Published in final edited form as:

Exp Eye Res. 2014 February ; 119: 106–110. doi:10.1016/j.exer.2013.11.003.

The *Lgr5* Transgene is Expressed Specifically in Glycinergic Amacrine Cells in the Mouse Retina

Kumar Sukhdeo^{1,2}, Catherine E. Koch¹, Tyler E. Miller¹, Hannah Zhou¹, Maricruz Rivera^{1,3}, Kenneth Yan¹, Constance L. Cepko⁴, Justin D. Lathia⁵, and Jeremy N. Rich^{1,*}

¹Department of Stem Cell Biology and Regenerative Medicine, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195

²Department of Pathology, Case Western Reserve University School of Medicine, Cleveland, OH 44106

³Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine at Case Western Reserve University, Cleveland, OH 44195

⁴Department of Genetics, Harvard Medical School, Boston, MA 02115

⁵Department of Cellular and Molecular Medicine, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195

Abstract

Retinal amacrine cells are a diverse set of interneurons within the inner nuclear layer. The canonical Wnt pathway is highly active within mature amacrine cells, but its role remains unclear. Leucine-rich repeat containing G-protein receptor 5 (*Lgr5*) is a newly identified component of the Wnt receptor complex that potentiates beta-catenin signaling. In multiple epithelial organs *Lgr5* marks adult tissue stem cells. We investigated the expression of this gene using *Lgr5-eGFP-IRES-CreER* transgenic reporter mice. In the eye, *Lgr5* was exclusively expressed in glycinergic amacrine cells in adult mice. Amacrine cells are post-mitotic and represent the first neuronal and non-stem cell lineage to express *Lgr5*. We further interrogated the spatiotemporal labeling of individual amacrine cells with controlled fluorophore expression. This “fluorofilling” technique provides a tool to study amacrine morphology and dissect neural networks.

The retina is a multilayered tissue comprised of Müller glia and six major neuronal cell types: rods, cones, bipolar, horizontal, ganglion and amacrine cells. Amacrine cells (ACs), located in the inner nuclear layer (INL), function to fine tune visual signals among bipolar and ganglion cells. They are among the most diverse neuronal type in the retina and demonstrate remarkable functional and morphological diversity. An estimated 20–40 distinct amacrine types exist classified by the span of their receptive field, depth of penetration into the inner plexiform layer (IPL) and type of neurotransmitter used (MacNeil and Masland, 1998).

*Correspondence: Jeremy N. Rich, M.D., Chair, Department of Stem Cell Biology and Regenerative Medicine, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Ave., NE3-301, Cleveland OH 44195, richj@ccf.org, P: (216) 636-0790, F: (216) 636-5454.

The highly patterned structure of the retina is developed and maintained as a result of tightly regulated intrinsic and extrinsic signals that vary within the tissue and across time. Among these cues, both canonical (β -catenin-dependent) and non-canonical Wnt signaling are active in the embryonic and adult eye. Activation of this pathway occurs mostly in a paracrine fashion as a means of cell-cell communication (Logan and Nusse, 2004). Secreted extracellular Wnt glycoproteins (Wnts) bind to surface receptors (Frizzled) on target cells to regulate the phosphorylation and activity of the co-receptors, LRP5 or LRP6. This results in a cascade of intracellular events that culminate in the liberation of cytosolic β -catenin from either cell surface sequestration or proteasomal degradation. β -catenin then translocates into the nucleus as a transcriptional cofactor to turn on the expression of Wnt target genes (Logan and Nusse, 2004). A number of Wnt agonistic and antagonistic (Secreted frizzled-related protein, Sfrps) ligands as well as receptors display coordinated and restricted expression within the retinal layers (Chang et al., 1999; Liu et al., 2003; Liu et al., 2006; Rattner et al., 1997; Wang et al., 1996). Using a canonical Wnt signaling-dependent reporter system, Liu et al. traced the dynamic activation of this pathway in the embryonic eye in the ciliary margin and prospective ciliary epithelium (Liu et al., 2003; Liu et al., 2006). Wnt reporter activity was seen in postnatal and adult retinas within the non-pigmented ciliary epithelium, retinal ganglion layer, and a subset of amacrine cells in the INL (Liu et al., 2006).

In this study, we examined the expression of a newly identified component of the canonical Wnt signaling pathway, Leucine-rich repeat containing G-protein receptor 5 (Lgr5). This gene was identified in the mouse intestine within an actively dividing population of stem cells at the base of the crypt that exclusively express the Wnt target gene *Lgr5* (Barker et al., 2010; Barker et al., 2007). In a series of elegant studies, Lgr5-positive intestinal stem cells were shown to reside long-term in the crypt, self-renew, and give rise to all populations of the intestinal mucosa (Barker et al., 2010; Barker et al., 2007; Snippert et al., 2010). These studies used a knock-in eGFP reporter allele under the control of the endogenous *Lgr5* promoter (*Lgr5-eGFP-IRES-CreER2*; hereafter *Lgr5^{eGFP/CreER}*). Remarkably, a single Lgr5-positive cell, identified by high eGFP expression in *Lgr5^{eGFP/CreER}* mouse intestines, is able to generate an entire crypt-villus organoid *in vitro* and functionally engraft *in vivo*, thus confirming the stem cell nature of these cells (Yui et al., 2012). Lgr5 also marks additional stem cell populations in other epithelial tissues, including the stomach, hair follicle, cochlea, embryonic kidney, mammary gland, uterus, and tooth bud (Schuijers and Clevers, 2012). Additionally, Lgr5-positive cells are the putative cells-of-origin and cancer stem cells in colon cancer (Schepers et al., 2012).

Lgr5 is closely related to its family members Lgr4 and Lgr6 and functional redundancy may be present among these molecules based on targeted deletion studies. Identification of Lgr5 ligands proved elusive until the R-spondin (R-spo) family of glycoproteins was found to modulate Wnt signaling through Lgr5 (Carmon et al., 2011; Gong et al., 2012; Ruffner et al., 2012; Schuijers and Clevers, 2012). R-spo binding to Lgr5 as part of the Wnt receptor complex causes an immediate increase in the amount of active β -catenin, followed by rapid degradation (Carmon et al., 2011). The precise role of this regulation of β -catenin by Lgr5 and perhaps other unknown signaling sequelae in stem cells is unknown.

To date, *Lgr5* has been primarily characterized in endodermally-derived tissues (Schuijers and Clevers, 2012). We hypothesized that *Lgr5* might be present within the retina in the same domain as other Wnt pathway members. Our analysis utilized *Lgr5^{eGFP/CreER}* mice to examine expression in this tissue.

In freshly isolated tissue the eGFP reporter signal was visible however fixation with paraformaldehyde quenched a majority of the fluorescence, a known limitation of eGFP in some applications (Morris et al., 2010). The signal was rescued in tissue sections by staining with an anti-GFP antibody that gave robust labeling indicative of *Lgr5* expression. Detection of *Lgr5* expression was also attempted with commercially available antibodies however none were successful in identifying endogenous levels in mouse tissue (data not shown).

Examination of *Lgr5*/eGFP in the retinas of *Lgr5^{eGFP/CreER}* transgenic mice revealed expression in a restricted subset of retinal cells residing in the innermost region of the INL (Figure 1A). These cells were morphologically distinct from bipolar and horizontal cells but were consistent with ACs on the basis of their small, pyramidal INL somata and axonal projections that extended into all layers of the IPL (Figure 1B).

To confirm the identity of the *Lgr5*-positive cells as amacrine, immunostaining for syntaxin, a pan-AC marker, was performed (Barnstable et al., 1985). Out of the *Lgr5*-positive cells, 99.5% (1841 of 1851 imaged cells) were also syntaxin-positive (Figure 1C and 1D, left). Interestingly, only 48% (1841 of 3835 imaged cells) of syntaxin-positive ACs were positive for *Lgr5*, suggesting that *Lgr5* marks only a subset of ACs. ACs can be further distinguished by their neurotransmitter expression - roughly half are glycinergic and half GABAergic (Cherry et al., 2009; Vardi and Auerbach, 1995). To determine if *Lgr5* marked either of these AC neurotransmitter subsets, immunostaining with GlyT1, a glycinergic amacrine-specific marker, was performed (Menger et al., 1998). Approximately half of the ACs were positive for GlyT1 while *Lgr5*-positive cells demonstrated near complete overlap with the marker (Figure 1C). In fact, 99% of the *Lgr5*-positive cells were positive for GlyT1 (1286 of 1299) and 99.8% of GlyT1-positive cells were positive for *Lgr5* (1286 of 1288) (Figure 1D). Immunostaining with GAD65/67, a GABAergic amacrine-specific marker, stained the remaining half of the ACs but was absent from the *Lgr5*-positive cells (Figure 1C right) (Vardi and Auerbach, 1995). Only 1.7% (12 of 883) of the *Lgr5*-positive cells were also positive for GAD65/67 (Figure 1D right). Taken together, these results support *Lgr5* as a specific marker of glycinergic ACs.

In the retina, Wnt signaling is dynamically regulated during early development but relatively stable in the INL from P7 onward (Liu et al., 2003; Liu et al., 2006). Consistent with prior reports of Wnt pathway activity in development and the relationship of *Lgr5* to canonical Wnt signaling, *Lgr5* expression was not detected in embryonic *Lgr5^{eGFP/CreER}* retinas (E8.5 onward; data not shown). In neonatal mice, expression of *Lgr5* was first detectable at P4 with relatively low expression levels in scattered cells of the developing INL (Figure 2A). The intensity of expression and number of *Lgr5*-expressing cells increased over the first week of life and expression at P7 was nearly equivalent with the pattern seen at P150 in fully adult mice (Figure 2A). Our findings were corroborated by *in silico* interrogation of developmental retinal cell populations at the single cell level by gene expression profiling.

Lgr5 mRNA expression was activated in developing ACs around P4 (Figure 2B) (Cherry et al., 2009; Dyer and Cepko, 2001; Kim et al., 2008; Roesch et al., 2008; Trimarchi et al., 2008; Trimarchi et al., 2007).

To examine the effects of *Lgr5* loss-of-function in the retina, we interbred heterozygous *Lgr5^{eGFP/CreER}* mice because the knock-in allele disrupts the first exon and eliminates *Lgr5* transcripts from this locus. Therefore, homozygous *Lgr5^{eGFP/CreER}* mice are *Lgr5*-null. In accordance with previous reports of *Lgr5* knockout mice of animals (Morita et al., 2004), homozygous *Lgr5^{eGFP/CreER}* mice died within 12 hours after birth, which is well before *Lgr5* expression is activated in the retina and precluded an examination of the phenotype. Accordingly, homozygous-null P0 retinas were identical to either wildtype or heterozygous animals (data not shown).

Lgr5^{eGFP/CreER} mice harbor the bi-cistronic *Lgr5-eGFP-IRES-CreER2* allele that, in addition to eGFP, also produces an inducible CreER recombinase that is inert in the absence of the synthetic estrogen tamoxifen. Upon binding to experimentally-delivered hormone, CreER translocates into the nucleus, whereupon it recognizes *loxP* sites to either excise or invert the intervening DNA sequence. This provides a useful tool for spatiotemporal control to genetically manipulate a gene-of-interest within CreER-expressing cells.

We crossed *Lgr5^{eGFP/CreER}* mice with a *Rosa26-loxP-STOP-loxP-dTomato* reporter strain (Madisen et al., 2010). The *Rosa26* locus is highly active in almost all tissues. Expression of dTomato red fluorescent protein is prevented by a preceding STOP element until it is removed by CreER recombinase. No dTomato fluorescence was seen in either wildtype or untreated adult *Lgr5^{eGFP/CreER};Rosa26-loxP-STOP-loxP-dTomato* mice as expected. In contrast, *Lgr5^{eGFP/CreER};Rosa26-loxP-STOP-loxP-dTomato* animals given tamoxifen underwent recombination in *Lgr5*-expressing cells and were rendered dTomato-positive. The stochastic nature of CreER recombination following low dose tamoxifen administration resulted in a subset of *Lgr5*-positive retinal ACs inducing the expression of dTomato (Figure 2C). In contrast to the amalgamation of eGFP signal from *Lgr5*-positive ACs, we were able to exploit this technical aspect in a process we termed ‘fluorofilling’ to define the morphology and specific dendritic branches within the IPL of individual dTomato-positive ACs (Figure 2C). This process could be used as an alternative to the photofilling method devised by Masland and others to label individual cells in order to subclassify AC subtypes using manually injected dyes (MacNeil and Masland, 1998; Menger et al., 1998). We observed dTomato-positive cells in the form of small-field AC subtypes including AII, Flag A, and Flag B, consistent with previous description of glycinergic ACs (Figure 2D) (Menger et al., 1998; Weiss et al., 2008). Collectively, these data show the utility of the *Lgr5* mouse model to selectively label and characterize glycinergic amacrine cells.

Lgr5 has garnered a significant amount of attention due to its restricted expression within the stem cell compartments of several adult somatic tissues. The study of this gene has been facilitated greatly by the availability of the *Lgr5^{eGFP/CreER}* reporter mouse developed by the Clevers lab. Moreover, we confirmed *Lgr5* expression in glycinergic amacrine cells using an independently-derived *Lgr5/eGFP* reporter strain (Tian et al., 2011). Our findings represent the first in-depth description of *Lgr5* expression in neuronal cells, following a preliminary

analysis of the gene using *in situ* hybridization (Hermey et al., 1999). The role of Lgr5 in neuronal cells is unclear as Wnt plays a myriad of roles in different tissues. One possibility is that Lgr5 may affect Wnt-dependent synaptogenesis in these cells that possess elaborate dendritic arborization (Park and Shen, 2012). The restricted expression of Lgr5 in the INL matches closely with the expression of Frizzled receptors, Sfrp, and Wnts, suggesting that Wnt signaling helps maintain homeostasis in these ACs and that Lgr5 may function as a regulator of this pathway (Liu et al., 2003; Liu et al., 2006). However, we did not evaluate a functional link between Lgr5 and the Wnt signaling so it remains possible that Lgr5 may regulate alternative pathways in this tissue.

ACs are the most diverse type of neuronal cell in the mammalian retina and each subtype serves a specific function in achieving appropriate visual signaling. Defining the precise networks between cell types and retinal layers will require a better understanding of the connectivity of these cells. In this regard, additional fluorophores can be used for fluorofilling individual cells utilizing other reporter strains such as *Confetti* mice (Snippert et al., 2010).

Currently only a limited number of specific AC markers have been identified (reviewed in (Dyer and Cepko, 2001)). Syntaxin, ChAT, calbindin, and parvalbumin are expressed in ACs as well as other retinal cell types. Similar to Lgr5, p57^{Kip}, calretinin, and tyrosine hydroxylase are only expressed in ACs. Our data show that Lgr5 is specifically expressed in glycinergic (GlyT1-positive) ACs and can be used as a marker for their identification. However, due to inadequate commercially available antibodies such studies are dependent on the usage of the *Lgr5* transgenic mice.

Glycinergic ACs are generated from retinal progenitor cells (RPCs) around E18, but do not display GlyT1 expression until P4 (Cherry et al., 2009). The majority of cells in the neonatal rodent retina are post-mitotic and those that are proliferating mostly generate photoreceptors (Alexiades and Cepko, 1996). The Lgr5-positive AC populations identified here likely do not serve as stem cells. Our data raise the possibility Lgr5 may function in a broader capacity to regulate Wnt and/or other signaling in stem and non-stem cell populations during mammalian development and homeostasis. These effects may be mediated through β -catenin (Schuijers and Clevers, 2012) or additional mechanisms yet to be defined. Proper regulation of the Wnt pathway in cells with high Wnt activity may be critical in balancing cellular maintenance rather than uncontrolled growth. In concordance, high levels of Lgr5 expression are found in malignancies with mutated Wnt pathway components, including β -catenin and the R-spondin ligand (Braeuning et al., 2007; McClanahan et al., 2006; Seshagiri et al., 2012; Tanese et al., 2008; Yamamoto et al., 2003).

Study of the Wnt pathway in the retina and neuronal tissues will also be informative for the understanding of disease states. Multiple pathologies are associated with deregulated Wnt signaling in the eye including familial exudative vitreoretinopathy, congenital hypertrophy of the retinal pigmented epithelium, Norrie's disease, and coloboma (de Jongh et al., 2006; Lad et al., 2009). Moreover, activation of the Wnt pathway is seen in response to retinal injury (Yi et al., 2007). As shown here, using *Lgr5^{eGFP/CreER}* mice and Cre-lox technology,

investigators can dissect amacrine cell diversity and manipulate these cells to assess the phenotype of selective perturbations.

All animal experiments were approved by the Cleveland Clinic Institutional Animal Care and Use Committee. *Lgr5-eGFP-IRES-CreER2* (Stock No. 008875) (Barker et al., 2007) and *Rosa26-lox-STOP-lox-tdTomato* (Stock No. 007914) mice were acquired from Jackson Labs. Double transgenic mice were heterozygous for *Lgr5^{GFP/CreER}* and homozygous for *Rosa26-lox-STOP-lox-tdTomato*. Standard PCR genotyping was performed according to the vendor's website. *Lgr5* homozygous-null animals were generated by breeding pairs of heterozygous *Lgr5^{GFP/CreER}* mice. Recombination was induced by administering 200 mg/kg body weight tamoxifen to 8–12 week old *Lgr5^{GFP/CreER}; Rosa26-loxP-STOP-loxP-tdTomato* mice (Barker and Clevers, 2010).

Tissues were fixed in 4% paraformaldehyde overnight and then transferred to 30% sucrose (w/v) for another 24 hours. Tissue was then embedded in Optimal Cutting Temperature (OCT) compound and sectioned at 7 microns on a Leica cryostat. Slides were washed in blocking buffer (PBS, 5% goat serum, 0.1% TritonX-100) for 10 minutes before the primary antibody (chicken anti-GFP IgY fraction, 1:250; Aves Labs, Inc.) was applied. The slides were then placed in a humidity chamber at room temperature for an hour, washed twice for 5 minutes in PBS and once in blocking buffer for 5 minutes. The secondary antibody (goat anti-chicken conjugated to AlexaFluor 488; Invitrogen) at 1:250 dilution was then applied to the slides, which were then placed in the humidity chamber for 1 hour at room temperature. Slides were kept in the dark and again washed twice in PBS for 5 minutes and once in blocking buffer for 5 minutes. Double immunofluorescence was done as above with antibodies: Syntaxin (Sigma clone HPC-1 S0664) 1:500; GAD65/67 (Abcam ab26113) 1:100; GlyT1 (Millipore AB1770) 1:100. Slides were mounted using Fluoromount Reagent (Calbiochem) mounting media and were stored at 4°C in the dark. Images were captured and processed as previously described (Lathia et al., 2010). Retinal tissue was harvested from *Lgr5^{GFP/CreER}; Rosa26-loxP-STOP-loxP-tdTomato* mice 5 days after tamoxifen injection. Retinal cell counts were performed by collecting at least 15 high-power fields each from 3 different mice. Individual cells were tallied manually by two independent observers. Undeterminable cells accounted for less than 1% of all counts and were not included in the final numbers.

Gene expression microarray analysis and identification of cellular lineages was performed using previously established datasets (Cherry et al., 2009; Kim et al., 2008; Roesch et al., 2008; Trimarchi et al., 2008; Trimarchi et al., 2007). For retinal stages E12 through P0, retinal cell microarrays were first clustered around known marker genes for retinal RPCs (neurofilament light; *NF68*), PRs (neural retina leucine zipper, *Nrl*), and ACs (transcription factor AP-2 beta; *TCFAP-2b*) to define a genetic signature composed of dozens of genes (Trimarchi et al., 2008; Trimarchi et al., 2007). For the identification of retinal cells from later time points, ACs were identified on the basis of expression of known AC markers (e.g. *Tcfap2b*, *Pax6*) and the absence of marker expression from other retinal cell types (rods: *Rho*, *Gcap2*; bipolar: *Cabp5*, *Og9x*; Muller glia: *ApoE*, *Aqp4*; ganglion: *Nefl*, *Pou4f2*; progenitor: *Sfrp2*, *Fgf15*). Once identified as ACs, another signature gene list was generated that could robustly identify specific cellular lineages (Cherry et al., 2009). A similar

approach was taken to identify genetic signatures for Muller glia (Roesch et al., 2008) and photoreceptors (Kim et al., 2008) using the inclusionary and exclusionary genes listed above. Normalized expression values for Lgr5 (Unigene ID 42103) were generated across samples and a value 1000 was used as a cutoff for positive expression.

Acknowledgments

K.S. is supported by the Case Western Reserve University MSTP, NIH T32 GM007250, and a Ruth Kirchstein National Research Service Award (NIH-NCI F30 CA165857). J.N.R. is funded National Institutes of Health grants CA112958, CA116659, and CA154130. We thank Dr. Neil Peachey (CCF), Dr. Andrew Fisher (Ohio State), and Dr. Richard Masland (Harvard) for valuable comments during preparation of the manuscript.

References

- Alexiades MR, Cepko C. Quantitative analysis of proliferation and cell cycle length during development of the rat retina. *Dev Dyn.* 1996; 205:293–307. [PubMed: 8850565]
- Barker N, Clevers H. Lineage tracing in the intestinal epithelium. *Curr Protoc Stem Cell Biol.* 2010; Chapter 5(Unit5A):4. [PubMed: 20443207]
- Barker N, Huch M, Kujala P, van de Wetering M, Snippert HJ, van Es JH, Sato T, Stange DE, Begthel H, van den Born M, et al. Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell Stem Cell.* 2010; 6:25–36. [PubMed: 20085740]
- Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ, Clevers H. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature.* 2007; 449:1003–1007. [PubMed: 17934449]
- Barnstable CJ, Hofstein R, Akagawa K. A marker of early amacrine cell development in rat retina. *Brain Res.* 1985; 352:286–290. [PubMed: 3896407]
- Braeuning A, Ittrich C, Kohle C, Buchmann A, Schwarz M. Zonal gene expression in mouse liver resembles expression patterns of Ha-ras and beta-catenin mutated hepatomas. *Drug Metab Dispos.* 2007; 35:503–507. [PubMed: 17220236]
- Carmon KS, Gong X, Lin Q, Thomas A, Liu Q. R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/{beta}-catenin signaling. *Proceedings of the National Academy of Sciences of the United States of America.* 2011; 108:11452–11457. [PubMed: 21693646]
- Chang JT, Esumi N, Moore K, Li Y, Zhang S, Chew C, Goodman B, Rattner A, Moody S, Stetten G, et al. Cloning and characterization of a secreted frizzled-related protein that is expressed by the retinal pigment epithelium. *Hum Mol Genet.* 1999; 8:575–583. [PubMed: 10072424]
- Cherry TJ, Trimarchi JM, Stadler MB, Cepko CL. Development and diversification of retinal amacrine interneurons at single cell resolution. *Proceedings of the National Academy of Sciences of the United States of America.* 2009; 106:9495–9500. [PubMed: 19470466]
- de Jongh RU, Abud HE, Hime GR. WNT/Frizzled signaling in eye development and disease. *Frontiers in bioscience: a journal and virtual library.* 2006; 11:2442–2464. [PubMed: 16720326]
- Dyer MA, Cepko CL. The p57Kip2 cyclin kinase inhibitor is expressed by a restricted set of amacrine cells in the rodent retina. *J Comp Neurol.* 2001; 429:601–614. [PubMed: 11135238]
- Gong X, Carmon KS, Lin Q, Thomas A, Yi J, Liu Q. LGR6 is a high affinity receptor of R-spondins and potentially functions as a tumor suppressor. *PLoS One.* 2012; 7:e37137. [PubMed: 22615920]
- Hermey G, Methner A, Schaller HC, Hermans-Borgmeyer I. Identification of a novel seven-transmembrane receptor with homology to glycoprotein receptors and its expression in the adult and developing mouse. *Biochemical and biophysical research communications.* 1999; 254:273–279. [PubMed: 9920770]
- Jaks V, Barker N, Kasper M, van Es JH, Snippert HJ, Clevers H, Toftgard R. Lgr5 marks cycling, yet long-lived, hair follicle stem cells. *Nat Genet.* 2008; 40:1291–1299. [PubMed: 18849992]
- Kim DS, Ross SE, Trimarchi JM, Aach J, Greenberg ME, Cepko CL. Identification of molecular markers of bipolar cells in the murine retina. *J Comp Neurol.* 2008; 507:1795–1810. [PubMed: 18260140]

- Lad EM, Cheshier SH, Kalani MY. Wnt-signaling in retinal development and disease. *Stem Cells Dev.* 2009; 18:7–16. [PubMed: 18690791]
- Lathia JD, Gallagher J, Heddleston JM, Wang J, Eyler CE, Macswords J, Wu Q, Vasanji A, McLendon RE, Hjelmeland AB, Rich JN. Integrin alpha 6 regulates glioblastoma stem cells. *Cell Stem Cell.* 2010; 6:421–432. [PubMed: 20452317]
- Liu H, Mohamed O, Dufort D, Wallace VA. Characterization of Wnt signaling components and activation of the Wnt canonical pathway in the murine retina. *Dev Dyn.* 2003; 227:323–334. [PubMed: 12815618]
- Liu H, Thurig S, Mohamed O, Dufort D, Wallace VA. Mapping canonical Wnt signaling in the developing and adult retina. *Invest Ophthalmol Vis Sci.* 2006; 47:5088–5097. [PubMed: 17065530]
- Logan CY, Nusse R. The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol.* 2004; 20:781–810. [PubMed: 15473860]
- MacNeil MA, Masland RH. Extreme diversity among amacrine cells: implications for function. *Neuron.* 1998; 20:971–982. [PubMed: 9620701]
- Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, Ng LL, Palmiter RD, Hawrylycz MJ, Jones AR, et al. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci.* 2010; 13:133–140. [PubMed: 20023653]
- McClanahan T, Koseoglu S, Smith K, Grein J, Gustafson E, Black S, Kirschmeier P, Samatar AA. Identification of overexpression of orphan G protein-coupled receptor GPR49 in human colon and ovarian primary tumors. *Cancer Biol Ther.* 2006; 5:419–426. [PubMed: 16575208]
- Menger N, Pow DV, Wassle H. Glycinergic amacrine cells of the rat retina. *J Comp Neurol.* 1998; 401:34–46. [PubMed: 9802699]
- Morita H, Mazerbourg S, Bouley DM, Luo CW, Kawamura K, Kuwabara Y, Baribault H, Tian H, Hsueh AJ. Neonatal lethality of LGR5 null mice is associated with ankyloglossia and gastrointestinal distension. *Mol Cell Biol.* 2004; 24:9736–9743. [PubMed: 15509778]
- Morris LM, Klanke CA, Lang SA, Lim FY, Crombleholme TM. TdTomato and EGFP identification in histological sections: insight and alternatives. *Biotech Histochem.* 2010; 85:379–387. [PubMed: 20109099]
- Park M, Shen K. WNTs in synapse formation and neuronal circuitry. *Embo J.* 2012; 31:2697–2704. [PubMed: 22617419]
- Rattner A, Hsieh JC, Smallwood PM, Gilbert DJ, Copeland NG, Jenkins NA, Nathans J. A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors. *Proceedings of the National Academy of Sciences of the United States of America.* 1997; 94:2859–2863. [PubMed: 9096311]
- Roesch K, Jadhav AP, Trimarchi JM, Stadler MB, Roska B, Sun BB, Cepko CL. The transcriptome of retinal Muller glial cells. *J Comp Neurol.* 2008; 509:225–238. [PubMed: 18465787]
- Ruffner H, Sprunger J, Charlat O, Leighton-Davies J, Grosshans B, Salathe A, Zietzling S, Beck V, Therier M, Isken A, et al. R-Spondin potentiates Wnt/beta-catenin signaling through orphan receptors LGR4 and LGR5. *PLoS One.* 2012; 7:e40976. [PubMed: 22815884]
- Schepers AG, Snippert HJ, Stange DE, van den Born M, van Es JH, van de Wetering M, Clevers H. Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. *Science.* 2012; 337:730–735. [PubMed: 22855427]
- Schuijers J, Clevers H. Adult mammalian stem cells: the role of Wnt, Lgr5 and R-spondins. *Embo J.* 2012; 31:2685–2696. [PubMed: 22617424]
- Seshagiri S, Stawiski EW, Durinck S, Modrusan Z, Storm EE, Conboy CB, Chaudhuri S, Guan Y, Janakiraman V, Jaiswal BS, et al. Recurrent R-spondin fusions in colon cancer. *Nature.* 2012; 488:660–664. [PubMed: 22895193]
- Snippert HJ, van der Flier LG, Sato T, van Es JH, van den Born M, Kroon-Veenboer C, Barker N, Klein AM, van Rheenen J, Simons BD, Clevers H. Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. *Cell.* 2010; 143:134–144. [PubMed: 20887898]
- Tanese K, Fukuma M, Yamada T, Mori T, Yoshikawa T, Watanabe W, Ishiko A, Amagai M, Nishikawa T, Sakamoto M. G-protein-coupled receptor GPR49 is up-regulated in basal cell

- carcinoma and promotes cell proliferation and tumor formation. *Am J Pathol.* 2008; 173:835–843. [PubMed: 18688030]
- Tian H, Biehs B, Warming S, Leong KG, Rangell L, Klein OD, de Sauvage FJ. A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable. *Nature.* 2011; 478:255–259. [PubMed: 21927002]
- Trimarchi JM, Stadler MB, Cepko CL. Individual retinal progenitor cells display extensive heterogeneity of gene expression. *PLoS One.* 2008; 3:e1588. [PubMed: 18270576]
- Trimarchi JM, Stadler MB, Roska B, Billings N, Sun B, Bartch B, Cepko CL. Molecular heterogeneity of developing retinal ganglion and amacrine cells revealed through single cell gene expression profiling. *J Comp Neurol.* 2007; 502:1047–1065. [PubMed: 17444492]
- Vardi N, Auerbach P. Specific cell types in cat retina express different forms of glutamic acid decarboxylase. *J Comp Neurol.* 1995; 351:374–384. [PubMed: 7706548]
- Wang Y, Macke JP, Abella BS, Andreasson K, Worley P, Gilbert DJ, Copeland NG, Jenkins NA, Nathans J. A large family of putative transmembrane receptors homologous to the product of the *Drosophila* tissue polarity gene *frizzled*. *The Journal of biological chemistry.* 1996; 271:4468–4476. [PubMed: 8626800]
- Weiss J, O'Sullivan GA, Heinze L, Chen HX, Betz H, Wassle H. Glycinergic input of small-field amacrine cells in the retinas of wildtype and glycine receptor deficient mice. *Mol Cell Neurosci.* 2008; 37:40–55. [PubMed: 17920294]
- Yamamoto Y, Sakamoto M, Fujii G, Tsuiji H, Kenetaka K, Asaka M, Hirohashi S. Overexpression of orphan G-protein-coupled receptor, Gpr49, in human hepatocellular carcinomas with beta-catenin mutations. *Hepatology.* 2003; 37:528–533. [PubMed: 12601349]
- Yi H, Nakamura RE, Mohamed O, Dufort D, Hackam AS. Characterization of Wnt signaling during photoreceptor degeneration. *Invest Ophthalmol Vis Sci.* 2007; 48:5733–5741. [PubMed: 18055826]
- Yui S, Nakamura T, Sato T, Nemoto Y, Mizutani T, Zheng X, Ichinose S, Nagaishi T, Okamoto R, Tsuchiya K, et al. Functional engraftment of colon epithelium expanded in vitro from a single adult Lgr5(+) stem cell. *Nature Medicine.* 2012; 18:618–623.

Figure 1. Lgr5 expression in the retina

A. Immunofluorescence sagittal section through the retina showing Lgr5/eGFP expression (green) and cell nuclei (blue). Some background autofluorescence is seen in the extra-ocular muscles in the retro-orbital space. The area in the white box is magnified in **B**. Note, as an internal positive control, some Lgr5 expression in the hair follicles at the top of the picture, in accord with previous publications (Jaks et al., 2008). Scale bar, 0.5 mm. **B.** Left: Magnification of boxed area in **A**. Amacrine cell bodies are positive for Lgr5/eGFP (white arrow) in *Lgr5^{eGFP/CreER}* mice. Right: Wildtype control retina does not show Lgr5/eGFP staining. Abbreviations: germinal cell layer, GCL; inner plexiform layer, IPL; inner nuclear layer, INL, outer plexiform layer, OPL; outer nuclear layer, ONL. Scale bars, 25 mm. **C.** Immunofluorescence of Lgr5/eGFP expression (green) and pan-amacrine cell marker syntaxin (left), glycinergic neuronal marker GlyT1 (center), and GABA-ergic neuronal marker GAD65/67 (right). **D.** Venn diagrams on bottom represent quantification of Lgr5 and marker expression. Scale bars, 25 mm; Inserts, 2.5 mm.

Figure 2. Perinatal activation of Lgr5 expression and fluorofilling

A. Immunofluorescence of Lgr5/eGFP expression (green) at time points P1 through P7 as well as P150 as an additional time point of a fully adult mouse. Nuclei are in blue. Scale bars, 25 μm. **B.** Single cell genomic profiling of the developing retina at indicated timepoints. Fractions indicate number of cells positive for Lgr5 expression over total number of cells profiled. Lgr5 expression began in 3 of 19 profiled P5 amacrine cells. **C.** Immunofluorescence image of two amacrine cells that have undergone recombination and express dTomato; “fluorofilling.” Image viewed in greyscale allows for fine detail of the cellular projections into the INL. **D.** Examples of Lgr5-dTomato fluorofilled cells imaged

with IFC and shown in greyscale, classified according to the criteria in Macdonald et al.
Scale bars, 15 mm.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript