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Spitz from the Retina Regulates Genes Transcribed in the Second Mitotic Wave, Peripodial Epithelium, Glia and Plasmatocytes of the *Drosophila* Eye Imaginal Disc

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Abstract

Proliferation, differentiation, and other processes must be coordinated during the development of multi-cellular animals. A discrete and regulated cell division, the Second Mitotic Wave (SMW), occurs concomitantly with early cell fate decisions in the *Drosophila* developing retina. Signals from the Epidermal Growth Factor Receptor (EGFR) are required to promote cell cycle arrest of specified cells and antagonize S-phase entry in the SMW. Cells that do not receive any EGFR activity enter S-phase in the SMW in response to the Notch pathway. To identify genes with potential roles in the SMW, we used microarrays and genetic manipulation of the EGFR pathway to seek transcripts regulated during the SMW. RNA in situ hybridization of 126 differentially transcribed genes revealed genes that have novel expression patterns in cells closely associated with the SMW. In addition, other genes' transcripts were regulated in the differentiating photoreceptor cells, retinal basal glia, the peripodial epithelium, and blood cells (plasmatocytes) associated with the developing retina. These novel targets suggest that during eye development, EGFR activity coordinates transcriptional programs in other tissues with retinal differentiation.

Introduction

The development of multicellular animals requires the co-ordination of multiple signals that control proliferation and differentiation. Often, the extracellular signaling pathways required for differentiation also regulate the cell cycle. How these signals are choreographed together to produce properly sized and patterned tissue is just beginning to be understood (Baker, 2007).

In the *Drosophila* eye, the cell-cell signals that control differentiation have been well characterized (Zipursky and Rubin, 1994; Freeman, 1997; Greenwood and Struhl, 1999; Curtiss and Mlodzik, 2000; Simon, 2000) and several of these signals are also required to regulate proliferation in the developing retina (Penton et al., 1997; Horsfield et al., 1998; Baonza and Freeman, 2005; Firth and Baker, 2005). The retina and other limbs each develop from epithelial sacs or imaginal discs (Auerbach, 1936; Cohen, 1993). Each imaginal disc is made of 2 opposing epithelial layers. The layer that forms the adult eye or limb is composed of columnar cells, the disc proper (DP) and overlaying the DP is a layer of squamous epithelial

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cells, the peripodial epithelium (PE) (Cohen, 1993) (Figure 6). Although the PE does not form part of the adult eye, signals from the PE to the DP are important for retinal development (Cho et al., 2000; Gibson and Schubiger, 2000).

Differentiation starts at the posterior edge of the presumptive retinal epithelium and progresses anteriorly (Wolff and Ready, 1993). The front of this wave of differentiation is marked by the apical constriction of cells called the morphogenetic furrow (MF). The MF separates the undifferentiated and differentiating portions of the eye. Anterior to the MF, cells are randomly proliferating. Just ahead of the furrow all cells undergo a prolonged G1 arrest during which time signals to specify early cell fate decisions are received and neurogenesis begins (Thomas et al., 1994). Some cells then re-enter the cell cycle and undergo a single round of proliferation, the Second Mitotic Wave (SMW) (Wolff and Ready, 1991). Not all cells enter the SMW however. Groups of cells remain arrested in G1, permanently withdraw from the cell cycle and differentiate (Wolff and Ready, 1993).

During eye development signals from the Epidermal Growth Factor Receptor (EGFR) are required at numerous stages. First, EGFR is activated by the ligand Spitz (Spi) in four cells surrounding the founding photoreceptor cell R8, resulting in the G1 arrest and recruitment of these four cells (R2, R5, R3 and R4) into the photoreceptor precluster (Freeman, 1994; Dominguez et al., 1998; Kumar et al., 1998; Baker and Yu, 2001). The surrounding cells, without any EGFR activity, reenter the cell cycle and perform DNA synthesis. After S-phase, progression from G2 phase into mitosis requires EGFR activity. EGFR is activated in G2 cells that are in contact with precluster cells by Spi (Baker and Yu, 2001). Later expression of Spi recruits these post-mitotic cells into the remaining retinal cell fates (Freeman, 1996). Interestingly, Spi protein travels down the photoreceptor axons into the brain lamina, where it triggers the differentiation of the synaptic cartridge units (Huang et al., 1998). Retinal basal glia cells are closely associated with the developing photoreceptor axons and are important for their guidance down the optic stalk to the brain lamina (Choi and Benzer, 1994; Rangarajan et al., 1999). Also, larval hemocytes/plasmatocytes are found on the outer surface of the eye disc. Both of these cell types are of a different linage to the imaginal disc.

Only some gene targets of EGFR signaling have been identified. The canonical Ras/MAPK pathway downstream of EGFR transduces the EGFR signal to the ETS transcription factor, Pointed (Pnt) (Brunner et al., 1994; O Neill et al., 1994; Yang and Baker, 2003). Phyllopod also appears to be a target during photoreceptor differentiation (Chang et al., 1995; Dickson et al., 1995; Wassarman et al., 1995). EGFR signaling targets the Cdc25 homolog, *String* (*Stg*), for progression from G2 to mitosis in the SMW (Baonza et al., 2002; Baker and Yu, 2001). The signal to enter S-phase in the SMW is dependant on the Notch (N) pathway; cells defective for N signaling are unable to progress from G1 phase into S-phase (Baonza and Freeman, 2005; Firth and Baker, 2005). In the absence of both EGFR and N, cells remain in G1 despite lacking EGFR activity. Both N and EGFR signaling are thought to regulate S-phase entry through transcriptional targets, but these targets remain unknown (Firth and Baker, 2005).

To understand the events surrounding the SMW we have taken a genome wide approach to isolate genes expressed within and around the SMW. Other groups have analyzed the gene expression profile during wild type eye development using microarrays or SAGE analysis of FACs sorted cells (Jasper et al., 2002; Klebes et al., 2002; Michaut et al., 2003) and showed that many hundreds of genes are transcriptionaly upregulated during eye development. It is likely that many are targets of EGFR or N regulation.

We designed a microarray gene expression screen to identify genes regulated specifically during the SMW, rather than all the targets of EGFR or N signaling during eye development.

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The SMW was eliminated genetically through the manipulation of the EGFR pathway and the gene expression profile was compared to similar retinas with a SMW. This approach gave a number of candidate genes. We then determined the mRNA expression patterns of differentially transcribed genes by RNA in situ hybridization. We have identified genes whose expression is regulated by EGFR signaling that are transcribed in cells participating in the SMW or the cell cycle arrested differentiating cells as expected, but also many genes transcribed in the PE, glia and the plasmatocytes. We think that our specific strategy also selected for genes that EGFR regulates in other tissues in response to the eye disc.

Materials and Methods

RNA isolation, probe preparation for array and data analysis

Total RNA was extracted from 100 eye/antennal discs using Trizol and analyzed for quality on the Agilent Bioanalyzer (www.chem.agilent.com). For both *GMR>Ras*^{V12} and *GMR>sSpi*, 3 independent samples of total RNA were prepared for the GeneChip® arrays according to the manufacturer's specifications and hybridized to DrosGenome1 expression arrays (www.affymetrix.com) (Platform accession no. **GPL72**). The raw data reported in this paper has been submitted to the NCBI Gene Expression Omnibus, www.ncbi.nlm.nih.gov/geo (accession series no. **GSE6300**). Data analysis was performed using Microarry Suite 5.0 (MAS 5.0), the Data Mining Tool software (Affymetrix) and Microsoft Excel. Single array analyses for each array were performed in MAS 5.0. All nine comparison replicates were performed in the Data Mining Tool with *GMR>sSpi* as the baseline and *GMR>Ras*^{V12} as the experiment. A Signal Log Ratio (SLR) greater than 1 is the same as a Fold Change of 2 (Wodicka et al., 1997). Gene expression changes that satisfied both the T-Test and the non-parametric Mann-Whitney Test ($p < 0.05$) were used to evaluate gene expression changes between the *GMR>Ras*^{V12} and *GMR>sSpi* (Affymetrix, 2003). A cut off was applied and genes that consistently had a Signal Log Ratio of 1 or greater were deemed significantly upregulated in *GMR>Ras*^{V12} (or downregulated in *GMR>sSpi*) and those with a Signal log ratio less than −1 were deemed significantly downregulated in *GMR>Ras*^{V12} (or upregulated in *GMR>sSpi*).

RNA in situ probe design, preparation and hybridization

RNA probes were designed against the contiguous cDNA sequence of differentially expressed genes. A PCR strategy for rapid generation of template DNA for synthesis of labeled RNA probes was used. Probes were designed to be between 400–800 base pairs long and correspond to unique sequences of the cDNA as determined by Blast (http://flybase.net/blast). Primers were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3). The following linkers were added to the 5′ end of each primer: Forward Primer: 5′ GGCCGCGG 3′; Reverse Primer: 5′ CCCGGGGC 3′. Primers for each sequence amplified can be found in the supplementary table 1. PCR was checked by gel electrophoresis. If a single product was obtained, 5μl of the PCR reaction was treated with ExoSAP-IT (USB Corp. #78202). 1–2μl of this was used for a second PCR reaction with universal primers containing sequences that hybridize to the linker sequence and promoter sequences for the T7 and SP6 polymerases. Universal forward primer sequence (adds T7 promoter and EcoR1 restriction site for subcloning): 5′GAGAATTCTAATACGACTCACTATAGGGCCGCGG 3′. Universal reverse primer sequence (adds SP6 promoter and a BamH1 restriction site for subcloning): 5′ AGGGATCCATTTAGGTGACACTATAGAACCCGGGGC 3′. A standard PCR program with an annealing temperature of 45°C was used. The second PCR products, containing the SP6 and T7 promoter sequences, were cleaned up by gel extraction. Sense (T7) and anti-sense (SP6) RNA DIG probes were made directly from this PCR product (Roche DIG RNA labeling Kit #1 277 073). In cases where the first PCR reaction gave multiple bands, the correct band was gel extracted before the second PCR reaction. DIG probe was precipitated with LiCl and

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re-suspended in 100μl of 50% Formamide in HSW solution (see below). RNA in situ hybridization was performed as described (Cornell et al., 1999).

Drosophila strains used

GMR Gal4 (Hay et al., 1994; Freeman, 1996); *UAS sSpi* (Schweitzer et al., 1995); *UAS* Ras^{V12} (Karim and Rubin, 1998).

Immunofluorescence

Labeling of eye discs was performed as described (Firth et al., 2006). Preparations were examined on the BioRad Radiance 2000 Confocal microscope. Images were processed using Adobe Photoshop 4.0 and NIH Image J software. Rabbit anti-phosphoHistone3 was from Cell Signaling Technology (#9701). Rabbit anti-Caspase 3 (CM1) (Srinivasan et al., 1998). Anti-MDP-1 (Hortsch et al., 1998). Anti-arm (N2 7A1), anti-Dlg (4F3), anti-Repo and anti-ElaV were from the Developmental Studies Hybridoma Bank, maintained by the University of Iowa, Department of Biological Sciences, Iowa City IA52242, USA under contract N01-HD-7-3263 from the NICHD.

Results

Screen design and strategy for identifying genes expressed in the SMW during Drosophila eye development

Using the *Drosophila* developing eye a microarray gene expression screen was designed to identify genes involved in the SMW. Since the cells in the SMW could not reliably be dissected out from the surrounding tissue, an approach where the SMW was ablated genetically was taken. The SMW was blocked in the developing eye by expressing the EGFR activating ligand, Spitz (Spi). Spi was over expressed in all cells in the differentiating part of the retina posterior to the MF using the UAS-Gal4 system; *GMR>sSpi* (*GMRGal4*/+; *UAS sSpi*/+). GMR Gal4 is active in all cells posterior to the MF (Hay et al., 1994; Freeman, 1996). Expression of sSpi promotes G1 arrest and as a result there is no SMW (Yang and Baker, 2003). Due to increased activity of the EGFR/Ras pathway ectopic differentiation also occurs (Fig. 1A) (Freeman, 1996). The program of eye development and photoreceptor differentiation leads to the induction of many genes (Jasper et al., 2002; Klebes et al., 2002; Michaut et al., 2003). To circumvent the expected enrichment of all EGFR targets throughout the retinal eye field in *GMR>sSpi*, the gene expression profile of *GMR>sSpi* eye/antennal discs was compared to that of *GMR>*Ras^{V12} (*GMRGal4/UAS* Ras^{V12}), rather than to wild type discs. Hyper-activation of the EGFR pathway with oncogenic Ras^{V12} results in a similar ectopic differentiation phenotype; the effects of *GMR>*Ras^{V12} are autonomous, however and the EGFR/Ras/MAPK pathway is activated after the SMW begins, so that *GMR>*Ras^{V12} does not prevent the SMW (Fig. 1B) (Baker and Yu, 2001). Due to the absence of cells participating in the SMW, we hypothesized that transcripts of genes expressed in the SMW would be downregulated in *GMR>sSpi* when compared to *GMR>*Ras^{V12}. There also might be other differences between *GMR>sSpi* and *GMR>*Ras^{V12}, such as differences in genes specific to the early columns of retinal specification that occur during the SMW. For example, *GMR>sSpi* contains very few R8 photoreceptors, whereas normal numbers of R8 cells are specified in *GMR>*Ras^{V12} (Lesokhin et al., 1999; Baker and Yu, 2001).

The mRNA expression profiles of *GMR>sSpi* and *GMR>*Ras^{V12} whole eye/antennal imaginal discs were determined using oligonucleotide arrays representing approximately 13,500 known and predicted genes in the *Drosophila* genome (GeneChip® expression array DrosGenome1, Affymetrix). For both genotypes, 3 independent RNA samples were prepared and hybridized to the arrays. Using Affymetrix Data Mining Tool Software whole genome gene expression changes between *GMR>sSpi* and *GMR>*Ras^{V12} were evaluated (Affymetrix, 2003). 140 genes

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were differentially transcribed between *GMR>sSpi* and *GMR>Ras^{V12}*. 72 of these genes were downregulated and 68 were upregulated in *GMR>sSpi* compared to *GMR>Ras^{V12}* (Figure 1C).

Characterized genes associated with eye development

Known transcripts downregulated in *GMR>sSpi* compared to *GMR>Ras^{V12}*—72 transcripts were downregulated in *GMR>sSpi* compared to *GMR>Ras^{V12}*. 14 of these genes have been previously characterized during eye development. Because the SMW is absent in *GMR>sSpi*, we expected genes expressed in the SMW cells to be included in this group. As expected 4 of the 14 genes characterized previously are known to be expressed more highly in the cycling cells of the SMW and the in the unspecified cells i.e. in cells for 2/3 columns at the posterior edge of the MF: *lozenge* (*lz*), *BarH1* (*B-H1*), *Traf1* and *Spn43A* (Higashijima et al., 1992; Daga et al., 1996; Flores et al., 1998; Lai et al., 2000; Preiss et al., 2001). Three of the downregulated genes are required for R8 photoreceptor development: *atonal* (*ato*), *bearded* (*brd)* and *big brain* (*bib*) (Jarman et al., 1994; Singson et al., 1994; Li and Baker, 2001). R8 specific transcripts were expected to be downregulated because there are fewer R8s in *GMR>sSpi (*Lesokhin et al., 1999*)*. Another 4 genes downregulated in *GMR>sSpi* are reported to be present in other differentiating photoreceptors: *SoxNeuro* (*SoxN*/*SoxB1*), *Fasciclin 2* (*Fas2*), *klingon* (*klg*) and *tartan* (*trn*) (Chang et al., 1993; Butler et al., 1997; Pignoni et al., 1997; Cremazy et al., 2001). Finally, transcripts of 4 downregulated genes are expressed in a pattern associated with the MF: *E(Spl)m2*, *brd*, *arc* (*a*) and *spineless* (*ss*) (Singson et al., 1994; Duncan et al., 1998; Lai et al., 2000; Liu and Lengyel, 2000).

As anticipated, the array analysis identified genes already known to be expressed in cells within the SMW or required for the development R8 photoreceptor cells. The remaining 58 genes, whose transcription was uncharacterized or genes uncharacterized with respect to eye development are described later in the results. They include both, SMW and R8-associated transcripts, as well as genes expressed in other tissues.

Known transcripts upregulated in *GMR>sSpi* compared to *GMR>Ras^{V12}*—68 transcripts were upregulated in *GMR>sSpi* compared to *GMR>Ras^{V12}*. Since *GMR>sSpi* promotes G1 arrest and interferes with the differentiation of R8 photoreceptors, we expect this group to contain genes required for photoreceptor neurons other than R8, possibly required for G1 arrest, or negative regulators of the SMW. Such genes should be expressed in groups of cells corresponding to the precluster cells just posterior to the MF. Six genes known to be necessary for eye development were upregulated in *GMR>sSpi*; 5 of these are expressed in differentiating cells in the eye and required for neuronal development: *kekkon* (*kek*), *seven-up* (*svp*), *βTub60D* and *neuroglian* (*nrg*) and *pointed* (*pnt*) (Mlodzik et al., 1990; Bellen et al., 1992; Desai et al., 1994; O Neill et al., 1994; Musacchio and Perrimon, 1996; Hoyle et al., 2000). Transcripts for one negative cell cycle regulator, *scribble* (*scrib*) (Bilder and Perrimon, 2000), were up regulated in *GMR>sSpi*.

As expected, the microarray analysis identified transcripts known to be expressed in differentiating photoreceptor cells. The remaining 62 transcripts enriched in *GMR>sSpi* were uncharacterized with respect to eye development and are described later in the results. They include not only genes transcribed in photoreceptors, but also genes expressed in other tissues (Figure 1C).

Identification of genes' expression patterns during Drosophila eye development

Using RNA in situ hybridization we examined the wild type expression patterns of 126 genes whose transcripts were determined to be significantly different between *GMR>sSpi* and *GMR>Ras^{V12}* (Fig. 1C). We found 27 genes downregulated and 30 genes upregulated in *GMR>sSpi* that were expressed in the cells of the DP (Figs. 2A–2E, 2K–2T). A single gene,

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CG31676, downregulated in *GMR>sSpi*, was expressed in the retinal basal glia (Fig. 2F). Surprisingly, 12 of the genes downregulated and 17 genes upregulated in *GMR>sSpi* were expressed in the PE of the eye imaginal disc (Figs. 2G and 2H). In addition, transcripts of 10 downregulated and 6 upregulated genes in *GMR>sSpi* were expressed in the larval plasmatocytes present on the basal outer surface of the eye imaginal disc (Figs. 2I and 2J) (Wolff and Ready, 1993). Some of the genes are expressed in overlapping locations. For example, 3 genes, *Pde8*, *CG8502* and *Neu3* were expressed in the DP, PE and plasmatocytes. The Venn diagram in fig. 1C summarizes the expression pattern data and tables 1 and 2 list the novel and known expression patterns of the differentially transcribed genes that are downregulated and upregulated in *GMR>sSpi* compared to *GMR>Ras*^{V12} respectively.

We have established the expression pattern of 35 uncharacterized genes expressed in the DP, 29 genes expressed in the PE, 15 genes expressed in the plasmatocytes and 1 uncharacterized gene expressed in glia, suggesting that EGFR signaling may regulate gene expression in these different cell types. The RNA in situ hybridization patterns of all genes examined can be found in Supplementary Table 2. This includes expression patterns in some other imaginal discs and larval tissues. 45 of the differentially regulated transcripts were not detected during normal eye development; 9 of these genes were expressed in other imaginal discs or tissues however including 2 (*Lim1* and *al*) in the antennal disc. The genes for which no transcript was detected in the eye disc are either expressed at levels too low to detect by in situ hybridization, not detected by the probe generated or else ectopically induced by either *GMR>Ras*^{V12} and *GMR>sSpi*. Seven genes remain untested due to technical difficulties.

Transcripts of uncharacterized genes associated with the SMW and MF—Six uncharacterized genes are expressed in a patterned band of cells associated with the SMW. Two of these genes, *CG15630* and *CG8502* appear to be expressed in the dividing cells, and are down regulated in the absence of the SMW in *GMR>sSpi. CG15630* is expressed in groups of 2 or 3 cells and *CG8502* in single cells, both at the posterior edge of the MF for 2–3 columns (Figs. 2A and 2B). The other four genes were upregulated when the SMW was blocked by *GMR>sSpi*, and are expressed in a band cells associated with the SMW: *CG11339*, *CG31176*, *CG11382* and *Lip1* (Figs. 2K–2N). *CG11339*, *CG11382* and *Lip1* are expressed in the non-dividing cells whereas *CG31176* appears to be expressed in all cells near the SMW. These four genes maybe associated with maintaining the G1 arrest of differentiating cells or early photoreceptor differentiation. The gene *CAP*, which was downregulated in *GMR>sSpi*, was expressed in the MF prior to the onset of the SMW. *E(Spl)m2*, *brd*, *arc* and *ss* are also reported to be expressed within the MF (Singson et al., 1994; Duncan et al., 1998; Lai et al., 2000; Liu and Lengyel, 2000).

We also report the expression of 8 uncharacterized genes in the anterior part of the eye disc ahead of the MF. Four of these, *SP1029*, *Pde8*, *CG13966* and *CG14598* were expressed a band of cells anterior to the MF (Figs. 2C, 2D and 2O). The other four genes, *GM130*, *CG5929*, *Neu3* and *Tsp42E1* were expressed throughout the anterior part of the eye disc (Fig. 2E).

Transcripts of uncharacterized genes expressed in differentiating cells—8 genes downregulated and 17 genes upregulated in *GMR>sSpi* are expressed in the differentiating photoreceptors (Tables 1 and 2). The expression of 7 out of the 8 photoreceptor expressed genes downregulated in *GMR>sSpi* is known (Table 1). These genes may be regulated specifically in cells that arrest and differentiate in the first 4–5 columns, before the SMW is complete. Transcripts of the remaining gene, *Obp44a*, were detected in the axons of late differentiating photoreceptors and as they exit the eye disc via the optic stalk. The expression of 11 out of the 17 genes upregulated in *GMR>sSpi* is novel and 8 of these genes are uncharacterized: *CG14275*, *CG15522*, *CG30337*, *CG30188*, *CG9487*, *SP2353*, *CG9336* and *CG32030* (Figs. 2P–2S); 2 genes, *squeeze* (*sqz*) and *couch potato* (*cpo*), have previously

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implicated in the development of the nervous system but not shown to be expressed in the photoreceptors (Fig. 2T) (Bellen et al., 1992;McGovern et al., 2003) and 1 gene, *Neu3* is known to be expressed in plasmatocytes but it's expression in the photoreceptors has not been reported before (Asha et al., 2003) (Table 2). Transcripts of *CG9336* and *CG14275* were both detected in the axons of differentiating cells (Fig. 2S). A noteworthy expression pattern is that of *CG15522* which was upregulated in *GMR>sSpi* and is expressed in the groups of cells along the posterior edge of the presumptive eye field (Fig. 2P). These groups of cells may correspond to a distinct subset of peripheral ommatidia; an expression pattern of this kind has not been previously reported.

Consequences of increased Spitz and Ras activity for cells outside of the retina

Many of the differentially regulated transcripts were predominantly expressed outside of the retinal epithelium: in the PE, retinal basal glia or larval plasmatocytes. We have therefore investigated the role of Spi and Ras signaling with respect to these different eye imaginal disc cell types.

Genes expressed in the peripodial epithelium—Transcripts of 29 differentially regulated genes were expressed in PE cells; 12 genes downregulated and 17 genes upregulated in *GMR>sSpi* (Tables 1 and 2). A change in the relative sizes of the retina and the PE would lead to a consistent increase or decrease in the relative abundance of PE-expressed genes, so these results instead suggest more specific regulation of particular genes. Consistent with this idea, there was no expansion or reduction of the PE in response to Ras^{V12} expression in the retina (data not shown). The morphology of the PE in *GMR>*Ras^{V12} and *GMR>sSpi* eye discs was also investigated. To examine the size and shape of the PE cells eye discs were labeled with Armadillo (Arm), which localizes to the adheren junctions (Riggleman et al., 1990). Although the PE of both *GMR>*Ras^{V12} and *GMR>sSpi* contained more cells than wild type no discernable difference in cell size or shape between the genotypes was observed (Figs. 3A–3C). The difference in genes expressed in the PE between *GMR>*Ras^{V12} and *GMR>sSpi* is not due to gross differences in cellular morphology, cell size or PE size.

The majority of the PE-expressed genes are uncharacterized or little characterized previously (Tables 1 and 2). Notably, all the transcripts were also expressed in the PE of other imaginal discs examined, the leg and or wing discs (Supplementary Fig. 1). At third instar the eye/antennal PE is composed of squamous epithelial cells. The leg and wing PE are made of up both squamous cells and at the margins of the PE, cuboidal cells (margin cells) (Auerbach, 1936;Cohen, 1993). The difference in squamous and margin cells is reflected in the expression of some of the PE specific transcripts. For instance, transcripts of *CG8502* are only present in the squamous cells (Supplementary Fig. 1, A–B). In contrast, *CG3893*, *CG2657*, *Osi23* and *dScam* transcripts were more readily detected in the margin cells (Supplementary Fig. 1, C–G). The other genes were transcribed throughout the PE (Supplementary Fig. 1, H–I).

The PE-expressed genes can be divided into 2 categories: (I). Genes expressed in PE cells only. This includes most of the PE-expressed genes. Nine genes downregulated in *GMR>sSpi: Cyp4e2*, *Osi23*, *CG11073*, *CG3893*, *beat-Ic*, *wbl*, *CG4408*, *CG2657* and *CG15370*, and 10 genes upregulated in *GMR>sSpi: CG32354*, *CG18854*, *sda*, *Rac2*, *Sulf1*, *CG9699*, *Aplip1*, *tun*, *Dscam* and *CG13890*. (II). Genes expressed in the PE and elsewhere in the eye disc and/or plasmatocytes. Three genes downregulated in *GMR>sSpi: Pde8*, *CG8502* and *CG13203*, and 7 genes upregulated in *GMR>sSpi: CG13532*, *CG13041*, *CG9487*, *Neu3*, *CG9336*, *eiger*, and *Lip1* were expressed in this manner (Tables 1 and 2). For example, *CG9487*, *CG9336* are expressed in the PE and DP whereas *CG13203* and *CG13041* are expressed in the PE and plasmatocytes. As these genes are also expressed outside of the PE, the microarray may have detected the altered expression levels due to either changes in the PE or elsewhere.

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Because GMR Gal4 drives UAS transgene transcription in DP cells and not PE cells (data not shown), changes in Group I genes that are only expressed in the PE, indicate EGFR-dependent signaling from the DP to the PE. The simplest hypothesis is that direct targets of EGFR are activated in PE cells by Spi from the DP. However, the results would also be consistent with indirect effects on the PE of EGFR signaling in the DP, if the PE is affected by the SMW, early- differentiating photoreceptors, or cell survival differences that distinguish *GMR>Ras^{V12}* from *GMR>sSpi*.

CG31676* is expressed in the retinal basal glia—CG31676* is downregulated in *GMR>sSpi* and is expressed in the retinal basal glial (Fig. 2F). *CG31676* message was also detected along the Bolwig nerve (Supplementary Table 2). In the differentiating eye disc, glia are present along the basal surface of the eye epithelium associated with photoreceptor axons where they are required for guiding axons into the optic stalk (Fig. 3D) (Choi and Benzer, 1994;Rangarajan et al., 1999). In situ hybridization of the *CG31676* probe to the original microarray genotypes revealed that the levels of *CG31676* mRNA were comparable to wild type in *GMR>Ras^{V12}* but greatly reduced in *GMR>sSpi* (Figs. 5G and 5H). Due to non-autonomous Spi secretion it is likely that *CG31676* was down regulated in the glia in response to EGFR/Ras signaling. We hypothesize that activation of EGFR in the glia by Spi blocked the expression of *CG31676*.

To investigate the effects of ectopic Spi on glia, we examined the retinal basal glia in *GMR>Ras^{V12}* and *GMR>sSpi* with the glial cell marker, Repo. The glia in *GMR>Ras^{V12}* retinas were comparable to wild type (Fig. 3E). The glia in *GMR>sSpi* displayed defects in both migration and localization (Fig. 3F). Normally the anterior border of glial cell migration is posterior to the MF around column 6, the point at which axons begin to turn posteriorly, known as the axonal boundary. Basal glia in *GMR>sSpi* retinas migrated anterior to the MF past differentiating photoreceptors (Fig. 3F′). In addition, glia were also found apical to the photoreceptors and along the Bolwig nerve (Fig. 3F″). We propose that activation of EGFR in glia, by Spi from the photoreceptor axons, promotes the motility of and alters the localization of retinal basal glia, possibly through *CG31676*.

Genes transcribed in the larval plasmatocytes—Ten genes downregulated in *GMR>sSpi* are expressed in the plasmatocytes present on the basal surface of the wild type retinal eye field: *Pde8, twe, CG12508, CG8502, CG13203, CG15911, PGRP-SC2, Smg1, robl62A* and *CG18547* (Figs. 2I and 2J). Seven genes enriched in *GMR>sSpi* were also expressed in the plasmatocytes: *CG13521, CG32406, CG14598, CG13041, CG30022, CG33275* and *Neu3*. Other genes expressed in larval plasmatocytes such as, *serpent* (*srp*), *croqumort* (*crq*) or *peroxidasin* (*pxn*) were not differentially regulated in our microarray analysis (Nelson et al., 1994; Franc et al., 1996; Rehorn et al., 1996). Although some genes, such as *CG8502* and *Pde8*, are expressed in other eye disc cells, 7 genes are expressed only in the plasmatocytes, implying a difference in the plasmatocytes between *GMR>Ras^{V12}* and *GMR>sSpi*.

To identify the plasmatocytes on the developing retina we labeled *GMR>Ras^{V12}* and *GMR>sSpi* discs with Arm. More plasmatocytes were present in both *GMR>Ras^{V12}* and *GMR>sSpi* than in wild type; there was little difference in number between *GMR>Ras^{V12}* and *GMR>sSpi*, however (Figs. 4A–C). To investigate further we labeled wild type, *GMR>Ras^{V12}* and *GMR>sSpi* developing retinas with the mature plasmatocyte marker MDP-1 (Hortsch et al., 1998). MDP-1 is present in plasmatocytes on the surface of wild type eye imaginal discs (Fig. 4D′) and plasmatocytes in *GMR>Ras^{V12}* and *GMR>sSpi* both expressed MDP-1 suggesting that they mature in both genotypes (Figs. 4E′ and 4F′).

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During development plasmatocytes are required for the phagocytosis of apoptotic cells (Rizki, 1978). EGFR signaling serves as a survival signal, and activation of the EGFR/Ras/MAPK pathway promotes cell survival in the developing retina (Bergmann et al., 1998; Kurada and White, 1998). To examine cell death in *GMR>Ras^{V12}* and *GMR>sSpi* we labeled developing retinas with the CM1 antibody that recognizes activated Drice (Srinivasan et al., 1998; Yu et al., 2002). As previously reported, no cell death occurred in *GMR>Ras^{V12}* (Fig. 4H) (Yang and Baker, 2003). Some cells in *GMR>sSpi* eye discs labeled positively for CM1, however; the apoptosis was observed in the posterior regions of the retinal eye field (Fig. 4I). Apoptosis in *GMR>sSpi* is one potential explanation for changes in gene expression in plasmatocytes.

Retinal EGFR signaling regulates basal lamina composition—Transcripts of the proteoglycan *papilin* (*ppn*) were downregulated in *GMR>sSpi* (Table 1). Ppn is transcribed by imaginal disc cells and accumulates in the extracellular matrix where it forms part of the basal lamina (Campbell et al., 1987;Kramerova et al., 2000) (Supplementary Table 2). Ppn is recognized by the monoclonal antibody MDP-1 that we used to label plasmatocytes (Hortsch et al., 1998) (J. Fessler, Pers. Comm.). In wild type retinas, Ppn was detected in the basal lamina under epithelial cells posterior to the MF (Figs. 4D′ and 4D″). Ppn was not detected in the basal lamina of *GMR>sSpi* eye imaginal discs (Fig. 4F″). In *GMR>Ras^{V12}* eye discs, Ppn was only present in the in the basal lamina next to cells at the posterior edge of the MF (Fig. 4E″). If the *ppn* mRNA expression level is a reflection of Ppn in the basal lamina in the microarray analysis, a difference in *ppn* expression in the DP between *GMR>Ras^{V12}* and *GMR>sSpi* may have been detected. Since Ppn in the basal lamina is anterior to ectopic Ras^{V12} activity in the DP, the increased activation of the EGFR pathway in the retinal epithelium possibly negatively regulates *ppn* transcription in retinal epithelial cells, thereby affecting the composition of the basal lamina.

Microarray predicted changes translate to alterations of transcript levels *in vivo*

To verify our array results, the RNA in situ hybridization pattern of six genes were re-examined in the original microarray genotypes, *GMR>Ras^{V12}* and *GMR>sSpi: CG8502, CG15522, SP1029, CG31676, Smg1* and *Dscam. CG8502*, *CG15522* and *SP1029* are all expressed in the DP. CG8502 was downregulated in *GMR>sSpi* with a signal log ratio (SLR) of −1.7. Transcripts of *CG8502* are expressed in 3 columns of cells at the posterior edge of the MF in both wild type and *GMR>Ras^{V12}* eye discs; in agreement with the array data, this expression was not detected in *GMR>sSpi* (Figs. 5A and 5B). The microarray analysis determined a SLR of +4 for *CG15522*. By in situ hybridization, we also observed an up regulation in the expression level and an expansion of cells expressing *CG15522* in *GMR>sSpi* compared to *GMR>Ras^{V12}* (Figs. 5C and 5D). The normal expression of *SP1029* in a band of cells anterior to the MF was absent in *GMR>sSpi* (Figs. 5E and 5F) also consistent with the predicted downregulation of the message in *GMR>sSpi* (SLR = −3.45). In wild type eye discs, *CG31676* is expressed in the retinal basal glia. We could not detect any message by in situ for *CG31676* in *GMR>sSpi* eye discs. The in situ for *CG31676* in *GMR>Ras^{V12}* was comparable to wild type. This is consistent with a SLR of −3.2 for *CG31676* in the array analysis (Figs. 5G and 5H). The microarray analysis predicted changes of *Smg1*, a plasmatocyte expressed gene, and *Dscam* a PE expressed gene (SLR of −1.44 and +1.36 respectively) were both consistent with changes observed between *GMR>sSpi* and *GMR>Ras^{V12}* by in situ hybridization (Figs. 5I–5L). Because the gene expression changes observed by microarray analysis, were confirmed in each of these cases, it is likely that the microarray results provide a generally accurate picture of transcriptional differences between *GMR>Ras^{V12}* and *GMR>sSpi* in the DP, PE, glia and plasmatocytes.

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Discussion

We have used microarray to identify genes transcribed in the SMW during *Drosophila* eye development. By manipulating the EGFR pathway, we blocked the SMW, and compared the RNA profile of these developing retinas to retinas with a SMW. Both genotypes activated EGFR signaling posterior to the SMW, controlling for many of the other roles of EGFR. We performed RNA in situ on most of the 140 differentially transcribed genes. Together this analysis has identified of uncharacterized genes that are expressed in SMW and differentiating cells.

Although our strategy avoided identifying many thousands of EGFR-dependant genes, many of the genes found were not expressed during the SMW. Instead, these genes are expressed in cells of the PE, larval plasmatocytes and glia. Having investigated the significance of ectopic EGFR signaling on these different cell types, we suggest that : (1) There are targets of EGFR signaling in the PE; (2) Ectopic EGFR signaling leads to the differential regulation of several genes expressed in plasmatocytes; (3) Ectopic EGFR signaling in the glia effects the migration and localization of glia in the developing retina and (4) EGFR signaling also regulates the composition of the the basal lamina. These genes are probably identified because they are targets of Spi secreted from the DP, although some could be regulated indirectly, in response to the SMW or other differences between *GMR>sSpi* and *GMR>Ras*^{V12} discs. Thus an unexpected bonus of the approach was to uncover genes that are candidates to mediate responses in other tissues to Spi secretion from the DP. In future it will be interesting to explore these predictions by direct investigation of the non-autonomous role of retinal Spi.

Identification of genes transcribed in cells associated with the SMW

Our primary goal was to identify genes transcribed in the SMW. By comparing eye discs with and without a SMW we have identified 10 genes that are expressed in cells associated with the SMW; 6 of these genes have novel expression patterns in the SMW. The SMW is a specific patterned cell cycle regulated by the developmental signaling pathways EGFR and N. The transcriptional targets of these pathways in the SMW remain unknown. Cell cycle regulators such as Stg, Cyclin B, or Dacapo (Dap) were not differentially regulated between the sSpi and Ras^{V12} expressing eye discs; this was probably due to the many cycling cells in the anterior part of the eye disc that are unaffected by *GMR>sSpi* and *GMR>Ras*^{V12}.

We expected genes expressed in the SMW cells to be transcribed in a patterned band of cells at the posterior edge of the MF for 2–3 columns of retinal development. The 6 uncharacterized genes are expressed in this manner: *CG8502* and *CG15630* were down regulated in *GMR>sSpi* and *CG11339*, *CG11382*, *CG31167* and *Lip* were upregulated in *GMR>sSpi*. The 4 characterized genes that were all downregulated in *GMR>sSpi* are *lz*, *B-H1*, *Traf1* and *Spn43A* their potential cell cycle roles have not been previously examined. Since early cell fate decisions occur simultaneously with the SMW, further work will be required to distinguish whether expression of these genes is associated with cell cycle progression or arrest, or the differentiation of R8 or other early-specified cells.

Ten genes were expressed ahead of the MF in the region of the eye disc where the cells are unspecified and asynchronously cycling: *Spn43A*, *SP1029*, *Pde8*, *Klg*, *CG13966*, *GM130*, *trn* and *CG5929* were downregulated in *GMR>sSpi*, and *CG14598* and *Tsp42E1* were upregulated in GMR>sSpi. One of these genes, *SP1029*, a metallopeptidase located 3′ of stg that exhibits a similar mRNA expression pattern to *stg* (Alphey et al., 1992). We speculate that *SP1029* may come under the regulation of the SMW enhancer of *stg*; this enhancer not yet been mapped and could be 3′ to *stg* (Lehman et al., 1999). Five of the genes downregulated in response to sSpi are expressed within the MF: *E(Spl)m2*, *CAP*, *Traf1*, *Brd* and *ss*. The EGFR pathway might have transcriptional targets anterior to and within the MF, although this has not

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been reported previously. Alternatively these targets may be indirect and depend on secreted signals produced during the SMW.

EGFR targets in differentiating cells

Even though *GMR>sSpi* and *GMR>Ras^{V12}* lead to ectopic photoreceptor specification to similar degrees, 25 genes expressed in the differentiating cells were uncovered. *SoxN*, *lz*, *Fas2*, *B-H1*, *thisbe* (*ths)*, *klg*, *trn* and *Obp44a* were downregulated in *GMR>sSpi*, and *CG14275*, *CG15522*, *CG30337*, *kek1*, *CG30188*, *CG9487*, *CG30022*, *Neu3*, *svp*, *CG9336*, *sqz*, *SP2353*, *CG32030*, *βTub60D*, *Nrg*, *cpo* and *pnt* were upregulated in *GMR>sSpi*. These genes are potential targets of the EGFR during photoreceptor differentiation. More of the genes expressed in differentiating cells are upregulated in *GMR>sSpi* than downregulated. Although photoreceptor differentiation occurs only slightly earlier in *GMR>sSpi*, this maybe sufficient to increase the proportion of transcripts from such cells; alternatively there may also be changes in the type of photoreceptor specified. For example, Spi interferes with R8 photoreceptor development (Lesokhin et al., 1999; Frankfort and Mardon, 2004).

The mRNAs of three genes affected by EGFR signaling, *Obp44a*, *CG9336* and *CG14275*, were detected in the axons of photoreceptors neurons. The transport of mRNAs to the axon of young neurons has an important role regulating nerve cell maturation (Mohr and Richter, 2000). It will be interesting to determine whether EGFR regulates the transport and/or localization of mRNAs.

EGFR activity in the DP also regulates the composition of extracellular structures preventing the addition of glycoprotein, Ppn, to the adjacent basal lamina. Eight genes expressed in the differentiating cells have putative or known roles either cell adhesion or cytoskeletal organization. EGFR signaling alters the adhesive properties of cells in the eye disc so that normal cell shape re-arrangements occur (Brown et al., 2006). These genes maybe downstream of EGFR in maintaining proper cell adhesion during the G1 arrest of differentiating cells.

Possible targets of the EGFR/Ras pathway in the peripodial epithelium

In this study we have identified 29 genes that were not previously known to be expressed in the PE. 17 are uncharacterized genes that are expressed exclusively in some or all cells of the PE and not elsewhere in the imaginal disc. Until now *Ultrabithorax* (*Ubx)* was the only gene expressed in all cells of the wing PE but not the DP, although *Ubx* is expressed in the DP other imaginal discs (Brower, 1987). Coronin-Gal4 is also detected in the wing PE only but in a subset of cells (Pallavi and Shashidhara, 2003). Thus, we uncovered an unexpected pool of PE-specific genes, suggesting that gene expression in the PE is regulated by Spi secreted from the DP, either directly or in response to the SMW or R8 differentiation.

It is well established that signals from the PE affect the development of the DP (Cho et al., 2000; Gibson and Schubiger, 2000; Pallavi and Shashidhara, 2003; Pallavi and Shashidhara, 2005). Our data suggests that the reverse may also be true; verticals signal from the DP to the PE have an important role in regulating the development and interactions between two disc epithelia. As PE cells do not change in size and morphology between the two genotypes it will be interesting to discover the nature of the response.

Spitz affects glial cell migration and localization

CG31676 was the only gene found to be altered in the retinal basal glia. We speculate that activation of EGFR in the glia, by Spi secreted from the axons, negatively regulates the expression of *CG31676*. Since only one glia gene was affected, this is unlikely to reflect a change in the relative number of glial cells after Spi expression. We found that migration and localization of glia was affected in *GMR>sSpi*. Glia normally migrate up to the axonal

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boundary, the point at which the axons of differentiating photoreceptors turn posteriorly to the optic stalk (Choi and Benzer, 1994). Glia in *GMR>sSpi* migrated beyond the axonal boundary and photoreceptor differentiation. In addition, glia were present apical to photoreceptor and along the Bolwig nerve. The actual molecular mechanism of glia migration is unknown (Rangarajan et al., 1999). We speculate that *CG31676* maybe directly involved in glia migration and/or localization in response to sSpi from photoreceptor cells, as it was the only gene whose transcription was affected.

Spitz affects larval plasmatocytes

Ectopic Spi in the retinal epithelium affected gene expression in plasmatocytes that are associated with the eye imaginal disc. 7 of 17 differentially transcribed genes were not expressed elsewhere in the eye imaginal discs. Most of the plasmatocyte expressed genes uncharacterized. However, *Neu3* and *PGRP-SC2*, have previously been demonstrated by microarray to be expressed in plasmatocytes (De Gregorio et al., 2001; Asha et al., 2003). We propose that Spi from the DP must affect the plasmatocytes directly or indirectly. Interestingly, we detected no difference in number or differentiation (as assessed by the MDP-1 antigen).

EGFR activity coordinates development of the eye disc with other associated tissues

In summary, combining microarray and RNA in situ hybridization has led to the identification of genes that are transcribed in cells associated with the SMW, but we also uncovered as many targets of EGFR in the other cell types associated with the eye imaginal disc. Because our microarray was designed to identify a narrow subset of targets, and appears to have excluded the majority of genes directly or indirectly regulated by EGFR in the eye disc as a whole, we think that most of these genes are different because they are targets of Spi in cells where GMRGal4 does not express. Thus, we inadvertently selected for genes in other tissues that respond to Spi made in the DP. These findings suggest that, in addition to regulating multiple aspects of retinal differentiation, and regulating brain differentiation in response to retinal innervation (Huang et al., 1998), changes in EGFR activity during eye disc differentiation could also serve to coordinate the developmental programs of the glia, PE, and plasmatocytes with the eye disc proper together comprising an organ system of cells from multiple origins (Fig. 6).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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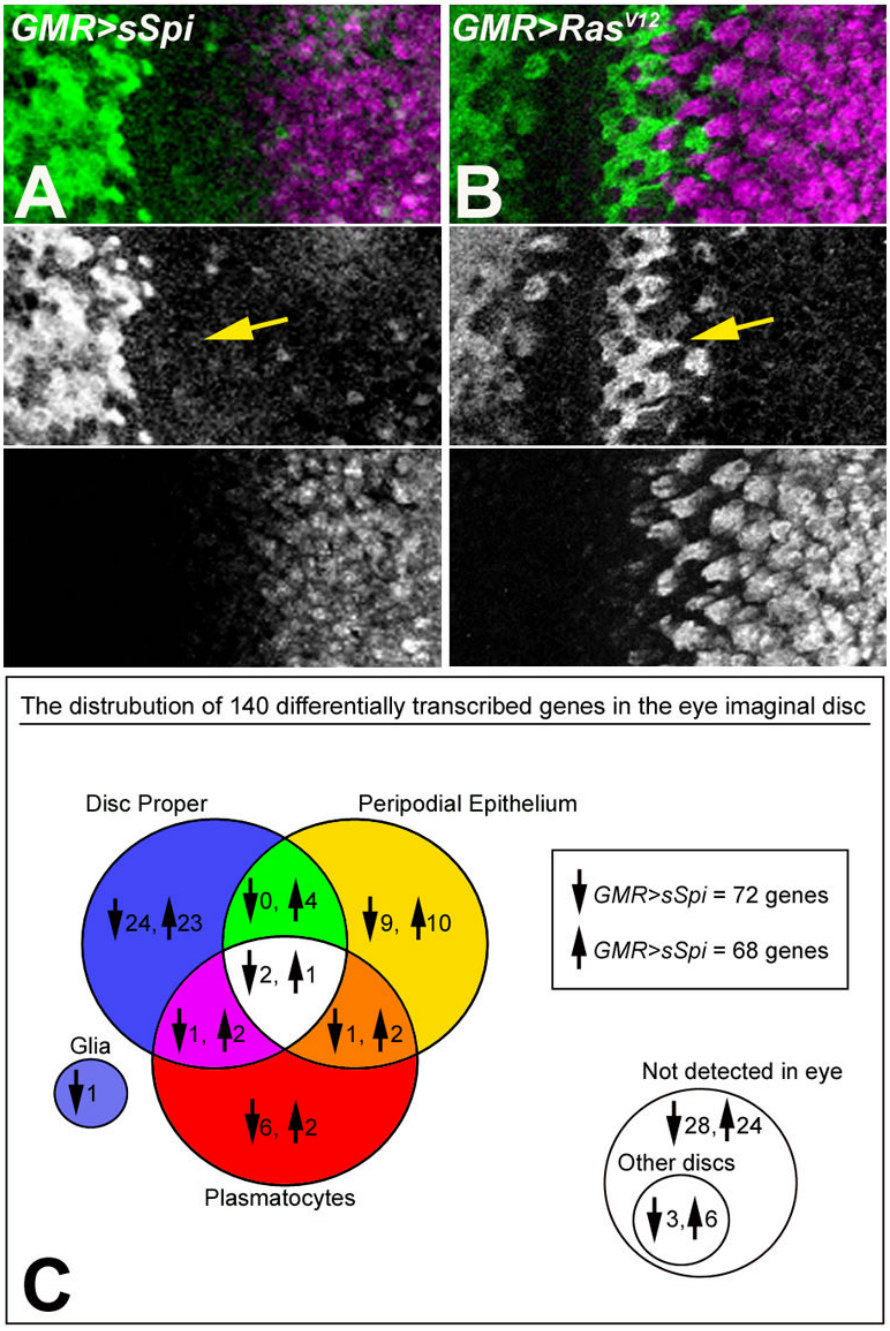

Figure 1.

Eye imaginal discs from *GMR>sSpi* (A) and *GMR>Ras^{V12}* (B) labeled with Cyclin B (green) to monitor the cell cycle and Elav (magenta) to label differentiating cells. The SMW occurs in *GMR>Ras^{V12}* expressing eye discs (arrow in B) but is absent in *GMR>sSpi* (arrow in A). (C) Venn diagram showing the distribution of genes that were up and downregulated in *GMR>sSpi* compared to *GMR>Ras^{V12}* and their location of expression in the DP, PE, plasmatocytes and glia of the eye imaginal disc. 54 genes were not expressed in the eye imaginal disc; 9 of these were expressed in other imaginal discs, including 2 expressed in the antennal disc. 7 genes remain untested due to technical difficulties.

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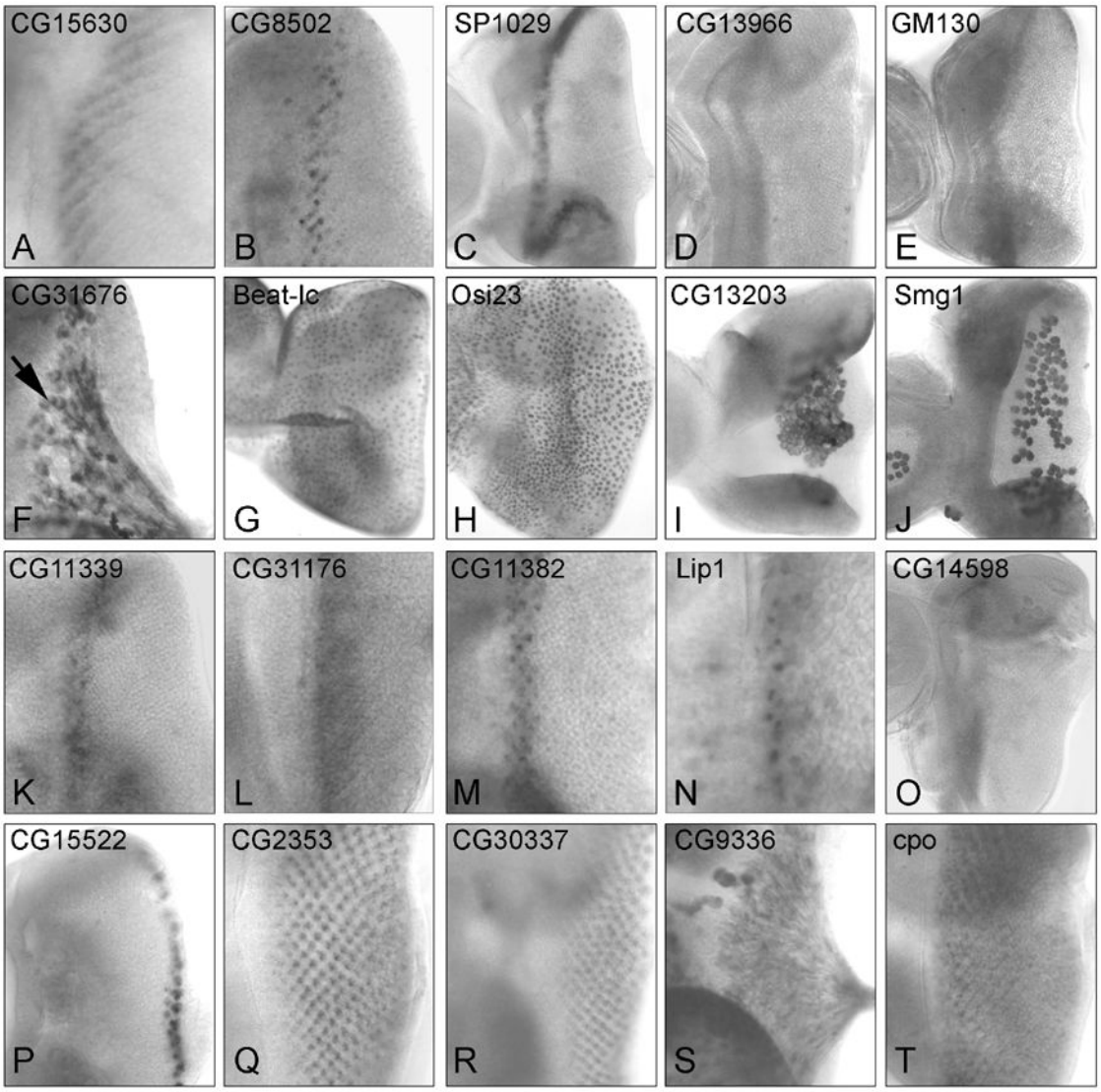

Figure 2.

RNA in situ hybridization of differentially expressed genes in 3rd instar eye imaginal discs: (A–J) Transcripts enriched in *GMR>Ras*^{V12}. (K–T) Transcripts enriched in *GMR>sSpi*. (A–E) Transcripts expressed in a band associated with the MF or SMW: (A) CG15630 and (B) CG8502 are expressed near the SMW; (C) SP1029,(D) CG13966 and (E) GM130 are expressed anterior to the MF; (F) CG31676 is expressed in glia; (G) Beat-Ic and (H) Osi23 are expressed in the peripodial epithelium; (I) CG13203 and (J) Smg are expressed in larval plasmatocytes on the basal surface of the eye imaginal disc. Transcripts for (K) CG11339, (L) CG31176, (M) CG11382 and (N) Lip are expressed in coincident with early fate specifications; (O) CG14598 is expressed ahead of the MF; (P) CG15522 transcripts are present in peripheral ommatidia; (Q) CG30337, (R) SP2353, (S) CG9336 and (T) Cpo are expressed in differentiating cells. The mRNA for CG9336 is present in the axons of photoreceptors.

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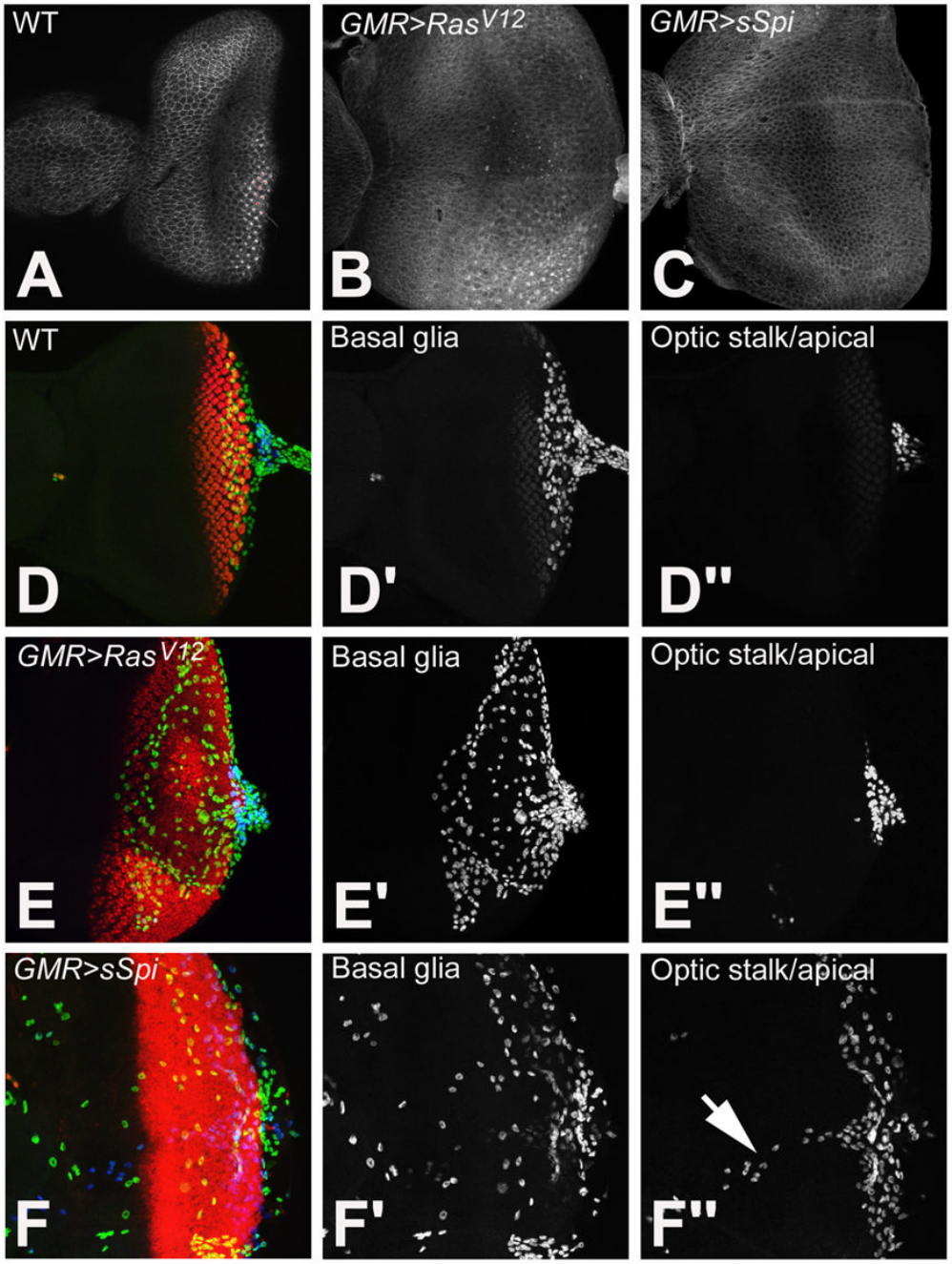

Figure 3.

Examination of the PE in wild type (A), *GMR>Ras*^{V12}, (B) and *GMR>sSpi* (C) eye imaginal discs with Arm. Retinal basal glia in wild type (D-D″), *GMR>Ras*^{V12} (E-E″) and *GMR>sSpi* (F-F″) eye imaginal discs. Differentiating photoreceptors are labeled with ElaV (red). Glia are identified with Repo. Glia in the basal part of the eye disc are in the green channel (D′, E′ and F′). Glia located apical and in the optic stalk are in the blue channel (D″, E″ and F″). Normally glia are located basal to photoreceptors and within the optic stalk (D′ and D″). This is unaffected in *GMR>Ras*^{V12} (E′ and E″). In *GMR>sSpi*, basal glia migrate more anteriorly (F′), some glia are also located apically and along the Bolwig nerve (arrow) (F″).

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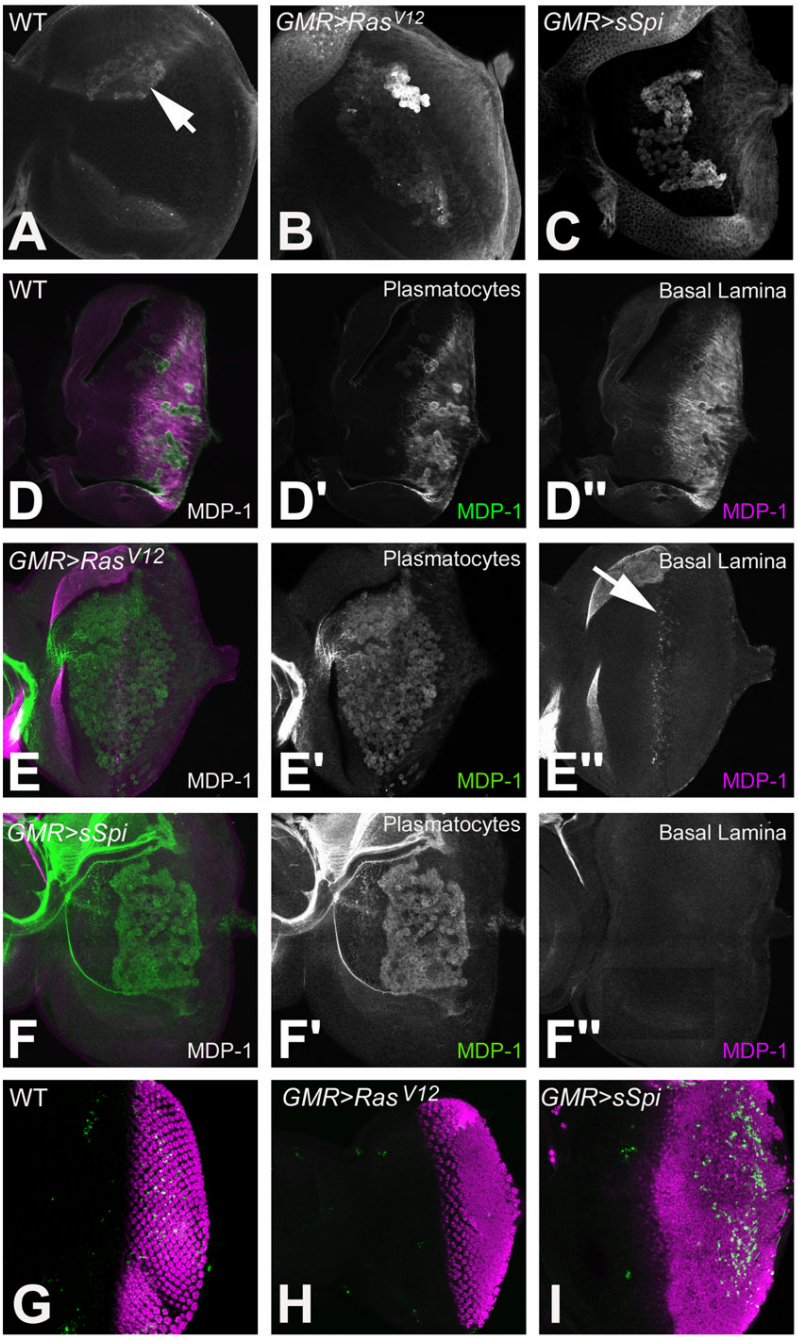

Figure 4.

Identification of plasmatocytes in 3rd instar eye imaginal discs. Plasmatocytes identified on the basal surface of the eye imaginal disc with anti-Arm in wild type (A), *GMR>Ras*^{V12} (B) and *GMR>sSpi* (C). MDP-1 labeling of wild type (D), *GMR>Ras*^{V12} (E) and *GMR>sSpi* (F) eye imaginal discs. MDP-1 in plasmatocytes is green (D′, E′ and F′). MDP-1 in the basal lamina is magenta (D″, E″ and F″). CM1 (green) and Elav (magenta) labels the dying and differentiating photoreceptors in wild type (G), *GMR>Ras*^{V12} (H) and *GMR>sSpi* (I) eye imaginal discs .

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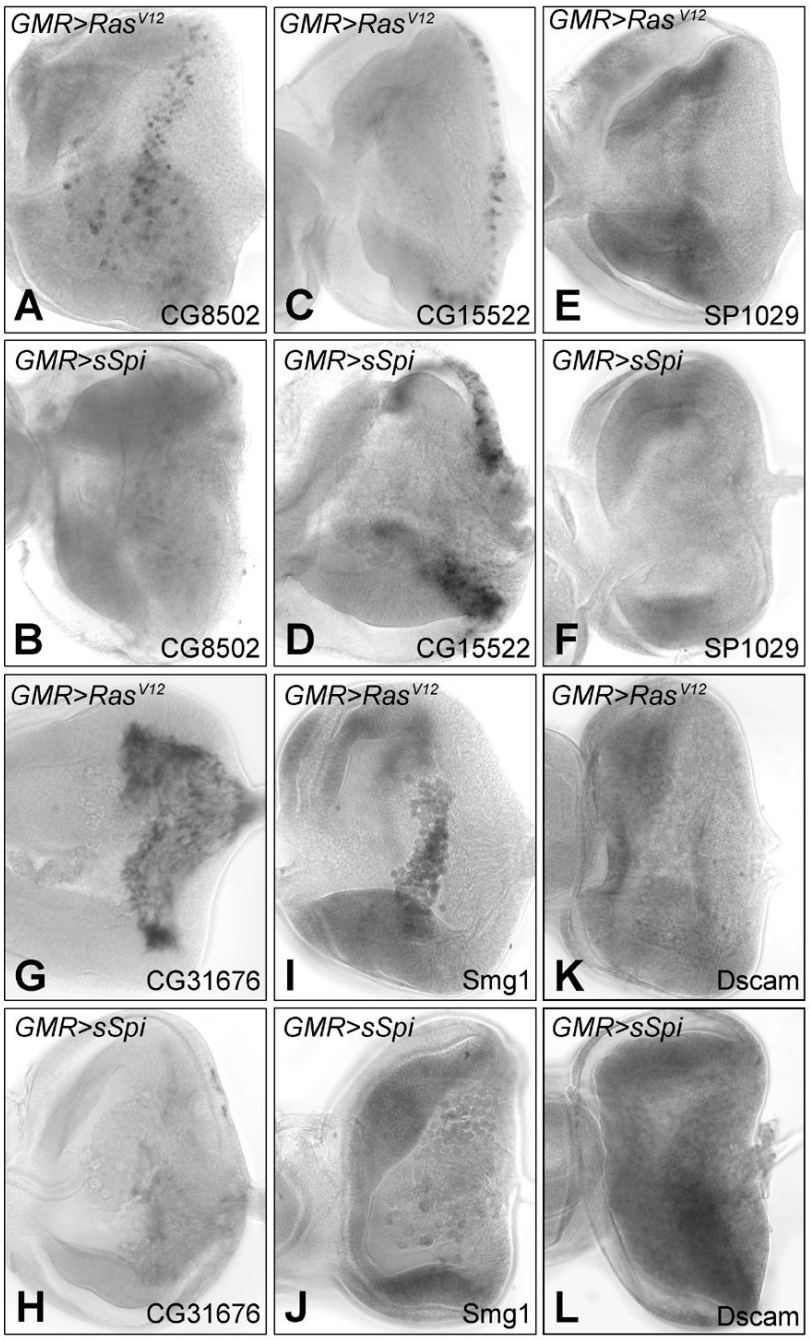

Figure 5.

mRNA in situ hybridization of differentially transcribed genes in *GMR>Ras*^{V12} and *GMR>sSpi* eye imaginal discs: *CG8502* in *GMR>Ras*^{V12} (A) and *GMR>sSpi* (B); *CG15522* in *GMR>Ras*^{V12} (C) and *GMR>sSpi* (D); SP1029 in *GMR>Ras*^{V12} (E) and *GMR>sSpi* (F). *CG8502*, *CG15522* and *SP1029* are expressed in the DP. *CG31676* in *GMR>Ras*^{V12} (E) and *GMR>sSpi* (F) in the glia. *Smg1* in *GMR>Ras*^{V12} (I) and *GMR>sSpi* (J) in the plasmatocytes. The mRNA levels of *Dscam* in the PE were lower in *GMR>Ras*^{V12} (K) than *GMR>sSpi* (L).

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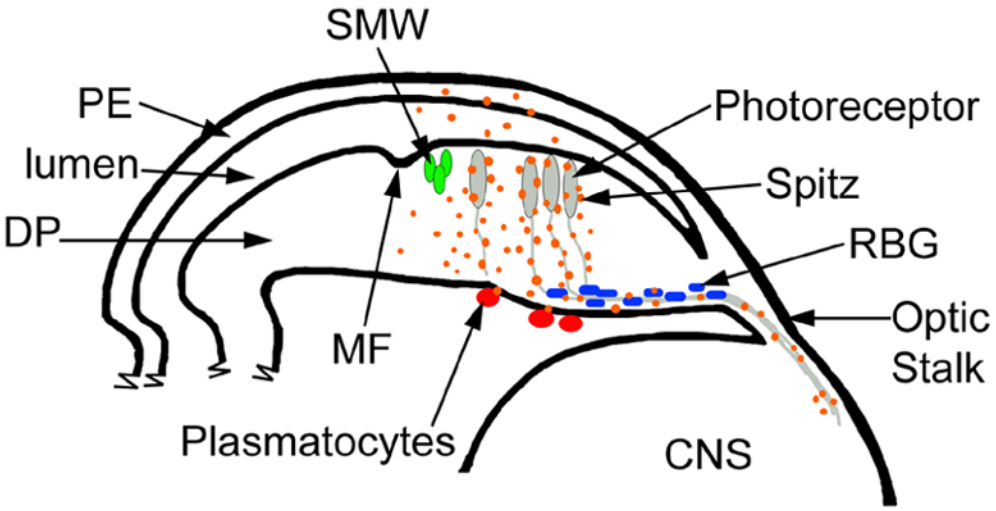

Figure 6.

EGFR signaling in the DP regulates the expression of genes in cells within and outside of the retinal epithelium. A cartoon depicting a cross section through the eye imaginal disc, optic stalk and the CNS; posterior is to the right. The PE overlays the apical surface of the DP. At the posterior edge of the MF the SMW occurs (green cells). Posterior to the MF, photoreceptors are differentiating (grey) and their axons extend to the lamina in the CNS via the optic stalk. Retinal basal glia (RBG; blue) are associated with the axons. Plasmatocytes (red) are found on the basal surface outside of the eye imaginal disc. Vertical EGFR signals from the DP to the PE alter gene expression in the PE; this maybe due to an increase of EGFR signaling in the DP or due to the non-autonomous signal Spi (orange). EGFR signaling from the DP regulates gene expression in plasmatocytes. Within the retinal epithelium, Spi signals from axons alter gene expression in the RBG and affect their localization and migration. EGFR also regulates the composition of the basal lamina (not shown).

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Table 1

RNA in situ hybridization patterns of genes downregulated in *GMR>sSpi vs. GMR>Ras*^{V12}

Gene	SLR	Expression pattern	Function/homology	Reference
Disc Proper (27)				
SoxN	−5.65	Differentiating photoreceptors posterior to MF	Nervous system development	(Cremazy et al., 2001)
lz	−4.06	Subset of cells posterior to the morphogenetic MF	Eye cell fate specification and differentiation	(Flores et al., 1998)
Spn43A	−3.67	Cells anterior to the MF and in posterior inter-ommatidial cells	Serine-type endopeptidase inhibitor activity	(Green et al., 2000)
CG15630	−3.49	Subgroups of cells in 3/4 columns posterior to MF (Figure 2A)	Cell adhesion	
SP1029	−3.45	Band anterior to the MF; inner tarsal ring of leg imaginal disc (Figure 2B)	Proteolysis	
CG31676	−3.22	Retinal basal glial cells (Figure 2F)	-	
Fas2	−2.91	Differentiating photoreceptors	Cell adhesion; axonal fasciculation; synaptic plasticity	(Pignoni et al., 1997)
B-H1	−2.87	Subset of photoreceptors and accessory cells	Photoreceptors R1 and R6, pigment and lens cell differentiation	(Higashijima et al., 1992)
thisbe	−2.81	Individual differentiating cells posterior to MF	FGFR ligand. FGFR signaling in mesoderm, hindgut and heart	
CG31291	−2.71	Ubiquitous	-	
Pde8	−2.37	Band just anterior to MF; PE; plasmatocytes; lumen and nuclei of salivary glands. Nuclei of fat body	Signal transduction; cyclic nucleotide metabolism; mesoderm dev.	
Klg	−2.14	Three rows of cells ahead of MF; ommatidial cluster; refines to R7	R7 photoreceptor cell fate commitment and differentiation	(Butler et al., 1997)
CG13966	−2.03	Band anterior to MF (Figure 2C)	-	
ato	−1.95	Stripe anterior to MF, refines to R8 cells only	R8 cell specification.	(Jarman et al., 1994)
GM130	−1.92	Anterior to MF (Figure 2D)	Golgi organization and biogenesis; mitosis; protein targeting	
trn	−1.91	Band of cells just anterior to MF refining to clusters of cells and a subset of differentiating cells.	Target of Egfr/PntP1 signaling in embryonic ventral ectoderm	(Chang et al., 1993)
Obp44a	−1.84	Photoreceptor axons as exiting optic stalk and optic lobe	-	
CG8502	−1.71	Individual cells near posterior edge of MF for 2/3 columns; eye, leg and wing PE; plasmatocytes (Figure 2E)	Structural constituent of larval cuticle	
CG5653	−1.71	Ubiquitous	-	
CG15911	−1.69	Anterior D/V margins of eye disc;plasmatocytes	-	
E(spl) m2	−1.68	Clusters of cells in the MF region	Notch signaling pathway	(Lai et al., 2000)
CAP	−1.64	Expressed within MF	MAPKKK cascade; cytoskeleton organization and biogenesis	
CG5929	−1.61	Broad band anterior to MF	Nucleic acid metabolism; RNA-dependent transcription	
Ppn	−1.58	Diffusely expressed in eye imaginal disc	Component of the ECM; metallopeptidase activity	(Kramerova et al., 2000)
Traf1	−1.56	SMW cells and clusters within MF; ZNP cells in the wing	In vivo regulator of JNK pathway	(Priess and Hirsch, 1986)
Brd	−1.5	Expressed within MF	Sensory organ precursor cell fate determination; Notch pathway	(Singson et al., 1994)
Bib	−1.5	Required during R8 specification	Lateral inhibition of R8 specification; embryonic neurogenesis	(Rao et al., 1990)
arc	−1.4	Clusters along MF	Adheren junction associated protein required for eye development	(Liu and Lengyel, 2000)
ss	−1.34	MF; expressed in all other imaginal discs	Antennal morphogenesis; transcription factor	(Duncan et al., 1998)
Peripodial Epithelium (12)				
Cyp4e2	−3.19	PE of the eye/antennal and wing imaginal discs	Electron transport; steroid metabolism	
Osiris 23	−3.16	PE of the eye and leg imaginal discs (Figure 2H)	-	
CG11073	−2.59	PE of the eye, leg and wing imaginal discs	-	
CG3893	−2.56	PE of the eye imaginal disc	-	

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Gene	SLR	Expression pattern	Function/homology	Reference
Pde8	−2.37	Band just anterior to MF; PE; plasmatocytes; lumen and nuclei of salivary glands; nuclei of fat body	Signal transduction; cyclic nucleotide metabolism; mesoderm development.	
beat-Ic	−2.33	Peripodial cell's nucleoli (Figure 2G)	Axon choice point recognition; cell adhesion; defasciculation	
wbl	−1.71	PE of the eye imaginal disc	Exocytosis; protein-Golgi targeting	
CG8502	−1.71	Individual cells posterior to MF for 2/3 columns; eye, leg and wing PE; plasmatocytes	Structural constituent of larval cuticle	
CG13203	−1.7	PE of the eye imaginal disc; plasmatocytes	-	
CG4408	−1.7	PE of the eye imaginal disc	Proteolysis: metallocarboxypeptidase activity	
CG2657	−1.5	PE of the eye and wing discs; cells in wing disc	Transport: Glutamate-gated ion channel activity	
CG15370	−1.34	PE of the eye imaginal disc	-	
Glia (1)				
CG31676	−3.22	Retinal basal glial cells (Figure 2F)	-	
Plasmatocytes (10)				
Pde8	−2.37	Band just anterior to MF; peripodial cells; plasmatocytes; lumen and nuclei of salivary glands; nuclei of fat body	Signal transduction; cyclic nucleotide metabolism; mesoderm dev.	
twe	−1.85	Plasmatocytes	Protein tyrosine phosphatase activity; mitosis	
CG12508	−1.73	Plasmatocytes	-	
CG8502	−1.71	Individual cells posterior to MF for 2/3 columns; eye, leg and wing peripodial cells; plasmatocytes	Structural constituent of larval cuticle	
CG13203	−1.7	PE of the eye imaginal disc; plasmatocytes (Figure 2I)	-	
CG15911	−1.69	Anterior D/V margins of eye disc;plasmatocytes	-	
PGRP-SC2	−1.55	Wing pleurites and plasmatocytes	Peptidoglycan recognition molecule	
Smg1	−1.44	Plasmatocytes (Figure 2J)	Nonsense-mediated decay; protein kinase activity	
robl62A	−1.44	Plasmatocytes	Microtubule-based movement; Dynein complex; ATPase activity	
CG18547	−1.39	Plasmatocytes	Oxidoreductase activity	
Other imaginal discs or tissues (5)				
Ugt86Di	−3.58	Expressed in the wing disc	-	(Butler et al., 2003)
Obp44a	−1.84	Photoreceptor axons as exiting optic stalk and optic lobe	-	
CG8483	−1.63	Expressed in the wing disc	-	(Butler et al., 2003)
PGRP-SC2	−1.55	Wing plurites and plasmatocytes	-	
ss	−1.34	MF; Expressed in all other imaginal discs	Antennal morphogenesis; transcription factor	(Duncan et al., 1998)

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Table 2

RNA in situ hybridization patterns of genes upregulated in *GMR>sSpi vs. GMR>Ras^{V12}*

Gene	SLR	Expression pattern	Function/homology	Reference
Disc Proper (30)				
CG14275	4.77	Axon tracts of differentiating neurons.	-	
CG15522	4	Peripheral ommatidia (Figure 2K)	-	
CG11339	2.87	Narrow band of cells posterior to MF; inner ring of antennal disc (Figure 2P)	Cytoskeleton organization and biogenesis	
CG31176	2.36	Band of cells at posterior edge of MF (Figure 2Q)	-	
T48	2.35	Ubiquitous	-	
CG4096	2.2	Expressed at the dorsal anterior margin in the eye disc and in wing and leg discs also	-	
CG14598	2.2	Anterior to MF; plasmatocytes (Figure 2T)	-	
CG30337	2.17	Later differentiating cells (Figure 2N)	Cell cycle; DNA metabolism; intracellular protein transport	
kek1	2.06	All cells posterior to MF; 2nd and 3rd antennal segments; presumptive femur in leg imaginal disc.	Negative regulation of EGF receptor activity	
CG30188	2.04	Differentiating cells posterior to MF	Cell adhesion; signal transduction	
CG9487	1.97	Differentiating cells; PE	-	
CG30022	1.95	Dorsal and ventral anterior eye margin; plasmatocytes	-	
Neu3	1.94	Differentiating cells; plasmatocytes; PE	Cell adhesion; proteolysis and peptidolysis; signal transduction	
svp	1.93	R1, R3, R4 and R6 photoreceptors	R3/R4, R1/R6 cell fate commitment; requires Ras signaling	(Mlodzik et al., 1990)
CG11382	1.92	Eye: 3/4 columns of ommatidia posterior to MF. Wing: pleurites, myoblasts, cells in wing pouch but restricted from presumptive margin (Figure 2R)	-	
CG9336	1.87	Axons projections; PE (Figure 2O)		
Tsp42El	1.85	Anterior to MF	Transmission of nerve impulse; neurogenesis; ectoderm dev.	
CG31163	1.84	Ubiquitous expression in eye; Wing pouch, restricted from presumptive margin	-	
ena	1.71	n.d. Ena receptor, Abl, is transcribed in all imaginal epithelial cells with increased levels over the differentiating photoreceptors.	Axonogenesis; regulation of actin polymerization; cytoskeleton organization	(Bennett and Hoffmann, 1992; Gertler et al., 1995)
scab	1.71	Random cells in eye and wing DP	-	
sqz	1.69	Late differentiating cells; nuclei of fat body; gut cells; salivary gland	Cell proliferation; regulation of transcription; oogenesis	
SP2353	1.67	Differentiating cells of the eye; maybe predominantly single cell type of ommatidium; optic lobe (Figure 2L)	Signal transduction; cell-matrix and cell-cell adhesion	
CG32030	1.63	Differentiating cells including clusters at posterior edge of MF	Cell organization and biogenesis	
βTub60D	1.57	Differentiating cells posterior to MF	Axonogenesis and guidance; GTPase activity; microtubule-based process	(Hoyle et al., 2000)
Nrg	1.51	Late differentiating cells	Neuron adhesion; Epidermal growth factor receptor signaling	(Hortsch et al., 1990)
scrib	1.5	MF	Negative regulation of cell proliferation; zonula adherens assembly	(Bilder and Perrimon, 2000)
eiger	1.5	Random cells in eye and leg PE and DP	Induction of apoptosis; JNK cascade; TNF receptor binding	(Igaki et al., 2002)
Lip1	1.41	In eye DP column of single cells at the posterior edge of MF express Lip1. Random cells of eye and wing DP, PE (Figure 2S)	Lipid metabolism	
cpo	1.34	Apical punctate expression on differentiating neurons and outer antennal ring; leg and wing discs; CNS (Figure 2M)	Peripheral nervous system development	(Bellen et al., 1992)
pnt	1.33	n.d.	Transcription factor target of Egf receptor/ MAPK signaling	(Brunner et al., 1994)
Peripodial epithelium (17)				
CG13532	2.37	PE eye; plasmatocytes; expressed within wing pouch	-	
CG13041	2.19	PE; plasmatocytes	-	
CG32354	1.99	PE eye/antennal imaginal disc.	Endopeptidase inhibitor activity	
CG9487	1.97	Differentiating cells; PE	-	

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Gene	SLR	Expression pattern	Function/homology	Reference
Neu3	1.94	Differentiating cells; plasmatocytes; PE	Cell adhesion; proteolysis and peptidolysis; signal transduction	
CG9336	1.87	Axons projections; PE	-	
CG18854	1.83	PE eye/antennal imaginal disc	Inositol-trisphosphate 3-kinase activity	
sda	1.68	PE eye/antennal imaginal disc	Proteolysis and peptidolysis; mechanosensory behavior	
Rac2	1.67	PE eye/antennal imaginal disc	Rhabdomere development; small GTPase signal transduction	
Sulf1	1.67	PE eye/antennal and wing disc	Pattern specification; metabolism	
CG9699	1.55	PE eye/antennal imaginal disc	Cytokinesis; mitosis; structural constituent of cytoskeleton	
eiger	1.5	Random cells in eye and leg PE and DP	Induction of apoptosis; JNK cascade; TNF receptor binding	(Igaki et al., 2002)
Aplip1	1.48	PE eye/antennal imaginal disc	Regulation of JNK cascade; rhodopsin-like receptor activity	
Lip1	1.41	In eye DP column of single cells at the posterior edge of MF express Lip1. Random cells of eye and wing DP, PE.	Lipid metabolism	
tun	1.4	Nuclei of peripodial cells	Olfactory learning	
Dscam	1.36	PE eye/antennal, wing and leg imaginal disc	PNS development; axon guidance; cell adhesion	
CG13890	1.22	PE	Fatty acid beta-oxidation	
Plasmatocytes (7)				
CG32406	3.2	Plasmatocytes; Expressed in posterior half of wing pouch	Intracellular signaling cascade; contains an SH2 motif	
CG13532	2.37	PE eye; plasmatocytes; expressed within wing pouch	-	
CG14598	2.2	Anterior to MF; plasmatocytes	-	
CG13041	2.19	PE; plasmatocytes	-	
CG30022	1.95	Dorsal and ventral anterior eye margin; plasmatocytes	Hydrolyase activity; lysase activity; defense response	
Neu3	1.94	Differentiating cells; plasmatocytes; PE	Cell adhesion; proteolysis and peptidolysis; signal transduction	
CG33275	1.8	Plasmatocytes	Guanyl exchange factor activity; MAPK cascade	
Other imaginal discs or tissues (15)				
CG3244	5.06	Specific pattern on CNS.	Sugar binding	
CG7900	3.43	Expressed in wing pouch	-	
CG32406	3.2	Plasmatocytes; Expressed in posterior half of wing pouch	Intracellular signaling cascade; contains an SH2 motif	
CG13606	2.98	Expressed in the wing DP	-	
CG13532	2.37	PE eye; plasmatocytes; expressed within wing pouch	-	
Al	2.37	Pattern in leg disc; 2nd antennal segment	-	(Schneitz et al., 1993)
CG4096	2.2	Expressed at the dorsal anterior margin in the eye disc; wing and leg discs.	-	
Lim1	2.95	3rd antennal segment; outer tarsal segments of leg disc	-	
CG11339	2.87	Narrow band of cells posterior to MF; inner ring of antennal disc; specific expression in wing disc	Cytoskeleton organization and biogenesis	
kek1	2.06	All cells posterior to MF; 2nd and 3rd antennal segments; presumptive femur in leg imaginal disc.	Negative regulation of EGF receptor activity	
CG11382	1.92	Eye: 3/4 columns of ommatidia just posterior to MF. Wing: pleurites, myoblasts, cells in wing pouch but restricted from presumptive margin	-	
scab	1.71	Random cells in eye and wing DP	-	
sqz	1.69	Late differentiating cells; nuclei of fat body; gut cells; salivary gland	Cell proliferation; regulation of transcription; oogenesis	
SP2353	1.67	Differentiating cells of the eye, maybe predominantly a single cell type per ommatidium; brain CNS	Signal transduction; cell-matrix and cell-cell adhesion	
CG31163	1.84	Ubiquitous expression in eye; Wing pouch, restricted from presumptive margin	-	

N.B.

(i) Transcripts of genes in bold face were detected in multiple tissue and cell types.

(ii) DP = disc proper; MF = morphogenetic furrow; PE = peripodial epithelium; SLR = Signal Log Ratio

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(iii) Transcripts were not detected for the following 24 *GMR>sSpi* downregulated genes: *mthl8; CG18278; Sox100B;Miple; CG9416; CG4914; CG3781; rn; CG33515; CG3525; CG9134; CG17278; pyr; CG1102; CG17919l dbe; disco-r; CG8965; CG14567; Mob1; CG4686; CG2264; prosalpha1; CG18600.*

(iv) Transcripts were not detected for the following 14 genes upregulated in *GMR>sSpi: CG4306; CG15756; CG8303; Obp56d; CG4341; CG4098; CG15117; comm.; CG14170; CG10433; lbm; Nrv1; sano; tna.*