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Two-step selection of a single R8 photoreceptor: a bistable loop between *senseless* and *rough* locks in R8 fate

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Patterning of sensory organs requires precise regulation of neural induction and repression. The neurocrystalline pattern of the adult *Drosophila* compound eye is generated by ordered selection of single founder photoreceptors (R8s) for each unit eye or ommatidium. R8 selection requires mechanisms that restrict R8 potential to a single cell from within a group of cells expressing the proneural gene *atonal* (*ato*). One model of R8 selection suggests that R8 precursors are selected from a three-cell 'R8 equivalence group' through repression of *ato* by the homeodomain transcription factor Rough (Ro). A second model proposes that lateral inhibition is sufficient to select a single R8 from an equipotent group of cells called the intermediate group (IG). Here, we provide new evidence that lateral inhibition, but not *ro*, is required for the initial selection of a single R8 precursor. We show that in *ro* mutants, ectopic R8s develop from R2,5 photoreceptor precursors independently of ectopic Ato and hours after normal R8s are specified. We also show that Ro directly represses the R8 specific zinc-finger transcription factor *senseless* (*sens*) in the developing R2,5 precursors to block ectopic R8 differentiation. Our results support a new model for R8 selection in which lateral inhibition establishes a transient pattern of selected R8s that is permanently reinforced by a repressive bistable loop between *sens* and *ro*. This model provides new insight into the strategies that allow successful integration of a repressive patterning signal, such as lateral inhibition, with continued developmental plasticity during retinal differentiation.

KEY WORDS: *Drosophila*, Eye, Lateral inhibition, Photoreceptor, Rough, Senseless

INTRODUCTION

The stereotyped pattern of adult *Drosophila* eyes results from exact specification of R8 precursors within the field of undifferentiated cells forming the larval eye imaginal disc (reviewed by Frankfort and Mardon, 2002; Hsiung and Moses, 2002). Once the R8 precursor is selected, it initiates ommatidial assembly by recruiting undifferentiated cells to the photoreceptor fate (Freeman, 1996; Tio et al., 1994). As there is no migration of cells in the eye, the establishment of the R8 cell array sets the pattern for the rest of eye development (White and Jarman, 2000). The proneural gene *atonal* (*ato*) is required for *Drosophila* eye development and resolution of its expression within the morphogenetic furrow (MF) determines the arrangement of R8 cells (Jarman et al., 1993b; Jarman et al., 1994). The MF is a physical marker of the wave of differentiation that progresses across the eye disc during the third larval instar and leaves a developing array of photoreceptors in its wake (Ready et al., 1976; Tomlinson and Ready, 1987). Ato is initially expressed in a dorsal-to-ventral stripe just anterior to and within the MF (Fig. 1A) (Jarman et al., 1994; Jarman et al., 1995). This stripe of Ato resolves to evenly spaced clusters of 10-15 cells known as intermediate groups (IGs) (Fig. 1B). This column of IGs is defined as column 0. From these IGs, a single cell is selected to continue to express Ato and begin R8 differentiation. The first column of selected R8s lies immediately posterior to the IGs and is identified as column 1. A new column of selected R8s emerges from the MF every 2-3 hours and is staggered out of phase with previous and subsequent columns, producing the characteristic hexagonal pattern of the adult eye (Wolff and Ready, 1991). R8 development also depends on the zinc finger transcription factor Sens (Frankfort et al., 2001; Nolo et al., 2000). Without *sens*, an R8 precursor is selected from the IG but fails to differentiate as an R8. Ato activates *sens* expression in a subset of IG cells, and then resolves together with Sens to a single R8 precursor in column 1. Ato and Sens are then co-expressed in the selected R8 until Ato is downregulated after column 3 (Frankfort et al., 2001; Jarman et al., 1994). Sens continues to be expressed in R8 through adult stages and is required for terminal R8 differentiation during pupation (Domingos et al., 2004; Sprecher and Desplan, 2008; Xie et al., 2007).

Two models have been proposed to explain how a single R8 is selected from an IG. The distinction between these models is important because they define different populations of cells with equal potential to become R8 precursors. One model is based on parallels between retinal differentiation and the early development of other peripheral nervous system (PNS) organs (reviewed by Bray, 2000; Ghysen and Dambly-Chaudiere, 1989). In this model, IGs are considered roughly equivalent to proneural clusters in PNS differentiation, R8 cells are considered analogous to sensory organ precursors (SOPs), and Notch-mediated lateral inhibition is necessary to select a single SOP or R8 precursor (reviewed by Lai, 2004; Voas and Rebay, 2004; Baker et al., 1996; Baker and Yu, 1997; Baker and Zitron, 1995; Cagan and Ready, 1989; Lee et al., 1996). Thus, if lateral inhibition is disrupted, clusters of R8 precursors are predicted to develop (Fig. 1C). However, mutations in genes outside the lateral inhibition pathway, such as *rough* (*ro*), which encodes a homeodomain transcription factor, are also capable of generating additional R8 photoreceptors, suggesting other mechanisms may be required (Cagan, 1993; Rawlins et al., 2003; Spencer and Cagan, 2003). Therefore, a second model was introduced, the 'R8 equivalence group' model (Dokucu et al., 1996). In this model, nuclei of three cells at the posterior edge of an IG migrate apically and continue to express Ato while in neighboring IG cells Ato expression

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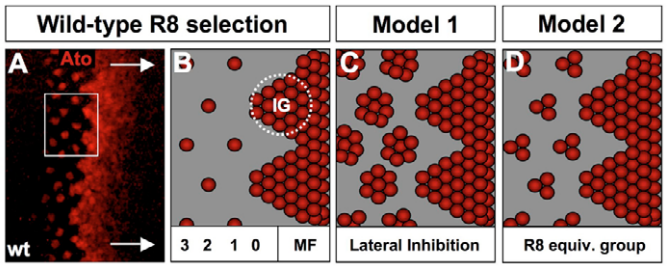

Fig. 1. Patterning of the eye depends on selection of a single atonal expressing R8 precursor cell per ommatidium. (**A**) In wild-type (wt) larval eye discs, Ato is expressed in a dorsal-ventral stripe within the morphogenetic furrow (MF) and resolves to single R8s. Posterior is towards the left and dorsal is upwards in all figures. Arrows indicate anterior progression of the MF. (**B**) Cartoon of boxed area in A. Column numbers are indicated. At the posterior edge of the MF, Ato is resolved to intermediate groups (IG), then to individual R8s in column 1. (**C**) When lateral inhibition is disrupted, clusters of Ato-expressing cells are present in column 1 instead of single R8s (Lee et al., 1996). (**D**) In the absence of *ro*, three cells of the R8 equivalence group express Ato (Dokucu et al., 1996).

is lost (Dokucu et al., 1996; Sun et al., 1998). Then, in two of the three R8 equivalence group cells, Ro represses Ato, leading to the selection of a single R8. Thus, in *ro*-null mutants up to three R8 precursors per ommatidium are predicted to develop (Fig. 1D) (Dokucu et al., 1996; Heberlein et al., 1991). Consistent with this model, ectopic Ro is capable of repressing Ato anterior to the MF and Ro is expressed in a pattern complementary with the posterior border of Ato expression in the MF (Chanut et al., 2000; Dokucu et al., 1996). Despite the differences between the two models, both are based on the premise that ectopic R8s are formed when the initial pattern of Ato expression is not refined to a single cell. In other words, an undifferentiated cell can only develop as an R8 in the context of ongoing Ato expression. Both models were proposed before *sens* was identified and its pivotal role in R8 development explored.

Sens is required in R8 to repress Ro expression and thereby allow R8 differentiation (Frankfort et al., 2001). In the absence of *sens*, Ro expression expands into the previously selected R8 precursor and this extra Ro-expressing cell switches fate to an R2,5 cell type. How Ro functions in this process is unknown as the molecular mechanisms controlling recruitment of the R2,5 precursors are poorly understood. In addition, ectopic Sens is capable of repressing endogenous Ro expression and ectopically inducing the R8 cell fate. Moreover, *ro* is probably a crucial early target of Sens repression as R8 differentiation is often restored in *sens*, *ro* double mutant ommatidia (Frankfort et al., 2001). The presence of three Ro-expressing cells in *sens* mutants also indirectly supports the presence of an R8 equivalence group, defined as three cells with equal potential to differentiate as R8 precursors. However, the issue of how a single R8 is selected from this potential equivalence group has not been specifically addressed or answered.

In this work, we show that *ro* is not required for the initial selection of a single R8 precursor. We show that in *ro*-null mutants, the single R8 fate persists for several hours and that ectopic Sens-expressing R8s develop in the absence of ectopic Ato 6 hours after normal R8 specification. Furthermore, we show that Ro directly represses *sens* transcription in the R2,5 precursors. Together with our previous report that Sens repression of Ro is required for R8 differentiation, our current findings suggest that the R8 and R2,5 cell precursors comprise the R8 equivalence group and that a negative regulatory loop between *sens* and *ro* is required to select the R8 vs. R2,5 cell fate from among these cells. We also report the identification of an enhancer that is necessary and sufficient for R8-specific *sens* expression and characterize distinct elements within the enhancer responding to inductive and repressive signals during R8 selection. Our data suggest that a two-step process is required for R8 selection. Initially, Ato directly activates *sens* expression in the IG and lateral inhibition transiently selects a single R8 from the IG cells. Then, after the correct pattern of R8 precursors is established by lateral inhibition, Ro is required to maintain this pattern by direct repression of *sens* in the R2,5 precursors.

MATERIALS AND METHODS

Preparation of transgenic wild-type and mutant reporter constructs

Twelve PCR products spanning the *sens* genomic locus were generated by PCR with 5′ *Eco*RI and 3′ *Bam*HI tails and cloned into *pH-Pelican* or *pH-Stinger* (Barolo et al., 2000). Mutations in predicted homeodomain binding sites and E-boxes were made by using mutagenic primers; sequences can be obtained upon request. The *sens-L* genomic rescue was prepared as described previously (Venken et al., 2006). The *ΔF2* genomic rescue construct was created by inducing recombination between the *sens-L* genomic rescue and a mutagenic PCR fragment that lacks the F2 region (see Fig. S1 in the supplementary material). *F2-sens* was generated in the *pCaSpeR-4* vector (D'Avino and Thummel, 1999). The F2 enhancer sequence and *hsp70* promoter were excised from *F2-GFP* and cloned upstream of the 2558 bp *sens* cDNA and an *SV40 poly A* sequence.

For transgenic fly generation, embryo progeny of *yw* virgins crossed to *yw; KiΔ2-3* males were injected with *pH-Stinger*-based constructs. Third instar larvae heterozygous for reporter constructs were dissected and stained (see Pepple et al., 2007).

Drosophila genetics, immunohistochemistry and microscopy

The following stocks were used: $sens^{E1}$: ro^{X63}: RM104: Dl^{6B}: Dl^{RF}: $sens^{E2}$ FRT80B/TM6B: FRT82D ato^{1}: FRT82D ro^{X63}: Fragment E1/CyO, hs-hid; $sens^{E2}$ FRT80B/TM6B: yw,hsflp; M(3),arm-lacZ,FRT80B/TM6B: yw,hsflp; FRT82D arm-lacZ/TM6B. Clones were generated by standard protocols (see Pepple et al., 2007) with minute heat shocks at 37°C for 1 hour 40-42 hours after egg laying. Dl^{ts} is $Dl^{6B/RF}$ (Baker and Zitron, 1995). Dl^{ts} animals were raised at 18°C until third instar then vials were submersed in a 31°C water bath for 6 hours. Larvae were immediately dissected and stained (see Pepple et al., 2007). Primary antibodies used were: rabbit anti-GFP (1:1000, Molecular Probes); guinea-pig anti-Ato (1:1000, a gift from Hugo Bellen) for DAB stains; rabbit anti-Ato (1:3000, a gift from Kwang Choi) for fluorescent stains; guinea pig anti-Sens (1:2000, a gift from Hugo Bellen); and mouse anti-β galactosidase (1:1000, Promega). Goat anti-rabbit Cy3 and goat anti-guinea pig Cy5 secondary antibodies were obtained from Jackson laboratories (West Grove, Pennsylvania, USA). Goat anti-mouse Alexa, goat anti-guinea pig Alexa and goat anti-rabbit Alexa secondary antibodies were obtained from Molecular Probes (Eugene, Oregon, USA). All secondary antibodies used at a 1:500 dilution in PAXDG (PBS with 1% BSA, 0.3% Triton X-100, 0.3% sodium deoxycholate and 5% NGS). Scanning electron microscopy was performed (see Pepple et al., 2007).

In Fig. 3I-J, Sens and Ato expression were counted only in ommatidia that could be unambiguously assigned a column designation. For each column, percentages of ommatidia containing single positive cells and multiple positive cells were determined. Ato data were generated from nine wild-type discs and 17 ro^{X63} discs. Sens data were generated from 13 wild-type discs and 17 ro^{X63} discs. An average percentage for each column was determined using the normalized percentage from each disc. Error bars represent the standard error of the mean. Student's *t*-tests were performed to determine *P* values.

Electrophoretic mobility shift assays

Ato/Da EMSAs were performed (see Jarman et al., 1993b). Wild-type probes for Ato/Da EMSAs are as follows: E1, *TT*AGTACCGGACCGACATATGGTCAAAAAGCCGA; E2, *TT*AAGCCGACGAAGACAGTTGCCAGAGTCCTTTG; E3, *TT*AGTCACTGTTCTTCAGCTGTT-

TATGTATAAAA; and E4, *TT*AATTCGTGCTTTACATCTGTTCACCATTGGAG. Italicized thymidines were added to probe sequence for radiolabeling with αP^{32}-dATP, core E-boxes sequence underlined. For all mutant probes, CANNTG was mutated to AANNTT. Ro EMSAs were performed (see Heberlein et al., 1994). Probes for Ro EMSAs are as follows: wt, ATTTATGTACAAATTACAATCATAATAATT*T*; H1*, ATTTATGTACAAGGGGCAATCATAATAATT*T*; H2*, ATTTATGTACAAATTACAATCATGGGGATT*T*; H1,2*, ATTTATGTACAAGGGGCAATCATGGGGATT*T*. Gels were dried before autoradiography.

RESULTS

Initial selection of a single Sens-expressing R8 does not require *rough*

The two models for R8 selection have been tested primarily using Ato expression as the R8 marker (Baker et al., 1996; Baker and Yu, 1997; Baker and Zitron, 1995; Dokucu et al., 1996; Heberlein et al., 1991; Lee et al., 1996). Sens is an additional, consistent and more specific marker of the R8 cell fate. We therefore re-evaluated the role of lateral inhibition and *rough* in R8 selection using Sens to mark R8s. Lateral inhibition was disrupted using a combination of *Delta* (*Dl*) alleles that generate Dl^{ts} animals ($Dl^{6B/RF}$) (Baker and Zitron, 1995). Previous work has demonstrated that, after disruption of lateral inhibition in $Notch^{ts}$ and Dl^{ts} animals, Ato is expressed in groups of cells in column 1 rather than in single R8s (Model 1) (Lee et al., 1996). In agreement with this data, we found that Sens expression also fails to resolve to a single cell in column 1 when lateral inhibition is disrupted (Fig. 2C,G). This supports the previous model that lateral inhibition is necessary for selection a single Ato- and Sens-expressing R8 precursor in column 1.

In contrast to the Dl^{ts} phenotype, in ro^{x63} null mutants only single Sens-expressing R8 cells are found in columns 1 and 2 (Fig. 2D,H,I). This is not consistent with a previous report that in *ro* mutants Ato expression is found in two or three cells in column 1, owing to failure of resolution of the R8 equivalence group (Model 2) (Dokucu et al., 1996). To evaluate this discrepancy, we closely re-examined Ato expression in ro^{x63} null mutants and found that 6±3% of ommatidia in column 1 do have two Ato-staining cells (data not shown). However, this is not significantly different (P=0.8) from wild-type discs, where two Ato-positive cells are found in 6±2% of column 1 ommatidia (data not shown). The occasional second Ato-expressing cell does not persist in either genotype, and by column 2, only a single Ato staining cell is seen in all ommatidia (data not shown). These data indicate that, although Ato expression is not limited to a single cell in column 1, by column 2 in both wild-type and ro^{x63} mutant discs, expression of Ato and Sens is always restricted to a single cell. Thus, *ro* is not required for selection of a single Ato- or Sens-expressing R8 photoreceptor.

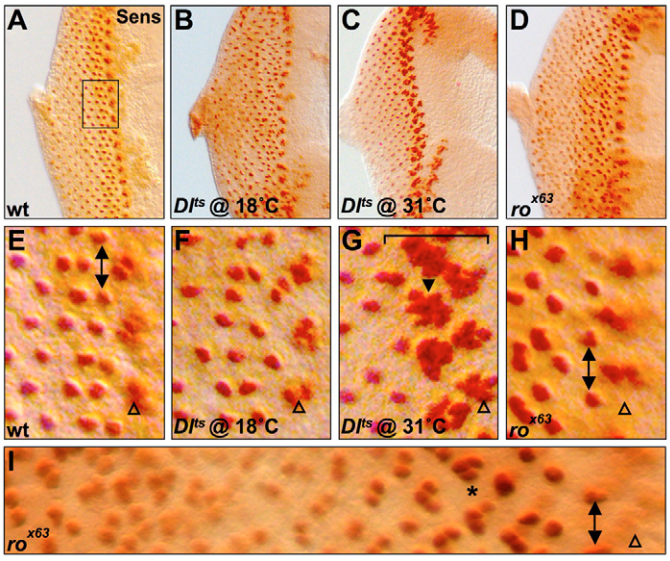

Fig. 2. *ro* is not required for initial selection of a single R8 precursor. (**A**,**E**) Wild-type (wt) Sens expression. The boxed area in A is shown in E. Sens is first expressed in a subset of IG cells in column 0 (open arrowhead). Single R8s are identified in column 1 (double arrow). (**B**,**F**) At permissive temperatures (18°C) Dl^{ts} has a mild effect with rare additional R8s. Open arrowheads indicate column 0 in all panels. (**C**,**G**) After a 6-hour heat shock at 31°C (affected columns bracketed), multiple Sens-positive cells form in column 1 (black arrowhead). (**D**,**H**) In ro^{X63} mutants (null allele), a single Sens-positive cell forms in column 1 (double arrow). (**I**) The developing eye field in a ro^{X63} mutant from column 1 (right) to the posterior of the disc (left). Additional Sens-positive cells are present in older ommatidia. An asterisk indicates two ommatidia in column 5 with three Sens-positive cells. Only single Sens-positive nuclei are observed in column 1 (double-arrow). Column 0 and the mid-section of the disc are out of the plane of focus.

Rough represses ectopic R8 development and Sens expression three columns after selection of a single Ato/Sens-expressing R8 precursor

In ro^{x63} null mutant discs, single Sens-expressing cells are selected and persist for two columns, demonstrating that *ro* is not required for selection of a single R8 precursor. However, in column 3, Sens expression is occasionally found in multiple cells per ommatidium and by column 5 many ommatidia have three Sens-positive cells (Fig. 2I, asterisk). This suggests that the *ro* phenotype may be due to a later effect on R8 differentiation than previously reported. To better characterize the ro^{x63} phenotype, we closely examined ro^{x63} discs using Ato and Sens expression as R8 cell markers. Initially, expression patterns of Ato and Sens are the same in both wild-type and ro^{x63} discs (Fig. 3A-H). In a subset of IG cells, Ato and Sens are co-expressed (circled in Fig. 3B-D,F-H) and in column 1 and 2 Ato and Sens colocalize to a single R8 precursor (open arrowhead in Fig. 3B-D,F-H). The first difference is found in column 3 where, in wild-type discs, Ato and Sens are expressed in only one cell per ommatidium. By contrast, in ro^{x63} discs, 9±3% of ommatidia have multiple Ato-positive cells and 21±5% have multiple Sens-positive cells (Fig. 3I). The difference between wild-type and ro^{x63} mutants further increases after column 4, where Sens is expressed in up to three cells per ommatidium in more than 50% of ommatidia (Fig. 3F, white arrowhead; Fig. 3J). Although Sens is a specific and consistent R8 marker, it is possible that not all extra Sens-positive cells in ro^{x63} mutants are equivalent to wild-type R8s. Therefore, in ro^{x63} mutants, supernumerary cells expressing Sens are considered putative R8s ('R8s'). To determine when extra 'R8s' form, we calculated the average percentage of ommatidia with extra Sens-expressing 'R8s' in the first seven columns of ro^{X63} mutant discs (Fig. 3J). We find that the ro^{X63} phenotype evolves gradually starting in column 3, where 21±5% of ommatidia contain extra 'R8' cells. By column 6, 60-70% of ommatidia have multiple 'R8s'. By contrast, in wild-type discs, a single R8 cell is found in every ommatidium. Therefore, in ro^{X63} mutants, additional 'R8s' develop from cells that begin to express Sens starting in column 3.

Rough directly represses *sens* expression

Ro is a homeodomain-containing protein and has been shown to bind DNA at two sites in its own enhancer containing an ATTA core sequence (Heberlein et al., 1994). To explore the possibility that Ro

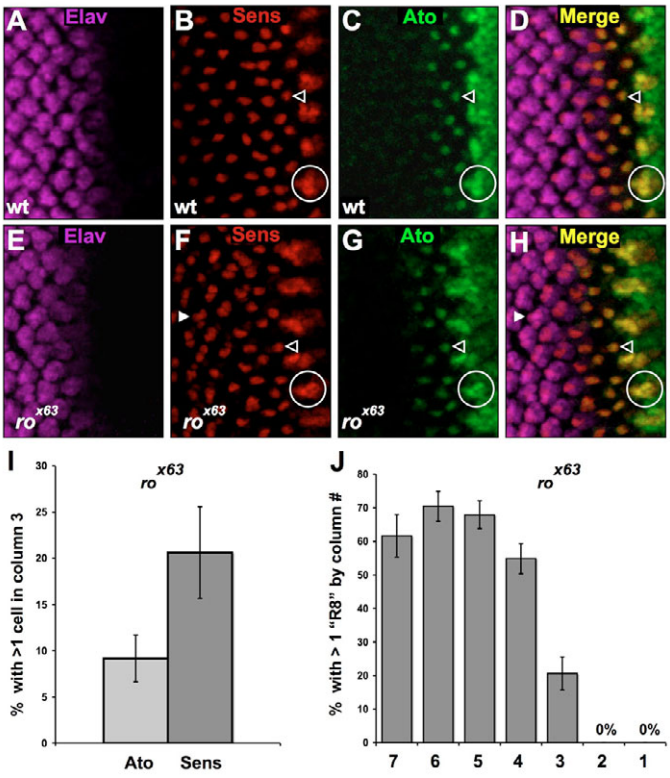

Fig. 3. *ro* is required to repress R8 differentiation in column 3. (**A**) Normally, the neuronal marker embryonic lethal abnormal vision (Elav) is expressed in all developing photoreceptors. (**B**) Wild-type Sens. (**C**) Wild-type Ato. (**D**) Merge of A-C shows that, in a wild-type disc, Ato and Sens are co-expressed in clusters of cells within an intermediate group (circled) and in single Ato- and Sens-positive R8s in column 1 (open arrowhead). In column 3, wild-type ommatidia always have a single Ato- and Sens-positive cell. (**E-H**) In ro^{X63} null discs, Elav (E) expression is delayed by one column whereas Sens (F) and Ato (G) expression are initially unchanged from wild type. (H) Ato and Sens are co-expressed within the IG (circled) and single Ato- and Sens-positive R8s are selected (open arrowhead). More posterior ommatidia often have additional Sens-positive cells (white arrowhead). (**I**) In column 3 of ro^{X63} mutants, 9±3% of ommatidia have extra Ato-positive cells and 21±5% of ommatidia have extra Sens-positive cells. Error bars represent the standard error of the mean in I and J. (**J**) The *ro* mutant phenotype develops starting in column 3 with 21±5% of ommatidia containing more than one Sens-expressing cell. The average percentage of ommatidia with multiple Sens-expressing cells for columns 1-7 in ro^{X63} mutants is shown.

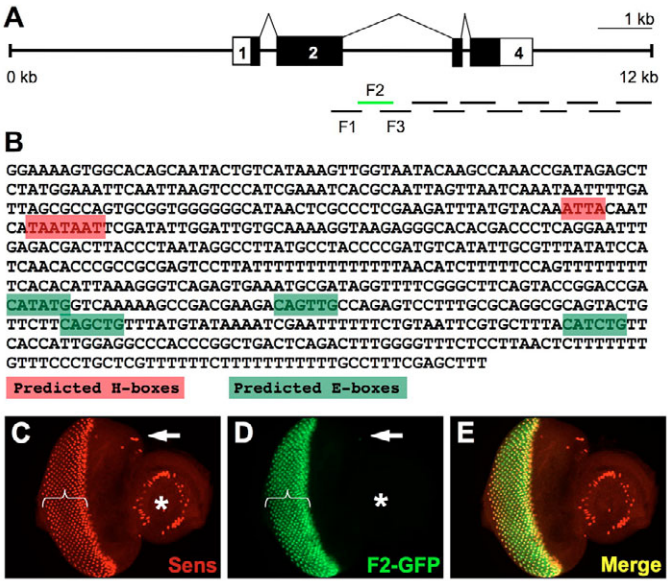

Fig. 4. Identification of the *senseless* eye-specific enhancer. A 645 bp fragment within the second intron of the *sens* genomic locus named F2 was identified that is sufficient to drive reporter expression specifically in the developing eye. (**A**) The position of F2 in the *sens* genomic locus among the 12 fragments tested is shown. Fragments F1 and F3 overlap F2, but do not drive reporter expression in the eye. (**B**) The 645 bp sequence of F2 contains two potential Ro-binding sites (highlighted in red) and four potential Ato-binding sites or E-boxes (highlighted in green). (**C**) In larval eye-antennal discs, Sens is expressed in the R8 photoreceptors (bracket), the ocelli (arrow) and antennal SOPs (asterisk). (**D**,**E**) F2-GFP is expressed only in photoreceptors.

directly represses *sens*, we identified the R8 specific *sens* enhancer and characterized the mechanisms regulating *sens* expression. A 645 bp fragment within the second intron of the *sens* genomic locus named F2 was identified that is sufficient to drive reporter expression specifically in photoreceptors of the developing eye-antennal imaginal disc (Fig. 4). To test whether the F2 region is necessary for R8-specific *sens* expression, the 645 bp region was specifically deleted from the *sens-L* genomic rescue construct generating *ΔF2* (see Fig. S1 in the supplementary material). In *sens*-null mutants, one copy of *ΔF2* rescues the null phenotype in all tissues except the eye (see Fig. S2 in the supplementary material). Thus, F2 is the *sens* eye enhancer and is necessary and sufficient for R8-specific *sens* expression.

F2 contains two potential Ro-binding sites known as H1 and H2, for homeodomain 1 and 2 (Fig. 4B, highlighted in red). To test for a direct interaction, electrophoretic mobility shift assays (EMSAs) were performed. A probe containing H1 and H2 is bound specifically by Ro protein in vitro (Fig. 5A). Complete loss of binding occurs with mutation of H2. Mutation of H1 does not prevent Ro binding, but there may be a mild decrease in binding compared with the wild-type probe. To test the in vivo significance of these interactions, each site was mutated in a reporter generated with the minimal R8-specific enhancer, B-short-GFP, and the effect on GFP was evaluated (Fig. 5D-L). Although H1 is not required for Ro binding in vitro, mutation of H1 in B-short (termed H1*) leads to consistent expression of GFP in two extra cells per ommatidium (arrowheads in Fig. 5H). These two cells were identified as the R2,5 photoreceptor pair by co-localization of GFP with β-galactosidase from the R2,5-specific enhancer trap RM104 (Fig. 5M-O). GFP expression is also expanded into the R2,5 pair with the H2 mutation (H2*) (Fig. 5K). Mutation of both H1 and H2 (H1,2*) results in a GFP expression pattern indistinguishable from H2* (see Fig. S3 in the supplementary material). To test whether the loss of *ro* function has the same effect on B-short-GFP expression as does mutation of the Ro-binding sites, ro^{X63} clones were generated. In the absence of *ro* function, both Sens and B-short-GFP expression are detected in two to three cells per ommatidium (Fig. 5Q-S, arrowheads). Together with the in vitro binding data, these in vivo results suggest that Ro directly represses *sens* expression in R2,5 photoreceptors.

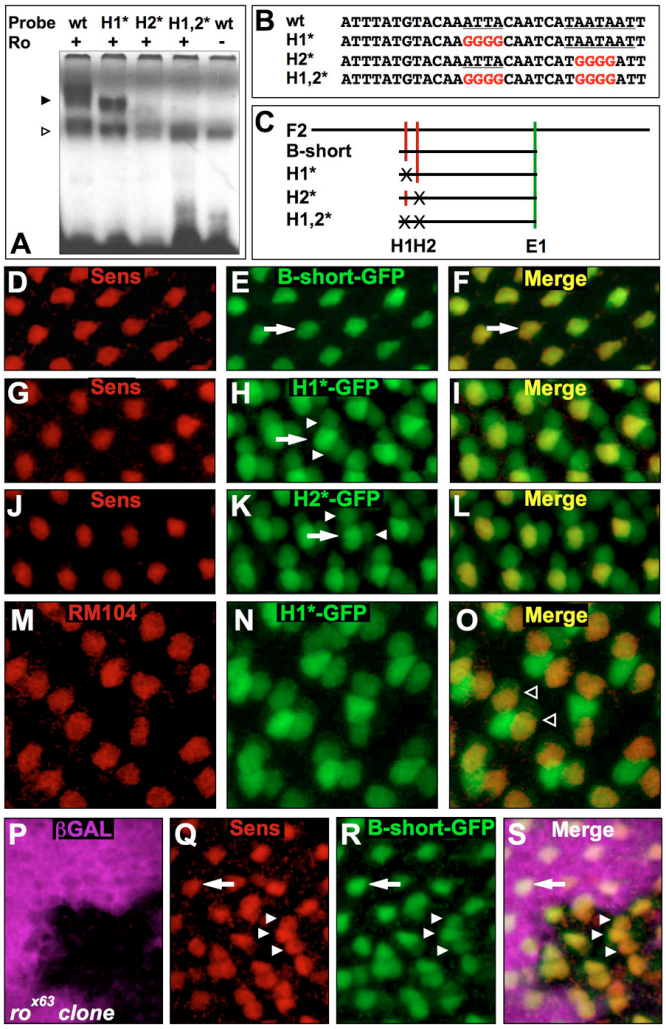

Fig. 5. Rough directly represses *sens* expression in R2,5 precursors. (**A**) EMSAs were performed using wild-type (wt) and mutant (*) probes for the predicted Ro-binding sites H1 and H2. Ro binds specifically to wild-type and mutant probe H1* (black arrowhead). Specific binding is lost with the H2 mutation (H2*) and with mutation of both H1 and H2 (H1,2*). The white arrowhead indicates non-specific binding. (**B**) Probe sequences. Predicted core sequences are underlined. Mutations are indicated in red. (**C**) Subfragments used for GFP reporter studies in vivo. B-short is the minimal R8-specific subfragment of F2. B-short contains two potential Ro-binding sites (H1, H2) and one potential Ato-binding site E-box 1 (E1). Red lines indicate the position of Ro-binding sites. Mutations are indicated by an X. (**D-F**) Expression of the B-short-GFP reporter. Sens and B-short-GFP colocalize to a single R8 per ommatidium (arrow). (**G-I**) Mutation of H1 in B-short (H1*) causes expansion of GFP expression to two additional cells per ommatidium (arrowheads). (**J-L**) Mutation of H2 in B-short (H2*) also expands GFP expression to two additional cells (arrowheads). (**M-O**) The additional GFP-expressing cells are R 2,5 precursors. (M) RM104-β-gal (red) is expressed in the R2,5 photoreceptors. (N) H1*-GFP is expressed in three cells per ommatidium. (O) Colocalization of the RM104-β-gal and H1*-GFP in R2,5 cells (open arrowheads). (**P-S**) *ro*^{x63} clones, marked by the absence of β-gal (P), Sens (Q) and GFP (R), expand to three cells per ommatidium (arrowheads). In wild-type tissue, Sens and GFP are expressed in a single cell (arrow).

Positive and negative regulation of *sens* expression in R8

In order to identify mechanisms activating *sens* expression in R8, we performed binding site analysis and functional assays with subfragments of F2 (Fig. 6). Sens expression in the eye is dependent on the proneural bHLH protein Ato (Frankfort et al., 2001). To activate target gene expression, Ato heterodimerizes with another bHLH protein, Daughterless (Da), and binds to the minimal E-box consensus sequence CANNTG (core nucleotides underlined) (Brown et al., 1996; Jarman et al., 1993a; Murre et al., 1989a; Murre et al., 1989b). Four potential E-boxes are present in F2 (E1-E4, identified by green boxes in Fig. 4B and green vertical lines in Fig. 6A), suggesting that Ato directly regulates *sens*. To test whether these potential binding sites are required for reporter expression, we generated three subfragments of F2 (fragments A, B and C). Fragment A lacks all E-box sequences and does not express GFP in the eye (Fig. 6F). Fragment B contains two E-boxes, E1 and E2, and drives GFP expression strongly starting in column 1, but lacks significant IG expression (Fig. 6L, IGs bounded by white vertical lines). The lack of IG GFP expression with fragment B suggests that E3 and E4 may also be required. Therefore, we predicted that fragment C, which contains all four E-boxes, would recapitulate the complete F2-GFP expression pattern. Owing to the deletion of the Ro-binding sites, additional expression in the R2,5 was also anticipated. As predicted, C-GFP is expressed at high levels in the IGs, suggesting that multiple E-boxes are required for the earliest expression of *sens* (Fig. 6R). However, GFP expression also expands to nearly every cell posterior to the IGs, suggesting that additional negative regulatory elements other than the Ro-binding sites are missing from fragment C (Fig. 6A, blue bracket).

In order to identify a minimal enhancer containing all positive and negative regulatory regions, fragments B-short (Fig. 6I) and B-long (Fig. 6O) were generated. Fragment B-short contains only E1 and is sufficient for expression in the selected R8s, but lacks IG expression. Fragment B-long contains all four E-boxes and is sufficient for both IG and selected R8 GFP expression. Thus, fragment B-long is the minimal enhancer containing all necessary negative and positive regulatory elements and recapitulates the complete eye specific *sens* expression pattern.

To test for a direct interaction between Ato/Da heterodimers and the four E-boxes present in the R8 enhancer, EMSAs were performed (Fig. 7A). Ato/Da heterodimers bind strongly to E1 with weaker binding detectable for E4. Binding is lost with mutation of the core E-box sequence. No binding is detected to probes containing E2 or E3. Da homodimers also bind to E1 and E4 (indicated by arrowhead). This binding is lost with mutation of the E-box core sequence. Interaction of Da homodimers with E-box sites has been described previously, but the significance of this interaction *in vivo* is unknown (Jarman et al., 1993b; Jarman et al., 1994). These data suggest that Ato/Da heterodimers directly regulate *sens* expression by binding E1 and E4 in the R8 enhancer. This supports the subfragment analysis that shows that both E1 and E4 are required in fragment B-long for IG reporter expression.

Fragment B-short contains a single E-box, E1, and is sufficient for R8-specific expression starting in column 1. To determine whether E1 is necessary for expression in vivo, two base pairs in the E-box core sequence were mutated in B-short, generating E1* (Fig. 7G). Mutation of the E-box does not abolish all R8 reporter expression in vivo but delays the onset of GFP expression by three or four columns (Fig. 7G, bracket). Deletion of the entire E-box has the same effect on GFP expression (data not shown) and suggests an Ato-independent enhancer that is sufficient to

maintain *sens* expression is present in fragment E1*. This is not an unexpected finding as Ato expression ends after column 3, whereas Sens continues to be expressed until early adult stages. No additional transcription factors with the ability to directly activate *sens* expression in an Ato-independent manner have been identified.

DISCUSSION

A repressive bistable loop between *sens* and *ro* specifies the R8 versus R2,5 cell fate decision

In this work, we show that Ro directly represses *sens* in developing R2,5 cells and that de-repression of Sens is sufficient to initiate R8 cell fate in the absence of ectopic Ato (Fig. 8B). Although there are a small number of ectopic Ato-expressing cells in column 3 in ro^{x63} mutants, it is not likely that the additional 'R8' cells are due to misregulation of Ato as the great majority of ectopic 'R8s' never express detectable Ato protein after the intermediate group stage. It is more likely that the extra Ato-positive cells are due to secondary Sens activation of proneural gene expression, a previously reported phenomenon (Acar et al., 2006; Nolo et al., 2000).

In a previously published report, we have shown that *sens* is required for R8 differentiation to occur through repression of Ro in R8, and that ectopic Sens is sufficient to repress endogenous Ro expression (Frankfort et al., 2001). Thus, in the absence of *sens*, three R2,5 cells develop and in the absence of *ro* up to three R8 cells form per ommatidium. This reciprocal phenotype supports the existence of the three cell R8 equivalence group and a mechanism of mutual repression between *sens* and *ro* that specifies opposite cell types (Fig. 8C). Although we have shown that one mechanism regulating this mutual repression is the direct repression of *sens* by Ro, other roles for Ro may exist. We observe that the Ro-binding site mutations do not produce the same level of GFP reporter protein expression elevation in R2,5 precursors that would be predicted from the level of GFP expressed in *ro* mutants. This suggests that Ro may also regulate *sens* by repressing an activator of *sens* expression in R2,5 precursors.

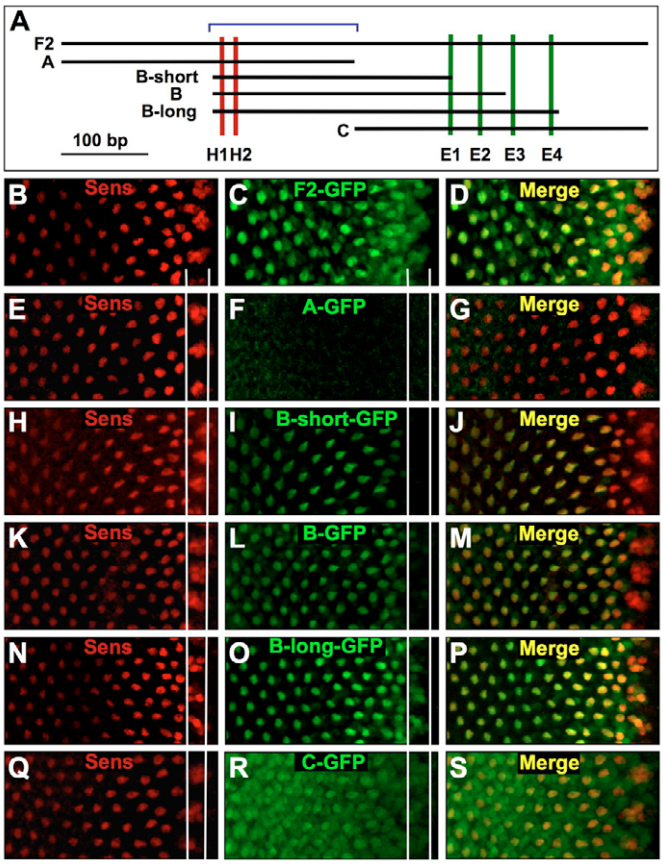

Fig. 6. Positive and negative regulatory regions of the eye enhancer identified by subfragment analysis. GFP reporter constructs were generated with subfragments of F2 and tested for in vivo expression. (**A**) Relationship of subfragments to F2. The blue bracket indicates the negative regulatory region containing the Ro-binding sites H1 and H2 shown as red vertical bars. Fragment sizes: F2, 647 bp; A, 324 bp; B, 324 bp; B-short, 266 bp; B-long, 383 bp; C, 324 bp. (**B-D**) High magnification image of Sens (B), F2-GFP (C) and their co-expression (D) in R8 photoreceptors. GFP perdurance marks additional cells of the IG not selected as the R8. White bars indicate IG boundaries. (**E-G**) Fragment A does not express GFP in the eye. (**H-J**) Fragment B-short is sufficient to drive GFP in single R8s but not in IGs. (**K-M**) Fragment B drives GFP strongly in Single R8s, and weakly in IGs. (**N-P**) Fragment B-long drives GFP robustly in both IGs and single R8s. (**Q-S**) Fragment C-GFP is expressed at high levels in IGs and in most cells posterior to the MF.

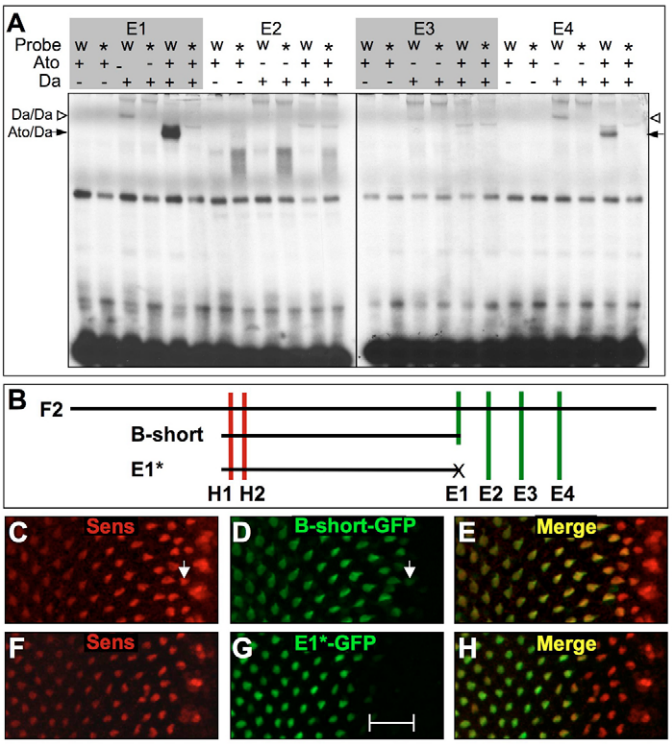

Fig. 7. Atonal directly regulates early *senseless* expression. (**A**) EMSAs were performed using wild-type (Wt) or mutant (*) probes for E-boxes 1-4. Ato/Da heterodimers bind E1 strongly and E4 weakly (black arrow). Specific binding to E1 and E4 is lost when the E-box core sequence is mutated to AANNTT. No binding is observed by Ato alone. Da homodimers bind to E1 and E4 (white arrowhead). This interaction is also lost with E-box mutations. No binding was detected to E2 or E3. An additional nonspecific band of higher molecular weight is present in all Ato/Da and Da reactions. (**B**) Fragment E1* was generated by mutation of E1, the sole E-box in B-short-GFP (indicated by an X). (**C-E**) Expression of Sens (C) and B-Short-GFP (D) beginning in column 1 (arrow). (**F-H**) E1*-GFP expression is delayed to column 4-5 (indicated by white bracket).

Regardless of the mechanism, the negative-feedback loop between *sens* and *ro* is secondary to the initial force driving R8 selection in which Ato and Sens are transiently repressed by lateral inhibition in all but one cell within an IG. Thus, lateral inhibition transiently represses neural differentiation in the eye, establishing the patterned array of precisely spaced ommatidia while retaining the potential for later recruitment of undifferentiated cells to the photoreceptor cell fate. If the effects of lateral inhibition were to repress permanently the potential for neuronal differentiation, further retinal development would be blocked. Therefore, the effects of lateral inhibition must be limited and our data indicate that column 3 is the boundary of its influence. As the effects of lateral inhibition diminish, the negative-feedback loop between *sens* and *ro* reinforces the pattern of selected R8s and ensures that only one Sens-expressing cell from the R8 equivalence group develops as an R8. This simple bistable loop translates the transient developmental signal of lateral inhibition into a committed irreversible fate (Ferrell, 2002).

In later R8 differentiation, another bistable loop is used to specify the 'pale' or 'yellow' subtypes of R8 photoreceptors (Mikeladze-Dvali et al., 2005). During this late developmental step, the bias for the 'pale' R8 fate is provided by a signal from a 'pale' R7. We propose that the bias signal that tips the fate decision in the *sens-ro* loop is provided by resolution of Ato to a single cell by lateral inhibition. Ato then directly activates Sens expression and biases that cell to the R8 cell fate. It is not yet known what activates Ro expression and thereby establishes the R2,5 cell fates. However, it has been suggested that epidermal growth factor receptor (EGFR) or Hedgehog signaling may be required for Ro expression (Dominguez, 1999; Dominguez et al., 1998). Ligands for both of these signaling pathways are expressed in developing R8s (Freeman, 1994; Heberlein et al., 1993; Ma et al., 1993; Tio et al., 1994; Tio and Moses, 1997). As a result, after the R8 bias is established, a signal such as the EGFR ligand Spitz could be sent from R8 to the two neighboring cells that bias their *sens-ro* loop towards Ro expression and the R2,5 fate. Once Ro expression is initiated in the R2,5 pair, the pattern of a single Sens-expressing R8 per ommatidium becomes irreversible.

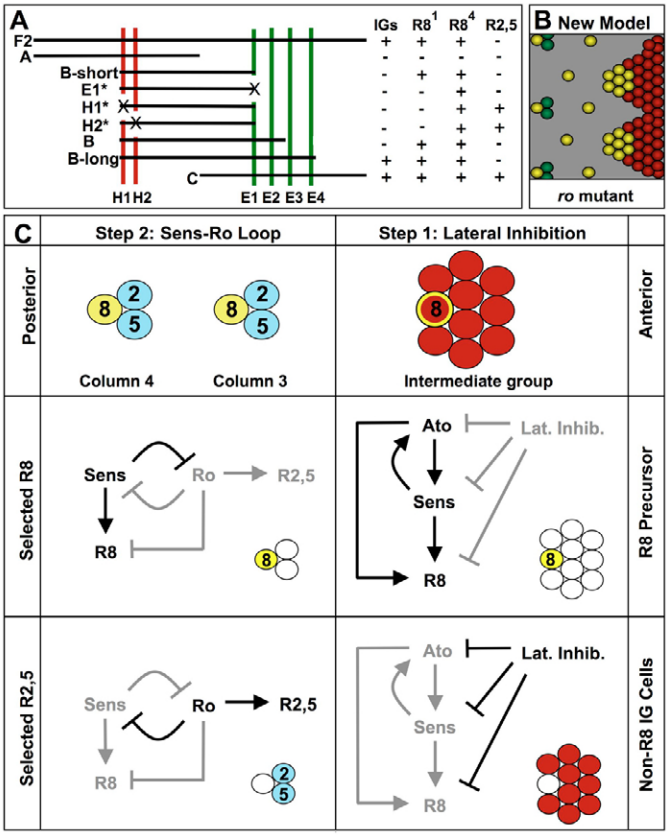

Fig. 8. Two-step selection of R8 by lateral inhibition and Rough. (**A**) Overview of reporter fragments with expression pattern indicated to the right. Intermediate groups (IG), R8s in column 1 ($R8^1$) and column 4 ($R8^4$), and ectopic expression in R2,5s. (**B**) Cartoon of Ato (red), Sens (green) and co-expression (yellow) in *ro* mutants. (**C**) Model of genetic interactions in the two-step selection of R8. Step 1 (right column): red colored circles represent Ato- and Sens-expressing cells in IGs. (Top) Generation of a single R8 per ommatidium initially requires selection of one R8 precursor from among the equipotent cells of the IG. (Middle) In one cell within the IG, Ato and Sens are not repressed by lateral inhibition and become the R8 precursor (yellow border). (Bottom) In cells not destined to adopt the R8 cell fate (red with black border) lateral inhibition represses neuronal cell fate and the expression of Ato and Sens. Step 2 (left column): yellow circles represent developing R8s and blue circles represent R2,5 precursor cells. (Top) By column 3, the selection event determined by lateral inhibition must be reinforced by the *sens-ro* loop to maintain the pattern of a single R8 per ommatidium and to specify the R2,5 cell fate. (Middle) In the developing R8, Sens blocks R2,5 differentiation by repression of Ro and locks in the R8 fate. (Bottom) In putative R2,5 cells, Ro is expressed and directly represses Sens to block R8 and promote R2,5 differentiation.

R8 cell fate potential is maintained despite transient repression by lateral inhibition

Proper patterning of the *Drosophila* eye requires precise selection of R8 precursors in a highly ordered array. Previously, the potential to assume the R8 fate was generally believed to reside in the single cell that achieved the highest balance of proneural induction by *ato* and escaped repression by lateral inhibition. This concept has influenced the interpretation of mutants that exhibit multiple R8 phenotypes, such as *ro*, by linking the extra R8s that form to cells that inappropriately maintain Ato expression. However, our data show that the expression pattern of Ato and Sens in a *ro*-null mutant is not altered in a manner consistent with this model. Our re-evaluation of the *ro* phenotype suggests the intriguing possibility that undifferentiated cells posterior to the furrow retain the developmental plasticity to develop as R8s even in the absence of ongoing Ato expression.

The *ro* phenotype demonstrates that, despite initial repression of the R8 cell fate by lateral inhibition, at least two additional cells have the potential to develop as R8s starting in column 3 if Sens expression is de-repressed. One of the subfragments of the *sens* eye enhancer, fragment C-GFP, is expressed in nearly all cells posterior to the MF, suggesting that *sens* could be de-repressed in cells other than the R2,5 cell precursors and initiate R8 development. The widespread expression of fragment C-GFP suggests that it lacks an important negative regulatory region distinct from Ro repression. One potential mechanism that may explain the fragment C-GFP expression pattern is that the stripe of Ato expression in the MF confers R8 potential to all cells and that this potential is only transiently repressed by lateral inhibition during patterning. Then, as the effects of lateral inhibition fade, secondary mechanisms repress *sens* expression and R8 differentiation in cells posterior to the MF. This model, demonstrated by the function of Ro and suggested by fragment C-GFP expression, is distinct from the previous concept that R8 cell fate is limited to cells of the IG.

The eye-specific *senseless* enhancer integrates positive and negative regulation of R8 differentiation

The minimal eye specific enhancer of *sens*, fragment B-long, contains at least four potentially discreet regulatory elements that balance the positive and negative inputs required to specify a single R8 precursor per ommatidium. The first positively acting element is under the direct control of Ato/Da heterodimers and contains E-boxes 1 and 4. This element is required for Ato-dependent *sens* expression in the IGs and in columns 1-3. Although *ato* is at the top of the genetic cascade required for eye differentiation, *sens* is only the third direct target identified in the eye after *bearded* (*brd*) and *dacapo* (*dap*) (Powell et al., 2004; Sukhanova et al., 2007). We find that Ato/Da heterodimers bind to two E-boxes (E1 and E4) to drive early *sens* expression in R8. This is in contrast to the previously described direct regulation of *sens* in SOPs of the embryonic and developing adult PNS by Ato and Scute at a single E-box in their common enhancer (Jafar-Nejad et al., 2003).

The second positively acting regulatory element resides within the boundaries of fragment E1*, although we did not specifically identify the minimal necessary sequence. This element responds to an Ato-independent mechanism that is sufficient to maintain Sens expression in selected R8 cells after column 3. Sens is known to respond to Ato-independent inductive cues much later in R8 development (48 hours after pupation) when Sens expression requires the *spalt* genes (Domingos et al., 2004). However, larval expression of Sens is not disrupted in *spalt* mutants, suggesting the existence of yet another unidentified positive regulator.

In addition to these two positively acting elements, there are also at least two negative regulatory elements. We specifically identified the Ro-binding element H2 that is responsible for repressing Sens expression in R2,5 cells. The second element was not specifically identified, but its presence is suggested by the nearly ubiquitous expression of fragment C-GFP. Together these positive and negative regulatory elements outline an elegant strategy for the multi-staged selection of a single R8 per ommatidium and highlights a model where blocking R8 cell fate potential with sequential, independent, repressive mechanisms is an important strategy for patterning and cell fate development in the *Drosophila* eye.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/24/4071/DC1

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