
Figures and figure supplements

Differentiation signals from glia are fine-tuned to set neuronal numbers during development

Anadika R Prasad *et al*

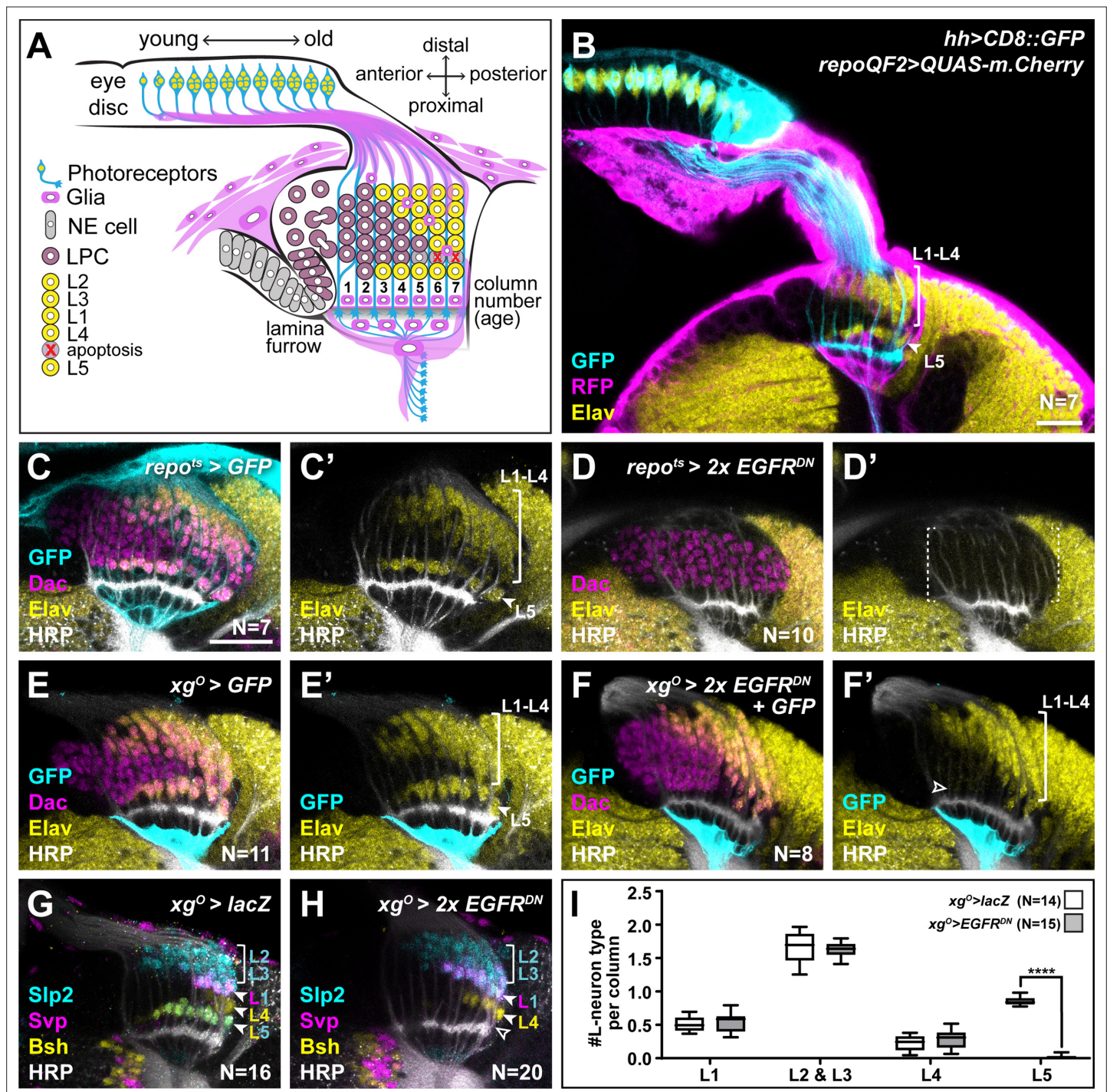


Figure 1. Epidermal growth factor receptor (EGFR) activity in the *xg^O* is required for the differentiation of L5 neurons. **(A)** Schematic of the developing lamina. Photoreceptors (blue) drive lamina precursor cell (LPC; purple) birth from neuroepithelial cells (NEs; grey) and their assembly into columns of ~6 LPCs, which differentiate into the L1-L5 neurons (yellow) following an invariant spatio-temporal pattern. The 'extra' LPC is cleared by apoptosis (red X). Several glial types (magenta) associate with the lamina. **(B)** A cross-sectional view of an early pupal (0–5 hr after puparium formation; APF) optic lobe where *hh-Gal4* drives *UAS-CD8::GFP* expression in photoreceptors (cyan). The pan-glial driver *repo-QF2* drives *QUAS-m.Cherry* (magenta) in all glia. Embryonic lethal abnormal vision (Elav) (yellow) marks all neurons. **(C)** A cross-sectional view of an optic lobe with pan-glial expression of *CD8::GFP* stained for GFP (cyan), Dachshund (Dac) (magenta), Elav (yellow), and Horseradish Peroxidase (HRP; axons; white). **(D)** Pan-glial expression of two copies of *EGFR^{DN}* stained for Dac (magenta), Elav (yellow), and HRP (white). **(E)** *xg^O*-specific expression of *CD8::GFP* stained for GFP (cyan), Dac (magenta), Elav (yellow), and HRP (white). **(F)** *xg^O*-specific expression of two copies of *EGFR^{DN}* and *CD8::GFP* stained for GFP (cyan), Dac (magenta), Elav (yellow), and HRP (white). The number of Elav+ cells in proximal row (L5s) decreased (empty arrowhead) relative to control **(E)**. **(G,H)** HRP (white) and L-neuron-

Figure 1 continued on next page

Figure 1 continued

type-specific markers Sloppy paired 2 (Slp2) (cyan), Brain-specific homeobox (Bsh) (yellow), and Seven-up (Svp) (magenta) in **(G)** control $xg^O>lacZ$ optic lobe and **(H)** $xg^O>2xEGFR^{DN}$. L2s and L3s express Slp2; L1s express Slp2 and Svp; L4s express Bsh and L5s express Bsh and Slp2. **(I)** Quantification of the number of L-neuron types per column for control and $xg^O>2xEGFR^{DN}$. Only L5 neurons were decreased significantly ($p^{L5}<0.0001$; Mann-Whitney U-test. Ns indicated in parentheses. Boxes indicate the lower and upper quartiles; the whiskers represent the minimum and maximum values; the line inside the box indicates the median). Scale bar = 20 μm .

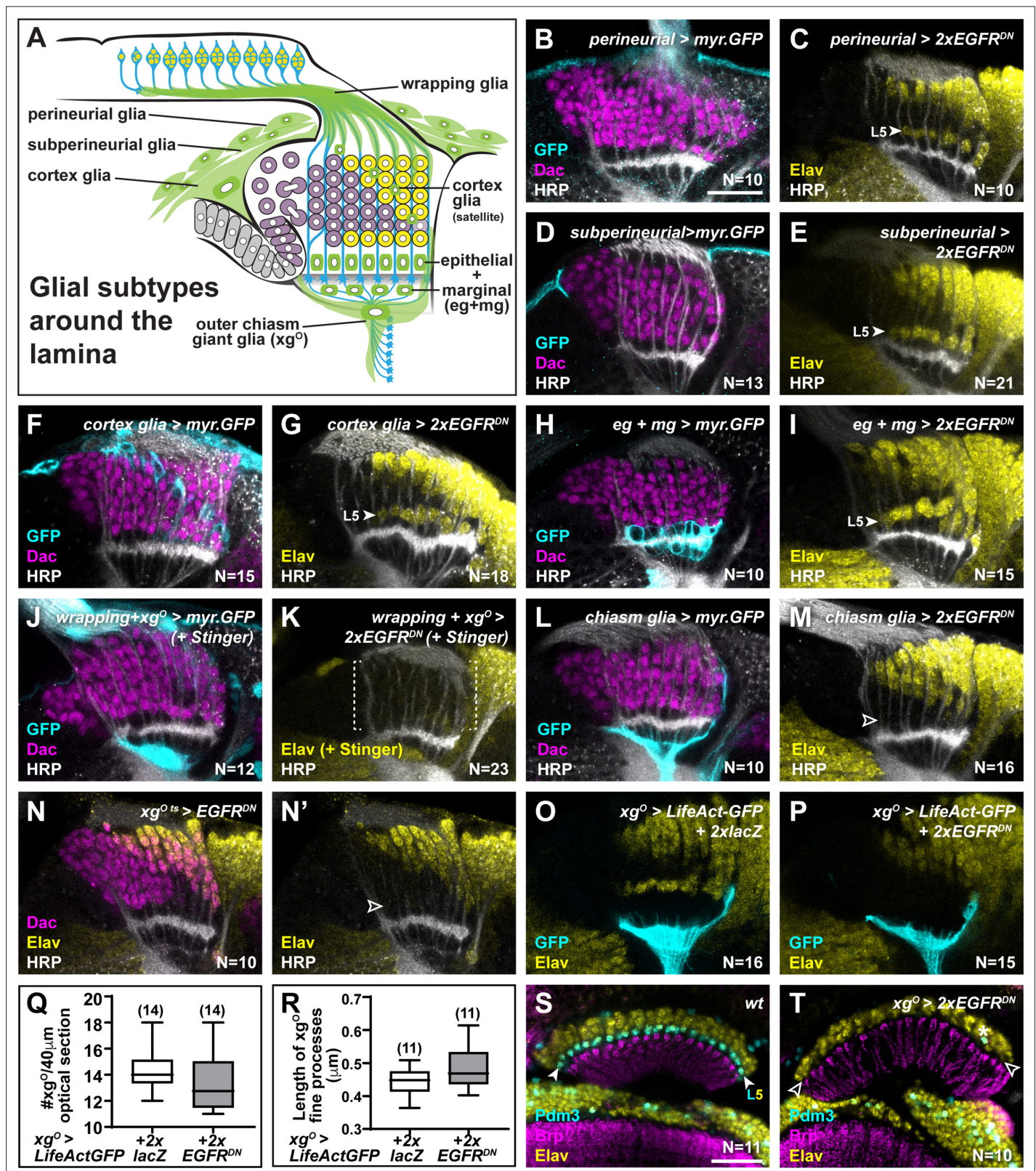


Figure 1—figure supplement 1. A Gal4 screen identifies xg^o as the glial subtype that regulates L5 neuronal differentiation. (A) Schematic of the developing lamina and associated glial types (green; labelled). (B) A perineurial glia-specific Gal4 drives expression of myr.GFP stained for GFP (cyan), Dachshund (Dac) (magenta), and Horseradish Peroxidase (HRP) (white). (C) Perineurial glia-specific expression of EGFR^{DN} stained for Embryonic lethal abnormal vision (Elav) (yellow) and HRP (white). L5 differentiation was not affected. (D) A subperineurial glia-specific Gal4 drives expression of myr.GFP

Figure 1—figure supplement 1 continued on next page

Figure 1—figure supplement 1 continued

stained for GFP (cyan), Dac (magenta), and HRP (white). **(E)** Suberineurial glia-specific expression of EGFR^{DN} stained for Elav (yellow) and HRP (white). L5 differentiation was not affected. **(F)** A cortex glia-specific Gal4 drives expression of myr.GFP stained for GFP (cyan), Dac (magenta), and HRP (white). **(G)** Cortex glia-specific expression of EGFR^{DN} stained for Elav (yellow) and HRP (white). L5 differentiation was not affected. **(H)** An epithelial and marginal glia (eg+mg) specific Gal4 drives expression of myr.GFP stained for GFP (cyan), Dac (magenta), and HRP (white). **(I)** Epithelial and marginal glia-specific expression of EGFR^{DN} stained for Elav (yellow) and HRP (white). L5 differentiation was not affected. **(J)** A wrapping glia- and xg^O-specific Gal4 drives expression of myr.GFP stained for GFP (cyan), Dac (magenta), and HRP (white). **(K)** Wrapping glia- and xg^O-specific expression of EGFR^{DN} stained for Elav (yellow) and HRP (white). L1-L4 and L5 differentiation were disrupted as observed by the lack of Elav+ cells in the lamina. **(L)** A chiasm glia (xg^O and xg^{inner}) specific Gal4 drives expression of myr.GFP stained for GFP (cyan), Dac (magenta), and HRP (white). **(M)** Chiasm glia-specific expression of EGFR^{DN} stained for Elav (yellow) and HRP (white). L1-L4 differentiation proceeded normally but L5 differentiation was disrupted as observed by the lack of Elav+ cells in the proximal lamina. **(N)** Gal80^{ts}-restricted Gal4 expression in xg^O, driving EGFR^{DN} during lamina development (see **Figure 3—source data 1**) stained for Dac (magenta), Elav (yellow), and HRP (white). L5 neurons were dramatically reduced. **(O,P)** LifeAct-GFP expression driven in xg^O in **(O)** controls and **(P)** when two copies of EGFR^{DN} are co-expressed. In both conditions, the fine processes from the xg^O are present. **(Q)** Quantification of xg^O numbers in control xg^O>LifeAct-GFP+2xlacZ and xg^O>LifeAct GFP+2xEGFR^{DN}. p>0.05; Mann-Whitney U-test. Ns indicated in parentheses. **(R)** Quantification of the length of xg^O fine processes in control xg^O>LifeAct-GFP+2xlacZ and xg^O>LifeAct GFP+2xEGFR^{DN}. p>0.05; Unpaired t-test. Ns indicated in parentheses. **(S)** Wild-type adult optic lobe stained for POU domain motif 3 (Pdm3) (L5 marker) (**Tan et al., 2015**), Bruchpilot (Brp; marks neuropils) and Elav (yellow). **(T)** xg^O>2xEGFR^{DN} adult optic lobe stained for Pdm3 (L5 marker) (**Tan et al., 2015**), Bruchpilot (Brp; marks neuropils) and Elav (yellow). Pdm3+ cells (L5s) are reduced dramatically. Scale bar = 20 μm. For all quantifications boxes indicate the lower and upper quartiles; the whiskers represent the minimum and maximum values; the line inside the box indicates the median.

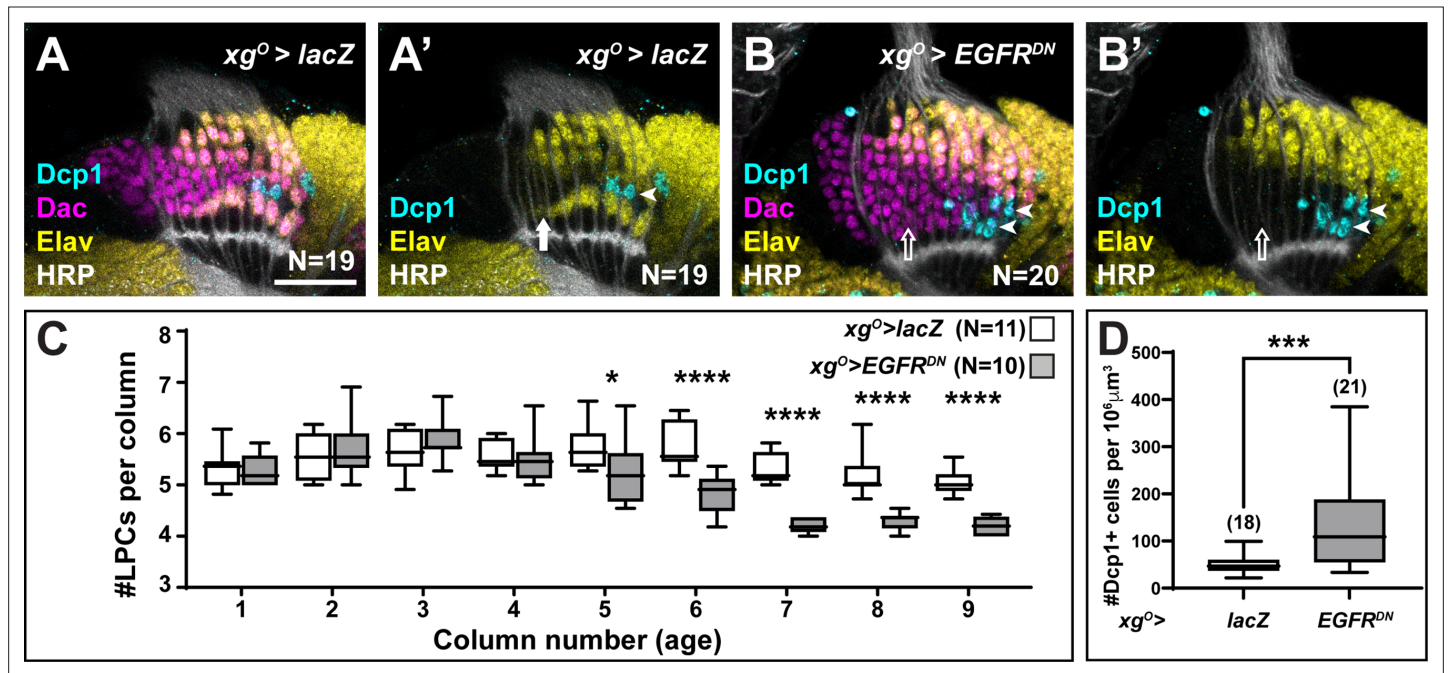


Figure 2. Lamina precursor cells (LPCs) that fail to differentiate into L5s undergo apoptosis. **(A)** Control $xg^O > lacZ$ optic lobe stained for Death caspase-1 (Dcp-1) (cyan), Embryonic lethal abnormal vision (Elav) (yellow), and Horseradish Peroxidase (HRP) (white). Dcp-1+ cells were always observed just distal to the most proximal row of cells (L5s). **(B)** $xg^O > EGFR^{DN}$ stained for Dcp-1 (cyan), Dachshund (Dac) (magenta), Elav (yellow), and HRP (white). Dcp-1 positive cells were observed in the most proximal row of LPCs as well as the row just distal to these. **(C)** Quantification of the number of LPCs/column (i.e., Dac+ cells/column) for control and $xg^O > EGFR^{DN}$. * $p < 0.05$, **** $p < 0.0002$; Mann-Whitney U-test. Ns indicated in parentheses. **(D)** Quantification of the number of Dcp-1 positive cells in **(A)** compared to **(B)**. *** $p < 0.0005$, Mann-Whitney U-test. Ns indicated in parentheses. Boxes indicate the lower and upper quartiles; the whiskers represent the minimum and maximum values; the line inside the box indicates the median. Scale bar = 20 μm .

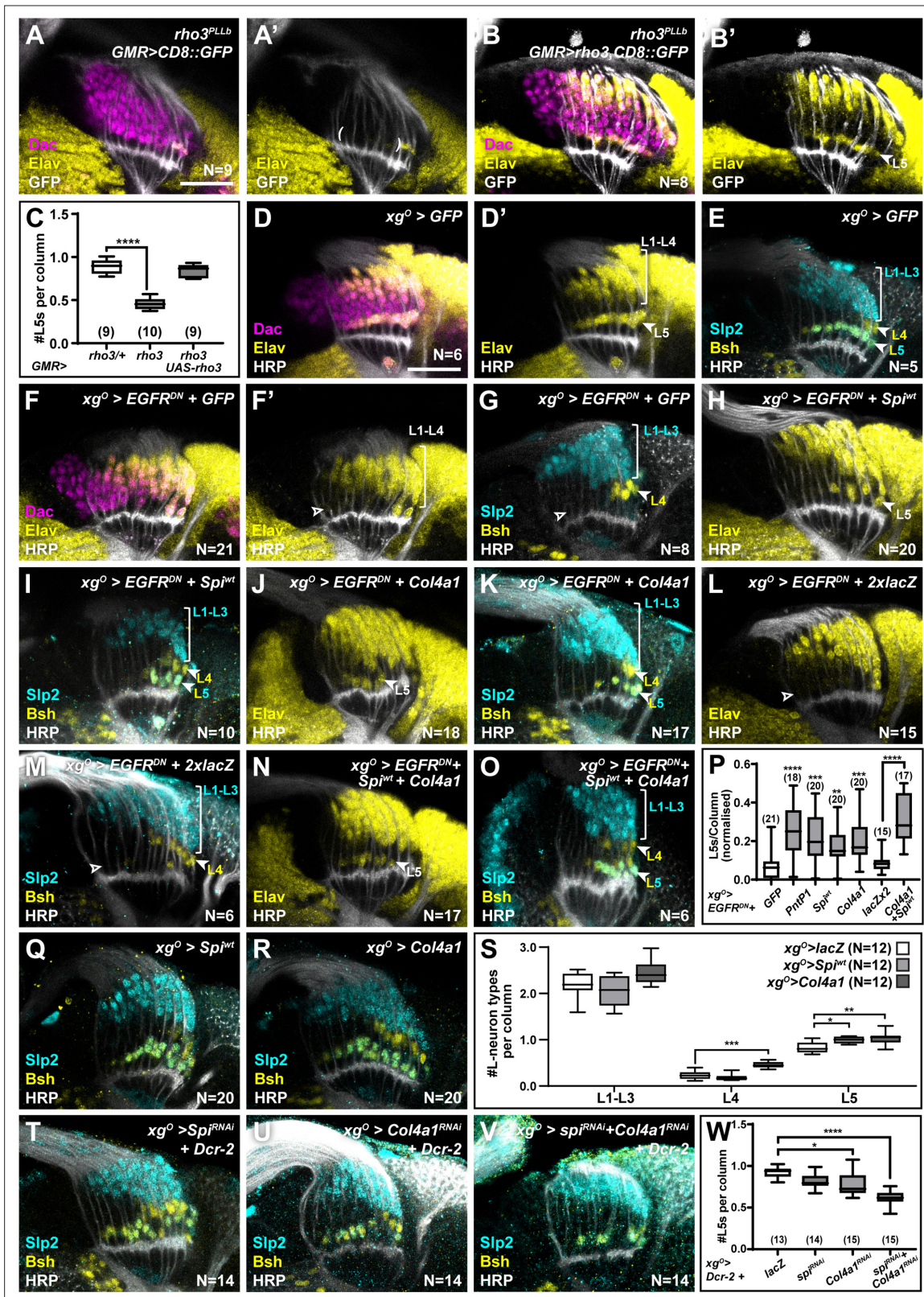


Figure 3. Xg° secrete multiple ligands to induce L5 neuronal differentiation in response to epidermal growth factor (EGF) from photoreceptors. (A) GMR-Gal4-driven CD8::GFP expression in photoreceptors in a *rho3^{PLLB}* background stained for GFP (white), Dachshund (Dac) (magenta), Embryonic lethal abnormal vision (Elav) (yellow). Few proximal Elav+ cells (L5s) were recovered in older columns only as previously published (Fernandes et al., 2017). (B) GMR-Gal4-driven Rho3 and CD8::GFP in a *rho3^{PLLB}* background stained for GFP (white), Dac (magenta), Elav (yellow) showed that L5

Figure 3 continued

neuronal differentiation was rescued (Elav+ cells in the proximal lamina). **(C)** Quantifications for number of L5 neurons/column in **(A)** and **(B)** compared to *rho3^{PLB}* heterozygotes (*rho3/+*). *****p*<0.0001, one-way ANOVA with Dunn's multiple comparisons test. Ns indicated in parentheses. **(D,E)** Control *xg^O>GFP* optic lobes stained for **(D)** Dac (magenta), Elav (yellow), and Horseradish Peroxidase (HRP) (white) or **(E)** HRP (white) and L-neuron-specific markers Sloppy paired 2 (Slp2) (cyan) and Brain-specific homeobox (Bsh) (yellow). **(F,G)** Gal4 titration control *xg^O>GFP + EGFR^{DN}* stained for **(F)** Dac (magenta), Elav (yellow), and HRP (white) or **(G)** HRP (white) and L-neuron-specific markers Slp2 (cyan) and Bsh (yellow). **(H,I)** Wild-type Spitz (Spi) (*Spi^{wt}*) co-expression with *EGFR^{DN}* specifically in *xg^O* stained for **(H)** Elav (yellow) and HRP (white) or **(I)** HRP (white) and L-neuron-specific markers Slp2 (cyan) and Bsh (yellow). **(J,K)** Col4a1 co-expression with *EGFR^{DN}* specifically in *xg^O* stained for **(J)** Elav (yellow) and HRP (white) or **(K)** HRP (white) and L-neuron-specific markers Slp2 (cyan) and Bsh (yellow). **(L,M)** Gal4 titration control *xg^O>EGFR^{DN} + 2xlacZ* stained for **(L)** Elav (yellow) and HRP (white) or **(M)** HRP (white), Slp2 (cyan), and Bsh (yellow). **(N,O)** Wild-type *Spi^{wt}* and Col4a1 co-expression with *EGFR^{DN}* specifically in *xg^O*. **(N)** stained for Elav (yellow) and HRP (white) or **(O)** HRP (white) and L-neuron-specific markers Slp2 (cyan) and Bsh (yellow). **(P)** Quantification of the number of L5s/column for the genotypes indicated compared to the appropriate titration control. For *pntP1*, *spi^{wt}*, and *Col4a1* co-expression with *EGFR^{DN}*, the titration control is *xg^O>EGFR^{DN} + GFP* (***p*<0.005, ****p*<0.0005; *****p*<0.0001; one-way ANOVA with Dunn's multiple comparisons test. Ns indicated in parentheses). For *spi^{wt}* and *Col4a1* simultaneous co-expression with *EGFR^{DN}*, the titration control is *xg^O>EGFR^{DN} + 2xLacZ* (*****p*<0.0001, Mann-Whitney U-test. Ns indicated in parentheses). **(Q,R)** Optic lobes stained for Slp2 and Bsh when *xg^O* overexpress **(Q)** *spi^{wt}* or **(R)** *Col4a1*. **(S)** Quantification of the number of L-neuron types/column in **(Q)** and **(R)** compared to controls, *xg^O>lacZ*. (**p*<0.05; ***p*<0.005; ****p*<0.001; one-way ANOVA with multiple comparisons test). **(T, U, V)** Optic lobes stained for Slp2, Bsh, and HRP when *xg^O* co-express Dcr-2 with **(T)** *spi^{RNAi}*, **(U)** *Col4a1^{RNAi}*, and **(V)** *Spi^{RNAi}* and *Col4a1^{RNAi}* simultaneously. **(W)** Quantifications of the number of L5s/column for genotypes indicated compared to the titration control *xg^O>Dcr-2+lacZ* (**p*<0.05, *****p*<0.0001, one-way ANOVA with Dunn's multiple comparisons test. Scale bar = 20 μm. For all quantifications boxes indicate the lower and upper quartiles; the whiskers represent the minimum and maximum values; the line inside the box indicates the median).

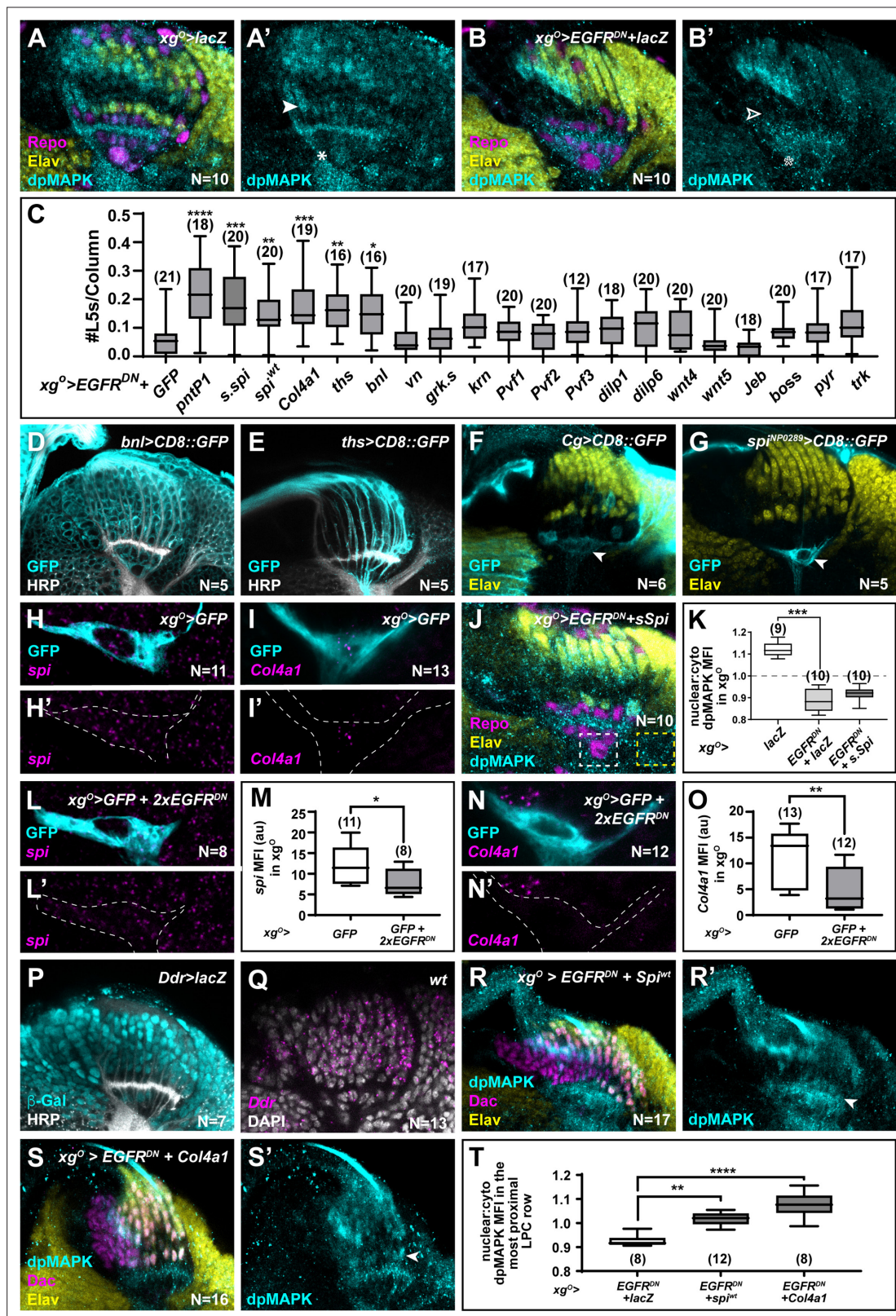


Figure 3—figure supplement 1. Multiple *xg°* secreted ligands activate mitogen-activated protein kinase (MAPK) signalling to drive L5 neuronal differentiation. (A,B) Optic lobes stained for Embryonic lethal abnormal vision (Elav) (yellow), Repo (magenta), and double phosphorylated MAPK (dpMAPK) (cyan) in (A) *xg°>lacZ* controls and (B) with *EGFR^{DN}* and *lacZ* expressed in *xg°*. dpMAPK levels decreased in the *xg°* (indicated by asterisk) and in cells in the proximal row of the lamina (indicated by arrowhead) when compared with *xg°>lacZ* controls. (C) Quantification of the number of

Figure 3—figure supplement 1 continued on next page

Figure 3—figure supplement 1 continued

L5s/column (based on Elav expression) when different ligands that can activate MAPK signalling were co-expressed with EGFR^{DN} in the xg^O (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.0005$; **** $p < 0.0001$; one-way ANOVA with Dunn's multiple comparison test. Ns indicated in parentheses). **(D)** *bnl*>*CD8::GFP* showed GFP (cyan) expression in all cells in the optic lobe; Horseradish Peroxidase (HRP) (white). **(E)** *ths*>*CD8::GFP* showed GFP (cyan) expression in photoreceptors; HRP (white). **(F)** *Collagen*>*CD8::GFP* drove GFP (cyan) expression in xg^O (arrowhead); Elav (yellow). **(G)** *spⁱNP0289*>*CD8::GFP* drove GFP (cyan) expression in xg^O (arrowhead); Elav (yellow). **(H, I)** xg^O >*GFP* lobes stained for GFP (cyan) and **(H)** *spi* mRNA (magenta) and **(I)** *Col4a1* mRNA (magenta) by *in situ* hybridisation chain reaction (HCR). **(J)** xg^O >*EGFR^{DN}* + *s.spi* lobes stained for Elav (yellow), Repo (magenta), and dpMAPK (cyan). Inset shows a magnified view of the xg^O nucleus. **(K)** Quantifications of nuclear:cytoplasmic ratios of dpMAPK mean fluorescence intensity (MFI) in the xg^O in indicated genotypes ($p < 0.0005$, one-way ANOVA with Dunn's multiple comparisons test. Ns indicated in parentheses). **(L)** *spi* mRNA (magenta) detected by HCR in xg^O >*GFP* + *2xEGFR^{DN}* lobes. **(M)** Quantification of *spi* MFI (arbitrary units) for **(H and L)**. ($p < 0.05$; Mann-Whitney U-test.). **(N)** *Col4a1* mRNA (magenta) detected by HCR in xg^O >*GFP* + *2xEGFR^{DN}* lobes. **(O)** Quantification of *Col4a1* MFI (arbitrary units) for **(I and N)** ($p < 0.005$; Mann-Whitney U-test). **(P)** *Ddr*>*lacZ* showed β -Galactosidase (β -Gal; cyan) expression in the lamina; HRP (white). **(Q)** *Ddr* mRNA (magenta) detected by HCR in wild-type lobes; DAPI (white). **(R,S)** Lobes stained for Dac (magenta), Elav (yellow), and dpMAPK (cyan) when **(R)** *Spⁱwt* is co-expressed with EGFR^{DN} in xg^O or **(S)** *Col4a1* is co-expressed with EGFR^{DN} in xg^O . Arrowheads indicate Elav+ cells in the most proximal row. **(T)** Quantifications of nuclear:cytoplasmic ratios of dpMAPK MFI in the most proximal row of lamina precursor cells (LPCs) in indicated genotypes (** $p < 0.005$, **** $p < 0.0001$; one-way ANOVA with Dunn's multiple comparisons test). Scale bar = 20 μ m. For all quantifications boxes indicate the lower and upper quartiles; the whiskers represent the minimum and maximum values; the line inside the box indicates the median.

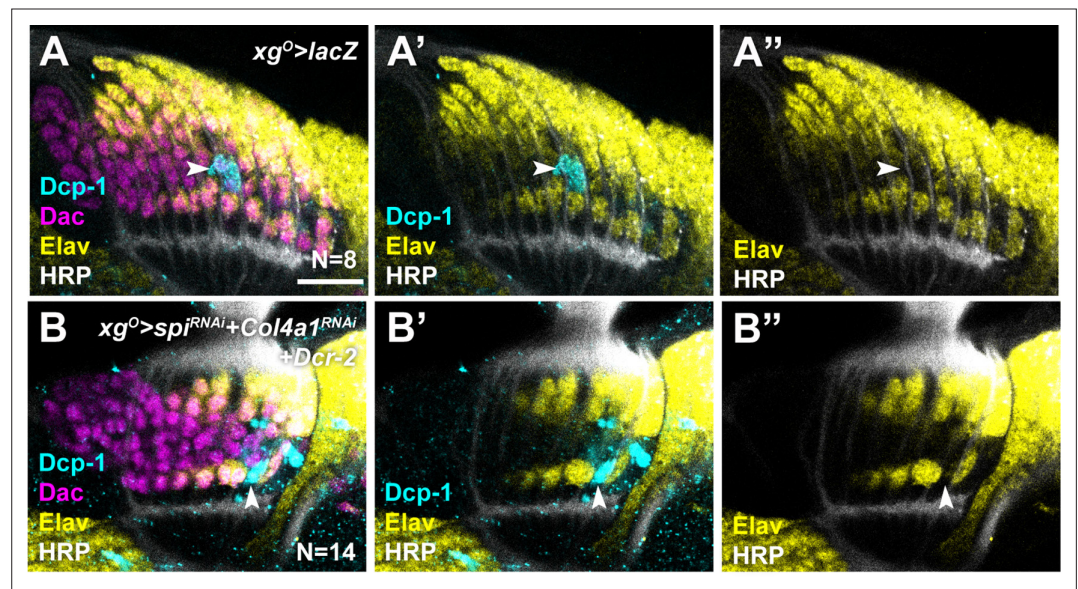


Figure 3—figure supplement 2. Spi and Col4a1 from xg^O promote cell survival in proximal lamina precursor cells (LPCs). (A) $xg^O>lacZ$ lobes stained for Death caspase-1 (Dcp-1) (cyan), Dachshund (Dac) (magenta), Embryonic lethal abnormal vision (Elav) (yellow), and Horseradish Peroxidase (HRP) (white). Dcp-1 positive cells (indicated by arrowhead) were located between L4s and L5s and corresponds to 'extra' LPCs which undergo apoptosis. (B) $xg^O>spi^{RNAi} + Col4a1^{RNAi} + Dcr-2$ lobes stained for Dcp-1 (cyan), Dac (magenta), Elav (yellow), and HRP (white). Dcp-1 positive cells were observed in the proximal row of L5s (indicated by arrowhead) which were never observed in controls.

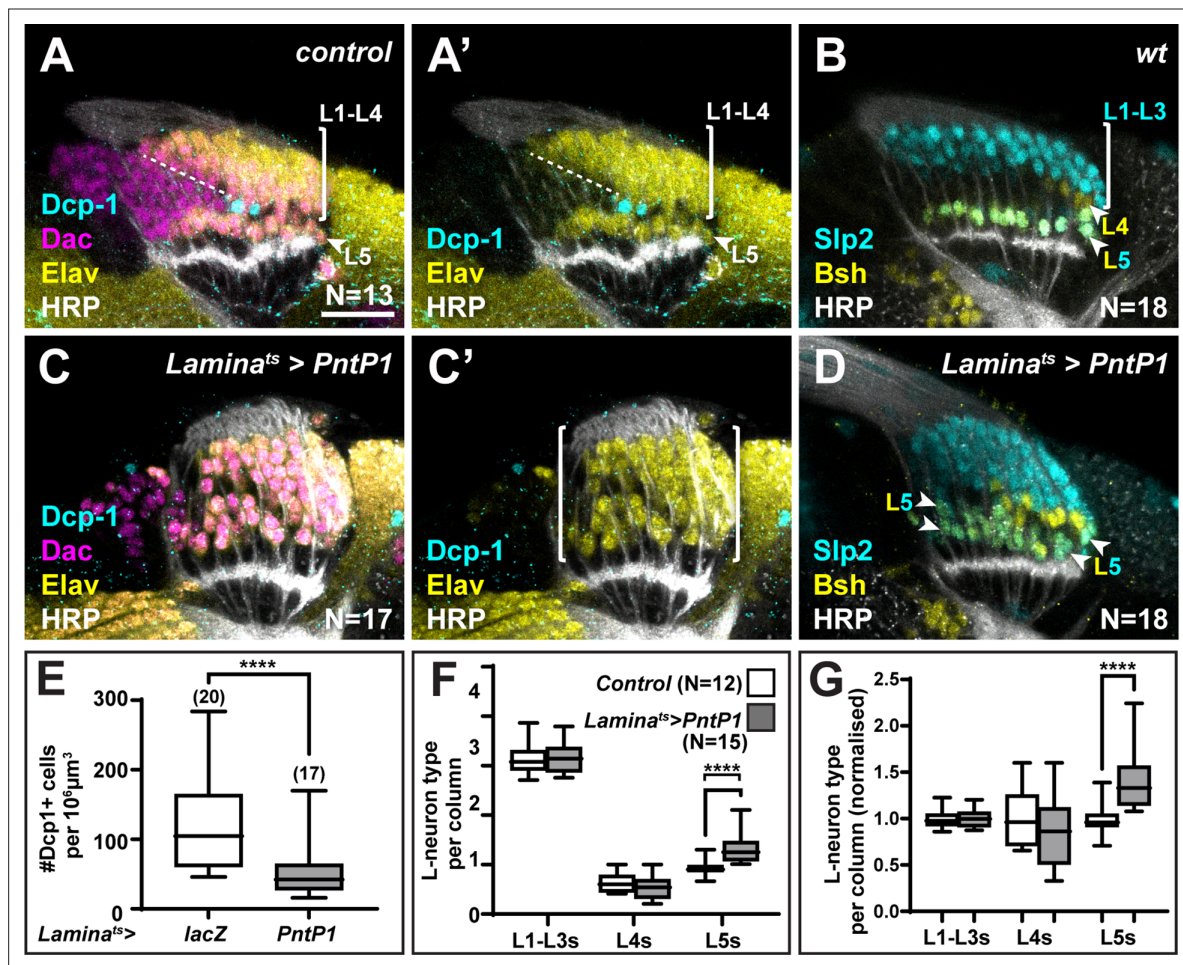


Figure 4. The 'extra' lamina precursor cells (LPCs) are specified as L5s. (A) Wild-type optic lobes stained for Dachshund (Dac) (magenta), Horseradish Peroxidase (HRP) (white), Embryonic lethal abnormal vision (Elav) (yellow), and cleaved Death caspase-1 (Dcp-1) (cyan). (B) Wild-type optic lobes stained for HRP (white) and L-neuron-type-specific markers sloppy paired 2 (Slp2) (cyan) and brain-specific homeobox (Bsh) (yellow). (C, D) Optic lobes with lamina-specific overexpression of PntP1 stained as in (A) and (B), respectively. (C) Fewer Dcp-1 positive cells were recovered compared with controls. (D) Roughly two rows of Slp2 and Bsh co-expressing cells (L5s) were recovered (arrowheads). (E) Quantification of the number of Dcp-1 positive cells in (B) compared with control *Lamina^{ts} > lacZ* (Figure 4—figure supplement 1A) ($p < 0.0001$; Mann-Whitney U-test). (F) Quantification of the number of L-neuron types per column based on Slp2 and Bsh expression from column 7 onwards shows an increase in the number of L5s/column in *Lamina^{ts} > PntP1* compared with controls; $p < 0.0001$; Mann-Whitney U-test. (G) Same as (F) but normalised to the mean of the control. The number of L5s/column in *Lamina^{ts} > PntP1* increase ~1.2-fold relative to controls; $p < 0.0001$; Mann-Whitney U-test. Ns indicated in parentheses. Scale bar = 20 μm. For all quantifications boxes indicate the lower and upper quartiles; the whiskers represent the minimum and maximum values; the line inside the box indicates the median.

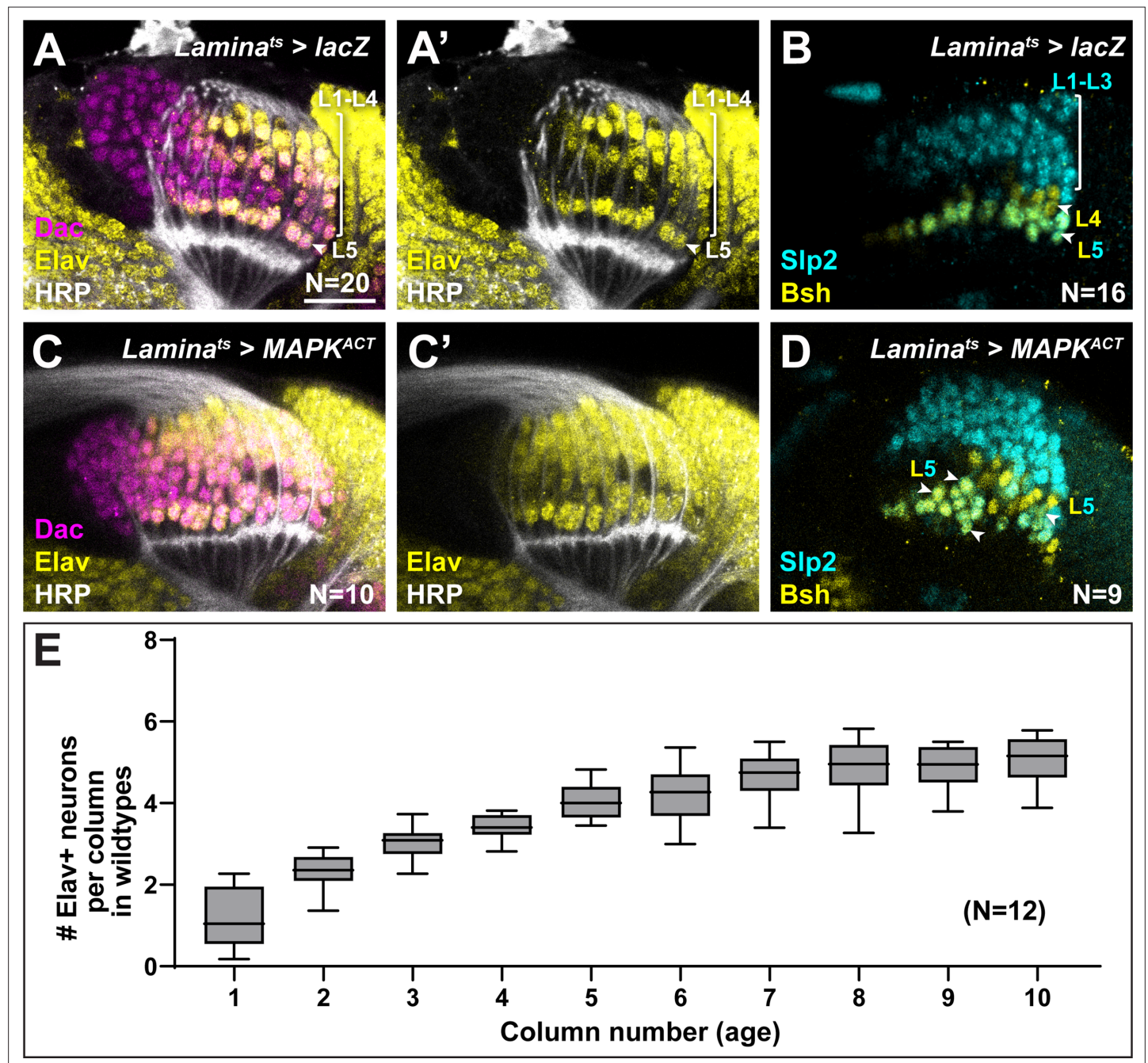


Figure 4—figure supplement 1. Hyperactivating Mitogen-activated protein kinase (MAPK) in the lamina drives ectopic L5 differentiation. (**A,B**) Control *Lamina^{ts}>lacZ* optic lobes stained for (**A**) Dachshund (Dac) (magenta), Horseradish Peroxidase (HRP) (white) and Embryonic lethal abnormal vision (Elav) (yellow), and (**B**) L-neuron-type-specific markers Sloppy paired 2 (Slp2) (cyan) and Brain-specific homeobox (Bsh) (yellow). (**C,D**) *Lamina^{ts}>MAPK^{ACT}* optic lobes stained for (**C**) Dac (magenta), HRP (white), and Elav (yellow), and (**D**) L-neuron-type-specific markers Slp2 (cyan) and Bsh (yellow). Ectopic Slp2 and Bsh co-expressing cells (L5s) were observed (arrowheads). (**E**) Quantification of the number of Elav+ cells per lamina column as a function of column number (age) in wild-type animals. Columns were fully differentiated (five Elav+ cells) by column 7. Boxes indicate the lower and upper quartiles; the whiskers represent the minimum and maximum values; the line inside the box indicates the median. Scale bar = 20 μ m.

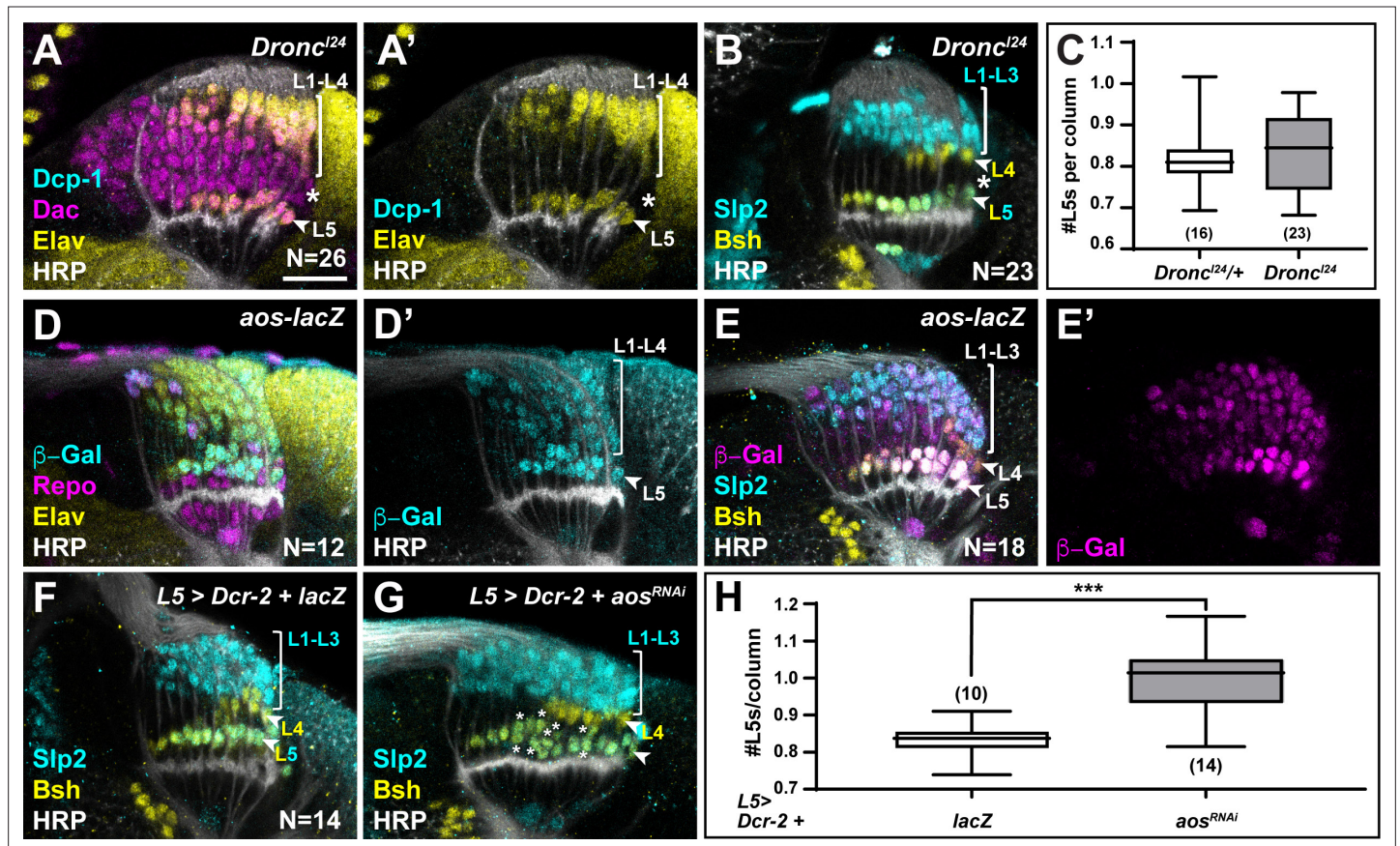


Figure 5. Newly induced L5 neurons secrete Aos to limit differentiation signals from xg^O . (A) *Dronc^{I24}* optic lobes stained for Death caspase-1 (Dcp-1) (cyan), Dachshund (Dac) (magenta), Embryonic lethal abnormal vision (Elav) (yellow), and Horseradish Peroxidase (HRP) (white). No Dcp-1 positive cells were recovered and Dac positive cells between L1-L4s and L5s persisted into the oldest columns (asterisk). (B) *Dronc^{I24}* optic lobes stained for L-neuron-type-specific markers Sloppy paired 2 (Slp2) (cyan) and Brain-specific homeobox (Bsh) (yellow). A space (negative for both markers; asterisk) was present between L4s and L5s. (C) Quantifications for number of L5s/column in *Dronc^{I24}* optic lobes compared to controls (*Dronc^{I24/+}*) ($p > 0.05$, Mann-Whitney U-test. Ns indicated in parentheses). (D,E) *aos-lacZ* expression in the lamina with (D) β-Galactosidase (β-Gal) (cyan), Repo (magenta), Elav (yellow), HRP (white), and with (E) β-Gal (magenta) and L-neuron-type-specific markers Slp2 (cyan), Bsh (yellow), as well as HRP (white). (F) An L5-specific Gal4 was used to drive expression of *Dcr-2* and *lacZ* in control lobes stained for Slp2 (cyan), Bsh (yellow), and HRP (white). (G) Optic lobes stained for HRP (white), Slp2 (cyan), and Bsh (yellow) when *Dcr-2* and *aos^{RNAi}* were expressed in developing L5 neurons specifically, which led to an increase in the number of Slp2 and Bsh co-expressing cells (L5s; asterisks). (H) Quantification of the number of L5s/column for (F) and (G). *** $p < 0.0005$; Mann-Whitney U-test. Ns indicated in parentheses. For all quantifications boxes indicate the lower and upper quartiles; the whiskers represent the minimum and maximum values; the line inside the box indicates the median. Scale bar = 20 μ m.

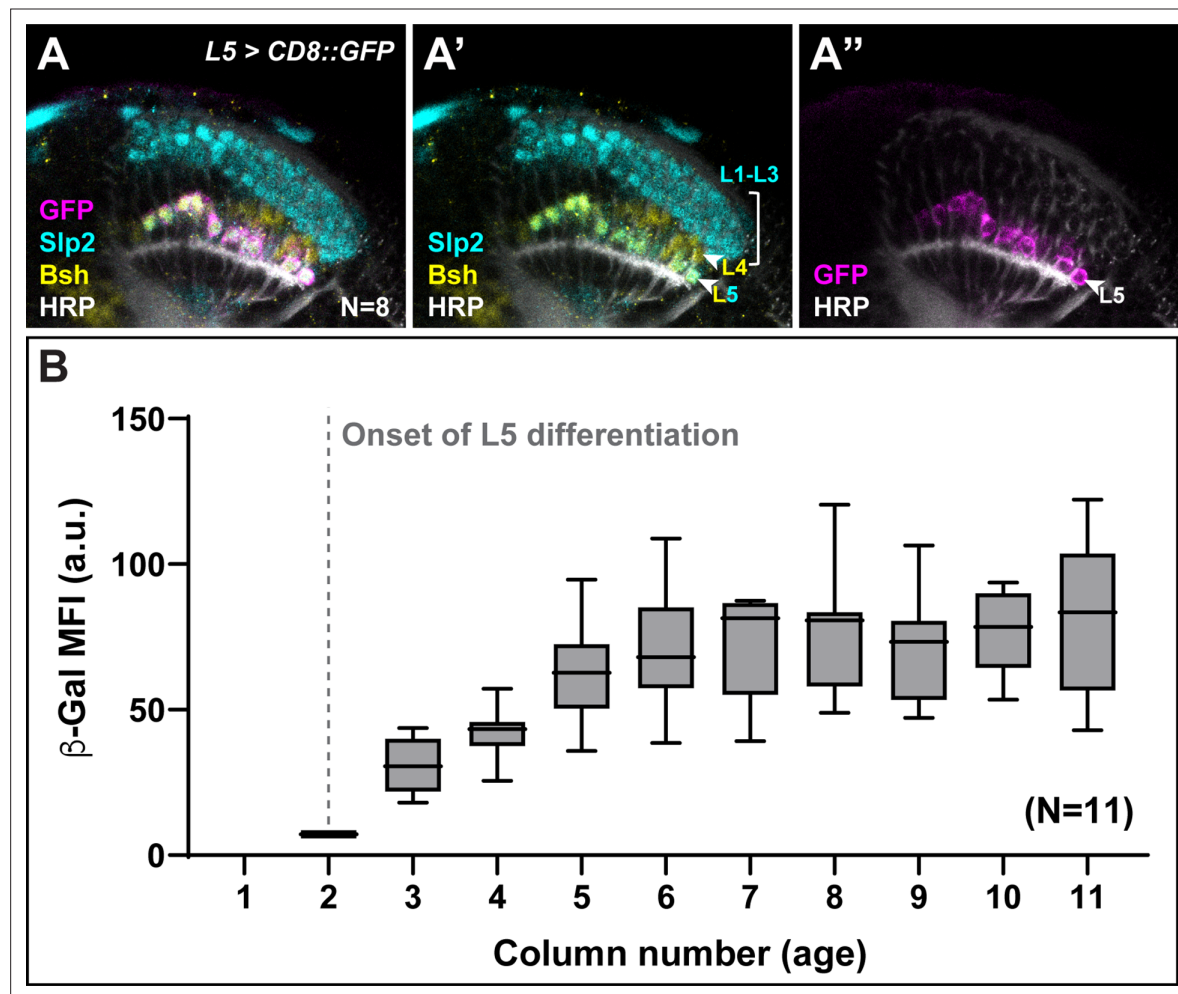


Figure 5—figure supplement 1. Aos expression is delayed in younger L5s. (A) An L5-specific driver was used to drive the expression of GFP (magenta) in the lamina; Horseradish Peroxidase (HRP) (white) and L-neuron-type-specific markers Sloppy paired 2 (Slp2) (cyan) and Brain-specific homeobox (Bsh) (yellow). (B) β -Galactosidase (β -Gal) mean fluorescence intensity (MFI) quantifications in the proximal row of L5s as a function of column number (age) in *aos-lacZ* lobes. β -Gal MFI is low in young columns and increases in older columns (from column 5). Boxes indicate the lower and upper quartiles; the whiskers represent the minimum and maximum values; the line inside the box indicates the median. Scale bar = 20 μ m.

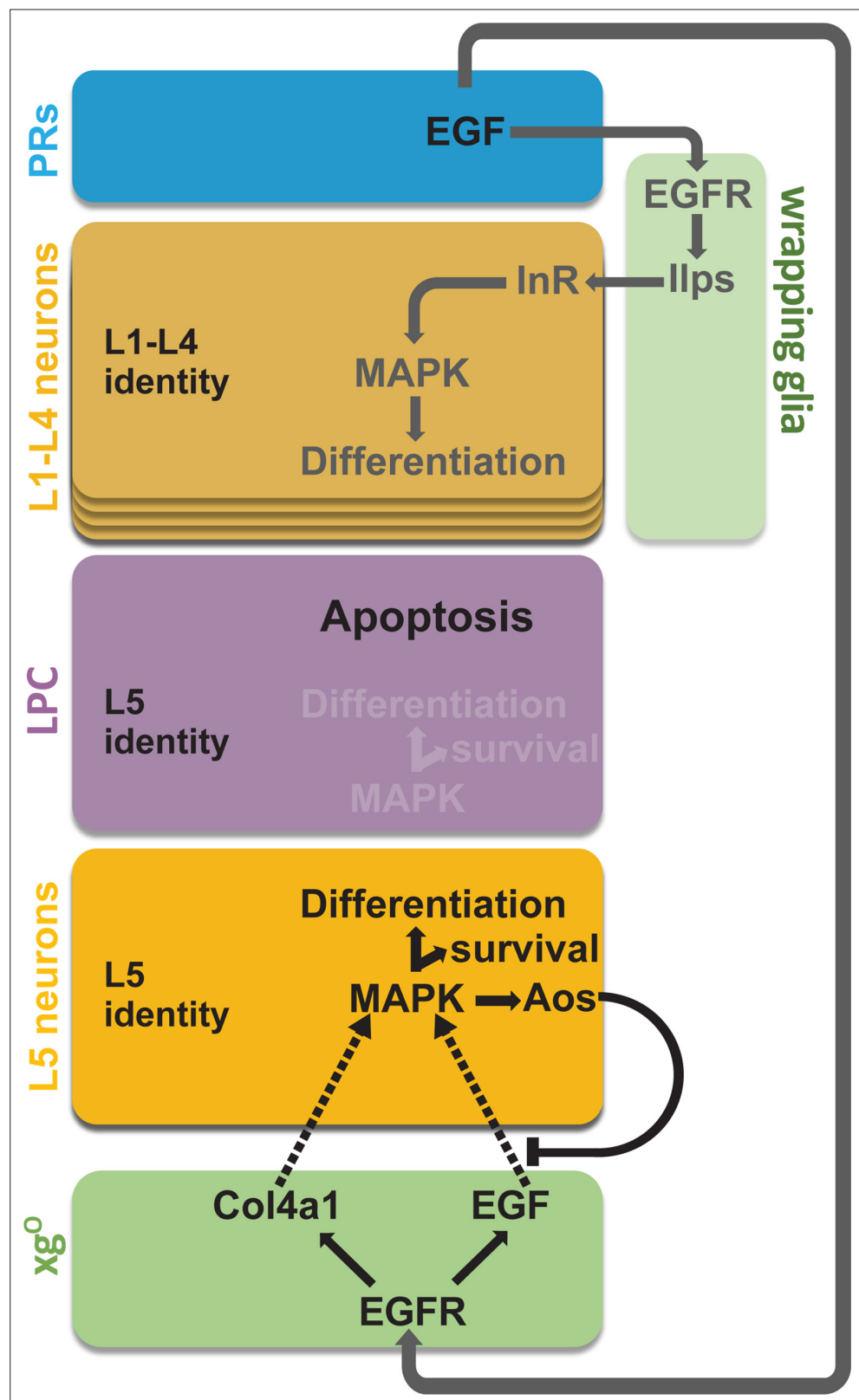


Figure 6. Summary schematic of neuronal differentiation in the lamina. In our model of lamina neuronal differentiation, lamina precursor cells (LPCs) are prepatterned with unique identities based on their positions within a column, such that the two most proximal cells are specified with L5 identity. Epidermal growth factor (EGF) from photoreceptors activates EGF receptor (EGFR) signalling in wrapping glia, which induce L1-L4 differentiation, and Figure 6 continued on next page

Figure 6 continued

in xg^0 , which induce L5 differentiation. Only a subset of the LPCs specified as L5s differentiate (i.e., those in the proximal row). We propose that this selective neuronal induction of L5s is due to tissue architecture and feedback from the newly born L5s, which limit available EGF (Spitz [Spi]) by secreting the antagonist Argos (Aos).