Localized Notch signal acts through *eyg* and *upd* to promote global growth in *Drosophila* eye

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Summary

Notch (N) signal is activated at the dorsoventral (DV) border of the *Drosophila* eye disc and is important for growth of the eye disc. In this study, we showed that the Pax protein Eyg is a major effector mediating the growth promotion function of N. *eyg* transcription is induced by N signaling occurring at the DV border. Like *N*, *eyg* controls growth of the eye disc. Loss of N signaling can be compensated by overexpressing *eyg*, whereas loss of the downstream *eyg* blocked the function of N signaling. In addition, we showed that *N* and *eyg* could induce expression of *upd*, which encodes the ligand for the Jak/STAT pathway and acts over long distance to promote cell proliferation. Loss of *eyg* or *N* can be compensated by overexpressing *upd*. These results suggest that *upd* is a major effector mediating the function of *eyg* and *N*. The functional link from *N* to *eyg* to *upd* explains how the localized Notch activation can achieve global growth control.

Key words: *Drosophila*, Eye, Cell proliferation, Growth, *Notch*, *upd*, *eyg*

Introduction

The size of an organ is controlled by multiple processes that have to be coordinately regulated. The *Drosophila* compound eye has been used extensively as an excellent model system to study the growth control of an organ. In this study, we provide a link from a localized signal to a long-range signal to coordinate the growth of the entire organ.

In *Drosophila*, the compound eye of adult fly is composed of about 750 ommatidia that each contains eight photoreceptors and 12 accessory cells (Ready et al., 1976). All these cells are derived from the eye-antennal disc that invaginates from the ectoderm of the embryo and grows inside the larva. In embryo stage, there are 6-23 cells determined as eye-antennal disc primordium. These cells rapidly proliferate without differentiation in the first and second instar. In early third instar, cells at the posterior margin of the eye discs start to progressively differentiate into ommatidial clusters in a posterior to anterior direction. The front of the differentiation wave is mark by an indent called the morphogenetic furrow (MF). The MF is a moving boundary that separates undifferentiated from differentiated tissue. The differentiation of eye is complete during metamorphosis. Loss-of-function and gain-of-function mutations in a number of genes can cause an alteration in the eye size. The study of these genes has provided some knowledge on the genetic control of eye size.

The activation of *Notch*-mediated signaling along dorsoventral (DV) midline can form an organizer of eye growth and patterning (Cho and Choi, 1998; Dominguez and de Celis, 1998; Kenyon et al., 2003; Papayannopoulos et al., 1998). The organizer is established by restricted expression of the *Notch* ligand, *Delta* (*Dl*) and *Serrate* (*Ser*). *Dl* is expressed in the dorsal side and *Ser* is detected preferentially in the ventral side along midline before the initiation of differentiation (Cho and Choi, 1998). This expression pattern creates the DV axis and specifies the expression domain of *Notch* (*N*) in the DV boundary (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). *N* activation in DV boundary is known to play an essential role both in promoting the growth and in regulating patterning during development of *Drosophila* wing and tetrapod limb (Irvine, 1999). In eye development, reducing *N*, as in N^{ts} mutant grown at the non-permissive temperature during the second instar stage, caused an eyeless to headless phenotype (Shellenbarger and Mohler, 1978). Blocking N signaling by misexpressing the antagonists *Hairless* (*H*) or a dominant-negative form of *N* (N^{DN}) can abolish the eye formation (Kurata et al., 2000). Conversely, targeted activation of *N* induced strong mitotic activity in eye discs and caused hyperplasia in adult eyes (Go et al., 1998; Kurata et al., 2000). These observations indicated that *N* is required for the growth of eye discs. But it is not clear which gene(s) is the downstream effector in this process. It is also not clear how a localized activation of Notch signaling at the DV border can affect the growth of the entire eye disc.

eye gone (*eyg*) is another gene that regulates eye size. It encodes a Pax transcription factor (Jun et al., 1998; Jones et al., 1998; Jang et al., 2003). Loss-of-function *eyg* mutants show a phenotypic series ranging from mild reduction of eye size to a headless phenotype that lacks structures derived from the eye-antennal disc (Jang et al., 2003; Dominguez et al., 2004). In *eyg* mutants, the eye disc is reduced even before photoreceptor differentiation. Blocking apoptosis by expressing the anti-apoptotic P35 (Hay et al., 1994) in eye disc

does not rescue the reduced size (Jang et al., 2003). These results suggest that *eyg* plays a role in the early growth of the eye disc. Consistent with this interpretation, targeted expression of *eyg* can cause overgrowth in the eye disc (Jang et al., 2003). Like *N*, *eyg* is expressed in the central region of eye discs in second instar (Cho and Choi, 1998; Jang et al., 2003). The similarity in expression and in mutant phenotype suggests that *eyg* may be a target of Notch and may be an effector of N signaling for eye growth. However, *eyg* encodes a transcription factor, so is expected to affect target gene expression autonomously. For the locally expressed *eyg* to affect global growth in the eye disc, it must induce some signaling molecule, which then promotes long-range cell proliferation.

The Unpaired (Upd) protein, a ligand for the Jak/STAT signaling pathway (Harrison et al., 1998), was recently shown to promote cell proliferation in eye disc (Bach et al., 2003; Tsai and Sun, 2004). Upd is expressed in the center of the posterior margin of eye disc (Tsai and Sun, 2004). This is the site where the DV border (N activation) intersects the posterior margin, making *upd* a candidate target for N. Loss-of-function *upd* mutations caused a reduction of eye size, while overexpression of *upd* caused enlargement of the eye (Bach et al., 2003; Tsai and Sun, 2004). Upd promotes cell cycle only in the undifferentiated cells anterior to the MF (Tsai and Sun, 2004), which mimics the early eye disc before MF initiated. In addition, Upd can induce cyclin D expression (Tsai and Sun, 2004). Thus, the proliferative function of Upd may be directly linked to the cell cycle genes. Mostly importantly, Upd protein can distribute over a long distance and can exert the proliferative effect over a long distance (Tsai and Sun, 2004). So, *upd* is an ideal candidate to turn the localized N activation into a global signal for proliferation.

In this study, we provide evidences to show that the N signal at the DV border induces *eyg* expression, which then induces *upd* expression. This functional link explains how the localized N signal from the DV organizer can non-autonomously affect the growth of an entire organ.

Materials and methods

Fly stocks

Fly culture and crosses were performed according to standard procedure at 25°C unless otherwise noted. In these experiments, transgenic lines used were *UAS-eyg* (Jang et al., 2003), *UAS-N^{act}* (Go et al., 1998), *UAS-N^{DN}* (Rebay et al., 1993), *UAS-H* (Go et al., 1998), *UAS-Su(H)*, *UAS-Ser*, *UAS-Dl*, *y w hsFLP^{12}; Sco/CyO, y w hsFLP^{22}; CxD/TM3*, *Sb* and *Act5C>y^{+}>*GAL4, *UAS-GFP^{S65T}* were kindly provided by G. Doughty, A. Nagel and J. E. Treisman, respectively. *UAS-upd* (Harrison et al., 1998; Zeidler et al., 1999) was from N. Perrimon. The GAL4 driver strains used were *dpp*-GAL4 (Staehling-Hampton et al., 1994) and *ey*-GAL4 (Quiring et al., 1994). Alleles used were *eyg^{1}* (weak mutant), *eyg$^{M3\text{-}12}$* (null mutant) (Jang et al., 2003) and *H^{E31}* (Bailey and Posakony, 1995). *upd-lacZ* (also called *PD*) (Sun et al., 1995) was identified by a P{*lacW*}-mediated enhancer/silencer screen (Sun et al., 1995).

Clonal induction

Positively labeled flp-out expression clones were generated by crossing *UAS*-lines to *hs-FLP^{22}; Act5C>y^{+}>*GAL4 *UAS-GFP^{S65T}* (Ito et al., 1997). Heat-shock induction of *hs-FLP^{22}* was at 37°C for 1 hour at 24-48 hours after egg laying for *UAS-eyg*, and at 48-72 hours after egg laying for *UAS-N^{act}*, *UAS-N^{DN}*, *UAS-Ser* and *UAS-Dl*. Mutant clones were induced by the FLP-FRT method (Xu and Rubin, 1993). For *Su(H)^{SF8}* mutant clones, *hs-FLP^{22};* 2XP[*ubi*-nls-GFP]*FRT40A* females were crossed to *Su(H)^{SF8} FRT40A* males. Heat shock induction of *hs-FLP^{22}* was at 37°C for 1 hour at 24-48 hours after egg laying.

Immunohistochemistry

Late third instar larval imaginal discs were dissected and stained. Primary antibodies were rat anti-Elav (1:500), mouse anti-WG, mouse anti-DAC (1:200, Developmental Studies Hybridoma Bank, University of Iowa) and rabbit anti-β-galactosidase (1:2000, Cappel). Secondary antibodies (Jackson ImmunoResearch) were Cy3 anti-rabbit, Cy5 anti-rabbit, Cy3 anti-rat, Cy5 anti-rat FITC anti-mouse and Cy5 anti-mouse.

In situ hybridization

upd antisense probe and hybridization procedure are as described previously (Tsai and Sun, 2004).

Results

eyg controls cell proliferation in the early eye disc

eyg is expressed in the embryonic eye primordium and in the larval eye disc (Jang et al., 2003; Dominguez et al., 2004). In loss-of-function *eyg* mutants, the eye disc is reduced. The size reduction is apparent in early third instar eye disc and cannot be rescued by blocking apoptosis (Jang et al., 2003), suggesting that *eyg* controls cell proliferation in early eye disc. We have identified a genomic fragment (E2-EX) from the *eyg-toe* locus that specifies *eyg* expression in the embryonic eye primordium to the second instar eye-antennal disc, but not in the third instar eye-antennal disc (not shown). When this fragment was used to drive the expression of *eyg* by the GAL4-UAS system (Brand and Perrimon, 1993), the *eyg* mutant phenotypes can be partially rescued. The *eyg^{1}*/*eyg$^{M3\text{-}12}$* has no eye (Fig. 1A), but can be rescued to have one small eye (37%; Fig. 1B) or two small eyes (42%). The *eyg$^{M3\text{-}12}$* homozygotes are headless (Fig. 1C), but can be rescued to have small head with antennae (Fig. 1D, 64%). These results indicate that *eyg* has a role in regulating cell proliferation in the early eye disc. When *eyg* is ectopically expressed by *dpp-GAL4* (Jang et al., 2003) or clonally induced by the flp-out method (Fig. 1E,F), it caused overgrowth in the eye disc and in proximal regions of the antennal disc. Clones posterior to the MF caused only one or two extra ommatidia, perhaps because the time before the passage of the MF is limited. Thus, *eyg* is a potent regulator of cell proliferation.

eyg expression is induced by Notch signaling

eyg is expressed at the equatorial region in the eye disc from late second instar to late third instar (Jang et al., 2003; Dominguez et al., 2004). The equatorial region is the dorsoventral boundary, which is also the region of N activation (Cho and Choi, 1998). N regulates cell proliferation in the eye disc (Cho and Choi, 1998; Dominguez and de Celis, 1998). In *N^{ts}* mutant that had grown at the non-permissive temperature during the second instar stage, the eye size is reduced (Shellenbarger and Mohler, 1978). In such flies, the expression of the mini-*white* reporter gene in the *eyg$^{M3\text{-}12}$* enhancer trap line (Fig. 2A) is also reduced in the eye (Fig. 2B). This result suggests that N signaling is required for *eyg* expression in the eye. Clonal expression of a constitutively activated *N* (*N^{act}*) in

the eye disc can induce overgrowth when located anterior to the MF (Fig. 2C,D). A clone near the lateral margin caused non-autonomous overgrowth, while a central clone caused only local overgrowth. Anterior to the MF, the *eyg-lacZ* (using the $eyg^{M3\text{-}12}$ enhancer trap line) was induced in the overgrown tissue (Fig. 2D,F, arrow) except when the clones were located within the *wg* expression domains in the dorsal and ventral margins (Fig. 2D,F, arrowhead). By contrast, WG level was enhanced by N^{act} only when the clone lies within the *wg* domain (Fig. 2D,E, arrowhead). Posterior to the MF, the N^{act} clones did not induce *eyg-lacZ* expression but inhibited neural differentiation (not shown), owing to the inhibition of *ato* expression by *N* (Baker et al., 1996). In the antennal disc (Fig. 2C-F) and wing disc (not shown), the N^{act} clones caused tissue overgrowth but did not induce *eyg-lacZ* expression. These results suggested that *eyg* transcription is specifically induced by N in the undifferentiated cells in the central equatorial region of the eye disc.

Delta (Dl) and Serrate (Ser) are two ligands for the N receptor. Dl^{rev10} and Ser^{RX82} transheterozyous mutant has normal eye size. In Dl^{rev10}, Ser^{RX82} and $eyg^{M3\text{-}12}$ triple heterozygous mutant, there is a reduction of eye size and mini-*white* reporter expression (Fig. 3A-C). Based on this genetic interaction, we tested which ligand contributed to this growth ability. When *Dl* or *Ser* are clonally expressed in the eye disc, *eyg-lacZ* was generally not induced within the clones (data not shown). However, when the *Dl*-expressing clone is located in the ventral part of the eye disc, *eyg* was non-autonomously induced at the border of the clone (Fig. 3G-I). Similarly, in dorsal *Ser*-expressing clones, *eyg* is induced non-autonomously at the clone border (Fig. 3D-F). The *eyg* induction only occurs when the *Dl*- and *Ser*-expressing clones are located near the central region of the eye disc. In eye discs, *Delta* is normally expressed in the dorsal side and *Serrate* is detected in the ventral side (Cho and Choi, 1998). Thus, the ventral *Dl*-expressing clones and dorsal *Ser*-expressing clones created novel DV borders, which should cause *N* activation. *eyg* induction is therefore consistent with N activation.

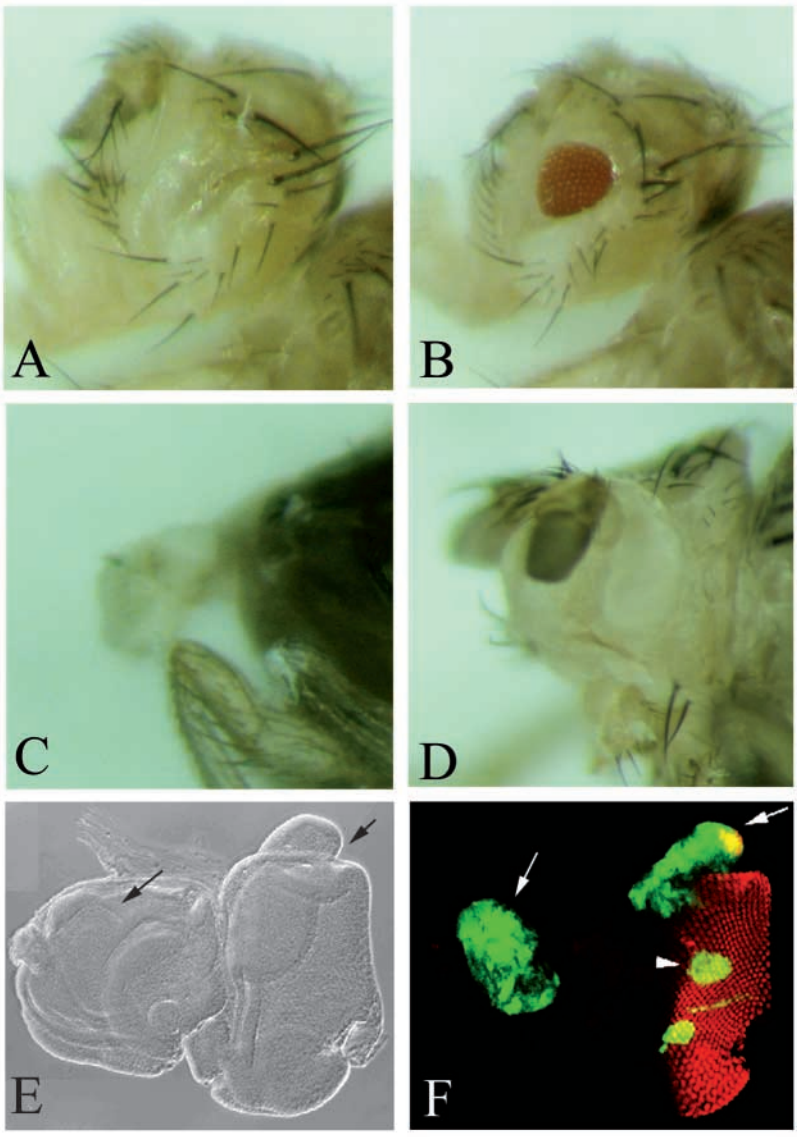

Fig. 1. *eyg* promotes growth of the early eye disc. The $eyg^{1}/eyg^{M3\text{-}12}$ mutants have no eye (A), but the phenotype can be rescued by expressing *eyg* driven by the E2-XS enhancer fragment (B). The $eyg^{M3\text{-}12}$ homozygotes are headless (C), but the phenotype can be rescued by expressing *eyg* driven by the E2-XS enhancer fragment (D). (E,F) Clonal expression of *eyg* (marked by GFP, green) can induce overgrowth (arrows) in the eye disc and antenna disc. Clones posterior to the MF caused only a few extra ommatidia (arrowhead). (E) DIC image.

Su(H) is a downstream component of N signaling and is required for the growth control of eye disc by N (Li and Baker, 2001). In $Su(H)^{SF8}$ mutant clones (marked by the absence of the GFP marker), *eyg-lacZ* is absent or reduced (Fig. 2G-L). This demonstrates that the activation of *eyg* is through the canonical N signaling pathway. In a few clones, *eyg-lacZ* is still expressed at the cells adjacent to wild-type tissue (Fig. 2G-I).

eyg mediates N signal to induce cell proliferation

As *eyg* expression is activated by N signaling, we asked whether *eyg* acts downstream of N signal to promote cell proliferation. The $eyg^{1}/eyg^{M3\text{-}12}$ mutants have no eye (Fig. 1D). Removing one copy of *Hairless* (*H*), an antagonist of N signaling, can partially restore the eye size (Fig. 4A). Targeted expression of N^{act} (Fig. 4B) or *Su(H)* (Fig. 4C) can also partially restore the eye size of $eyg^{1}/eyg^{M3\text{-}12}$ mutants. One interpretation is that N acted through an *eyg*-independent mechanism to affect cell proliferation, which can compensate for the loss of *eyg*. Another interpretation is that N enhanced the *eyg* expression in the hypomorphic eyg^{1} allele. So we tested with the $eyg^{M3\text{-}12}$ null mutant. $eyg^{M3\text{-}12}$ is a deletion that deleted the *eyg* transcription unit but does not extend to the adjacent *toe* gene (Jang et al., 2003). Removing one copy of *H* resulted in flies with a complete head except the eye (Fig. 4D; compare with Fig. 1F). In the third instar larva, expression of *eyg-lacZ* is detected in the antennal disc but not in the eye disc, which

is still highly reduced (Fig. 4E). Therefore, when *eyg* is null, increasing N signaling cannot promote cell proliferation in the eye disc. The rescue of $eyg^{1}/eyg^{M3\text{-}12}$ mutant by N signaling must be through the hyper-activation of the eyg^{1} allele.

We further tested whether expression of the activated N in eye disc ($ey>N^{act}$) can rescue $eyg^{M3\text{-}12}$ homozygous mutant. Most animals of such genotype die at the larval stage, only 6% survived to the pharate stage. In these pharates, the head development can be rescued, but the eye is usually absent (Fig. 5A). In about 20% of these pharates, the eye is partially rescued (Fig. 5B). Very rarely, the antenna is duplicated (Fig. 5C). When the discs were examined, the antenna disc is always present and has *eyg-lacZ* expression (Fig. 5D-F). A few of the antennal disc has duplicated or triplicated antennal field, as indicated by the *eyg-lacZ* expression domain (Fig. 5F). The endogenous eye field (based on the location of the optic stalk and Bolwig nerve; Fig. 5D-F, arrow) is highly reduced, lacks *eyg-lacZ* expression and has no photoreceptor differentiation (Fig. 5D-F), but an extra eye field is induced dorsal to the endogenous eye field in about 36% of these discs (Fig. 5D,E). The extra eye field can have *eyg-lacZ* expression and photoreceptor differentiation (Fig. 5E), which accounts for the presence of small adults eyes. These results suggest that *eyg* is required for the N-mediated proliferation in the endogenous eye field. Outside of the *eyg* domain in the eye disc, N signaling can induce cell proliferation by an *eyg*-independent mechanism. As these cells have *ey*, they are competent for eye development.

If *eyg* acts downstream of N signaling to control growth, then when N signaling is blocked, overexpression of *eyg* should be able to rescue the phenotype. Targeted expression of a dominant-negative N (N^{DN}) by the *ey*-GAL4 caused an 'eyeless' phenotype (Fig. 4F) (Kurata et al., 2000). Co-expression of *eyg* and N^{DN} restored the eye size to nearly normal (Fig. 4G). Similarly, expression of *H* driven by the *ey*-GAL4 completely blocked eye development (Fig. 4H), while co-expression of *eyg* with *H* can restore the eye size (Fig. 4I). These results indicate that *eyg* is a major effector mediating N signal to promote growth in the eye disc.

N and *eyg* activates *upd* expression

Another gene known to promote cell proliferation is the *unpaired* (*upd*) gene, which encodes a ligand for the Domeless (Dome) receptor and signals through the Jak/STAT pathway (Harrison et al., 1998). In second and early third instar eye disc,

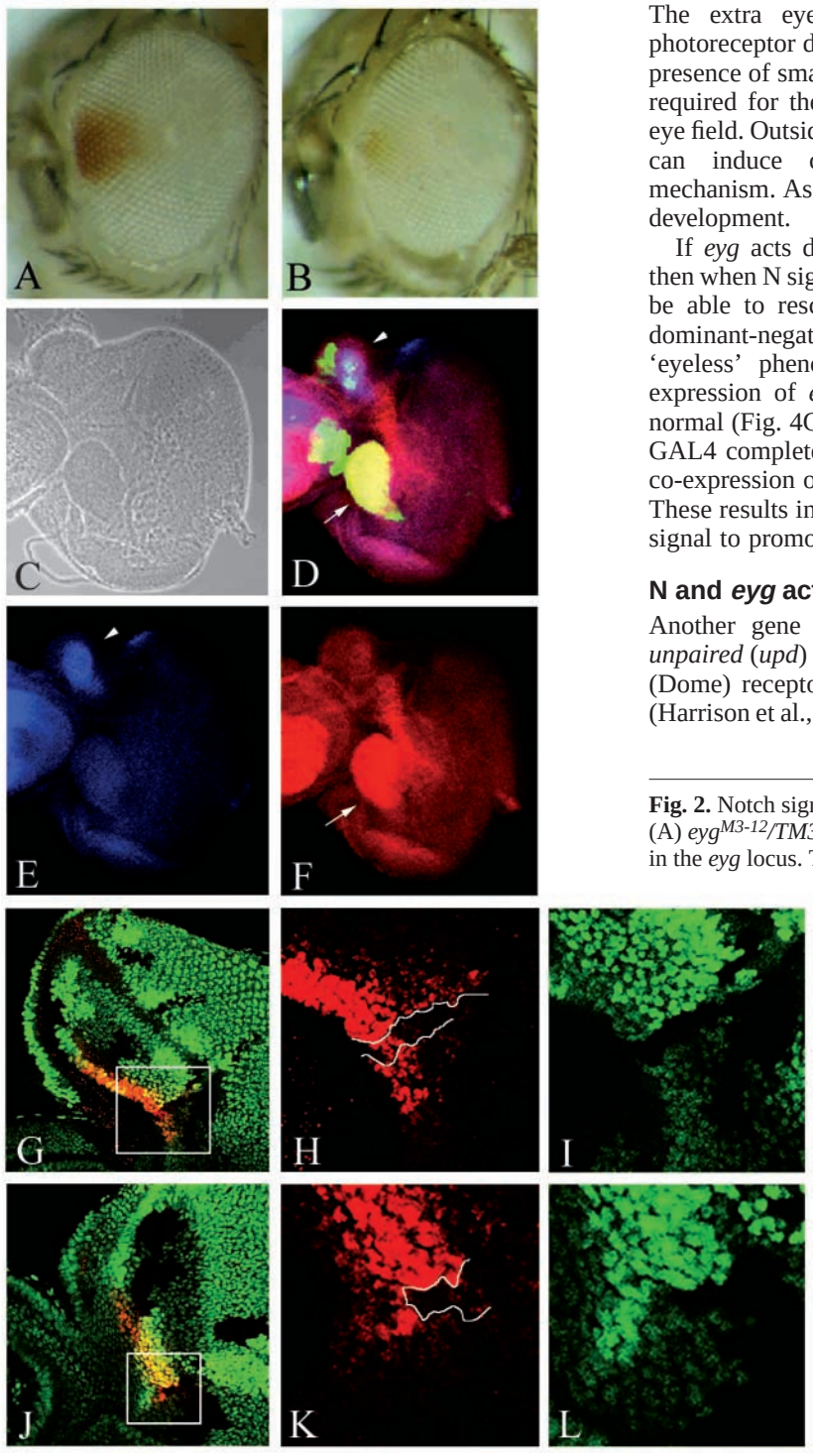

Fig. 2. Notch signaling induces *eyg* transcription in eye disc. (A) $eyg^{M3\text{-}12}/TM3$ fly is an enhancer trap line with a P[*lacW*] inserted in the *eyg* locus. The mini-*white* reporter in P[*lacW*] reflected the *eyg* expression pattern and in $eyg^{M3\text{-}12}/TM3$ shows the eye pigmentation pattern in an anterior-central region of adult eye. (B) The reporter expression in $eyg^{M3\text{-}12}/TM3$ is reduced in the N^{ts} mutant background. The eggs were laid at 18°C, raised at 18°C for 5 days, then shifted to 29°C until adults eclosed. The eye size is reduced. (C-F) Clones of ectopic N^{act} (marked by GFP, green) caused tissue overgrowth in eye-antennal disc. (C) DIC image. (D,F) *eyg-lacZ* ($eyg^{M3\text{-}12}$, stained by anti-β-galactosidase, red) expression is activated (arrow) within the N^{act} clones in eye discs except in the *wg* domain (arrowhead). (E) WG (stained by anti-WG, blue) is enhanced (arrowhead) within the *wg* endogenous expressing domain. (G-L) In most $Su(H)^{SF8}$ clones (marked by the absence of GFP, green) *eyg-lacZ* ($eyg^{M3\text{-}12}$, stained by anti-β-galactosidase, red) expression were not detected. But in a few clones, the mutant cells at the border of the clone still expressed *eyg-lacZ*.

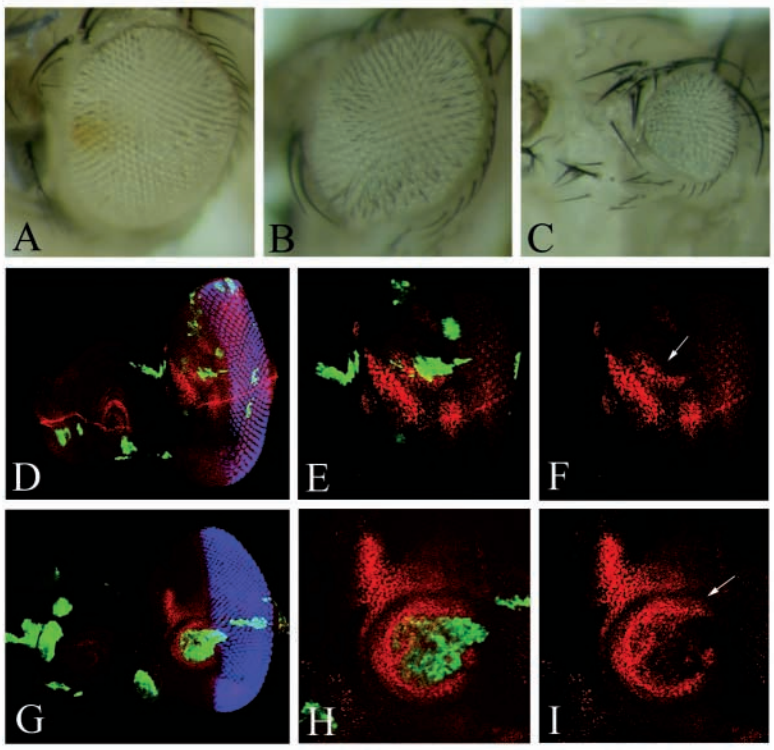

Fig. 3. *eyg* is induced at new DV border created by *Ser*- or *Dl*-expressing clones. (A-C) In Dl^{rev10}, Ser^{RX82} and $eyg^{M3\text{-}12}$ triple heterozygous mutant, the eye size and *eyg* expression (eye pigmentation based on the mini-*white* reporter in $eyg^{M3\text{-}12}$) were both reduced (1% of flies are like A; 98% are like B; 1% are like C). (D-I) Ectopic expressing clones were marked by GFP (green). Discs were stained using anti-Elav (blue) and anti-β-galactosidase ($eyg^{M3\text{-}12}$, red) antibodies. (D-F) When the *Ser*-expressing clone is located in the dorsal side near the DV midline of eye disc, *eyg-lacZ* is induced non-autonomously at the border of the clone (arrow). E,F are higher magnifications of D. (G-I) When the *Dl*-expressing clone is located in the ventral side near the DV midline of eye disc, *eyg-lacZ* is induced non-autonomously at the border of the clone (arrow). H,I are higher magnifications of G.

upd mRNA is expressed at the junction of eye disc and the optic stalk (Tsai and Sun, 2004). Loss-of-function *upd* mutants have small eyes, whereas misexpression of *upd* can induce non-autonomous proliferation of the undifferentiated cells of the eye disc (Bach et al., 2003; Chen et al., 2002; Chen et al., 2003; Tsai and Sun, 2004). The site of *upd* expression is where the posterior margin intersects with the DV border, which corresponds to N activation. So we tested the relationship between *upd* and the *N*/*eyg* pathway.

An *upd-lacZ* enhancer trap line was used to monitor the *upd* expression (Faucheux et al., 2001; Tsai and Sun, 2004). Although *upd* mRNA was not detectable in late third instar eye disc, the *upd-lacZ* can be detected in the pattern reflecting the expression in the early eye disc (Fig. 6A), probably owing to perdurance of the reporter protein. Clonal induction of N^{act} can induce *upd-lacZ* expression, but only in cells that are near the posterior margin (Fig. 6B,C). Conversely, clonal induction of N^{DN} can suppress the *upd-lacZ* expression (Fig. 6D,E). However, not all cells expressing N^{DN} have lost the *upd-lacZ* expression (Fig. 6D,E), perhaps because of the perdurance of the reporter protein. Alternatively, the *upd-lacZ* in some cells is induced by short-range signal from neighboring cells. To avoid these problem, we examined *upd* expression by in situ hybridization in N^{ts} mutant eye disc. In N^{ts} shifted to the restrictive temperature during eye development, the eye disc is reduced in size and has no detectable *upd* mRNA (Fig. 6G,H). Both the gain-of-function and loss-of-function experiments showed that *N* acts upstream to induce *upd* expression.

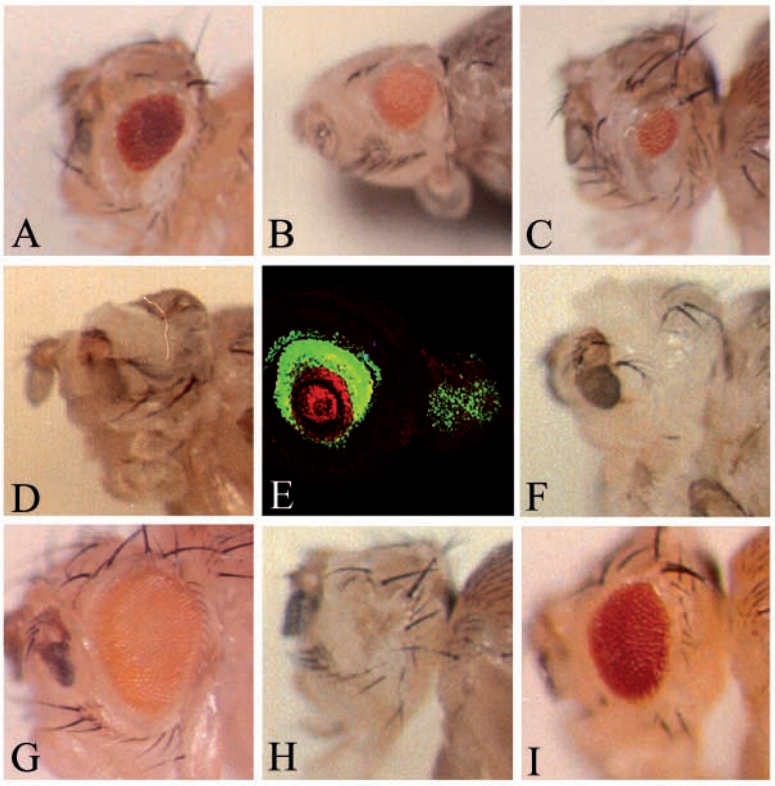

Fig. 4. *eyg* mediates the growth promoting function of Notch signaling. $eyg^{1}/eyg^{M3\text{-}12}$ mutants are eyeless with complete penetrance (see Fig. 1D). (A) In eyg^{1}, $H^{E31}/eyg^{M3\text{-}12}$ the eye size is partially restored. The eye size is also partially restored in (B) *dpp-GAL4 UAS-*N^{act} (abbreviated as *dpP>*N^{act}) and (C) *dpp>Su(H)* in the $eyg^{1}/eyg^{M3\text{-}12}$ mutant background. (D,E) When one copy of *H* was removed in $eyg^{M3\text{-}12}$, $H^{E31}/eyg^{M3\text{-}12}$, the flies have complete head structures but no eye (D) and *eyg-lacZ* (anti-β-galactosidase, red) is detected in antennal discs, but not in eye discs (E). The disc is also stained with anti-DAC (green) to mark the antennal disc. (F) In *ey>*N^{DN} flies, the eye formation is completely inhibited. (G) When *eyg* and N^{DN} were co-expressed (*ey>*N^{DN}*+eyg*), the eye size was partially restored. (H) In *ey>H* flies, the eye formation is completely abolished. (I) In *ey>H+eyg* flies, the eye size is partially restored.

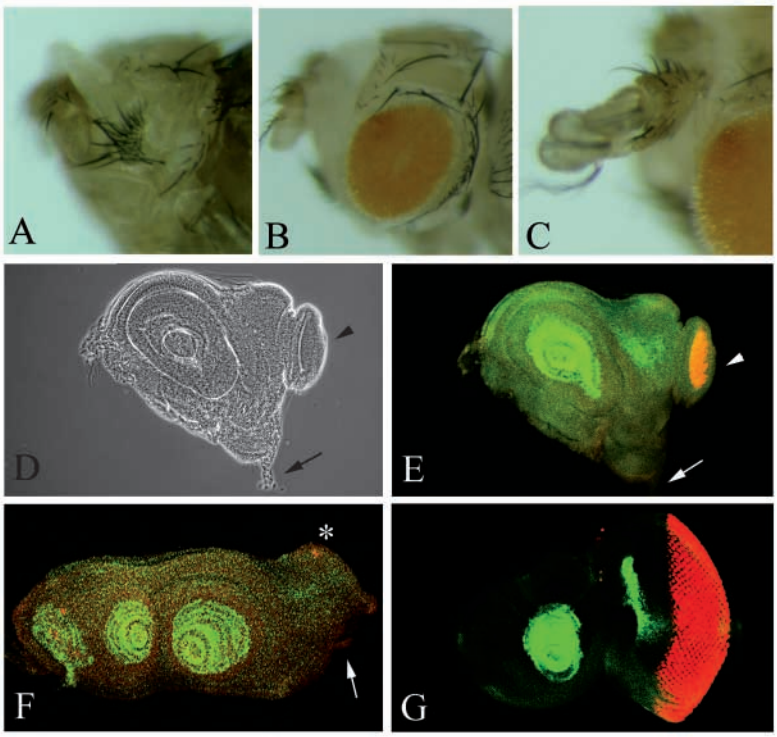

Fig. 5. Effect of N induction in *eyg* null mutant. N^{act} was induced by *ey*-GAL4 (abbreviated as *ey> N^{act}*) in *eyg^{M3-12}* homozygous mutant background. (A) Eighty percent of the pharates have head with no eyes. (B) Twenty percent have partially restored eye. (C) A few flies have a duplicated antennae. (D-F) The antennal discs are restored in size and have *eyg-lacZ* expression (green). (F) A disc has triplicated antennal fields (indicated by the *eyg-lacZ* expression). Occasionally one of the antennal fields can be recognized morphologically but lacks *eyg-lacZ* expression (not shown). (D-F) The endogenous eye field (based on the location of the optic stalk and Bolwig nerve; arrow) is highly reduced, lacks *eyg-lacZ* expression and has no photoreceptor differentiation (ELAV-staining, red). (D,E) In about 36% of the clones, an extra eye field (arrowhead) is induced dorsal to the endogenous eye field. The extra eye field can have *eyg-lacZ* (green) expression and photoreceptor differentiation (ELAV, red). The ocellus is marked by ELAV (*). (G) The *eyg-lacZ* expression pattern (green) in wild type is used for comparison.

Targeted expression of N^{DN}, driven by the *ey*-GAL4, completely blocked eye development (Fig. 4F). Co-expression of *upd* and N^{DN} rescued eye development and caused a slightly enlarged eye (Fig. 6F). Thus, *upd* is epistatic to *N*.

upd also genetically interacts with *eyg*. os^1 is a hypomorphic allele of *upd* and exhibits small eyes (Fig. 7A). eyg^1 is a hypomorphic *eyg* allele and also exhibits small eyes. os^1; eyg^1 double mutants have no eye (Fig. 7B). Removing one copy of *eyg* (in eyg^{M3-12}/+ heterozygotes) also enhanced the small eye phenotype of os^1 (Fig. 7C). In eyg^1 mutant eye disc, *upd-lacZ* expression is absent (Fig. 7D). Clonal expression of *eyg* can induce *upd-lacZ* expression when the clone is at the eye disc margin (Fig. 7E,F). So *eyg* acts upstream to regulate *upd* expression. In eyg^1/eyg^{M3-12} mutant, which has no eye (Fig. 1D), targeted expression of *upd* (*dpp>upd*) can completely restore the eye size (Fig. 7G). Conversely, in the os^1 mutant, targeted expression of *eyg* (*ey>eyg*) did not rescue the small eye phenotype (Fig. 7H). This is in contrast to the enlarged eye because of *ey>eyg* in wild-type background (Fig. 7I). These results suggest that *eyg* acts upstream to induce *upd* transcription and its effect on cell proliferation is largely mediated through *upd*.

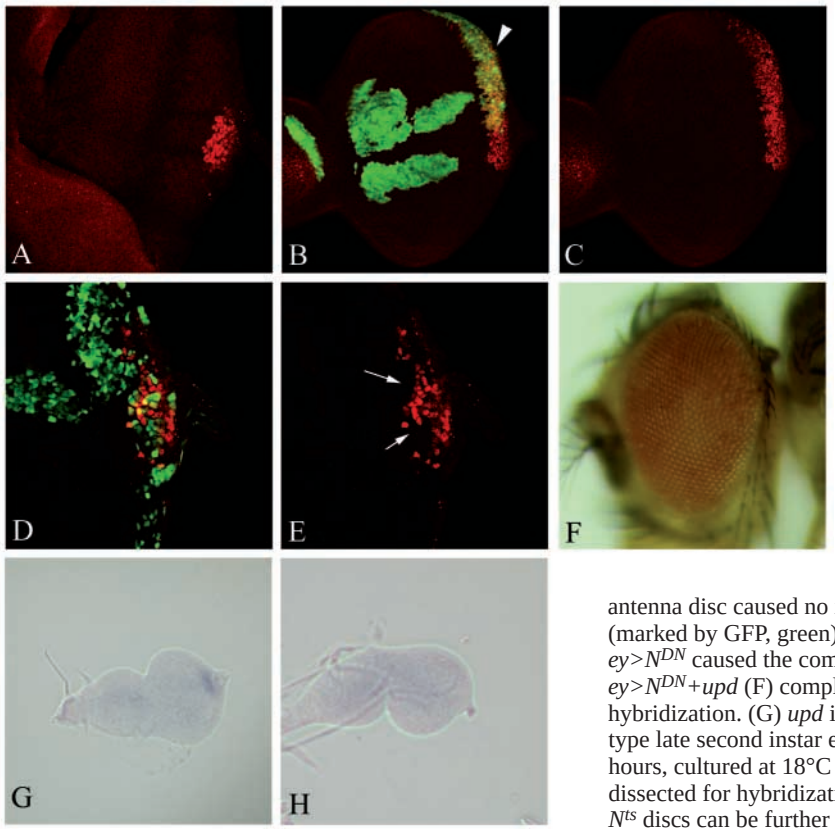

Fig. 6. Notch induces *upd* expression. (A) *upd-lacZ* (anti-β-galactosidase, red) is expressed at the junction of optic stalk and the posterior margin of eye disc. (B,C) *upd-lacZ* (red) is induced (arrowhead) in the N^{act}-expressing clones (marked by GFP, green), but only when the clone is located at the posterior margin of the eye disc. Even large clones in the central region of the eye disc and in antenna disc caused no induction on *upd-lacZ*. (D,E) N^{DN}-expressing clone (marked by GFP, green) inhibited *upd-lacZ* (red) expression (arrow). Whereas *ey>N^{DN}* caused the complete absence of eye development (see Fig. 4F), *ey>N^{DN}+upd* (F) completely restored the eye size. (G,H) *upd* RNA in situ hybridization. (G) *upd* is expressed at the center of the posterior margin in wild-type late second instar eye-antenna disc. (H) N^{ts} eggs were laid at 18°C for 24 hours, cultured at 18°C for 6 days, then shifted to 29°C for 24 hours and dissected for hybridization. Only males were selected for disc dissection. The N^{ts} discs can be further identified by the reduction of eye disc size.

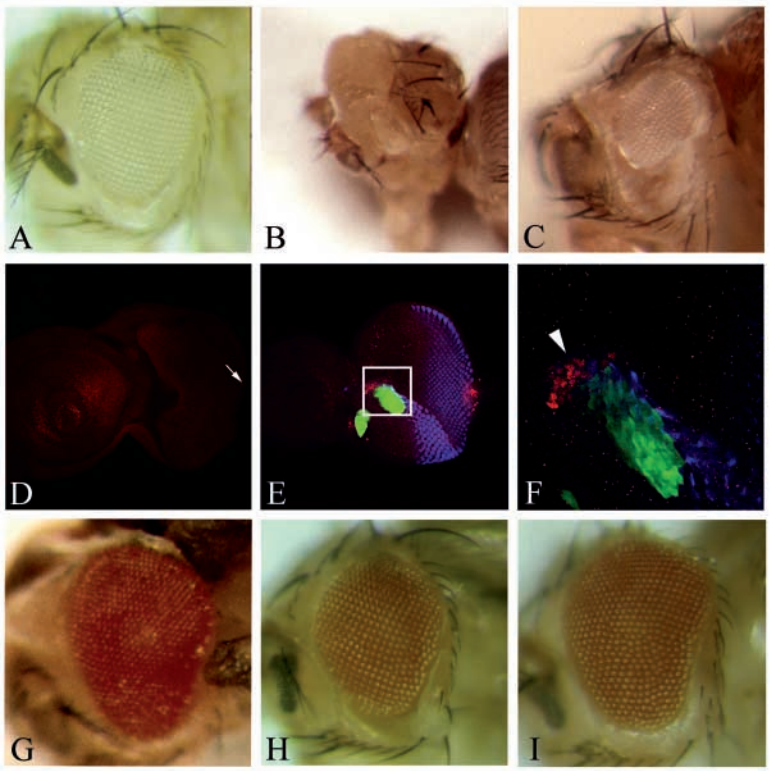

Fig. 7. *upd* expression is induced by *eyg* and mediates *eyg* function. (A) The hypomorphic *upd* allele os^1 has small eyes. Similarly, the hypomorphic eyg^1 mutants also have small eyes. (B) The os^1; eyg^1 double mutants have total absence of eyes. (C) The os^1 eye phenotype was enhanced by removing one copy of *eyg* in $eyg^{M3\text{-}12}$/+ heterozygote. (D) *upd-lacZ* (anti-β-galactosidase, red) expression is not detected in eyg^1 mutant eye disc. Arrow indicates its normal expression site. (E,F) Clonal expression of *eyg* (marked by GFP, green) at the eye disc margin non-autonomously induced *upd-lacZ* (red) expression (arrowhead in F, magnified view of fromed area in E. (G) *dpp>upd* fully rescued the eye development which is absent in $eyg^1/eyg^{M3\text{-}12}$. The eyes are slightly larger than wild-type (compare with Fig. 2A). (H) In os^1 mutant, the overexpression of *eyg* (*ey>eyg*) did not cause eye enlargement, which *ey>eyg* did in wild-type background (I).

Discussion

Turning a localized signal to a global growth signal

Notch signaling controls the growth of the eye disc. In this study, we showed that the Eyg Pax protein is a major effector mediating the growth promoting function of N. N is activated at the DV boundary of the early eye disc. This equatorial N signal then activates *eyg* expression at the transcriptional level. When N signal is reduced, *eyg* expression is reduced (Fig. 2B,G-J). When N signal is elevated, *eyg* expression is induced (Fig. 2C-F). Induction of *eyg* expression occurs at the DV border between the dorsal *Dl*-expressing and the ventral *Ser*-expressing cells (Fig. 3). Furthermore, when the upstream N signal is blocked, overexpression of *eyg* can rescue the growth defect in the eye (Fig. 4F-I), whereas increasing N signaling cannot rescue the eye-growth defect caused by the downstream *eyg* gene (Fig. 4D,E). Our analysis showed that the induction of *eyg* by *N* is dependent on the ligands *Dl* and *Ser*, and involves the effector *Su(H)* and the antagonist *H*. Thus, the localized activation of N signal is transmitted to the induction of a transcription factor, Eyg, which then promotes cell proliferation. A recent paper (Dominguez et al., 2004) came to the same conclusion.

Eyg is a transcription factor, so must activate the transcription of some genes that promote cell proliferation. Upd is reported to act through the Jak/STAT signaling pathway to promote cell proliferation (Tsai and Sun, 2004). We demonstrate that *upd* expression is dependent on *eyg* and N signaling (Fig. 6B-E, Fig. 7D-F). Furthermore, when the upstream N signaling or *eyg* is reduced, overexpression of *upd* can rescue the growth defect (Fig. 6F, Fig. 7G). The overgrowth effect due to overexpression of the upstream *N* or *eyg* is blocked when the downstream *upd* is defective. Our results suggest that *upd* is a major effector for the growth promotion by *N* and *eyg*.

Our results have demonstrated the functional link from *Notch* to *eyg* to *upd* in the promotion of eye growth. The link to *upd* solved a long-standing problem. N signaling is activated locally at the border between the dorsal Dl-expressing cells and the ventral Ser-expressing cells. How does a localized activation of N signal promote cell proliferation throughout the entire eye disc? The finding of *eyg* as the major mediator of N function did not solve the problem, as Eyg is a transcriptional factor and is expected to affect target gene expression autonomously. The link from *eyg* to *upd* provided a solution, as Upd is a diffusible signaling molecule. Upd protein can distribute over a long distance and exert long-range non-autonomous effect to promote cell proliferation (Tsai and Sun, 2004). So the localized N activation can locally activate *eyg*, which then turns on *upd* expression, probably through a short-range signal. The Upd signal then acts over a long range to promote cell proliferation in the early eye disc.

Mechanisms of induction

Although we demonstrated that *N* activates *eyg*, and *eyg* activates *upd*, these transcriptional activation may be direct or indirect. When novel DV borders were created by ectopic expressing *Dl* or *Ser*, *eyg* was induced non-autonomously at the border of these clones (Fig. 3D-I). We also noted that in *Su(H)* mutant clones, mutant cells at the border of the clone can still express *eyg-lacZ* (Fig. 2G-I). These observations suggest that *N* may induce a short-range signal, which then activates *eyg* expression. Alternatively, the apparent non-autonomous induction may be due to perdurance of the reporter protein in cells that were once close to the clone border. The induction of *upd* by *eyg* also may be indirect. Clonal expression of *eyg* also induced *upd* expression non-autonomously (Fig. 6E,F). In addition, based on RNA in situ hybridization, *eyg* expression in the eye disc did not extend to the posterior margin (Jang et al., 2003), so does not overlap with the expression domain of *upd* (Tsai and Sun, 2004). These suggested that the effect of *eyg* on *upd* expression may be indirect. However, an *eyg* enhancer trap line showed reporter expression extending to the posterior margin (Dominguez et al., 2004). Thus, we do not exclude the possibility that Eyg can directly activate the expression of *upd*.

The activation of *eyg* and *upd* are context dependent. N^{act} does not induce *eyg* expression in antenna and wing discs. In the eye disc, N^{act} induced *eyg* expression only in the region anterior to the MF, and not within the *wg* expression domain in the lateral margin (Fig. 2C-F). Similarly, N^{act} and *eyg* can only induce *upd* expression at the margin (Fig. 5B,C; Fig. 6E,F), but not in the center of the eye disc. N^{act} induce *upd* at the posterior margin but not lateral margins, while *eyg* can induce *upd* in the lateral margins but not in the posterior margin. The context dependence indicates that additional factors are involved to determine the specificity of induction.

In a late third instar eye disc, *eyg* is expressed in an equatorial domain that does not overlap with the disc margin, so cannot induce *upd*. In early eye disc, *eyg* expression domain comes closer to the posterior margin (Jang et al., 2003). Thus, the induction of *upd* by *eyg* is likely at second instar, which is consistent with the timing of *upd* expression (Tsai and Sun, 2004).

More than a linear pathway

Although *eyg* plays an important role in mediating the growth-promoting N signal, it is probably not the only effector. In the $eyg^{M3\text{-}12}$ null mutant, $ey{>}N^{act}$ does not rescue the endogenous eye field, but can still induce proliferation to provide the antennal disc and an extra eye field (Fig. 5). Thus, N can induce proliferation by an *eyg*-independent mechanism. The effect on antenna and on eye seems to be separate, because $ey{>}N^{act}$ can induce a large antenna disc with duplicate or triplicate antennal field without rescue of the eye disc (Fig. 5E). As N can induce *upd*, but not *eyg*, in the posterior margin, the induction of *upd* can also be through an *eyg*-independent mechanism.

N^{act} can induce overgrowth in the central domain of the eye disc (Fig. 2C,D). In these, *eyg*, but not *upd*, is induced. In addition, the overgrowth does not extend much beyond the clone. Ectopic *eyg* in the central domain also induced proliferation without inducing *upd*. In eyg^{1} mutant, there is no *upd-lacZ* expression in eye disc (Fig. 6D), but the eye is only slightly reduced. These results suggest that the N signaling and *eyg* can induce local proliferation independent of *upd*.

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