, 1997). It was thought that domains of Ato and Hairy expression reflected increasing concentrations of Hh and Dpp (Greenwood and Struhl, 1999; Lee and Treisman, 2002). Our data shows that, in the eye, the combination of signals is important. Differentiation is triggered where Dl and/or Hh synergize with Dpp, regardless of where the source of Dpp is. The additional requirements limit Dpp to initiating differentiation at the same locations that Hh does.

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