Senseless represses nuclear transduction of Egfr pathway activation

Benjamin J. Frankfort[1] **and Graeme Mardon**[1,2,3,4,5,*]

[1]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA
[2]Department of Pathology, Baylor College of Medicine, Houston, TX 77030, USA
[3]Department of Ophthalmology, Baylor College of Medicine, Houston, TX 77030, USA
[4]Department of Neuroscience, Baylor College of Medicine, Houston, TX 77030, USA
[5]Program in Developmental Biology, Baylor College of Medicine, Houston, TX 77030, USA
*Author for correspondence (e-mail: gmardon@bcm.tmc.edu)

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Summary

The Epidermal growth factor receptor (Egfr) pathway controls cell fate decisions throughout phylogeny. Typically, binding of secreted ligands to Egfr on the cell surface initiates a well-described cascade of events that ultimately invokes transcriptional changes in the nucleus. In contrast, the mechanisms by which autocrine effects are regulated in the ligand-producing cell are unclear. In the *Drosophila* eye, Egfr signaling, induced by the Spitz ligand, is required for differentiation of all photoreceptors except for R8, the primary source of Spitz. R8 differentiation is instead under the control of the transcription factor Senseless. We show that high levels of Egfr activation are incompatible with R8 differentiation and describe the mechanism by which Egfr signaling is actively prevented in R8. Specifically, Senseless does not affect cytoplasmic transduction of Egfr activation, but does block nuclear transduction of Egfr activation through transcriptional repression of *pointed*, which encodes the nuclear effector of the pathway. Thus, Senseless promotes normal R8 differentiation by preventing the effects of autocrine stimulation by Spitz. An analogous relationship exists between Senseless and Egfr pathway orthologs in T-lymphocytes, suggesting that this mode of repression of Egfr signaling is conserved.

Key words: Senseless, Egfr, Pointed, Spitz, *Drosophila melanogaster*, R8

Introduction

Interactions between receptors and their ligands are frequently used during development to distinguish one cell type from another. While much is known about the consequences of ligand/receptor binding in the signal-receiving cell, the mechanisms by which autocrine effects are prevented in the signal-producing cell are less clear. Ligand/receptor interactions are of critical importance during *Drosophila* eye development, as recruitment and differentiation of almost all photoreceptors are dependent on high levels of activation of the Epidermal growth factor receptor (Egfr) pathway (Freeman, 1996; Kumar et al., 1998; Lesokhin et al., 1999; Yang and Baker, 2001). Binding of activating ligand to Egfr results in a cascade of membranous and cytoplasmic phosphorylation events, ultimately resulting in the translocation of phosphorylated ERK to the nucleus where it phosphorylates and activates the P2 isoform of Pointed, an ETS domain-containing transcription factor (Brunner et al., 1994; Kumar et al., 2003; O'Neill et al., 1994). Pointed-P2 is then thought to induce transcription of a second *pointed* isoform, *P1*, which encodes the final nuclear effector of the Egfr pathway (O'Neill et al., 1994). Egfr activation in the *Drosophila* eye is induced by the Spitz (Spi) ligand (Freeman, 1994; Tio et al., 1994; Tio and Moses, 1997). Interestingly, while Spi is produced initially and primarily by R8, the founding photoreceptor of each ommatidium, R8 itself is the only photoreceptor that does not require Egfr activation to differentiate (Fig. 1A) (Kumar et al., 1998; Yamada et al., 2003; Yang and Baker, 2001).

Although Egfr pathway signaling is not required for R8 differentiation, several lines of evidence indicate that the uppermost tiers of the Egfr pathway are activated at a high level in the differentiating R8 photoreceptor: (1) R8 is very probably exposed to high levels of secreted Spi, the Egfr ligand in the eye, as R8 itself is the major source of Spi during photoreceptor recruitment; (2) Egfr protein is ubiquitously expressed at high levels at the time when early photoreceptor fates are assumed; and (3) high levels of ERK activation, as assayed by an antibody to dpERK (dual phosphorylated ERK), are detected in R8 intermediate groups and R8 itself, but only in the cytoplasm (Chen and Chien, 1999; Freeman, 1994; Kumar et al., 2003; Kumar et al., 1998; Lesokhin et al., 1999; Rawlins et al., 2003; Spencer and Cagan, 2003; Tio et al., 1994; Zak and Shilo, 1992). However, despite this apparent activation of the pathway at the level of the receptor and within the cytoplasm, a number of nuclear indicators of Egfr activation are either not detected or decreased in R8. First, Rough (Ro), a homeodomain-containing protein that is an early target of Egfr activation, is not expressed in R8 (Dokucu et al., 1996; Frankfort et al., 2001; Kimmel et al., 1990). Second, transcription of *argos* (*aos*), which encodes a negative regulator of Egfr signaling, is decreased in R8 relative to other photoreceptors (Lesokhin et al., 1999). As *aos* expression is

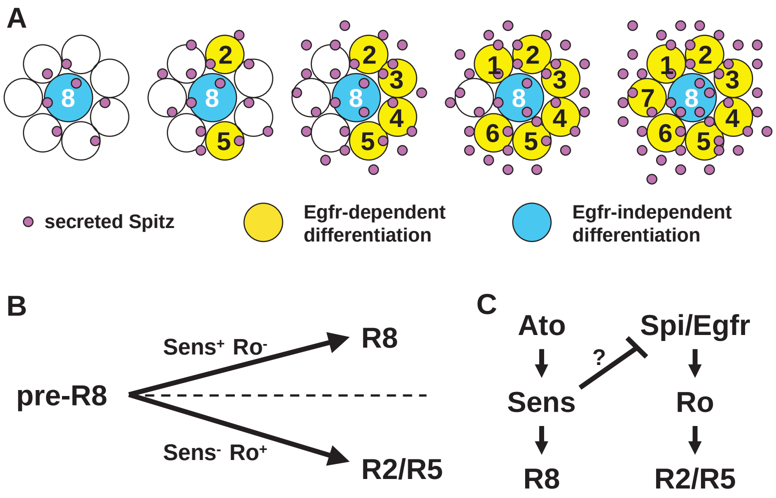

Fig. 1. R8 and non-R8 photoreceptors differentiate according to distinct developmental paradigms. (A) Non-R8 photoreceptors are recruited by Egfr signaling. Spitz (Spi, purple circles) is initially secreted by R8 and binds to the Egf receptor to induce photoreceptor differentiation (yellow). As the field of Spi expands outward from R8, photoreceptors differentiate in a stepwise fashion (R2/R5, R3/R4, R1/R6, R7). R8 differentiation does not require Egfr pathway activation (blue). (B) In wild-type ommatidia (top), Senseless (Sens) is expressed in the presumptive R8 (pre-R8) cell and Rough (Ro) expression is repressed. The pre-R8 cell then differentiates as an R8 photoreceptor. In *sens* mutant ommatidia (bottom), the pre-R8 cell expresses Ro and differentiates as a cell of the R2/R5 subtype. (C) R8 differentiation requires Ato and Sens function, while R2/R5 differentiation requires Spi/Egfr activation, which in turn induces Ro expression. Since Sens is a repressor of Ro in R8, it is possible that this prevention of Ro expression occurs via Sens-mediated repression of the Spi/Egfr pathway.

thought to occur proportionately to the degree of Egfr signaling in that cell, this implies that the Egfr pathway is activated at lower levels in R8 than in non-R8 photoreceptors (Golembo et al., 1996). It is probable that this reduction in Egfr signaling from cytoplasm to nucleus in R8 is developmentally important because high levels of activation of Egfr signaling induced by either ectopic expression or the *Egfr*^{Elp} mutation result in the development of very few R8 photoreceptors, suggesting that high levels of Egfr signaling are not compatible with R8 differentiation (Dominguez et al., 1998; Kumar et al., 1998). Thus, R8 serves as the signal-producing epicenter for Egfr-dependent recruitment in developing ommatidia, yet must remain refractory to the very signaling events that it initiates.

R8 development requires the actions of the proneural gene *atonal* (*ato*) and its downstream effector, *senseless* (*sens*), which encodes a conserved C2H2 zinc finger transcription factor (Frankfort et al., 2001; Jarman et al., 1994; Nolo et al., 2000). Several specific substages of R8 development have been identified (reviewed by Frankfort and Mardon, 2002). In particular, there is a distinction between R8 selection, the choosing of an R8 precursor from a group of developmentally equivalent cells, and R8 differentiation, a later process by which R8 fate is ‘locked’ and the expression of neural markers is initiated. *sens* is required only in R8, and mutations in *sens* result in a total failure of R8 differentiation despite normal selection of R8 precursor cells (called presumptive R8s, or pre-R8s) (Frankfort et al., 2001; Nolo et al., 2000). Furthermore, in *sens* mutants, the pre-R8 cell instead consistently differentiates as a founder photoreceptor of the R2/R5 subtype (Fig. 1B) (Frankfort et al., 2001; Nolo et al., 2000). R2/R5 is normally the first subtype of paired non-R8 photoreceptors to be recruited by Egfr activation via Spi secretion from R8 (Fig. 1A) (Tomlinson and Ready, 1987). Like wild-type R2/R5 cells, the R2/R5 cells that develop from the pre-R8 in *sens* mutants express Ro, which is required for R2/R5 differentiation (Frankfort and Mardon, 2002; Kimmel et al., 1990; Tomlinson et al., 1988).

R8 differentiation is restored when *ro* function is removed in *sens* mutant tissue, suggesting that Sens-mediated repression of *ro* is a critical event during R8 differentiation. However, complete loss of *ro* function does not rescue R8 differentiation in all *sens* mutant ommatidia. Therefore, it is probable that Sens also has a function in R8 which is distinct from its role as a repressor of *ro* (Frankfort et al., 2001). Since the pre-R8 cell in *sens* mutants consistently differentiates as a cell type that is normally Egfr dependent (R2/R5) and Egfr pathway activation appears incompatible with R8 differentiation, it is possible that this additional function of Sens may involve repression of Egfr signaling in R8 (Fig. 1C).

We show that Sens prevents transduction of Egfr signaling to the nucleus of R8, despite both Egf receptor activation and ERK phosphorylation. This is accomplished via a novel regulatory mechanism – Sens causes the transcriptional repression of the P1 isoform of *pointed*. This ensures that the ommatidial signaling center is protected from the effects of autocrine stimulation by secreted Spi, and that R8 differentiation and normal ommatidial organization are preserved. Finally, analogous relationships that exist between Sens and Egfr pathway orthologs in T-lymphocytes may establish R8 development as a novel system with which to study lymphomagenesis, apoptosis and cancer.

Materials and methods

Drosophila genetics and clonal analysis

All *Drosophila* crosses were carried out at 25°C on standard media. For clonal analyses in Figs 2 and 3, *GMRp35; rho-3*^{1} *rho-1*^{7M43} *FRT80B/TM6B* (Wasserman et al., 2000), *w; sens*^{E2} *FRT80B/TM6B* (Frankfort et al., 2001), *w; egfr*^{Elp} *px/CyO; sens*^{E2} *FRT80B/TM6B* (this work), or *GMRp35; rho-3*^{1} *rho-1*^{7M43} *sens*^{E2} *FRT80B/TM6B* (this work) were crossed to *y w hsFLP122; P[w*$^{+}$*=ubiGFP]61EF M(3)i(55) P[w*$^{+}$*]70C FRT80B/TM6B* (Frankfort et al., 2001); *GMRp35; spi*^{SC1} *FRT40A/CyO P[w*$^{+}$*=hshid]; sens*^{E1} *FRT80B/TM6B* (this work) (Tio et al., 1994) was crossed to *y w hsFLP; P[w*$^{+}$*=arm-lacZ] FRT40A; P[w*$^{+}$*=arm-lacZ] FRT80B* (this work); or *GMRp35; rho-3*^{1} *rho-1*^{7M43} *sens*^{E2} *FRT80B ro*^{X63}*/TM6B* (this work) was crossed to *y w hsFLP122; P[w*$^{+}$*=ubiGFP]61EF M(3)i(55) P[w*$^{+}$*]70C FRT80B ro*^{X63}*/TM6B* (Frankfort et al., 2001). Clones were induced as previously described (Frankfort et al., 2001). Misexpression of *sens* in the *egfr*^{Elp} background was accomplished through a cross between *w; egfr*^{Elp} *px/CyO; ey-GAL4* and *w; egfr*^{Elp} *px/CyO; UAS-sens*. The *flpout-GAL4* clones shown in Fig. 4 were generated as previously described (Dominguez et al., 1998) with crosses between *y w P[w*$^{+}$*=act<cd2<GAL4]; hsFLP MKRS/Tb* and either *UAS-Egfr*^{act} *UAS-lacZ* (Dominguez et al., 1998), *UAS-Egfr*^{act} *pnt*^{1277} (this work), or *UAS-sens; UAS-Egfr*^{act} *pnt*^{1277} (this work). Clonal misexpression experiments in Fig. 5 were performed essentially as described, except

with crosses between *y w hsFLP; FRT40A sca-GAL4/CyO P[w$^+$=hshid]* females and *w; M(2)24F P[w$^+$=arm-lacZ] P[w$^+$=tub-GAL80] FRT40A/+; UAS-gene/TM6B* males, where 'gene' represents *Egfr^{act}*, *pnt-P1*, or *ro* (this work) (Pappu et al., 2003).

pnt^{1277} (*pnt-lacZ*) is an enhancer trap line in the *pointed* locus which is expressed in many cells posterior to the morphogenetic furrow (see Fig. 3). *pnt-P1* transcript is normally detected in the morphogenetic furrow in intermediate groups and posteriorly in developing ommatidia (Rawlins et al., 2003). While the expression patterns posteriorly are very similar, *pnt-lacZ* cannot reflect the entire expression pattern of *pnt-P1* because it is not expressed in the intermediate groups. It is also not clear whether *pnt-lacZ* expression is specific to the P1 or P2 isoform of *pnt*, or if it represents a combination of the expression patterns of both.

The *sca-GAL4* line was a gift from Yash Hiromi. This line is expressed at high levels in R8, beginning with the first column of single R8 cells and at lower levels in both other cells in the morphogenetic furrow and some non-R8 cells posteriorly (our unpublished observations).

Immunohistochemistry and visualization of adult eyes

All antibodies were used and confocal microscopy performed as previously described (Frankfort et al., 2001). Adult eyes were fixed, embedded and sectioned as previously described (Frankfort et al., 2001). Whole adult eyes were examined using a Leica MZ16 stereomicroscope and processed with Image-Pro Plus image analysis software.

Generation of *UAS*-rough

proc4-2 (Tomlinson et al., 1988), was digested with *Eco*RI to yield a 1.2 kb *ro* cDNA lacking the coding sequence for the first four amino acids. This fragment was subcloned into pBluescript containing an *Eco*RI site, which was modified with the following adapters: 5′-AATTGCCTCAAACGAAATGCAG and 5′-AATTCTGCATTT-CGTTTGAGGC. This created a modified *ro* cDNA that encodes a protein N terminus of MQNSSK instead of the wild-type MQRHK. Several protein and biochemistry prediction programs were used to assess the modified *ro* cDNA and no changes in behavior compared to wild-type were predicted. The *ro* cDNA was then excised from pBluescript with an *Xho*I/*Xba*I digestion and directionally cloned into pUAST. Vector DNA was injected into *Drosophila* embryos according to standard protocols. Ro protein was detected in wing and leg imaginal discs by antibody staining when *UAS-ro* was misexpressed with *dpp-GAL4*, and transgenic *UAS-ro* animals were sufficient to rescue the *ro^{X63}* mutant phenotype when misexpressed with *hsGAL4*, suggesting that the encoded Ro protein is functional in vivo (Kimmel et al., 1990).

Results

Pre-R8 differentiation in *sens* mutants is dependent on Spi-mediated Egfr pathway activation

In our analysis of *sens* function in R8 differentiation, we found that the extra R2/R5 cell that develops from the pre-R8 in *sens* mutants expresses Ro, which is normally expressed in R2/R5 but not R8 (Dokucu et al., 1996; Frankfort et al., 2001; Kimmel et al., 1990). Ro is expressed downstream of Egfr pathway activation, and both *ro* function and high levels of Egfr pathway activation are required for R2/R5 differentiation (Dominguez et al., 1998; Freeman, 1996; Hayashi and Saigo, 2001; Tomlinson et al., 1988; Yang and Baker, 2001). Since the pre-R8 cell consistently expresses Ro and differentiates as an R2/R5 cell in *sens* mutants, we hypothesized that this transformation occurs as a consequence of high levels of Egfr activation in the pre-R8 cell.

We tested this hypothesis by simultaneously removing *sens* function and blocking Egfr activation in the developing *Drosophila* eye (Fig. 2A-C). We blocked Egfr activation by removing function of both *rhomboid-1* (*rho-1*) and *rhomboid-3* (*rho-3*; FlyBase: *roughoid*, *ru*). Loss of both *rho-1* and *rho-3* function prevents processing of secreted Egfr ligands, including Spi, and results in the loss of all ERK (MAP kinase) activation (Urban et al., 2002; Wasserman et al., 2000). Furthermore, loss of *rho-1* and *rho-3* phenocopies *Egfr* loss-of-function in that only R8 cells differentiate (Fig. 2A) (Wasserman et al., 2000). Loss of *sens* function results in pre-R8 differentiation as a founder R2/R5 cell which is sufficient to recruit a reduced number of photoreceptors (Fig. 2B). However, the absence of *rho-1*, *rho-3* and *sens* together causes total photoreceptor loss, except for a few photoreceptors near the clonal boundary that are rescued non-autonomously by neighboring wild-type cells that produce and process Spi appropriately (Fig. 2C) (Frankfort et al., 2001). A similar phenotype is detected in tissue mutant for both *spi* and *sens* (Fig. 2D). This loss of photoreceptors seen in *rho-1 rho-3 sens* and *spi sens* mutants is not due to cell death because apoptosis was prevented in these experiments by expression of *GMR-p35* (see Materials and methods) (Hay et al., 1994). Furthermore, pre-R8 selection still occurs in both *rho-1 rho-3* and *rho-1 rho-3 sens* mutant tissue, suggesting that a potential founding photoreceptor is present (Fig. 2E,F). Therefore, our interpretation of these results is that, in the absence of *sens* function, pre-R8 differentiation as a founder R2/R5 photoreceptor requires activation of the Egfr signaling pathway via the Spi ligand. In other words, in *sens* mutants, the pre-R8 switches from a Spi/Egfr-independent R8 differentiation pathway to a Spi/Egfr-dependent R2/R5 differentiation pathway.

Sens is a negative regulator of the Egfr pathway

When *rho-1* and *rho-3* function are removed in *sens ro* double mutants, R8 differentiation does not occur (Fig. 2G). This suggests that the requirement in the pre-R8 cell for Egfr activation remains even when *ro* function is removed, and that the *ro*-independent function of *sens* may involve a relationship with the Egfr pathway. Specifically, as the pre-R8 normally does not require Egfr activation but becomes completely dependent on Egfr activation when *sens* function is removed, we hypothesized that *sens* normally acts as a repressor not only of *ro*, but also of Egfr pathway activation in R8. This potential function of *sens* as a repressor of Egfr signaling is supported by genetic interactions between *sens* and the gain-of-function *Egfr^{Elp}* mutation. *Egfr^{Elp}* homozygotes have a greatly reduced number of ommatidia with large gaps of pigmented tissue between them (Fig. 3A) (Baker and Rubin, 1989). In contrast, *sens* mutant tissue is disrupted in appearance but does not contain undifferentiated gaps between ommatidia (Fig. 3B) (Frankfort et al., 2001). However, when clones of *sens* mutant tissue are induced in a background that is heterozygous for the *Egfr^{Elp}* mutation, gaps of undifferentiated tissue appear between ommatidia, a phenotype very similar to that of *Egfr^{Elp}* homozygotes (Fig. 3C). Thus, loss of *sens* function strongly enhances the *Egfr^{Elp}* heterozygous phenotype such that it closely approximates that of *Egfr^{Elp}* homozygotes. If this enhancement occurs by derepression of Egfr signaling by the loss of *sens* function, then misexpression of *sens* in an *Egfr^{Elp}*

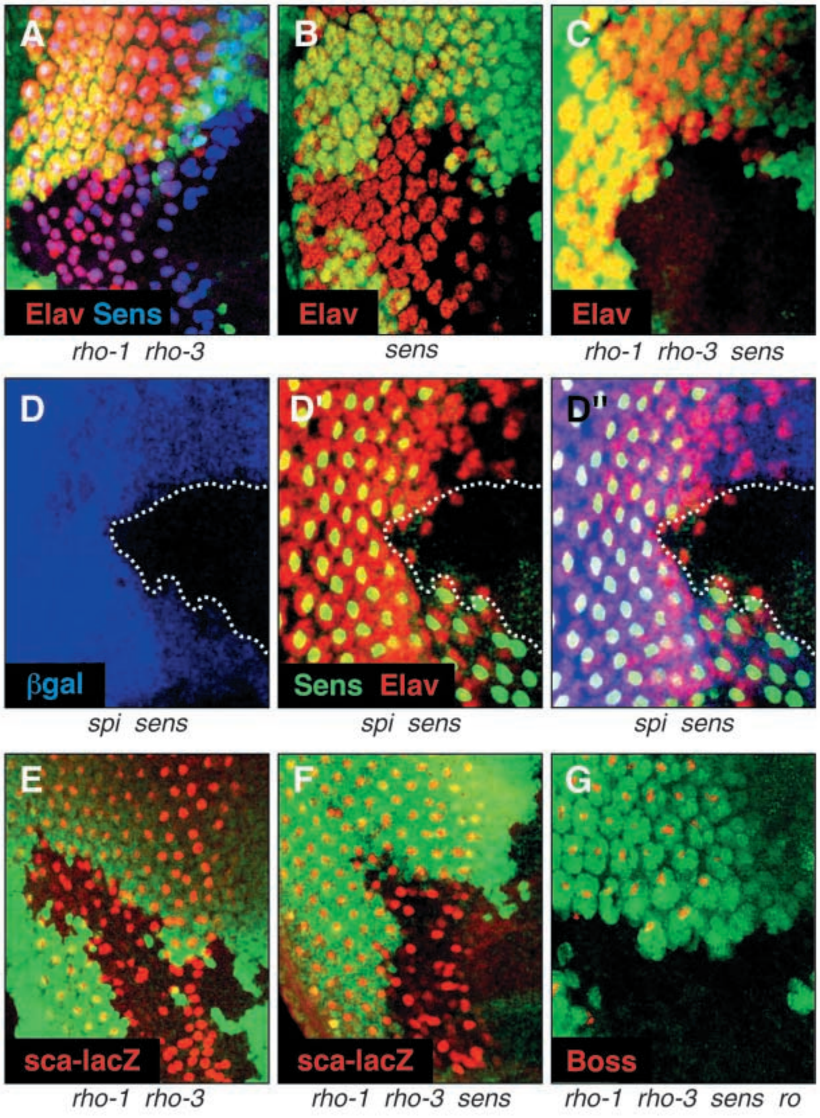

Fig. 2. The presumptive R8 cell requires Spitz-mediated activation of the Egfr pathway to differentiate in *sens* mutants. Third instar eye imaginal discs are presented with posterior to the left in this and subsequent figures. (A-C,E-G) Mutant tissue is negatively marked by the absence of GFP (green). (A-C) Neuronal differentiation is indicated by an antibody to Elav (red). (A) Single R8 neurons (overlap with Sens, blue) are detected in *rho-1 rho-3* mutant tissue, which lack all activation of the Egfr pathway. This suggests that R8 differentiation does not require Egfr activation. (B) Clusters of variable numbers of neurons are detected in *sens* mutant tissue. (C) Neurons are not detected in *rho-1 rho-3 sens* mutant tissue except at the clonal border, where non autonomous effects cause photoreceptor differentiation. This suggests that neuronal differentiation of the pre-R8 as an R2/R5 cell in *sens* mutants is dependent on Egfr activation. (D) *spi sens* double mutant tissue is identified by the absence of β-gal (blue) and outlined with the dotted line. (D′) *sens* mutant tissue is also marked by the absence of Sens (green). Elav (red) marks neurons. (D′′) Overlay of D and D′. Tissue that lacks both *spi* and *sens* function does not contain Elav-positive cells except near the borders of the clone, where non autonomous function of *spi* is sufficient to induce some neuronal differentiation. Differentiation of the pre-R8 as an R2/R5 cell in *sens* mutant tissue is therefore also dependent on *spi* function. (E-F) R8 selection (pre-R8) is marked by *sca-lacZ* (red). (E) Pre-R8s are selected in *rho-1 rho-3* mutant tissue. (F) Pre-R8s are still selected in *rho-1 rho-3 sens* mutant tissue, indicating that the loss of neuronal differentiation in these mutants is not secondary to a failure of R8 selection. (G) Boss (red), a marker for R8 differentiation, is absent in *rho-1 rho-3 sens ro* mutant tissue, suggesting that the R8 rescue seen in *sens ro* double mutants cannot occur when Egfr signaling is absent.

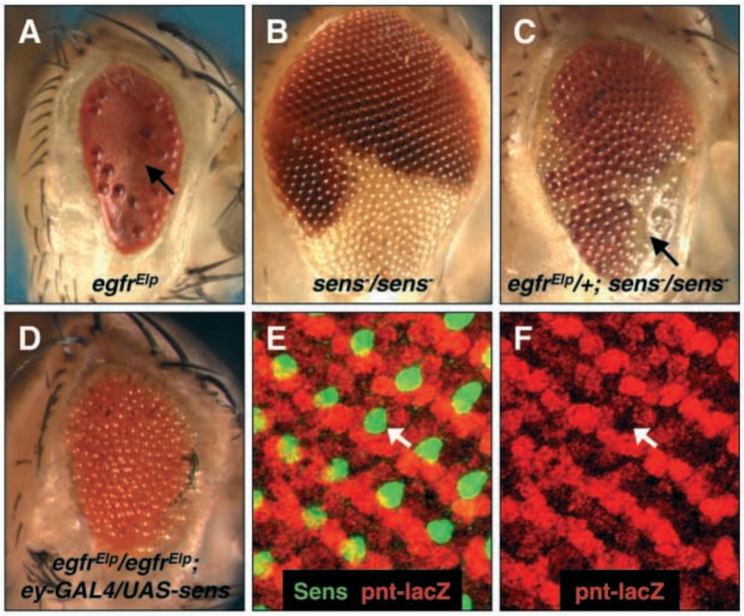

Fig. 3. *sens* is a repressor of the Egfr pathway. (A-D) Light micrographs of adult *Drosophila* eyes. (A) *Egfr*^{Elp} homozygotes have few ommatidia as well as prominent gaps of tissue between ommatidia (arrow). (B) *sens* mutant clone (unpigmented). The *sens* homozygous mutant tissue is roughened in appearance compared to *sens* heterozygous tissue, which is wild type in appearance (pigmented). (C) *sens* mutant clone (unpigmented) induced in an *Egfr*^{Elp} heterozygous background. Overall the eye is smaller, suggesting a dominant interaction between *sens* and *Egfr*^{Elp}. In the *sens* homozygous mutant area, there are reduced numbers of ommatidia, as well as gaps of tissue between ommatidia, similar to *Egfr*^{Elp} homozygotes (arrow, compare with A). (D) Expression of *UAS-sens* with *ey-GAL4* in an *Egfr*^{Elp} homozygote is sufficient to suppress the *Egfr*^{Elp} phenotype. These results suggest that *sens* acts as a repressor of the Egfr pathway. (E,F) Third instar expression of an enhancer trap in the nuclear effector of the Egfr pathway, *pnt*^{1277} (*pnt-lacZ*, red). *pnt-lacZ* is not expressed in Sens-expressing R8 cells (green, arrow), suggesting that the Egfr pathway is not activated to a high degree in the nucleus of R8.

homozygote might have the opposite effect and suppress the phenotype of ommatidial loss and interommatidial gaps. Indeed, misexpression of *UAS-sens* with *ey-GAL4* has precisely these effects on $Egfr^{Elp}$ homozygotes (Fig. 3D). Together, these gain- and loss-of-function experiments suggest that *sens* functions as a powerful negative regulator of the Egfr pathway during *Drosophila* eye development. Since the expression of Sens is tightly restricted to R8 and the primary *sens* mutant phenotype occurs in the pre-R8 cell, it is most likely that this repression occurs specifically in the differentiating R8 photoreceptor. We therefore looked at expression of an enhancer trap in *pointed* (*pnt-lacZ*), which encodes the nuclear effector of the Egfr pathway. Consistent with our hypothesis, *pnt-lacZ*, while expressed in many non-R8 photoreceptors as they differentiate, is not expressed in Sens-expressing R8 cells (Fig. 3E,F; Materials and methods) (Scholz et al., 1993).

Sens blocks activation of Egfr signaling at the nuclear level

While the Egfr pathway is probably activated at a high level at the cell membrane and in the cytoplasm of R8, expression of nuclear outputs of the pathway is low (Fig. 3E,F, see Introduction). Moreover, whereas loss-of-function mutations in all major Egfr pathway members have no effect on R8 differentiation, high levels of activation of Egfr signaling as a result of either ectopic expression or $Egfr^{Elp}$ mutations result in the development of very few R8 photoreceptors (Dominguez et al., 1998; Kumar et al., 1998; Lesokhin et al., 1999; Yamada et al., 2003; Yang and Baker, 2001). Thus, the reduction in Egfr activation from high cytoplasmic levels to low nuclear levels in R8 may be of developmental importance. Since Sens acts as a negative regulator of the Egfr pathway, we hypothesized

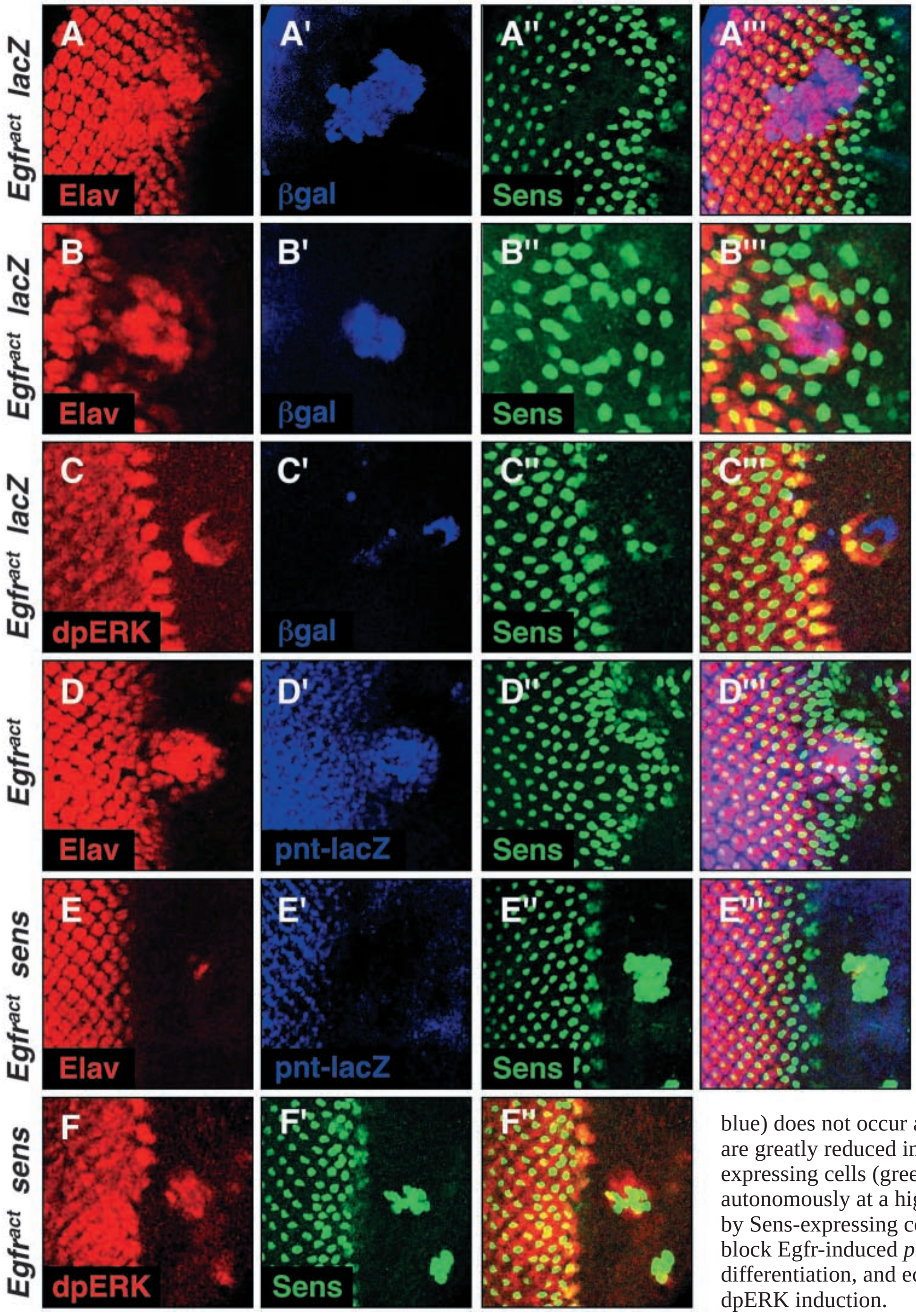

Fig. 4. Sens prevents Egfr pathway activation in the nucleus. *UAS* constructs were ubiquitously expressed in clones using *flpout-GAL4*. (A-A′′′) Co-misexpression of *UAS-*$Egfr^{act}$ and *UAS-lacZ* posterior to the morphogenetic furrow (MF). Elav (red) is expressed in almost all cells within the clone (blue). Sens (green) is not detected within the clone. (B,C) Co-misexpression of *UAS-*$Egfr^{act}$ and *UAS-lacZ* anterior to and within the MF. (B-B′′′) Elav (red) is expressed within and surrounding the clone (blue). Sens (green) is not expressed within the clone but is ectopically induced non autonomously. (C-C′′′) dpERK (red) and Sens (green) are expressed non-autonomously. Together, B and C are consistent with the presence of ectopic MFs surrounding areas of Egfr activation. (D-D′′′) Misexpression of *UAS-*$Egfr^{act}$ anterior to the MF. *pointed* (*pnt*) transcription (*pnt-lacZ*, blue) occurs in most ectopic Elav-positive (red) cells. (E,F) Co-misexpression of *UAS-*$Egfr^{act}$ and *UAS-sens* anterior to the MF. (E-E′′′) *pnt* transcription (*pnt-lacZ*, blue) does not occur and numbers of Elav-positive cells (red) are greatly reduced in the clone, which is marked by the Sens expressing cells (green). (F-F′′′) dpERK (red) is expressed autonomously at a high level within the clone, which is marked by Sens-expressing cells (green). Thus, *sens* is sufficient to block Egfr-induced *pnt* transcription, photoreceptor differentiation, and ectopic MF generation, but does not prevent dpERK induction.

that Sens mediates this critical decrease in Egfr signaling from membrane/cytoplasm to nucleus in R8.

To test this hypothesis, we ubiquitously expressed an activated form of Egfr (*Egfr*^{act}) in small clones using *flpout-GAL4*. We first looked at *Egfr*^{act} clones positioned posterior to the morphogenetic furrow (MF). Neural differentiation occurs throughout such clones (Fig. 4A). Since ectopic Egfr activation is sufficient to induce photoreceptor differentiation prior to passage of the MF, these clones probably represent a field of cells that had already differentiated as neurons by the time the MF reached it (Dominguez et al., 1998). Consistent with the hypothesis that high levels of Egfr activation are not compatible with R8 differentiation, these clones show a cell-autonomous lack of Sens expression (Fig. 4A). Anterior to the MF, activation of Egfr signaling causes precocious neural development autonomously, and induces ectopic MFs non-autonomously. These ectopic MFs express Ato, Sens and dpERK appropriately (Fig. 4B,C) (Dominguez et al., 1998). Furthermore, the ectopic neurons generated with this system express *pnt-lacZ* at a high level, indicating that the nuclear target of Egfr activation is being induced and the canonical Egfr signaling pathway is probably the cause of neural differentiation (Fig. 4D). However, when *sens* is co-misexpressed along with *Egfr*^{act}, *pnt-lacZ* expression is prevented and neural differentiation is severely reduced in the cells that express *sens*, and ectopic MFs are not established (Fig. 4E,F). In contrast, dpERK expression still occurs when *sens* is co-misexpressed with *Egfr*^{act}, indicating that the Egfr pathway is being activated at the cell membrane and within the cytoplasm (Fig. 4F). This suggests that Sens cell-autonomously blocks transduction of the activated Egfr pathway to the nucleus and is sufficient to prevent the effects of cell membrane activation of the Egfr pathway. These results are also consistent with our proposed role for Sens as a repressor of high levels of Egfr signaling in R8.

Sens represses *pointed-P1* in R8

If Sens acts to reduce Egfr signaling from the cytoplasm to the nucleus of R8, we hypothesized that activation of Egfr signaling downstream of the point at which Sens blocks the pathway could disrupt normal R8 differentiation, whereas activation of the pathway upstream of this point would have little or no effect. To test this hypothesis, we misexpressed members of the Egfr pathway in R8 using *sca-GAL4* (Materials and methods). When *Egfr*^{act} or *ras1*^{val12} (an activated form of Ras that functions in the cytoplasm upstream of ERK) is expressed in R8 with this system there is no appreciable effect on Sens, Boss, or Ro expression in third instar eye imaginal discs (Fig. 5A-C, not shown). This suggests that R8 differentiation proceeds normally when the Egfr pathway is activated at the cell membrane or within the cytoplasm of R8 and is consistent with our proposed role for Sens in R8. Since Sens acts as a repressor of *pnt* transcription, we also misexpressed both isoforms of *pnt* in R8. Interestingly, misexpression of the P2 isoform of *pnt* (*pnt-P2*), or an activated form of *pnt-P2*, also has no effect on R8 differentiation (not shown) (Halfon et al., 2000). However, misexpression of *pnt-P1* causes a disruption in Sens expression such that Sens-expressing nuclei are displaced apically in the imaginal disc (Fig. 5D,E). Since photoreceptor nuclei move basally during neuronal differentiation, this implies that misexpression of *pnt-P1* in R8 may disrupt R8 differentiation. Consistent with this, many *sca-GAL4* × *UAS-pnt-P1* adult ommatidia do not contain small rhabdomeres, suggesting an absence of R8 (Fig. 5F). Ommatidia also contain a variable number of photoreceptors and these adult phenotypes are very similar to the *sens* loss-of-function phenotype. These results imply that *pnt-P1*, but not *pnt-P2*, may be a target of *sens* repression. Misexpression of *ro*, an early target of Egfr signaling, has a more profound effect on R8 development as both Sens and Boss expression are absent

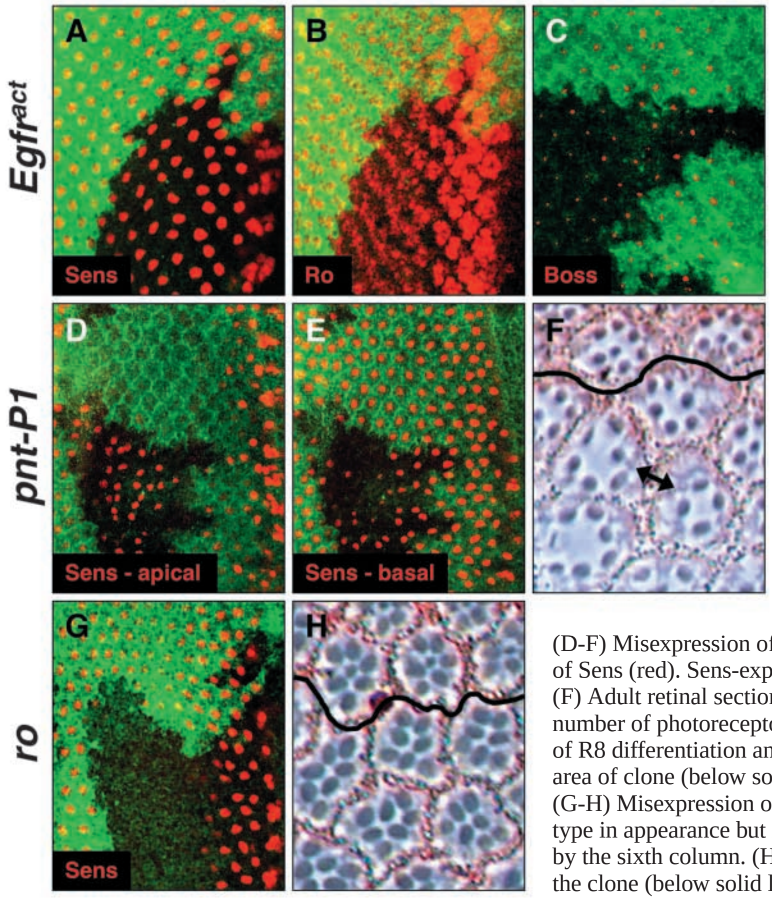

Fig. 5. Expression of nuclear effectors of the Egfr pathway prevents R8 differentiation. (A-H) Misexpression clones. *sca-GAL4* was used to induce expression of *UAS-Egfr*^{act}, *UAS-pnt-P1* or *UAS-ro* in R8. Third instar clones (A-E,G) are negatively marked by the absence of βgal (green). (A-C) Misexpression of *UAS-Egfr*^{act} in R8. Expression of Sens (A), Ro (B) and Boss (C), are not disrupted, suggesting that robust activation of the Egfr pathway at the level of the cell membrane is not sufficient to perturb R8 differentiation. *UAS-Egfr*^{act} induced predicted phenotypes in other tissues (not shown), indicating that the transgene was active in this assay. (D-F) Misexpression of *UAS-pnt-P1* in R8. (D,E) Apical (D) and basal (E) expression of Sens (red). Sens-expressing nuclei are not evenly spaced and are apically displaced. (F) Adult retinal sections at the level of R8 show ommatidia with a variably reduced number of photoreceptors and a lack of small rhabdomeres, consistent with a disruption of R8 differentiation and similar to *sens* loss-of-function phenotypes (arrow). Compare area of clone (below solid line) to neighboring wild-type tissue (above solid line). (G-H) Misexpression of *UAS-ro* in R8. (G) Expression of Sens (red) is initially wild type in appearance but is reduced by the fourth column of R8 differentiation and absent by the sixth column. (H) At the level of R8, no small rhabdomeres are detected within the clone (below solid line), phenocopying *sens* loss of function.

(Fig. 5G, not shown). Adult ommatidia lack small rhabdomeres but are otherwise of relatively normal construction (Fig. 5H). These results are consistent with the known function of *ro* as a critical determinant of R2/R5 cell fate determination, as well as with our previous model in which Sens acts as a repressor of *ro*. Together, these data strongly suggest an important additional role for Sens as a novel nuclear repressor of *pnt-P1* (Fig. 6).

Discussion

Senseless represses Spi/Egfr-mediated cell differentiation in R8

Our work suggests that Sens acts to ensure that the organizing center of each ommatidium is refractory to the developmental signals it produces – the R8 cell can secrete Spi and even activate Egfr on its own cell membrane, yet remains protected from the deleterious effects of activation of Pnt and other Egfr targets, such as Ro, in R8.

The mechanism by which Sens regulates the discrepancy between levels of Egfr activation at the receptor/cytoplasmic and nuclear levels in R8 is probably through repression of *pnt* transcription. This is supported by the observation that *pnt* transcription is not induced by misexpression of an activated form of *Egfr* when *sens* is co-misexpressed (Fig. 4). Furthermore, expression of the *pnt-P1* isoform in R8 disrupts R8 differentiation (Fig. 5D-F). As misexpression of *pnt-P2* has no effect on R8 differentiation, this suggests that Sens negatively regulates transcription of *pnt-P1*, but not *pnt-P2*. This mode of regulation is consistent with established models for transduction of the Egfr signal to the nucleus. Specifically, ERK phosphorylates Pnt-P2, which is thought to be a transient positive regulator of *pnt-P1* transcription (Brunner et al., 1994; O'Neill et al., 1994). In our model, transduction of Egfr activation occurs all the way into the nucleus of R8, but Sens represses the pathway at the final step – positive regulation of *pnt-P1* by Pnt-P2 (Fig. 6). When *sens* function is removed, the block on *pnt-P1* transcription is relieved, and Pnt-P1 can exert its transcriptional effects on the nucleus, including *ro* induction.

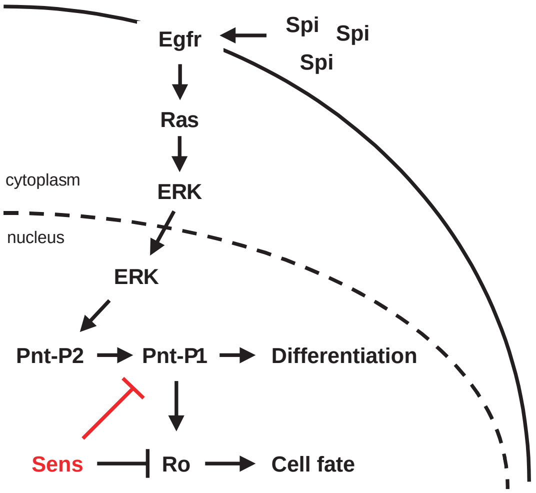

Fig. 6. Model for Sens action in R8. Spi induces Egfr activation and the signal transduction cascade is induced normally. However, Sens prevents transcription of *pnt-P1*, thereby blocking the pathway at the final step. This relationship is likely to specifically mediate cell differentiation in R8 (see text). Sens also represses *ro*, an early target of the Egfr pathway. This second relationship regulates the cell fate decision in the founder photoreceptor between R8 and R2/R5 (see text).

There is evidence that *pnt-P1* transcription can be regulated by Egfr signaling independently of *pnt-P2* during *Drosophila* embryogenesis (Gabay et al., 1996). If this is the case during eye development, our model would remain essentially the same – Sens would still act as a negative regulator of *pnt-P1* in R8. However, this regulation would occur independently of *pnt-P2* rather than downstream of *pnt-P2*.

Sens is also a potent negative regulator of *ro* and this relationship appears to specifically affect the cell fate decision between R8 and R2/R5 differentiation (Fig. 5G,H) (Frankfort et al., 2001). Several lines of evidence suggest that Sens-mediated repression of *ro* is distinct from other effects of Sens in R8. First, loss of *ro* function does not rescue R8 differentiation in all ommatidia in *sens* mutants (Frankfort et al., 2001). Second, even those R8 cells that do differentiate in *sens ro* double mutants require Spi/Egfr pathway activation (Fig. 2G). Third, misexpression of *ro* in R8 causes a different phenotype than misexpression of *pnt-P1* in R8. Specifically, even though Egfr pathway activation is necessary and sufficient for Ro expression, misexpression of *pnt-P1* in R8 does not cause an obvious cell fate transformation from R8 to R2/R5, while misexpression of *ro* in R8 does (Fig. 5D-H) (Dominguez et al., 1998; Hayashi and Saigo, 2001). Indeed, R8 markers are still expressed when *pnt-P1* is misexpressed in R8. However, aberrant nuclear movements and the absence of small rhabdomeres at the level of R8 in adults suggest that misexpression of *pnt-P1* does perturb R8 differentiation (Fig. 5D). Together, these results suggest that Sens repression of *pnt-P1* occurs independently of Sens function as a repressor of *ro*, and that Sens-mediated repression of *pnt-P1* is probably required for normal R8 differentiation upstream or independently of cell fate determination (Fig. 6).

Since Sens acts as a transcription factor and its mammalian homolog, Gfi-1, binds directly to enhancer regions of *Ets1* and *Ets3*, two mammalian orthologs of *pnt*, is it possible that Sens repression of *pnt-P1* expression occurs directly (Duan and Horwitz, 2003; Nolo et al., 2000; Zweidler-Mckay et al., 1996). Gfi-1 also interacts with nuclear matrix proteins to repress transcription (McGhee et al., 2003). Thus, it is possible that Sens represses transcription of Egfr nuclear effectors via a similar mechanism. Future experiments are required to determine which of these or other mechanisms are important during R8 differentiation. However, it is likely that Sens does not act as a positive regulator of Edl/Mae, a proposed cell-autonomous repressor of Egfr signaling, because *edl/mae* function is not required for normal R8 differentiation (Yamada et al., 2003). Finally, it is also unlikely that *sens* functions as an activator of *yan*, which encodes a nuclear repressor of the Egfr pathway, because *yan* loss-of-function mutations also do not impact R8 differentiation (Lai and Rubin, 1992).

Conservation of Sens/Egfr antagonism?

The positioning of Sens repression downstream of ERK activation may help explain interactions observed between *sens* and Egfr pathway homologs in T-lymphocytes. In Jurkat T-cells, activation induced cell death (AICD), a process that is required to prevent non-specific activation of T-cells, is dependent, in part, on ERK1/2 activation (van den Brink et al., 1999). Intriguingly, high levels of Gfi-1 have been shown to inhibit AICD despite high levels of ERK1/2 activation (Karsunky et al., 2002). The antagonistic relationship between Sens and the Egfr pathway in R8, in conjunction with the observation that Gfi-1 can bind to the enhancer regions of *Ets1* and *Ets3*, suggest that this inhibition of AICD may occur via Gfi-1-mediated repression of ERK1/2 targets (such as *Ets/pnt*) in T-cells (Duan and Horwitz, 2003). Thus, our results may establish R8 development as a powerful and novel system with which to study mechanisms of lymphomagenesis, apoptosis and cancer.

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