of PKR2-positive cells in the rostral portion of the RMS and OV of $PK2^{-/-}$ mice (fig. S12), also shown by PSA-NCAM immunostaining (Fig. 3E). Analysis of PKR2 mRNA expression in $PK2^{-/-}$ mice showed an overall decrease in neuronal progenitors migrating away from the OV into the GL and PGL (fig. S12). The compaction of PKR2-positive cells in the OV indicates that, in the absence of PK2 signaling, chained neuronal progenitors are either not detached properly or disoriented about the direction of migration. To evaluate whether PK2 is a genuine chemoattractant for SVZ neuronal progenitors, we performed SVZa explants coculture assay with the GL of the OB, where PK2 is primarily expressed (Fig. 1, D and E). Cell migration was directed toward the GL tissue from WT OB, whereas the corresponding tissue from $PK2^{-/-}$ OB exhibited no chemotaxis activity (proximal/distal ratio: 1.87 ± 0.31 versus 0.99 ± 0.06, WT versus $PK2^{-/-}$, respectively; $n = 6$ explants, $P < 0.05$) (fig. S13). Taken together, these results indicate that the migration of neuronal progenitors mediated by PK2 signaling is essential for the normal development and maintenance of the OB.

Thus, PK2 serves as a chemoattractant for SVZ-derived neuronal progenitors, and the establishment of normal OB architecture requires PK2 signaling. Together with other signals (*12*, *13*, *15*), PK2 appears to guide the migration of neuronal progenitors from the SVZ through the RMS to their final layers in the OB. The similar response of PKR1 and PKR2 to PK2 (*22*) implies that these receptors may mediate a redundant role for OB development. As with endothelin-3 signaling for the migration of enteric neurons (*33*) and orphan receptor GPR56 in the regional development of the cerebral cortex (*34*), our results further indicate that G protein–coupled receptors may be crucial for the establishment of the layered structures in the nervous system.

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35. We thank H. van Praag, O. Steward, and C. Zhang for discussions; C. Tu and H. Shen for technical assistance; and the laboratories of C. Cotman and F. LaFerla for access to equipment. Supported by a University of California Discovery grant. K.N. is a recipient of a UCI Medical Scientist Training Program training grant.

Supporting Online Material

www.sciencemag.org/cgi/content/full/308/5730/1923/DC1
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10 March 2005; accepted 2 May 2005
10.1126/science.1112103

GDF11 Controls the Timing of Progenitor Cell Competence in Developing Retina

Joon Kim,[1,2] Hsiao-Huei Wu,[1,2]* Arthur D. Lander,[2,3] Karen M. Lyons,[4] Martin M. Matzuk,[5] Anne L. Calof[1,2]†

The orderly generation of cell types in the developing retina is thought to be regulated by changes in the competence of multipotent progenitors. Here, we show that a secreted factor, growth and differentiation factor 11 (GDF11), controls the numbers of retinal ganglion cells (RGCs), as well as amacrine and photoreceptor cells, that form during development. GDF11 does not affect proliferation of progenitors—a major mode of GDF11 action in other tissues—but instead controls duration of expression of *Math5*, a gene that confers competence for RGC genesis, in progenitor cells. Thus, GDF11 governs the temporal windows during which multipotent progenitors retain competence to produce distinct neural progeny.

The vertebrate neural retina comprises seven neural cell types, all derived from one population of multipotent progenitors (*1*, *2*). Retinal cell types do not arise synchronously but are generated in a stereotyped sequence (*3*, *4*). In vitro results imply that retinal progenitors at different stages differ in their competence to produce distinct cell types (*5*–*7*). Such changes in potential are likely dictated by changes in expression of the transcription factors encoded by proneural genes (*8*, *9*), but mechanisms of proneural gene regulation are poorly understood. An important role for cell-cell signaling is suggested by the fact that production of at least two retinal cell types, retinal ganglion cells (RGCs) and amacrine cells, can increase to compensate for losses of mature cells in either population (*10*, *11*). This process has been postulated to be mediated by a feedback signal produced by mature cells (*12*), but the identity of the signal(s) is unknown.

GDF11, a member of the transforming growth factor-β superfamily of secreted signaling molecules, is expressed in several regions of a developing nervous system, including the retina (*13*). In olfactory epithelium (OE), GDF11 negatively regulates neuron number by causing cell-cycle arrest of the progenitor cells that give rise to olfactory receptor neurons (ORNs) (*14*). Here, we demonstrate that GDF11 is also a negative regulator of neuron number in neural retina, but through a completely different mechanism: GDF11 controls

[1]Department of Anatomy and Neurobiology, [2]Developmental Biology Center, [3]Department of Developmental and Cell Biology, University of California, Irvine, CA 92697, USA. [4]Department of Molecular, Cell, and Developmental Biology, University of California, Los Angeles, CA 90095, USA. [5]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA.

*Present address: Department of Biochemistry, Vanderbilt University, Nashville, TN 37232, USA.
†To whom correspondence should be addressed. Email: alcalof@uci.edu

the period during which retinal progenitor cells are competent to produce certain progeny, thus governing the relative numbers of neural cell types that arise.

In mouse retina, *Gdf11* expression begins about embryonic day (E) 12.5, when RGCs begin to differentiate (Fig. 1A). *Gdf11* mRNA is observed throughout the retina, including the neuroblastic layer (NBL), until at least the first postnatal day (P0), although by E15.5, expression is highest in the developing ganglion cell layer (GCL). Expression of *follistatin* (*Fst*), which encodes a secreted GDF11 antagonist (*15*), is first detected at E13.5. From E15.5 on, *Fst* expression is highest in the nascent GCL but also evident in the NBL and presumptive amacrine cells. Putative receptors for GDF11 (*14*, *16*–*18*) are also expressed in appropriate patterns in the neural retina from E12.5 to 13.5 onward (fig. S1).

To investigate the role of *Gdf11* in retinal development, we examined mice homozygous for the null allele $Gdf11^{tm2}$ (*14*). $Gdf11^{tm2/tm2}$ retinas show obvious changes as early as E14.5, when closure of the optic fissure is incomplete (fig. S2). By E17.5, the presumptive GCL of mutant embryos has an abnormally high cell density, and the inner plexiform layer (IPL), well demarcated in wild-type littermates, is not observed (Fig. 1B). Increased cell density in the mutant GCL is accompanied by widening of the cell layer expressing *Brn3b* [$Gdf11^{tm2/tm2}$, 49.5 ± 3.3 μm (SD); wild-type, 38.5 ± 0.4 μm (SD)], which encodes a POU-domain transcription factor specific for differentiated RGCs (*19*, *20*). By P0, the latest time at which the mutant is viable, $Gdf11^{tm2/tm2}$ GCLs contain ~50% more cells than wild types (Fig. 1C). The excess RGCs that form in $Gdf11^{tm2/tm2}$ animals appear to differentiate normally, extending axons through the optic chiasm and tracts, which also appear abnormally thick (Fig. 1D). By neurofilament immunohistochemistry, we estimate a 37% increase in the cross-sectional areas of optic nerves in $Gdf11^{tm2/tm2}$animals (Fig. 1E and fig. S2).

These changes, observed in all mutant mice examined (>32), imply that *Gdf11* is a negative regulator of RGC genesis. In this respect, the changes in $Gdf11^{tm2/tm2}$ retinas recall those in OE, in which $Gdf11^{tm2/tm2}$ mice also have excess differentiated ORNs (*14*). However, unlike the situation in OE (*14*), $Gdf11^{tm2/tm2}$ retinas display no increase in overall thickness, nor are the distribution or number of proliferating cells significantly altered (fig. S3). These observations suggest that the mechanism by which *Gdf11* regulates neurogenesis in the retina differs from that in the OE.

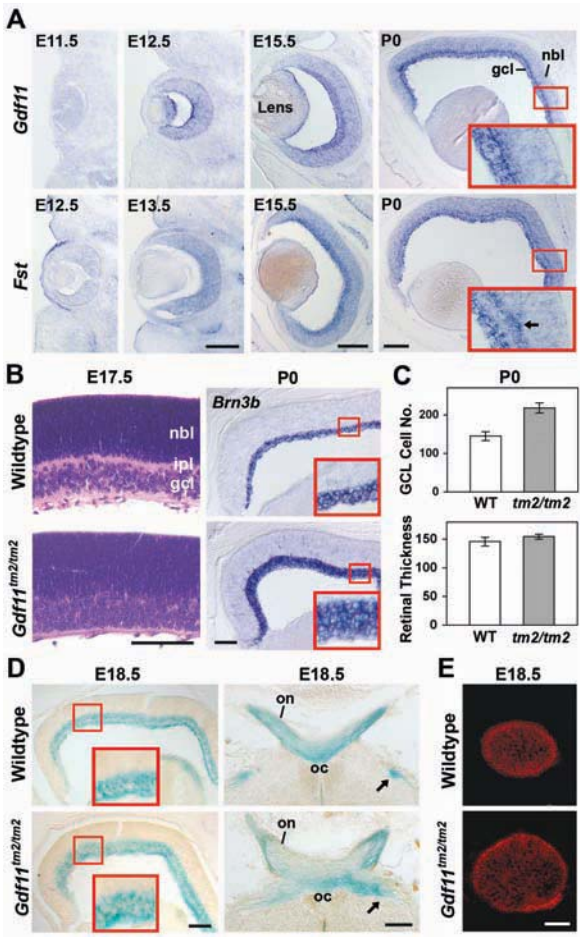

Fig. 1. Retinal abnormalities in *Gdf11* mutants. (**A**) In situ hybridization (ISH) for *Gdf11* and *Fst* in developing mouse retina. nbl, neuroblastic layer; gcl, ganglion cell layer. Arrow in inset indicates *Fst* expression in presumptive amacrine cells. Scale bars, 200 μm. (**B**) Left, hematoxylin-eosin-stained paraffin sections of retina. Right, ISH for *Brn3b*. Insets, higher magnification of $Brn3b^{+}$ gcl . Scale bars, 100 μm. (**C**) Top, increased cell number ($P < 0.01$, Student's *t* test) in $Gdf11^{tm2/tm2}$ retinas. Total cell nuclei in GCL + IPL were counted in 300 μm of central retina in P0 cryosections stained with Hoechst. Bottom, no significant change in central retina thickness. Histograms show mean ± SEM of measurements from 4 to 5 animals of each genotype. (**D**) B-galactosidase (X-gal) staining of sections of $Gdf11^{tm2/tm2}$;*Tattler-1* and $Gdf11^{+/+}$;*Tattler-1* littermate embryos (*33*). Scale bars, 200 μm. on, optic nerve; oc, optic chiasm. Arrows point to optic tract. (**E**) Cross sections of dissected optic nerves stained with antibodies to neurofilament. Scale bar, 50 μm.

Because Fst is known to antagonize GDF11 function in vivo and in vitro (*14*, *15*), we also examined $Fst^{-/-}$ and $Gdf11^{tm2/tm2}$;$Fst^{-/-}$ retinas. $Fst^{-/-}$ retinas showed a 26% reduction in the number of cells in the GCL and a large decrease in thickness of the $Brn3b^{+}$ cell layer (Fig. 2, A and B), which indicates that *Fst* is a positive regulator of RGC development. $Gdf11^{tm2/tm2}$;$Fst^{-/-}$ retinas showed an expanded *Brn3b*+ GCL, comparable to that observed in $Gdf11^{tm2/tm2}$ retinas, consistent with the primary role of Fst as an inhibitor of GDF11 (Fig. 2B). Just as in $Gdf11^{tm2/tm2}$ retinas, the level and pattern of progenitor cell proliferation was unaltered in $Fst^{-/-}$ retinas (fig. S3). The fact that cell proliferation is normal in $Gdf11^{tm2/tm2}$ and $Fst^{-/-}$ retinas suggests that the size of the progenitor pool is not regulated by GDF11. Moreover, expression of several genes involved in early eye specification, patterning, and expansion is also normal in $Gdf11^{tm2/tm2}$ mice (fig. S4).

During development, RGCs are born at the outer margin of the neural retina and migrate inward to the GCL during a defined period (*21*). Detailed examination of $Gdf11^{tm2/tm2}$ and $Gdf11^{tm2/tm2}$;$Fst^{-/-}$ retinas at E17.5 revealed that the NBL of these mutants contains three times as many $Brn3b^{+}$ cells (migrating RGCs) as do wild types (Fig. 2B, insets, and fig. S5). This suggested that, in $Gdf11^{tm2/tm2}$ retinas, RGC production may be prolonged beyond its normal period. To test this, we performed birthdating experiments. The results, shown in Fig. 2C, show an abnormally large number of bromodeoxyuridine-positive ($BrdU^{+}$) cells in the GCL of $Gdf11^{tm2/tm2}$ animals pulsed with BrdU from E15.5 to E17.5. Conversely, $BrdU^{+}$ cells in the GCL of $Fst^{-/-}$ animals pulsed over this same time course were strongly decreased in number, as expected if Fst acts to inhibit GDF11. These differences were not seen when pulse labeling was done at earlier ages (Fig. 2E). Thus, although onset of RGC production appears unaffected by loss of *Gdf11* or *Fst*, its down-regulation is delayed in $Gdf11^{tm2/tm2}$ retinas (and accelerated in $Fst^{-/-}$). A lengthened period of RGC production likely explains why $Gdf11^{tm2/tm2}$ retinas accumulate abnormally large numbers of RGCs.

To determine whether *Gdf11* regulates production of other retinal cell types, we examined rod photoreceptors and amacrine cells, two cell types whose peak periods of differentiation follow that of RGCs. *Crx1*, a marker for early photoreceptors, is normally up-regulated around birth when rod photoreceptor production peaks, and expands to cover much of the NBL (*22*). In $Gdf11^{tm2/tm2}$ retinas, up-regulation and expansion of *Crx1* expression are not observed (Fig. 3A). Amacrine cells may be visualized by expression of syntaxin (*23*), as well as *Pax6* and *Prox1* (*24*, *25*). In the amacrine cell layer of $Gdf11^{tm2/tm2}$ retinas, expression of all three markers was reduced

(Fig. 3A). Altogether, these results suggest that prolonged production of RGCs in *Gdf11*$^{tm2/tm2}$ retinas occurs at the expense of cell types (amacrine cells and photoreceptors) that normally differentiate after RGC production has declined. We observed no obvious increase in amacrine cell or photoreceptor production in *Fst*$^{-/-}$ animals, possibly because excess GDF11 activity in *Fst*$^{-/-}$ retina is mitigated by the reduction in RGC cells, which express the highest levels of *Gdf11* (Fig. 1).

We further tested the idea that GDF11 controls amacrine and photoreceptor cell number, as well as RGC number, by using retinal explant cultures to examine effects of exogenous GDF11 on wild-type retinas (*26*). E13.5 retinal explants grown in GDF11 exhibited a large reduction in *Brn3b*$^{+}$ RGCs, whereas expression of both *Crx1* and syntaxin were increased with GDF11 treatment (Fig. 3B). These findings support the idea that GDF11 is an important regulator of all three retinal cell types.

The finding that RGC genesis is increased, whereas amacrine and rod production are decreased, in *Gdf11* nulls led us to hypothesize that *Gdf11* regulates induction of cell-intrinsic changes by which progenitor cells lose competence to produce RGCs and acquire competence to produce later-born cell types. If GDF11 directly controls progenitor cell competence, *Gdf11* mutants might exhibit changes in expression of factors that determine competence states. *Math5* is among the first such factors expressed during retinal neurogenesis and is required for competence to produce RGCs (*27*–*29*). *Math5* expression is initiated normally in *Gdf11*$^{tm2/tm2}$ retinas, but mutants maintain high levels of expression in the NBL for an abnormally long period. Normally, *Math5* expression is down-regulated in central NBL by E16.5 and is essentially absent by E18.0; in *Gdf11*$^{tm2/tm2}$ retinas, however, *Math5* expression is still evident at these ages (Fig. 4A). Conversely, down-regulation of *Math5* expression occurs prematurely in *Fst*$^{-/-}$ retinas (Fig. 4B) and is accelerated when retinal explants are cultured in GDF11 (Fig. 4C). The prolonged period of *Math5* expression in *Gdf11*$^{tm2/tm2}$ retinas corresponds to the period of prolonged RGC genesis (Fig. 2).

The alteration in the period of *Math5* expression in *Gdf11*$^{tm2/tm2}$ retinas is accompanied by a shift in onset of expression of two other proneural genes, *Mash1* and *NeuroD*, which are involved in the development of bipolar and amacrine cells (*30*, *31*). In *Gdf11*$^{tm2/tm2}$ embryos, expression of both genes is barely detectable at E14.5, when substantial levels are seen in wild types (Fig. 4D). Conversely, *Mash1* expression occurs prematurely in *Fst*$^{-/-}$ retinas, at E13.5, when wild-type littermates express only low levels of *Mash1* (Fig. 4E). By E17.5, both *Mash1*

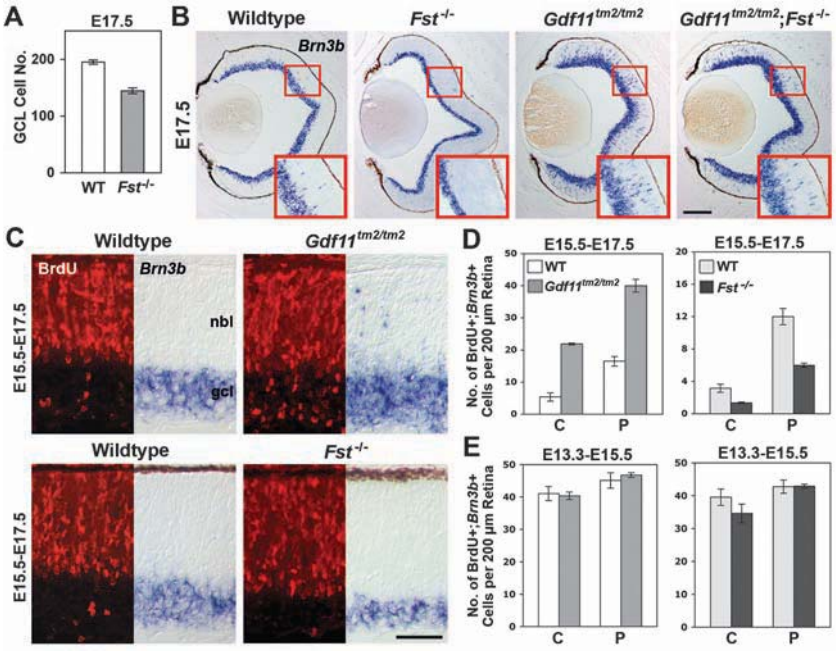

Fig. 2. The period of RGC genesis is altered in *Gdf11*$^{tm2/tm2}$ and *Fst*$^{-/-}$ retinas. (**A**) Total cell nuclei per 300 µm GCL of *Fst*$^{-/-}$ central retina counted after Hoechst staining. (**B**) ISH for *Brn3b* on cryosections of *Fst*$^{-/-}$, *Gdf11*$^{tm2/tm2}$, and *Gdf11*$^{tm2/tm2}$;*Fst*$^{-/-}$ retinas. Insets show superabundance of *Brn3b*$^{+}$ cells in NBL of *Gdf11*$^{tm2/tm2}$ and *Gdf11*$^{tm2/tm2}$;*Fst*$^{-/-}$ retinas. Scale bar, 200 µm. (**C**) Dividing progenitor cells were labeled in vivo by BrdU injection into pregnant dams at E13.5 or E15.5, and retina cryosections (20 µm) were processed 2 days later for *Brn3b* ISH and BrdU immunostaining. Scale bar, 50 µm. (**D**) Quantitative analyses of experiments illustrated in (C). Histograms show mean ± SEM of total *Brn3b*,BrdU double-positive cells in GCL per 200 µm of retina. $N = 2$ to 3 animals of each genotype at each age. $P < 0.05$ (Student's t test, all comparisons). C, central retina; P, peripheral retina (200 µm from peripheral margin). (**E**) Quantitative analysis of RGC birthdating following BrdU injection at E13.5 and analysis at E15.5. No significant differences were observed.

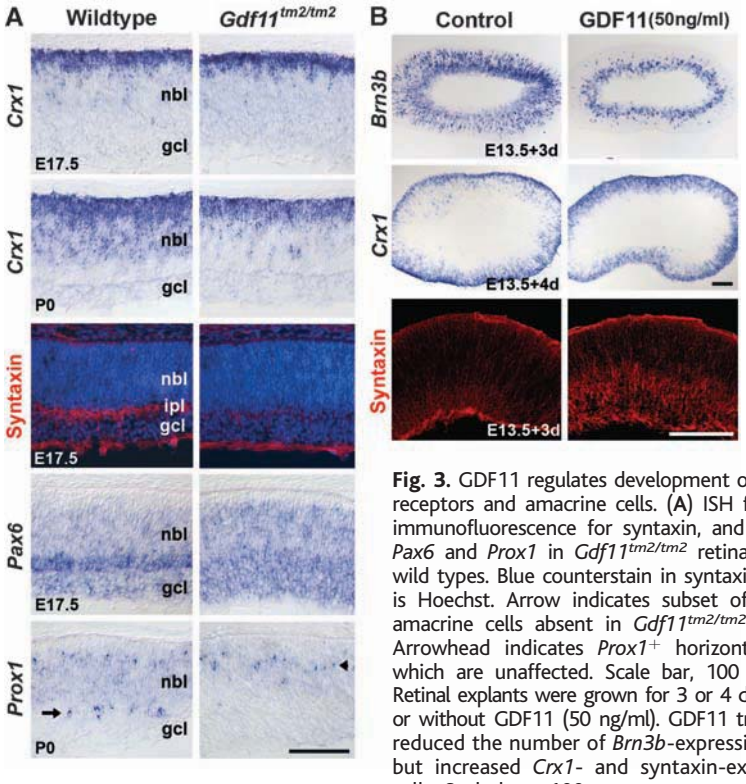

Fig. 3. GDF11 regulates development of photoreceptors and amacrine cells. (**A**) ISH for *Crx1*, immunofluorescence for syntaxin, and ISH for *Pax6* and *Prox1* in *Gdf11*$^{tm2/tm2}$ retinas versus wild types. Blue counterstain in syntaxin panels is Hoechst. Arrow indicates subset of *Prox1*$^{+}$ amacrine cells absent in *Gdf11*$^{tm2/tm2}$ retinas. Arrowhead indicates *Prox1*$^{+}$ horizontal cells, which are unaffected. Scale bar, 100 µm. (**B**) Retinal explants were grown for 3 or 4 days with or without GDF11 (50 ng/ml). GDF11 treatment reduced the number of *Brn3b*-expressing cells, but increased *Crx1*- and syntaxin-expressing cells. Scale bars, 100 µm.

and *NeuroD* expression recover to normal levels in *Gdf11*$^{tm2/tm2}$ retinas (Fig. 4D), which suggests that progenitor cells can acquire competence to produce later-born cell types even though *Math5* expression (and RGC genesis) remain elevated. Altogether, these observations suggest that GDF11 regulates the timing of progenitor competence by controlling the expression of genes crucial for progenitor cell fate determination.

Does GDF11 regulate generation of all retinal cell types, or only some? Because *Gdf11*$^{tm2/tm2}$ animals die at birth (*14*), this question cannot yet be answered with certainty. Expression of Lim1, a horizontal cell-specific transcription factor (*32*), appears to be normal in *Gdf11*$^{tm2/tm2}$ retinas (fig. S6), although changes in expression of a number of other regulatory genes expressed by retinal progenitors are observed (fig. S7). However, the absence of an effect on horizontal cells indicates that GDF11 signaling does not regulate production of all cell types in the retina. Instead, it must govern either a specific subprogram of retinal neurogenesis or act on only a subset of multipotent progenitor cells. This last idea suggests that early retinal progenitors, despite possessing the potential to give rise to all retinal cell types, are nonetheless heterogeneous, at least with respect to their capacity to respond to GDF11.

Our finding that GDF11 governs retinal progenitor cell fate without altering proliferation supports the idea—suggested by retroviral lineage studies—that regulation of cell division and cell-type determination occur independently in the retina (*2*). Moreover, our results highlight the difference in feedback mechanisms employed in different regions of the developing nervous system to effect proper neuron number. In retina, feedback regulation of neural cell number, mediated by GDF11 expressed by the earliest born neurons, is accomplished by altering the fates of multipotent progenitor cells independent of proliferation. In other regions, such as OE, neuronal GDF11 feeds back to regulate progenitor cell proliferation, independent of changes in cell fate (*14*). Finally, these studies demonstrate the diversity of action of GDF11 itself. In OE, GDF11 exerts its anti-neurogenic action by inducing reversible cell-cycle arrest in committed progenitors through increased expression of the cyclin-dependent kinase inhibitor p27^{Kip1} (*14*). In retina, by contrast, GDF11 controls the time course of expression of genes that regulate competence to produce RGCs, but neither p27^{Kip1} levels nor cell proliferation are affected (figs. S3 and S8). Thus, GDF11 acts as a negative feedback regulator of neurogenesis during development by altering either progenitor cell proliferation or progenitor cell fate in different tissues.

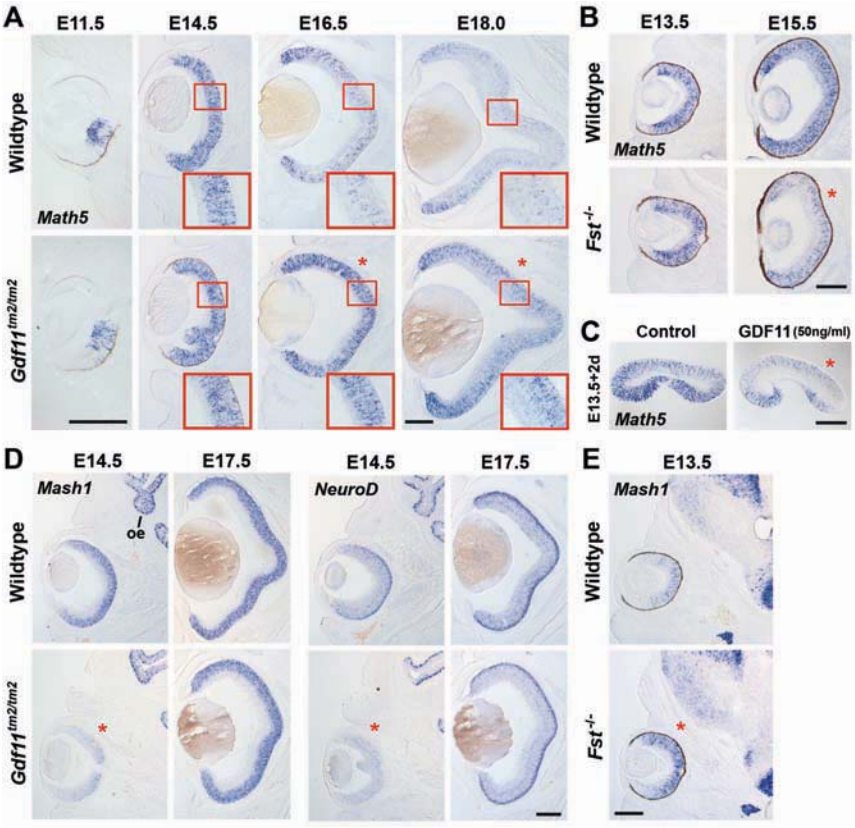

Fig. 4. GDF11 and Fst regulate temporal changes in *Math5* expression. (**A**) ISH for *Math5* in retinas of *Gdf11*$^{tm2/tm2}$ and wild-type littermates. (**B**) ISH for *Math5* expression in *Fst*$^{-/-}$ and wild-type littermate retinas. (**C**) E13.5 retinal explants grown in presence or absence of GDF11 (50 ng/ml) for 2 days (E13.5 + 2 days), then hybridized with probe to *Math5*. (**D**) Expression of *Mash1* and *NeuroD* in retina in *Gdf11*$^{tm2/tm2}$ mice and wild-type littermates. oe, olfactory epithelium. (**E**) Early onset of *Mash1* expression in *Fst*$^{-/-}$ retinas. Asterisks in (A) to (E) indicate reproducible differences from wild-type controls. Scale bars, 200 μm.

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33. Materials and methods are available as supporting material on *Science* Online.

34. This work was supported by the March of Dimes Birth Defects Foundation, NIH grants DC03583 and HD38761 to A.L.C. A.D.L. is supported by HD38761, K.M.L. by AR44528, and M.M.M. by HD32067.

Supporting Online Material

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25 January 2005; accepted 29 April 2005
10.1126/science.1110175