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Cell-type-specific transcription of *prospero* is controlled by combinatorial signaling in the *Drosophila* eye

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In *Drosophila*, Notch and Egfr signaling regulate the determination of many cell types, and yet how these common signals generate cell-specific transcription is not well understood. In the compound eye, *prospero* (*pros*) is transcribed specifically in R7 photoreceptors and cone cells. We show that the transcription of *pros* is activated by two visual-specific transcription selectors, Glass and Sine Oculis, that bind to an enhancer and promote its activation. Together with the pre-patterning transcription factor Lozenge, these factors work in a highly combinatorial manner, such that cells missing any one factor transcribe *pros* only weakly, if at all. However, the factors are not sufficient to activate the enhancer because of an additional requirement for both Notch and Egfr signals. The loss of Notch signaling produces a 'salt and pepper' effect, with some cells expressing near-normal levels and others expressing no detectable *pros* at all; thus, the signaling loss does not produce a uniformly reduced level of transcription activity in cells. This suggests a probabilistic mechanism, in which Notch signals influence the likelihood that the enhancer is inactive or fully active in any given cell. The activity level, therefore, is dictated by the proper combination of highly cooperative selector and pre-pattern factors present in the cell.

KEY WORDS: Notch, Egfr, Photoreceptor, *Drosophila*

INTRODUCTION

The differentiation fates of cells are often influenced by the intercellular signals that cells receive. This influence is usually manifested by a coordinated change in gene expression, often through the regulation of transcription factors within cells. A number of mechanisms have been described that are used during signaling to regulate the activities of transcription factors. These include phosphorylation, the mobilization of factors to novel cell compartments, and targeted protein-protein interaction. It remains unclear how these types of regulation manifest gene expression changes specifically within a cell when it selects a particular developmental program.

The *Drosophila* compound eye is an excellent model system with which to study this mechanism. Eye development is divided into two distinct phases, proliferation and differentiation. In the first phase, cells in the eye field asynchronously proliferate from the earliest larval stage until the third instar stage of larval life (Wolff and Ready, 1993). Although these cells are not differentiating, they express retinal determination (RD) genes that restrict their potency to eye-specific fates (Pappu and Mardon, 2004). RD genes encode transcription factors that promote the development of eyes throughout the animal kingdom, and they are organized in a hierarchical transcription network. The canonical members of the *Drosophila* RD network are Eyeless (Ey), Sine Oculis (So), Eyes Absent (Eya) and Dachshund (Dac) (Pappu and Mardon, 2004; Silver and Rebay, 2005).

The differentiation phase of eye development begins in the early third instar larva, when cells situated at the posterior end of the eye disc start to differentiate, followed progressively by more anterior cells (Fig. 1A). This wave of differentiation is marked by a morphogenetic furrow (MF), which traverses the eye disc from posterior to anterior for the remainder of the third instar stage up to the early pupal stage (Voas and Rebay, 2004). Prior to entering the MF, cells adopt a pre-proneural (PPN) state and they express Ey, So, Eya and Dac, which are a prerequisite to enter the proneural state (Greenwood and Struhl, 1999; Pappu and Mardon, 2004). The proneural state is hallmarked by the expression of a bHLH transcription factor called Atonal. So and Eya activate Atonal transcription, which then establishes the differentiation program by specifying R8-type photoreceptor neurons (Zhang et al., 2006). R8 cells then induce their neighbors to differentiate into other cell types (Voas and Rebay, 2004). Induction requires a short-range signal that activates the EGF receptor (Egfr) in neighboring cells (Nagaraj and Banerjee, 2004; Voas and Rebay, 2004). Egfr acts in many tissues and stages of development where it predominantly activates the Ras-MAPK signal transduction pathway (Rubin et al., 1997). Activated MAPK triggers the inhibition of an ETS-domain transcription repressor, Yan (Aop – FlyBase), and the stimulation of the ETS transcription factor Pointed (Pnt).

R8 cells both directly and indirectly induce the differentiation of seven other photoreceptors and four non-neuronal cone cells (Wolff and Ready, 1993). Photoreceptor neurons differentiate first, followed by cone cells (Fig. 1A). The fates of photoreceptors and cone cells are determined by the expression of cell-type-specific genes that encode transcription factors (Nagaraj and Banerjee, 2004; Voas and Rebay, 2004). One of the best-characterized examples of such a factor is encoded by the *prospero* (*pros*) gene. Pros is a homeodomain transcription factor that is required in R7 photoreceptors to differentiate them from R8 photoreceptors; the loss of *pros* results in a transformation of photoreceptor characteristics to an R8-state (Cook et al., 2003). The *pros* gene is expressed specifically in R7 photoreceptors and cone cells (Kauffmann et al., 1996). An enhancer located about 10 kb upstream of the promoter is essential for this transcription response (Xu et al., 2000). The enhancer requires binding of the RUNX transcription factor Lozenge (Lz) for activity in the eye; the loss of Lz completely abolishes enhancer activity (Xu et al., 2000). Lz protein is localized

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in precursor cells of the eye after the first wave of photoreceptor determination (R2, R3, R4, R5 and R8) has occurred (Flores et al., 1998).

The transcription of *pros* is stimulated by Egfr signal transduction (Kauffmann et al., 1996), which acts through several Yan/Pnt-binding sites within the enhancer (Xu et al., 2000). Although the Yan/Pnt-binding sites are necessary for Egfr stimulation of the enhancer, disabling these sites does not eliminate the enhancer's activity. Moreover, the transcription activity of the enhancer is not completely impaired when Egfr signaling or signal transduction is blocked (Xu et al., 2000). This suggests that Egfr acts either redundantly or additively with other inputs. Curiously, when Egfr signaling is blocked, *pros* expression and enhancer activity are nearly normal in some cells but are undetectable in others (Xu et al., 2000). This 'salt and pepper' phenotype suggests that the Egfr signal increases the probability of transcription but not the amount of transcription activity generated by the enhancer. The fraction of cells in which the enhancer is able to drive transcription reflects one aspect of the transcriptional strength of the enhancer. In this study, we further explore the mechanism by which the *pros* enhancer functions to control cell-type-specific expression in the eye.

MATERIALS AND METHODS

Genetics

Standard genetic methods were used. Flies were reared at 25°C. Mutant alleles and transgenic lines used in this study were as follows: N^{ts1}, N^{ts2}, N^{ts3}, UAS-N^{ECN} (=UAS-N^{DN}) (Kidd et al., 1998), *sev-GAL4*, *Lz-GAL4*, *ey-GAL4*, UAS-*Su*(*H*)-*VP16* (Morel and Schweisguth, 2000), UAS-*H* (Go et al., 1998), *sev-*N^{act} (Fortini et al., 1993), UAS-so^{DN}, gl^{60j} (Moses and Rubin, 1991), *sev-Svp* (Kramer et al., 1995), and *ey-FLP*. The *eya*(*ey*) genotype is: *y w ey*-FLP/*w; GMR-hid* FRT40A/eya^{cli1} FRT40A; *ey-GAL4*/UAS-*eya*. The *so*(*ey*) genotype is: *w;* FRT42D *GMR-hid*/FRT42D so^{3}*; ey-GAL4* UAS-FLP/UAS-*so*.

Plasmid construction and germline transformation

The *pros* enhancer regions were amplified by PCR and subcloned into pBluescript. After sequencing, the enhancers were subcloned into pwHZ128 (Flores et al., 2000) or pPRβE, in which the *hsp27* promoter of pWnβE was substituted with the 913-bp endogenous *pros* promoter (–789 to +124). Su(H), Gl and So binding sites were mutated as follows (underlined).

Su(H) sites: pros1, ATTAGAA to ATTATTA; pros2+pros3, TTACCATTATCAC to TTACCGGGATCAC; pros4, TTGTCAT to AAGTCAT; pros5+pros6, TTCCCCCCCAC to TTCCACACCAC; pros7, TTCTCAG to TAATCAG; pros8, TCCCCAC to TCAACAC; and pros9+pros10, AAGGGAGAA AAGGAATAA.

Gl site: CGAAACAATTAAAGGGTTTCGAG to CGAAACAATTA-AAACCGGACGAG.

So site: pros1, GGAAGACGAAACA to GGAAGACTCAACA; pros2, GGTTTCGAGTTGC to GGTTTCGAGATGA; and pros3, GCAGCCA-GACAAA to GCAGCCATCCAAA.

Multiple lines were examined for each transgenic construct. *lacZ* expression was examined in the eye discs of females carrying two copies of a transgene.

To make the UAS-so^{DN} construct, the full-length *so* cDNA (a gift from F. Pignoni, Harvard University, Cambridge, MA) was mutagenized as described (Roederer et al., 2005), and was amplified by PCR, with *Bam*HI and *Xba*I sites added at the 5′ and 3′ end, respectively. The amplified cDNA containing the entire coding sequence plus the last –80 bp of the 5′ UTR was inserted into the *Bgl*II/*Xba*I site of the pUAST vector to make the UAS-so^{DN} plasmid.

Immunohistochemistry

Antibody staining of the eye disc was carried out essentially as described previously (Hayashi et al., 1998). The antibodies used were: mouse anti-Eya (Bonini et al., 1993), rat anti-Elav (Robinow and White, 1988), rabbit anti-lacZ (Cappel), mouse anti-Pros (Kauffmann et al., 1996), mouse anti-Lz (Flores et al., 1998) and mouse anti-Arm (Peifer et al., 1994).

Electrophoretic mobility-shift assay

Electrophoretic mobility-shift assays were performed essentially as described by Pauli et al. (Pauli et al., 2005). So, Su(H) and Gl proteins were synthesized in a reticulocyte lysate-coupled transcription/translation system (Promega) from the full-length *so, Su*(*H*) and *gl* cDNAs inserted into pET vectors. The Kozak sequence (GCCACC) was added in front of the first ATG codon. Oligonucleotides were 5′ end-labeled. Molar excess (50× or 100×) of unlabeled oligonucletoide was added for competition in each binding reaction. The sequences of oligonucleotides used were as follows:

soAE, AAACTCTGGTAATTCGATATCATTGTT;
soAEmut, AAACTCTGGTAATTCCCCATCATTGTT;
lz,
ATGCGTAAATTGATATCAATAATTGTTATTGATATCAACGCACG;
prosSo1, GGCGAAGTCAGGAAGACGAAACAATTA;
prosSo2, TAAAGGGTTTCGAGTTGCCTTAATGAAC;
prosSo3, TAATGAACCGCAGCCAGACAAAAGGTC;
E(spl)m4S1, TATCCTTGTAGTTTCCCACACTGGGTGTTTT;
E(spl)m4S1mut, TATCCTTGTAGTTTGCCACACTGGGTGTTTT;
pros1, GAGGCCGCTTTATTAGAATATCCGGCAGCTT;
pros2+pros3, AGCAGCACCATTACCATTATCACTATCTGCCCT;
pros4, CCCTGGTCCACATTGTCATAATTCATAAATC;
pros5+pros6, AACAAATAGCTTCCCCCCCACCCAAAAAAAA;
pros7, TGAGTCGAAAAGTTCTCAGGCCTGGCGAAGT;
pros8, AACAAAGGTGTGTCCCCACACACACACACAC;
pros9+pros10, AACAAAAGCGAAAGGGAGAAGACGTCACGGC;
Rh1, TTAAAGGCATTTCAAGGGTTTCCACTGG;
prosGl, GACGAAACAATTAAAGGGTTTCGAGTTG; and
prosGlmut, GACGAAACAATTAAAACCGGACGAGTTTG.
Each oligonucleotide has two additional Ts (TT) at the 5′ end for labeling.

RESULTS

Analysis of the *pros* enhancer

The *pros* enhancer was previously identified as a 1.2 kb region that is approximately 10 kb upstream of the transcription start site (Xu et al., 2000). Its ability to drive expression of a hsp27-promoter>lacZ transgene in the eye was limited; four tandem copies of the enhancer achieved a modest reproduction of the *pros* expression pattern (Xu et al., 2000) (Fig. 1B). We tested whether the enhancer worked more effectively in combination with other promoters by linking the enhancer to pros-promoter>lacZ and hsp70-promoter>lacZ transgenes. Indeed, both promoters gave much stronger responses, such that only one copy of the enhancer was sufficient to strongly drive transgene expression in the proper pattern (Fig. 1B). Using the hsp70>lacZ reporter, we began to dissect the enhancer region that retains complete capacity to reproduce the *pros* expression pattern. The Yan/Pnt-binding sites and the Lz-binding sites are concentrated in the 5′ half of the 1.2 kb region. Indeed, a 465 bp fragment that encompasses the 5′ binding sites was able to reproduce completely the *pros* expression pattern (Fig. 1B). We refer to this fragment as the core-enhancer. Deletion analysis further indicated that the 5′-terminal 206 bp of the core-enhancer was dispensable, whereas the 3′-terminal 40 bp was essential. Using this information, we constructed a mini-enhancer in which sequences from 232-321 bp were fused to sequences from 420-465 bp of the core-enhancer. This 125 bp mini-enhancer induced reporter gene expression in the *pros* pattern with a little variegation (Fig. 1B). We aligned the sequences of the core- and mini-enhancers from several different *Drosophila* species (Fig. 2). We noted broad regions of strong sequence conservation that corresponded to previously characterized Yan/Pnt- and Lz-binding sites. There were also other conserved regions, implying other functional binding sites with these enhancers. We used the core-enhancer and the mini-enhancer for further genetic and biochemical analysis.

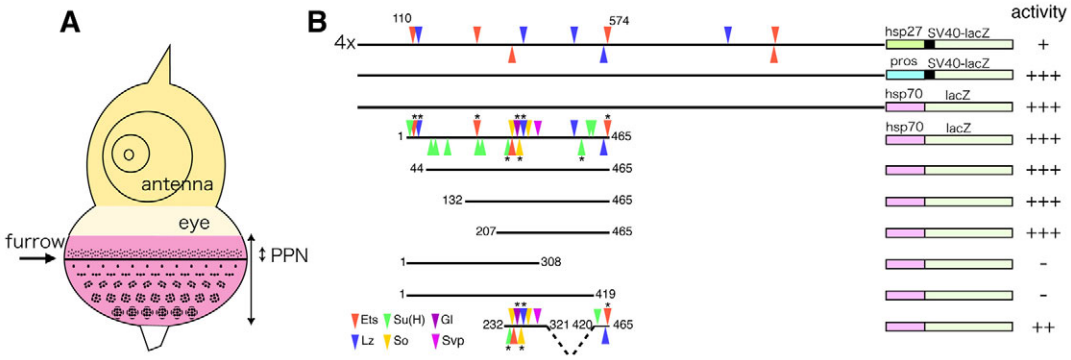

Fig. 1. The *pros* enhancer and eye development. (**A**) Schematic of the developing third instar eye-antennal disc complex. Anterior, top; posterior, bottom. The So/Eya expression domain is colored red. Positions of the morphogenetic furrow and PPN zone are shown. Black dots represent differentiating photoreceptor and cone cells, and show the progressive differentiation of cells from the furrow to the posterior disc margin. (**B**) Molecular analysis of the *pros* enhancer and transcription factor-binding sites. The original *pros* enhancer reporter is shown at the top, as a fusion of four tandem copies with *hsp27-lacZ*. Various constructs used in this study are shown below, including the full-size enhancer fragment fused to *pros-lacZ* and *hsp70-lacZ*. Deletion mutants of the fragment are presented below those. The transcription activity of each enhancer construct is presented as a qualitative scale from a β-gal pattern that completely mimics the Pros pattern (+++), to a β-gal pattern that exhibits variegated expression in R7 and cone cells (+ and ++), to no expression (–). Multiple independent transgenic lines were assayed for each construct. Colored triangles indicate putative transcription factor-binding sites based on their similarity to consensus-binding sequences. Their identities and locations in the *pros* enhancer region are indicated. Putative sites for which there are experimental binding data that confirm their binding-site activity (this paper) (Xu et al., 2000) are marked by an asterisk.

The *pros* enhancer is dependent on So and Eya

The RD proteins So and Eya act as a transcription heterodimer, in which the DNA-binding domain of So and the transactivation domain of Eya cooperate to trigger the transcription of target genes. Recent studies have identified a number of DNA sequence motifs that bind specifically to So protein with high affinity, and these sites mediate the transcriptional effects of So/Eya on target genes (Pauli et al., 2005). We used a consensus of these sequences to search for potential So-binding sites in the *pros* mini-enhancer and found three putative So motifs (Fig. 3A,B). No other potential motifs were detected in the core-enhancer (Fig. 1B). To test whether the three motifs bound to So protein, we performed EMSA assays using recombinant So protein. (Fig. 3C). The So protein formed a stable complex with a labeled DNA oligonucleotide that contained a consensus So-binding site. Moreover, binding was specific in that it was competed by unlabeled DNA with an intact So-binding site but not by DNA with a mutated So-binding site. We used this competition assay as a means to determine whether the motifs within the mini-enhancer had high affinity for So binding. Competition analysis found that So protein bound strongly to one of the three motifs in vitro (Fig. 3C). To test whether the So-binding sites were required to activate the *pros* enhancer, we mutated the sites in the enhancer and then placed the mutant enhancer upstream of a reporter *lacZ* gene. When this transgenic reporter was assayed for expression in the eye, it was found that expression was abolished (Fig. 3D,E).

We next examined whether So and Eya regulate *pros* expression. So and Eya are expressed in eye disc cells beginning when they enter the PPN/proneural state (anterior to the MF), and expression is subsequently maintained in cells posterior to the MF (Pappu and Mardon, 2004; Silver and Rebay, 2005) (see Fig. 1A). This complicates genetic analysis because the loss of either gene in cells when they are in the PPN/proneural state perturbs the subsequent differentiation of the cells. To uncouple the early and late functions, we generated *so* or *eya* mutant eyes in which their early functions (PPN/proneural) were rescued by transient transgene expression (Fig. 3F). These *so*(*ey*) and *eya*(*ey*) eyes showed qualitatively similar effects on *pros* expression, with *eya*(*ey*) showing a stronger effect than did *so*(*ey*). In these eyes, the number of cells expressing *pros* was greatly reduced (Fig. 3G-I). However, photoreceptor differentiation was also perturbed, and it was unclear whether the residual *pros*-positive cells were due to the perdurance of So/Eya. Therefore, we overexpressed a dominant-negative form of So in cells posterior to the MF (Fig. 3J). Such eye discs showed greatly reduced numbers of *pros*-positive cells and those that were positive showed reduced expression levels. These observations indicate that So and Eya are strongly required for *pros* expression in the eye.

It had been found that Lz expression is inhibited in clones of mutant *so* cells (Yan et al., 2003). This suggested to us that the loss of *pros* expression in *so*(*ey*) might be caused by a lack of Lz expression. However, both early and late functions of So were lost in the mutant clones of Yan et al.'s study. Thus, it was possible that *lz* expression requires early So function but not late So function, whereas we found that *pros* requires late So function. To explore this issue, we examined *lz* expression in *so*(*ey*) and *eya*(*ey*) eyes (Fig. 4). Although expression was weakly reduced in mutant eye discs, the degree of inhibition was minor in comparison with the effects of *so*(*ey*) and *eya*(*ey*) on *pros* expression. These results indicate that the dependence of *lz* and *pros* on So and Eya can be uncoupled, and that So and Eya act on *pros* in parallel to Lz. This interpretation is wholly consistent with the cis-mutagenesis of So- and Eya-binding sites causing a loss of *pros* enhancer activity in vivo.

Glass activates the *pros* enhancer

Although So and Eya are eye-specific transcription factors, they are not the only such factors that are present at the onset of *pros* expression. *glass* (*gl*) encodes a zinc finger DNA-binding transcription factor that is expressed specifically in the visual systems of *Drosophila* (Ellis et al., 1993; Moses and Rubin, 1991). In the eye disc, Gl is expressed in all cells (precursors and differentiating cells) posterior to the MF. Gl is required for the differentiation of all photoreceptors, but it is not necessary for these cells to become neurons (Moses et al., 1989; Moses and Rubin, 1991).

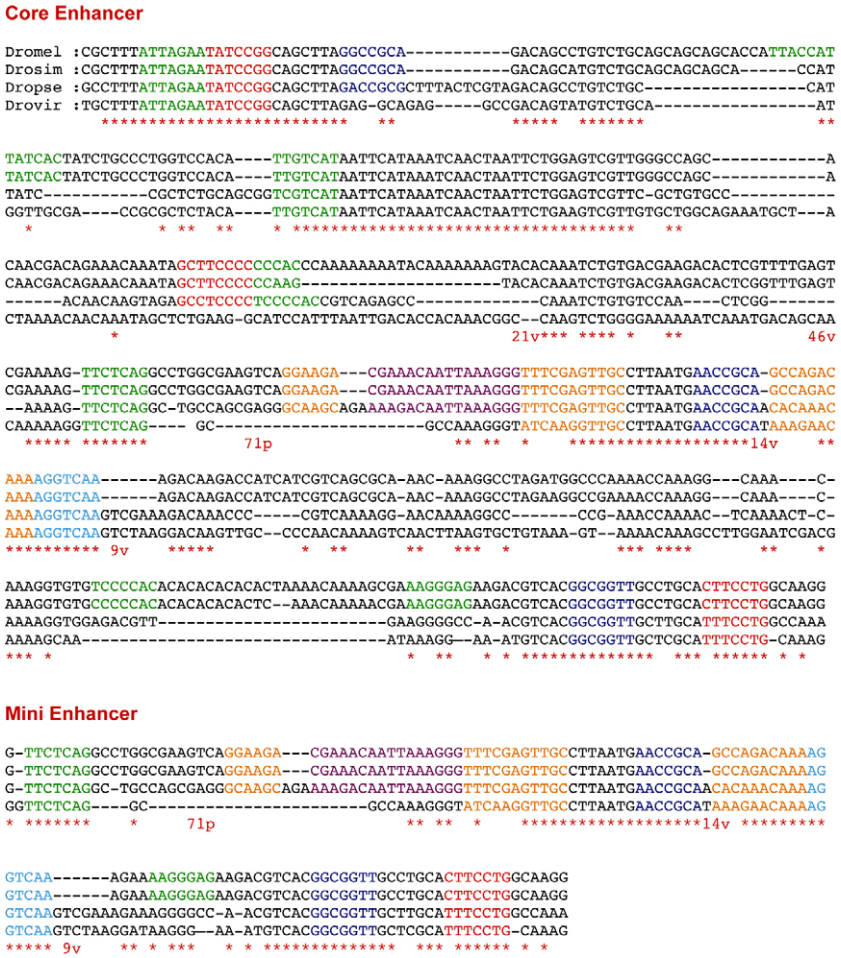

Fig. 2. Alignment of the *pros* enhancer from four *Drosophila* species. CLUSTAL alignment of sequences from the core-enhancer and the mini-enhancer used in experiments. Sequences conserved in all four species (*D. melanogaster*, *D. simulans*, *D. pseudoobscura*, *D. virilis*) are marked below the alignment by red asterisks. Long stretches of sequence found in only one species are not included, and their lengths are indicated by numbers of bases and a species identifier, below the alignment. Putative factor-binding sites are colored. These are: Su(H), green; Yan/Pnt, red; So/Eya, orange; Gl, purple; Lz, blue; Svp, cyan.

The *pros* mini-enhancer contains one putative Gl-binding motif based on sequence homology to known Gl-binding sites (Fig. 5A). No other Gl motifs were detected in the core-enhancer. Using competition-EMSA assays with recombinant Gl protein, we observed that the motif bound to Gl protein, though its affinity was slightly weaker than it is for a site within the Rh1 opsin promoter (Fig. 5A). To determine the effect of *gl* on *pros* expression in vivo, we made clones of *gl* mutant cells in the eye. Most mutant cells either weakly expressed Pros or did not detectably express Pros (Fig. 5B). However, some *gl* mutant cells expressed normal levels of Pros protein. Mutant cells also showed abnormal cone cell differentiation (Fig. 5C).

Although these results indicate that *pros* expression is positively regulated by *gl*, it does not necessarily mean that the regulation is direct. The functional importance of the Gl-binding site in the mini-enhancer was tested by mutating the site and assaying enhancer activity in vivo. This resulted in a strong and uniform reduction of enhancer activity (Fig. 5D), which indicates that the Gl-binding site is required for the enhancer to activate transcription. In total, our data indicate that Gl activates *pros* expression by directly binding to the *pros* enhancer.

Notch activates *pros* expression

Notch (*N*) encodes a transmembrane receptor molecule involved in intercellular signaling (Artavanis-Tsakonas et al., 1995). Delta (Dl) is a ligand for N, and is also a membrane-bound protein. Thus, Dl-N interactions are limited to neighboring cells. Dl in R1/R6 cells activates N in neighboring precursors and this is necessary for them to differentiate into R7 photoreceptors and cone cells (Cooper and Bray, 2000; Flores et al., 2000; Tomlinson and Struhl, 2001; Tsuda et al., 2002). Moreover, this signal is a key feature that distinguishes R7 and R1/R6 fates; precursor cells that become R1/R6 photoreceptors do not receive a Dl-N signal, whereas cells that become R7 cells do (Cooper and Bray, 2000; Tomlinson and Struhl, 2001).

We hypothesized that Dl-N signaling might be required for *pros* expression. We generated animals that carried a temperature-sensitive (ts) mutation to partially inactivate endogenous *N*, and that misexpressed a dominant-negative *N* isoform in R7 and cone cells. The result was that the number of *pros*-positive cells was reduced (Fig. 6A,B). We confirmed this *N* dependence in two other ways. We performed experiments in which dominant-negative *N* was driven by *Lz-GAL4* in all R1, R6, and R7 photoreceptors, cone cells, and their precursors (data not shown). This resulted in variegated Pros-positive cells in these discs, with both R7 and cone cells being equally affected. We also performed experiments without the dominant-negative *N*, in which N^{ts1} mutants were shifted from a permissive to a non-permissive temperature for 24 hours before analysis. In this case, there was a reduction in the number of Pros-positive R7 and cone cells in the anterior portion of the eye disc, and a weaker effect in the posterior portion (data not shown). These loss-of-function analyses indicate that *pros* expression in R7 and cone cells is dependent on *N*.

Upon Dl-N binding, an intracellular domain of N (N^{icd}) is cleaved and translocates to the nucleus (Mumm and Kopan, 2000). In the nucleus, the N^{icd} interacts with a DNA-binding transcription factor

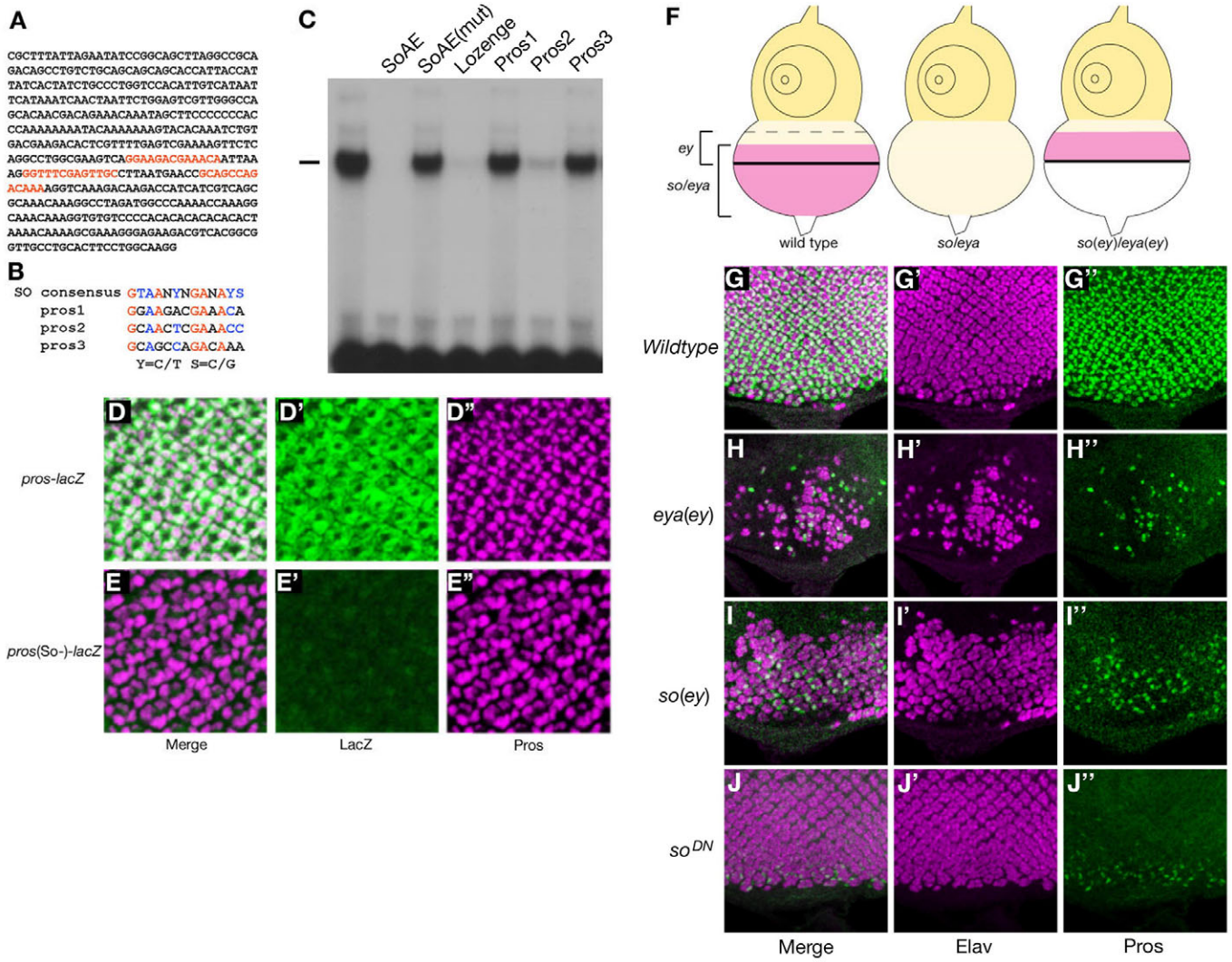

Fig. 3. So binds to the *pros* enhancer and activates transcription. (**A**) The three putative So-binding motifs are highlighted in red within the core-enhancer. These are also present in the mini-enhancer. (**B**) Alignment of a consensus So-binding site and the three putative So-binding sites. Essential residues are colored red; conserved residues are colored blue. (**C**) A mobility-shift gel showing a complex that contains So protein and labeled SoAE DNA. SoAE is a So-binding site identified in the *so* gene autoregulatory element. Each reaction also contained a 50-fold molar excess of an unlabeled competitor DNA as indicated, except for the reaction loaded into the leftmost lane, which did not contain competitor. The SoAE(mut) competitor has a mutated So-binding site; the Lozenge competitor contains a So-target sequence identified in the *lz* enhancer. The Pros competitors contain the three putative So-binding sites from the *pros* enhancer. (D,E) Transgenic core-enhancer-β-gal reporter expression (green) in eye discs counterstained for Pros (purple). (**D-D″**) The wild-type core-enhancer induces β-gal expression in the same cells that express Pros. (**E-E″**) The core-enhancer with mutant So sites does not induce β-gal expression. (**F**) Schematic of the approach to make *so*(*ey*) and *eya*(*ey*) mutant eye discs. The expression patterns of So/Eya in wild-type (left), *so*/*eya* mutant (middle) and *so*(*ey*)/*eya*(*ey*) (right) mutant eye discs are shown in red. In *so*(*ey*) or *eya*(*ey*) eye discs, So/Eya expression is limited to cells anterior to the MF (black line). (G-J) Expression of neural-specific Elav (purple) and Pros protein (green) in a wild-type eye disc (**G-G″**), an *eya*(*ey*) disc (**H-H″**), a *so*(*ey*) disc (**I-I″**), and an *Lz-GAL4*/+; UAS-so^{DN}/+ disc (**J-J″**).

Suppressor of Hairless [Su(H)]. In the absence of N^{icd}, Su(H) (or its mammalian homolog, CBF1) associates with a co-repressor and represses downstream genes (Barolo et al., 2000; Hsieh and Hayward, 1995; Morel and Schweisguth, 2000). N^{icd} de-represses target genes by displacing the co-repressor from Su(H), although some target genes only respond to N signaling when N^{icd}-Su(H) binds and activates their transcription (Morel and Schweisguth, 2000). To test whether Su(H) is required to regulate *pros* expression in vivo, we turned Su(H) into a constitutive repressor. When Hairless (H) protein is overexpressed, it binds to Su(H) and turns it into an N-independent constitutive repressor (Morel et al., 2001). Overexpression of H in the eye disc resulted in a reduced number of *pros*-positive cells, which was comparable to that observed following the loss of N activity (Fig. 6C). Thus, Su(H) regulates *pros* expression in a manner that phenocopies N.

N signaling does not occur in R1/R6 cells, which do not express *pros*. The lack of *pros* in R1/R6 cells might be due to the absence of Dl-N signaling. To test this possibility, we examined the effect of ectopically activating N in R1/R6 cells. The *sev-N*(*Act*) transgene expresses a constitutively active form of N in a subset of photoreceptors, including R1 and R6. In the presence of the *sev-N*(*Act*) transgene, one or two additional photoreceptors expressed

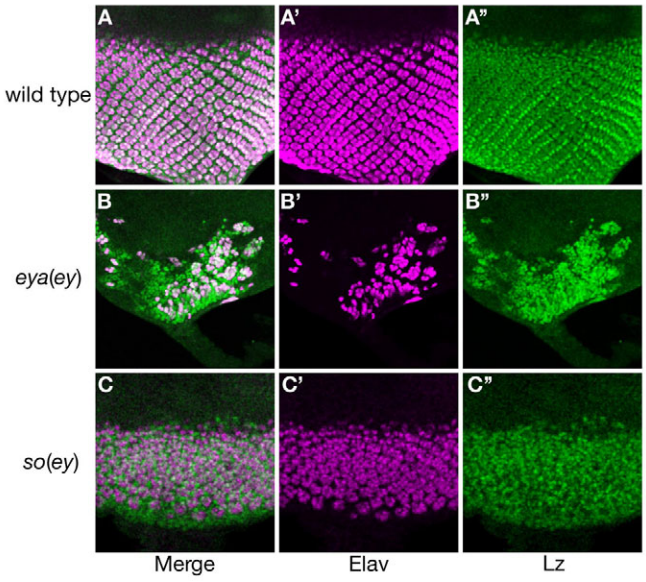

Fig. 4. Lz is expressed in *so(ey)* and *eya(ey)* eye discs. Elav protein (purple) and Lz protein (green) in wild-type (**A-A″**), *eya*(*ey*) (**B-B″**) and *so*(*ey*) (**C-C″**) eye discs.

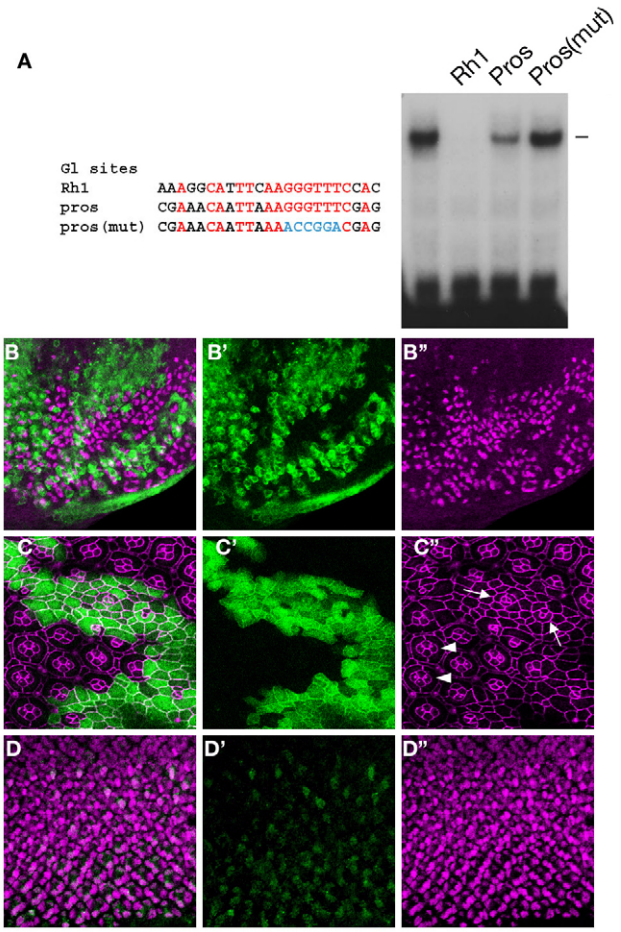

Fig. 5. Gl regulates *pros* transcription. (**A**) Gl protein binds specifically to a sequence present in the mini-enhancer. (Left) The sequence is aligned with a characterized Gl-binding site in the Rh1 promoter. Conserved nucleotides are highlighted in red. A mutated version of the *pros* site is shown, with altered nucleotides in blue. (Right) A mobility-shift gel showing a complex that contains Gl protein and labeled Rh1 DNA. Each reaction also contained a 100× molar excess of an unlabelled competitor DNA, as indicated at the top, except for the leftmost lane, which did not contain any competitor. (**B-B″**) gl^{60j} mutant cells are marked with cytoplasmic GFP (green), whereas Gl^{+} cells are unmarked in a genetically mosaic larval eye disc. Nuclear Pros protein was visualized by antibody (purple). Gl^{+} cells that normally express Pros appear as non-ringed purple, whereas gl^{60j} cells that normally express Pros appear as white or purple, ringed in green. gl^{60j} cells that express undetectable or very low levels of Pros appear as hollow green rings or green rings with a weak white signal (see B″). (**C-C″**) A large clone of gl^{60j} mutant cells is marked by GFP (green) in the pupal eye. Cell morphology is visualized by the β-catenin protein Arm (purple). Arrowheads indicate the morphologies of wild-type cone cells. Arrows indicate gl^{60j} cone cells that exhibit altered sizes and morphologies. (**D-D″**) Transgenic mini-enhancer-lacZ reporter expression (green) in an eye disc counterstained for Pros protein (purple). The mini-enhancer has its Gl-binding site mutated, which induces very weak or no β-gal expression in Pros-positive cells.

pros in each ommatidium (Fig. 6D,E). The position of these cells indicated that they were either R1 or R6 photoreceptors. Thus, N activation has the potential to drive *pros* expression in R1 and R6, although its effect is incomplete.

We further tested the capability of N signaling to stimulate *pros*. We misexpressed a constitutively active form of Su(H), with the VP16 activation domain fused to the protein. This resulted in the ectopic expression of *pros* in R1 and R6 (Fig. 6F). The similarities of gain-of-function and dominant-negative *Su*(*H*) phenotypes with gain- and loss-of-function *N* mutant phenotypes suggest that Su(H) mediates the positive effect of N on *pros* expression.

N signaling via Su(H) and Svp

The core- and mini-enhancer driven reporters were expressed in fewer eye disc cells in a N^{ts} background, indicating that N promotes the potential of the *pros* enhancer to activate transcription (data not shown). We wondered whether Su(H) directly mediates this effect. Su(H) binds to the sequence motif RTGRGAR (where R=A or G) (Lai, 2002). The *pros* mini-enhancer contains two motifs (pros7 and pros10) with one mismatch from the consensus (Fig. 1B). We examined whether Su(H) protein bound to pros7 and pros10 by performing competition-EMSA assays using recombinant Su(H) protein (Fig. 7A). The protein formed a stable complex with a labeled DNA oligonucleotide that contained a consensus Su(H)-binding site. Competition analysis found that Su(H) bound strongly to pros7 but not pros10. We tested the function of the pros7 motif by mutating the motif in a mini-enhancer-lacZ transgenic reporter. The pros7-mutant reporter did not exhibit a significant change in its expression pattern (Fig. 7B,C). To confirm that the pros7 mutant did not impair N signaling, we expressed constitutively activated *N* in R1/R6 cells and observed ectopic activity of the pros7-mutant reporter in R1/R6 cells (Fig. 7D). To ensure that we were not missing another active Su(H) site, we also analyzed the activities of eight other potential Su(H)-binding motifs. Competition-EMSA analysis found that only one (pros8) had Su(H)-binding activity in vitro (Fig. 7A). We mutated all ten motifs (including pros7 and pros10) within the core-enhancer, and tested whether the mutated enhancer could drive reporter expression in the eye disc. Indeed, there was no significant effect on the expression pattern of the reporter in vivo (data not shown). Thus, N signaling can activate the *pros* enhancer independently of direct Su(H) regulation.

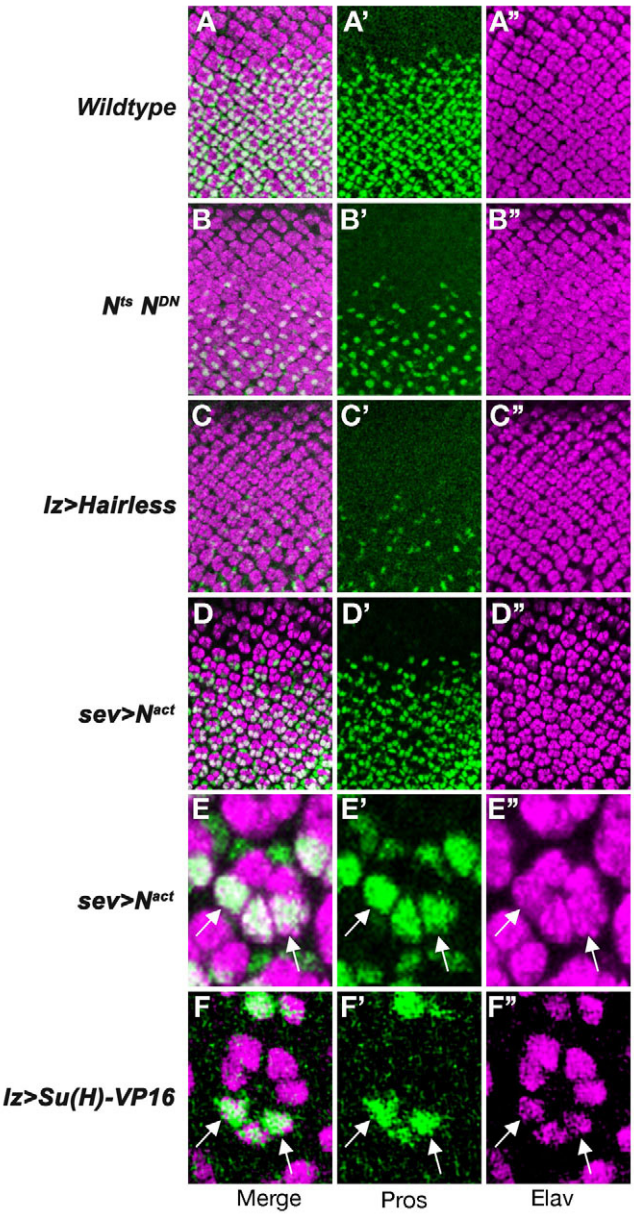

Fig. 6. Notch signaling activates *pros* expression. The expression of Pros protein (green) and neural-specific Elav protein (purple) are shown in eye discs. Co-expression is seen as white images in the merged panels. (**A-A″**) A wild-type eye disc from an animal raised at 25°C. (**B-B″**) A N^{ts3}/Y; UAS-N^{ECN} /+; *sev-GAL4* /+ eye disc from an animal raised at 25°C. (**C-C″**) A *Lz-GAL4* UAS-*H*/UAS-*H* eye disc. (**D-D″**) A *sev-*N^{act}/+ eye disc at a focal plane where only photoreceptor cells are visible. Two to three photoreceptors express Pros protein in each ommatidium. (**E-E″**) A high magnification view of one *sev-*N^{act}/+ ommatidium showing ectopic Pros in R1/R6 photoreceptors (arrows). (**F-F″**) A high magnification view of one *Lz-GAL4* /+; UAS-*Su*(*H*)-*VP16*/+ ommatidium. Ectopic Pros in R1/R6 is seen (arrows).

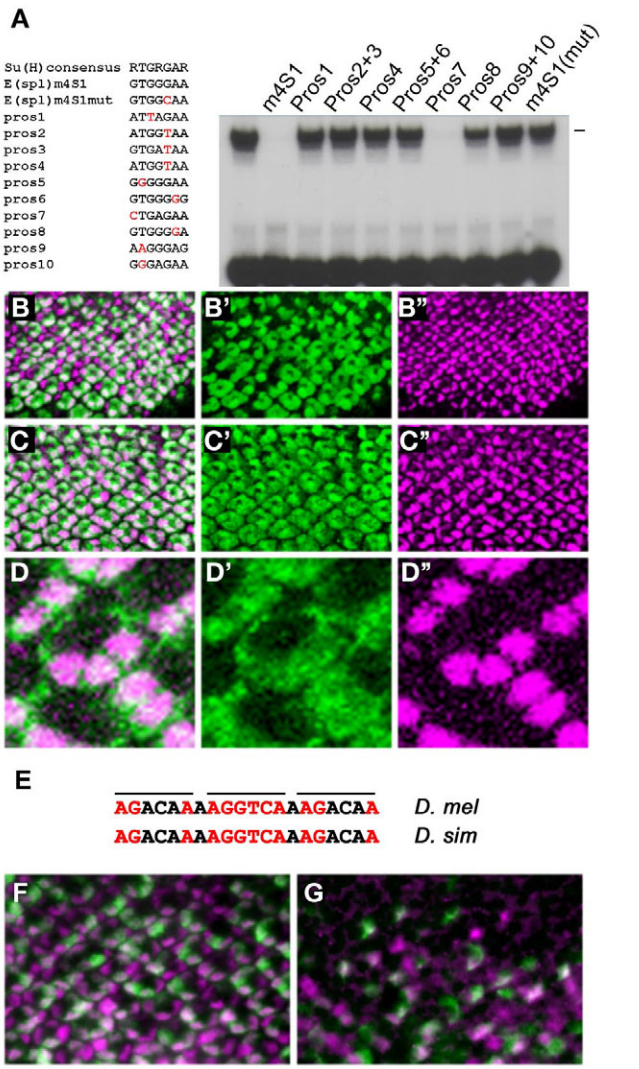

Fig. 7. Svp regulates the *pros* enhancer. (**A**) Alignment of the consensus Su(H)-binding sequence with the S1 site in the *E*(*spl*)*m4* gene and the ten putative-binding motifs found in the core-enhancer. Mismatched residues are colored in red. Two of these motifs (pros7 and pros10) are also present within the mini-enhancer. (Right) A mobility-shift gel showing the complex that contains Su(H) protein and labeled *E*(*spl*)*m4*S1 DNA (*m4S1*). Each reaction contained a 100× molar excess of an unlabeled competitor DNA as indicated, except for the reaction loaded into the leftmost lane, which did not contain competitor. (**B-C″**) Transgenic mini-enhancer-β-gal reporter expression (green) in eye discs counterstained for Pros protein (purple). (B-B″) β-gal expression induced by the wild-type mini-enhancer is weakly variegated. (C-C″) β-gal expression induced by the mutated pros7 mini-enhancer is not variegated but is still restricted to R7 and cone cells. (**D-D″**) Transgenic core-enhancer-β-gal reporter (green) which has 10 mutated Su(H)-binding sites in a *sev-*N^{act}/+ mutant eye disc. *sev-*N^{act} induces ectopic β-gal expression in R1/R6 cells. Endogenous Pros protein (purple) is also induced ectopically in these cells. (**E**) Sequence alignment of a Svp-binding site in the *pros* mini-enhancer from *D. melanogaster* and *D. simulans*. Response elements correspond to two half-sites (consensus AGGTCA) spaced one bp apart as a direct repeat (Zelhof et al., 1995). The position of the three tandem half-sites in the enhancer is shown with a bar over each half-site. (**F**,**G**) Transgenic mini-enhancer-β-gal reporter expression (green) in eye discs counterstained for Pros protein (purple). (F) A wild-type eye disc. (G) An eye disc carrying four copies of the *sev-Svp2* transgene. Although some cells are positive for both markers, other cells are positive for one marker but not the other.

These data indicate that another transcriptional effector of N signal transduction directly regulates the enhancer. One possibility is that this effector is encoded by a gene that is a downstream target of the N pathway. Such a candidate is the transcription factor Seven up (Svp). Svp is the *Drosophila* homolog of COUP-TF, and both fly and vertebrate factors repress transcription through a specific DNA-binding domain (Domingos et al., 2004; Kanai et al., 2005; Lo and

Frasch, 2001; Mettler et al., 2006; Zelhof et al., 1995). Svp is expressed in R1/R6 photoreceptors in the eye disc, and expression is repressed in R7 and cone cells by N signaling (Kauffmann et al., 1996; Mlodzik et al., 1990). To determine whether Svp regulates the *pros* enhancer, we looked for potential Svp-binding sites. Indeed, there is a highly conserved Svp-binding site located in the mini-enhancer of the *pros* gene (Fig. 2, Fig. 7E). When Svp was misexpressed in R7 and cone cells, there was a reduction in the number of cells expressing *pros* protein and exhibiting *pros* enhancer transcription activity (Fig. 7F). Interestingly, the resulting 'salt and pepper' patterns of endogenous and reporter gene expression were not completely overlapping. This suggests a probabilistic mechanism that is not coherent for all copies of a gene within a given cell.

DISCUSSION

The *pros* gene is a model for studying how cell-specific gene transcription is regulated in the *Drosophila* eye. Expression is activated by several distinct transcription factors, including the pre-patterning factor Lz, and the eye selectors So/Eya and Gl. Each of these factors is required to activate the *pros* enhancer; absence of any single factor-binding activity results in weak or no detectable enhancer activity in all eye cells. Thus, *pros* transcription is only fully active when all three factors are present in a retinal cell (Fig. 8). This positive combinatorial mechanism implies strong cooperativity between the factors to enable the enhancer to activate transcription. This mechanism also explains why *pros* is not transcribed in most imaginal disc cells outside of the eye, as most non-retinal cells do not contain So/Eya and Gl proteins, and therefore are unable to activate the *pros* enhancer. Thus, the roles of So/Eya and Gl on *pros* transcription are likely to be as eye selectors. Interestingly, So/Eya and Gl are also present in a few tissues outside of the eye (Ellis et al., 1993; Fabrizio et al., 2003), but these tissues lack the presence of Lz.

Such a combinatorial mechanism does not fully explain the restricted pattern of *pros* transcription in the eye. If it did, then many retinal precursor cells would also express *pros*. We have shown that cell-cell signaling is also required for *pros* transcription in retinal cells. Egfr promotes both *pros* expression and activity of the *pros* enhancer when Egfr is activated by ligand binding on precursor cells (Xu et al., 2000). In our present study, we have found that Notch signaling also promotes activity of the *pros* enhancer. It is known that Dl protein from R1/R6 cells signals to Notch receptors on their neighbors, inducing the neighbors to become R7 and cone cells (Cooper and Bray, 2000; Tomlinson and Struhl, 2001). This Dl-N signal appears to activate *pros* transcription in these cells. Thus, *pros* transcription is under the control of the Egfr pathway and the N pathway (Fig. 8).

From our knowledge of Egfr and N pathways in eye development, both pathways are coincidentally active in few retinal cells: R3, R4 and R7, and cone cells. A simple combinatorial model would assume that the *pros* enhancer is only active in cells with Lz, So/Eya and Gl, and that receive both signals. Indeed, this would explain completely the restricted expression pattern of *pros* in the eye. Some genes rely upon powerful cooperativity between cell-cell signals and selector transcription factors to generate a highly combinatorial response (Flores et al., 2000; Grienenberger et al., 2003; Guss et al., 2001; Neves et al., 2007; Walsh and Carroll, 2007). We find that to activate *pros*, selectors behave with strong cooperativity with other transcription factors, but not with the Egfr and N signals. Individual loss of Egfr or N signaling activities does not eliminate *pros* transcription, but reduces the number of cells that are transcriptionally active. Moreover, mutation of either Egfr or N transcriptional effectors has a similar effect on the transcription activity of the *pros* enhancer. Loss of either effector reduces the number of cells that exhibit enhancer activity, whereas ectopic effector activity increases this number. In both cases, some cells nevertheless appear to be independent of the signal. We interpret these data to mean that these signals do not operate like simple binary switches for *pros* transcription, where both switches need to be 'on' to trigger the enhancer.

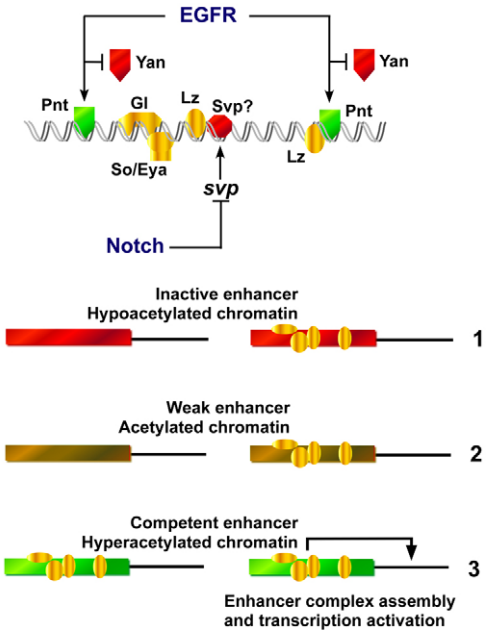

Fig. 8. Model for cell-type-specific transcription of *pros*. At the top is a schematic of the *pros* mini-enhancer arrayed with the different transcription factors on their binding sites. Several factors act downstream of Notch and Egfr signals. Notch acting through Su(H) inhibits production of the Svp repressor (red). Svp might repress the enhancer directly, or it might function through an intermediary. Egfr signaling inhibits the Yan repressor (red) and stimulates Pnt (green). Several factors work cooperatively to increase the transcription activity of the enhancer, and these are colored yellow. They are Lz, Gl and So/Eya. At the bottom is a model in which the enhancer becomes competent prior to its activation of *pros* transcription. (1) The inactive enhancer is in a repressive chromatin environment when neither Notch nor Egfr signals are received. (2) It transits towards competence when either Egfr or Notch alone is active. (3) It is competent when both Notch and Egfr signals are received by a cell. Competence may be expressed as a relaxation of chromatin structure, or the ability of the enhancer to connect to the promoter and facilitate transcription, or both. The formation of a cooperative enhancer complex (yellow ovals) composed of Lz, Gl and So/Eya is required for the enhancer to set the level of transcription. Competence may enable the cooperative enhancer complex to form (left). Alternatively, the enhancer complex may form on a non-competent enhancer but may only connect with the promoter when competence is achieved (right).

It is important then to note that transcriptional output is not being tuned by these signals. Loss of signaling does not produce a uniformly reduced level of expression in R7 and cone cells. Rather, it produces a 'salt and pepper' effect, with some cells expressing near-normal levels and other cells expressing no detectable *pros*. This suggests a mechanism in which signaling activity influences the probability that the enhancer switches on transcription. We suggest that the switching (and the level of transcription within each cell) is dictated by the constellation of cooperative selector and pre-

pattern factors present in the cell. Therefore, two distinct classes of input regulate *pros* transcription. One class (Lz, So/Eya, Gl) operates in a highly combinatorial manner to affect the level of transcription in retinal cells. The other class (Egfr and N signals) determines the probability that the enhancer will activate transcription in any retinal cell.

What might be the basis for such a probabilistic mechanism? The signals could promote the assembly of Lz-So/Eya-Gl proteins into productive and stable enhancer complexes within an otherwise repressive chromatin environment (Fig. 8). Based on our model of enhancer action, retinal cells lacking an Egfr signal would have Yan occupying the *pros* enhancer. This ETS factor and its mammalian orthologs recruit histone deacetylases to modify chromatin into a repressive state (Mavrothalassitis and Ghysdael, 2000). Retinal cells receiving an Egfr signal would have Pnt but not Yan occupying the enhancer. As Pnt homologs recruit histone acetylases to modify chromatin into an open state (Foulds et al., 2004), it is likely that the Egfr signal alleviates chromatin-mediated repression. Cells receiving an Egfr signal but not an N signal (e.g. R1 and R6) would presumably have Svp occupancy on the *pros* enhancer, whereas those cells receiving both signals would not have occupancy of Svp. As Svp and its orthologs recruit histone deacetylases (Beckstead et al., 2001; Smirnov et al., 2000), this would imply that the N signal also alleviates chromatin-mediated repression. However, it remains possible that Svp does not affect the enhancer directly, but rather through an intermediary. And what of the observation that in the absence of a signal, there is a fraction of cells that have normal levels of transcription? In such cells, fluctuations in weak chromatin structure might permit the spontaneous formation of functional enhancer complexes.

In a normal eye, variation in *pros* expression is not observed; only R7 and cone cells transcribe the gene (Kauffmann et al., 1996). How is this achieved using signals that use a probabilistic mechanism? Signaling can be prone to variation in strength or transience, and thus requires robust mechanisms to ensure uniform cellular response (Freeman, 2000). In the eye, both Egfr and N signaling use feedback and feedforward mechanisms to enhance developmental stability against fluctuation (Li and Carthew, 2005) (X. Li and R.W.C., unpublished). Systems also employ redundancy to ensure robustness (Kitano, 2004). We think that redundancy in Egfr and N signaling to promote *pros* transcription is a means by which variation in patterned gene expression is minimized during development.

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