RESEARCH ARTICLE

Twenty-seven Tamoxifen-Inducible iCre-Driver Mouse Strains for Eye and Brain; Including Seventeen Carrying a New Inducible-First Constitutive-Ready Allele

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Abstract

To understand gene function, the cre/loxP conditional system is the most powerful available for temporal and spatial control of expression in mouse. However, the research community requires more cre recombinase expressing transgenic mouse strains (cre-drivers), which restrict expression to specific cell types. To address these problems, a high-throughput method for large-scale production that produces high-quality results is necessary. Further, endogenous promoters need to be chosen that drive cell type specific expression, or we need to further focus the expression by manipulating the promoter. Here we test the suitability of using knock-ins at the docking site 5′ of Hprt for rapid development of numerous cre-driver strains focused on expression in adulthood, using an improved cre tamoxifen inducible allele (icre/ERT2) and testing a novel inducible-first, constitutive-ready allele (icre/f3/ERT2/f3). In addition, we test two types of promoters either to capture an endogenous expression pattern (MaxiPromoters) or to restrict expression further using minimal promoter element(s) designed for expression in restricted cell types (MiniPromoters). We provide new cre-driver mouse strains with applicability for brain and eye research. In addition, we demonstrate the feasibility and applicability of using the locus 5′ of Hprt for the rapid generation of substantial numbers of cre-driver strains. We also provide a new inducible-first constitutive-ready allele to further speed cre-driver generation. Finally, all these strains are available to the research community through The Jackson Laboratory.
Introduction

To understand gene function, the cre/loxP conditional system is the most powerful available for temporal and spatial control of expression in mouse (Hoess et al. 1986; Bradley et al. 2012; Murray et al. 2012; Rosen et al. 2015; Kaloff C et al. 2017). Large-scale efforts such as the International Knockout Mouse Consortium (IKMC) have targeted nearly 18,500 genes in mouse embryonic stem cells (ESCs), which have conditional potential in mice, dependent upon inactivation of their alleles by Cre recombinase catalyzing site-specific DNA recombination between 34 bp loxP recognition sites (Kaloff C et al. 2017). The IKMC has recently pointed out that “ideally, cre-driver mice should be at hand for every adult cell type to dissect pleiotropic gene functions related to human disease” (Kaloff C et al. 2017). To that end, the research community requires for spatial and temporal control of gene inactivation: 1) more cre recombinase expressing transgenic mouse strains (cre-drivers), and 2) cre-drivers that restrict expression to specific cell types.

Temporal control of cre activation can be further increased beyond that of the promoter used, by the inclusion of an inducible cre protein such as the tamoxifen responsive cre/ERT2 fusion protein (Indra et al. 1999). This inducible system has cre fused to a mutated estrogen receptor (ERT2) such that Cre/ERT2 is normally sequestered in the cytoplasm and inactive, but when tamoxifen binds ERT2 the cre fusion protein translocates to the nucleus where it is active (Indra et al. 1999). Therefore, the expression pattern of cre from the inducible system reports on a combination of the promoter driving cre, but only during the “window of time” when tamoxifen is present. Inducible alleles have found some application in development, although the toxicity of tamoxifen has been a limitation. However, in adulthood, their application is broad since
tamoxifen toxicity is less of a concern and the reduction in expression complexity, by not reporting on development, is profound. In contrast to the inducible allele, constitutive cre reflects only the promoter used (Zinyk et al. 1998). Cre is always present in the nucleus, and thus is a “historical” reporter of any expression from development through adulthood. Such alleles have found substantial application in development, but since many genes express more broadly in development and become more restricted in adulthood, the final additive labelling in adulthood can be undesirably complex.

A large-scale effort has been underway, employing several different strategies, to produce both inducible and constitutive cre-drivers for use with the IKMC conditional alleles (Murray et al. 2012; Rosen et al. 2015; Kaloff C et al. 2017). These span from generation of ESCs only, through animals, to extensively characterized cre-driver strains; the latter being the most immediately valuable to the community. Supporting the usability of these resources by the community is easy access through a repository, including information as to where the resource is and whether it is held as a mouse strain, alive or cryopreserved. The mouse genome informatics (MGI) cre portal (www.creportal.org) is a resource that allows researchers to retrieve a list of all recombinase-containing transgenes and knock-in alleles. There are currently 1,777 strains of mice listed that are available from an International Mouse Strain Resource (IMSR) repository, this represents strains made from 833 different genes. These strains correspond to 1,011 random-insert transgenic and 766 knock-in strains, of the knock-in 32 are at Hprt, 25 at Gt(ROSA), and 57 are enhancer traps. Of these strains, 339 MGI cre alleles are listed in the IMSR as live mice, representing 234 genes.
To address the first problem of generating more cre-driver strains, a high-throughput method of large-scale production that produces high-quality results is necessary. Three strategies were considered for this trial: random insertion, knock-in at the endogenous locus, and a “docking site” in the mouse (Murray et al. 2012). The often-used random insertion approach for cre-driver mice generates strains quickly, but is uncontrolled for number of cre transgenes inserted and their location in the genome, both factors that influence the expression of the transgene and necessitate characterization of many strains per construct. More desirable would be a predictable homologous recombination knock-in strategy, so that only one strain need be generated and characterized per construct. Knock-in of a cre transgene in the mouse gene to capture the endogenous expression was very attractive, but before the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology, generating many large homologous recombination constructs, and selecting hundreds of ESC clones, was deemed prohibitively slow and daunting. In addition, this strategy generates a new, possibly deleterious allele in the very gene whose normal expression is to be captured. Instead, we choose to use one of the two docking sites in the mouse genome; Gt(ROSA)26Sor on Chromosome 6 and Hprt on the X Chromosome. Although both are ubiquitous genes, the Gt(ROSA)26Sor locus is known to drive strong ubiquitous expression (Soriano 1999; Muzumdar et al. 2007; Madisen et al. 2010), whereas the region 5’ of Hprt has been shown to provide a relatively neutral environment in which the expression pattern of the knocked-in promoter is primarily maintained (Farhadi et al. 2003; Portales-Casamar et al. 2010; Schmouth et al. 2013; de Leeuw et al. 2014). Critically, this location also remains the only site in the mouse genome for docking bacterial artificial chromosomes (BACs) constructs, even in excess of 200 kb (Schmouth et al.
2012b; Schmouth et al. 2013; Peeters et al. 2018). When used with human DNA, the ‘high-throughput human genes on the X Chromosome’ (HuGX) strategy, designed for single copy, site specific, repetitive knock-in of transgenes using an established set of homology arms for constructs (Yang et al. 2009), which undergo relatively high-frequency homologous recombination (average 48% of selected clones) (Schmouth et al. 2013), was the most attractive for this study.

To address the second problem of generating cre-drivers that restrict expression to specific cell types, endogenous promoters must be chosen that drive such focused expression or we need to restrict expression further by manipulating the promoter. The average size of a human genomic region per gene is ~150 kb (3x10^6 kb in the genome/~20,000 genes), thus to capture all regulatory regions for a gene of interest a "MaxiPromoter" (human BAC with a reporter gene inserted at the start codon) may achieve this. We have previously used this strategy to capture brain and eye expression from the human genes AMOTL1, MAOA, NOV, and NR2F2 (Schmouth et al. 2013). To restrict expression to less than that of the endogenous gene, we have previously used bioinformatic approaches to identify brain and eye-specific regulatory elements to produce “MiniPromoters” (Portales-Casamar et al. 2010; de Leeuw et al. 2014). Plasmid based MiniPromoters were designed for restricted region- and cell-type expression and to be amenable for use in gene therapy as they are less than 4 kb and made entirely from human DNA. We have published 45 MiniPromoters driving a reporter by knock-in 5’ of the Hprt locus (Portales-Casamar et al. 2010; de Leeuw et al. 2014), and 33 in recombinant adeno-associated virus (rAAV) (de Leeuw et al. 2016; Hickmott et al. 2016; Simpson et al. 2018). Employing human DNA for promoters relies heavily on conservation of DNA-sequence and expression, and
presumably reduces our success when testing in mice. However, it allows us to delineate regions of human DNA in MaxiPromoters needed for expression, and facilitates the design of MiniPromoters, thereby increasing translation to gene therapy.

In this work, we detail the CanEuCre project, a successor of the Pleiades Promoter Project in cooperation with EUCOMMTOOLS and IKMC Projects, in which we address the need for more high quality cell type specific cre-driver mice. Here we test the suitability of using knock-ins at the docking site 5’ of Hprt for rapid development of numerous cre-driver strains focused on expression at adulthood, using a previously characterized improved cre (icre) tamoxifen inducible allele (icre/ERT2) (Erdmann et al. 2007) and testing a novel inducible-first, constitutive-ready allele (icre/f3/ERT2/f3) so that constitutive expression can also be studied. In addition, we test two types of promoters either to capture an endogenous expression pattern (MaxiPromoters) or to restrict expression further using minimal promoter element(s) designed for expression in restricted cell types (MiniPromoters). In the process, we have added to the mouse strain collection by developing 27 icre-driver strains for the brain and eye, available through The Jackson Laboratory. These 27 strains represent 10 MaxiPromoter strains driving icre/ERT2, 14 MiniPromoter strains driving icre/f3/ERT2/f3, and three control strains driving icre/f3/ERT2/f3. All strains were characterized by RT-PCR, with two inducible MaxiPromoter strains chosen for detailed characterization by histology, and four inducible and constitutive MiniPromoter strains chosen for detailed characterization by histology.
Materials and Methods

MaxiPromoter Design and Retrofitting

The BAC constructs came from the RPCI-11 human male BAC library (http://bacpac.chori.org/hmale11.htm (Table 1). BACs were modified by two sequential steps of retrofitting using the lambda recombination system (Yu et al. 2000). The first retrofitting step allowed the insertion of Hprt homologous recombination targeting arms as we have described previously (Schmouth et al. 2013). The second step allowed the insertion of the reporter cassette icre/ERT2 at the ATG encoding start codon of the specified gene. Two 50 bp oligonucleotide recombination arms were designed for the insertion of the reporter. The left arm targeted immediately upstream of the endogenous ATG encoding start codon. Ideally, the right arm targeted immediately after the end of the same exon. Because of sequence composition challenges for retrofitting, in some cases the right arm oligonucleotides designs were altered to target further downstream. For the reporter cassette (pEMS1725) an icre/ERT2 sequence (1,983 bp) was designed using a previously published icre sequence (Shimshek et al. 2002), a 3 amino acid linker sequence (Metzger and Feil 1999), and an ERT2 sequence (Feil et al. 1997). The retrofitting of the reporter cassette was done as we have described previously (Schmouth et al. 2013). Briefly, the reporter cassette was designed to contain a kanamycin gene, allowing the selection of correctly retrofitted clones. This resistance gene was designed with flanking full frt sites (Lyznik et al. 1993), which were used to excise the kanamycin gene via induction of Flp recombinase (Schlake and Bode 1994; Anastassiadis et al. 2009). The entire icre/ERT2 cassette, including targeting arms, was sequence verified for each construct.
MiniPromoter Design and Cloning

MiniPromoter bioinformatics has been described in detail previously (Portales-Casamar et al. 2010; de Leeuw et al. 2016). The MiniPromoter backbone plasmid (pEMS2001) was produced by three sequential steps. First, we had a new plasmid synthesized (pEMS1925) building on pJ344 (DNA2.0, Menlo Park, CA) and containing: I-SceI (for linearization), BamHI, an insulator (Sakurai et al. 2010), a multiple cloning site for introduction of promoters (AvrII, FseI, MluI, and Ascl), an intron (optimized chimeric (pCI, Promega, Madison, MI)), icre/ERT2 (with frt-f3 sites flanking the estrogen receptor sequences), a WPRE (Zanta-Boussif et al. 2009), a SV40 poly(A) signal, another insulator, EcoRI, and Sall. Second, the mouse 5′ Hprt homology arm (Portales-Casamar et al. 2010) was isolated from Pleiades plasmid (pEMS1314) and introduced into pEMS1925 using BamHI. Finally, the human HPRT complimentary sequence (Portales-Casamar et al. 2010) and mouse 3′ Hprt arm (Portales-Casamar et al. 2010) were isolated from Pleiades plasmid (pEMS1314) and introduced using EcoRI/Sall to produce the final pEMS2001. MiniPromoters were generated by direct synthesis (DNA2.0), or isolated from previous Pleiades Promoter plasmids (Portales-Casamar et al. 2010; de Leeuw et al. 2014; de Leeuw et al. 2016), and cloned into the multiple cloning site of pEMS2001. All new DNA junctions, whether for the reporter cassette, Hprt arms, or MiniPromoters, were sequence verified.

Mouse Strain Generation, Husbandry, and Breeding

The new mouse strains were generated using a variation of the previously described strategy to insert constructs 5′ of Hprt on the mouse X Chromosome (Bronson et al. 1996; Heaney et al. 2004; Yang et al. 2009; Schmouth et al. 2013). Briefly, either MaxiPromoter DNA was purified using the Nucleobond BAC 100 kit (Clontech laboratories, Mountain View, CA), or MiniPromoter
DNA was purified using the Qiagen MiniPrep kit (Qiagen, Hilden, Germany), and both MaxiPromoters and MiniPromoters were linearized with I-SceI. The constructs were then electroporated into ESCs using the following conditions: voltage, 190 V (MaxiPromoter), 270 V (MiniPromoter); capacitance, 500 μF (MaxiPromoter), 50 μF (MiniPromoter); resistance, 350 ohms (both); using a BTX ECM 630 Electro cell manipulator (BTX, San Diego, CA) (Schmouth et al. 2013). A male ESC line from a C57BL/6NTac-A\(^{w-j}/A^{w-j}\), Hprt\(^{b-m^3}/Y\) mouse (mEMS4855 or mEMS4857) was used. The agouti gene originated from JAX stock 00051 (C57BL/6J-A\(^{w-j}/J\)) and was backcrossed onto C57BL/6NTac (B6/NTac, Taconic, Hudson, NY) for 4 generations. The Hprt gene originated from JAX stock 002171 (B6.129P2-Hprt\(^{b-m^3}/J\)) and was backcrossed to B6/NTac for 6 generations. Following the backcrossing to B6/NTac, the agouti and Hprt strains were intercrossed for 4 generations to produce the final ESC line from a generation-equivalent NE4.75F4 mouse blastocyst. ESC derivation and culture was conducted as we have described previously (Yang et al. 2009). Targeted ESC clones were selected in hypoxanthine aminopterin thymidine (HAT) media, isolated, expanded, frozen, and DNA purified. Human-specific PCR assays were designed and used to initially screen the ESC clones, and verify the integrity of the MaxiPromoter and MiniPromoter inserted in the mouse genome (Yang et al. 2009; Schmouth et al. 2013). A minimum of three correctly-targeted ESC clones per construct were microinjected into C57BL/6J blastocysts (B6/J, JAX Stock 000664) to generate chimeras that were subsequently bred to B6/J females to obtain female germline offspring. A mixed background of B6/NTac and B6/J is designated B6.

A typical breeding strategy is detailed in Figure S1. To produce tm#a (#, stands for all numbers in the collection) strains for histology, N1 or N2 females were bred to the 129-ROSA-Stop-
tdTomato cre reporter strain (JAX Stock 007914 (minimum N1 backcrossed to 129S1/SvImJ (129, JAX Stock 002448))) to generate both male and female hybrid B6129F1 mice that carried both the icre/ERT2 and ROSA-Stop-tdTomato alleles. One exception was Ple274 (CRH) mice, which were bred to B6/J-ROSA-Stop-tdTomato cre reporter strain (JAX Stock 007905) to generate both male and female B6/J mice that carried both the icre/ERT2 and ROSA-Stop-tdTomato alleles. To produce tm#b strains for histology, N1 or N2 females were bred to the B6/J-CAG-flpo strain (MMRRC Stock 032247-UCD) a germline deleter used to remove the ERT2 portion of the allele. Following this, tm#b females were bred to the 129-ROSA-Stop-tdTomato reporter strain to generate both male and female adult hybrid B6129F1 mice that carried both the icre and ROSA-Stop-tdTomato alleles. As a tm#a control, the UBC (human ubiquitin C) promoter driving cre/ERT2 strain was used (Ruzankina et al. 2007). As a tm#b control, the ACTB promoter driving cre strain was used (JAX Stock 003376).

Mice were maintained in the pathogen-free Centre for Molecular Medicine and Therapeutics mouse facility on a 7 am–8 pm light cycle, 20 ± 2°C with 50 ± 5% relative humidity, and had food and water ad libitum. All procedures involving mice were in accordance with the Canadian Council on Animal Care and UBC Animal Care Committee (Protocol A10-0267 and A10-0268). Ple274 (CRH) mice were also maintained in the Technische Universität Dresden facility on a 12 am-12 pm light cycle, 22 ± 1°C with 55 ± 5% relative humidity, and had food and water ad libitum. All procedures involving mice were in accordance with the Guide of the Care and Use of Laboratory Animals of the Government of Bavaria, Germany.
RT-PCR

A minimum of three N2 male mice were used for each strain for RT-PCR experiments. Male N2 mice of at least 6 weeks of age were euthanized by cervical dislocation; brain and eyes were dissected and rapidly frozen in liquid nitrogen. The brain dissection was into seven regions: olfactory bulbs, telencephalon (without the hippocampus), hippocampus, diencephalon, midbrain, hindbrain (without the cerebellum), and cerebellum. Two to three regions were chosen for each strain. RNA was extracted using the PerfectPure RNA Cell & Tissue kit (5 Prime, Hilden, Germany) and converted to cDNA using the SuperScript III First-Strand Synthesis (Thermo Fisher Scientific, Waltham, MA). For MaxiPromoter strains, assays were designed within ERT2 and for each specific BAC. For MiniPromoter strains, assays were designed within ERT2, ERT2 to WPRE, and for each specific promoter expect Promoterless, CAGGS, and Ple198 where specific promoter designs were not possible.

Tamoxifen Induction

Adult mice were fed a diet of either tamoxifen (TD.120629, Harlan Laboratories, Madison, WI) or no-tamoxifen (control, TD.07570) food for a period of four weeks. Following this period, mice were put back on a regular diet for three weeks before harvest. Adult Ple274 (CRH) mice were fed a diet of tamoxifen (LAS CRdiet CreActive TAM400, LASvendi GmbH, Soest, Germany) during postnatal weeks 10-12. Following this period, mice were put back on a regular diet for two weeks before harvest.

Histology – TdTomato Epifluorescence and Immunofluorescence

Mostly, mice were sacrificed by avertin (MilliporeSigma, Burlington, MA) overdose and transcardially perfused for 2 mins with 1x Phosphate Buffer Saline (PBS), and 10 mins with 4%
paraformaldehyde (PFA) in PBS buffer at a flow rate of 5 ml/min. Tissues were removed and post-fixed for 2 hours in 4% PFA at 4°C and then cryoprotected with 25% sucrose overnight at 4°C. Tissues were then embedded in optimal cutting temperature compound (OCT) on dry ice and 20 μm cryosections were directly mounted onto slides. For Ple274 (CRH), mice were sacrificed by isoflurane (Floren®, Abbott, Lake Bluff, IL) overdose and transcardially perfused for 1 min with 1× PBS, 5 min with 4% PFA (w/v) in PBS, pH 7.4, and 1 min with PBS at a flow rate of 10 ml/min. Brains were removed and post-fixed overnight in 4% PFA in 1× PBS at 4°C. Brains were washed with 1× PBS and embedded in agarose (Invitrogen, Carlsbad, CA) in PBS and 50 μm coronal sections were prepared using a vibratome (MICROM HM 650V, ThermoScientific).

Mostly, TdTomato epifluorescence was visualized using an Olympus BX61 fluorescent microscope (Olympus, Tokyo, Japan). Images were processed using Image J (http://rsbweb.nih.gov/ij/), Photoshop, and Illustrator (Adobe, San Jose, CA). For Ple274 (CRH), TdTomato epifluorescence was visualized using a Zeiss Axioplan2 fluorescent microscope (Zeiss, Oberkochen, Germany). Images were digitalized using AxioVision Rel 4.5 and processed using Photoshop and Illustrator.

For immunofluorescent staining, sections were blocked in PBS + 0.4% Triton X-100 (A2058, MilliporeSigma, Burlington, MA) + 0.3% bovine serum albumin (T8787, MilliporeSigma) for 20 minutes, and incubated in primary antibody at 4°C overnight. Primary antibodies used were anti-CLDN5 (35-2500, Thermo Fisher Scientific, Waltham, MA), anti-PCP2 (sc-68356, Santa Cruz Biotechnology, Dallas, TX), anti-POU4F1 (also known as anti-BRN3A, sc-8429, Santa Cruz Biotechnology), anti-GAD65/67 (AB1511, MilliporeSigma), anti-TH (MAB318, MilliporeSigma),
anti-calretinin (AB1550, Millipore Sigma), and anti-SOX9 (AB5535, MilliporeSigma). Slides were washed 3x with PBS + 0.4% Triton X-100 and incubated with secondary antibody and Hoechst 33342 for 2 hours at RT (room temperature). Secondary antibodies used were from Thermo Fisher Scientific: anti-mouse IgG (A11029), anti-rabbit IgG (A11034), and anti-goat IgG (A21222). Sections were washed 3x in 0.1 M Phosphate Buffer (PB) buffer, 1x in 0.01 M PB buffer, mounted in ProLong Gold (P36930, Thermo Fisher Scientific) and coverslipped. Z-stack images were taken on an TCS SP8 Super-Resolution Confocal Microscope (Leica Microsystems, Wetzlar, Germany), deconvolved using Huygens Professional (Scientific Volume Imaging, Hilversum, Netherlands), and processed as described above. Images with only partial Hoechst overlay were processed as previously described (Hickmott et al. 2016). For POU4F1, GAD65/67, TH, and calretinin imaging, antigen retrieval was performed as follows: sections were incubated in citrate acid buffer + 0.05% Tween20 (BP337, Thermo Fisher Scientific), pH 6 for 40 minutes at RT, sections in buffer were then heated to 54°C for 20 minutes before immunofluorescent staining was performed as described above.

**Histology – Double In Situ Hybridization**

Mice were sacrificed by isoflurane (Floren®) overdose. Brains were carefully removed and rapidly frozen on dry ice and 20 μm at -20°C coronal sections were prepared using a cryostat (Thermo Fisher Scientific). The sections were thaw mounted onto slides, dried, and kept at -80°C. Double ISH was performed as previously described (Refojo et al. 2011). The following riboprobes were used: Crh (3’ UTR): 2,108-2,370 bp of AY128673; tdTomato: 740-1,428 bp of AY678269.
Data Availability

Plasmids used for cloning, controls, and Pleiades MiniPromoters are available to the research community through Addgene (www.addgene.org). Mouse strains are available through The Jackson Laboratory (www.jax.org/jax-mice-and-services). The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

Results

Ten MaxiPromoter Strains –icre/ERT2 (inducible)

Therapeutically interesting regions and cell types of the brain and eye were identified as described in Table 1 and below for selected lines, and eleven genes that had not previously been used for inducible strain development and with restricted expression that included those cells were chosen. Human BACs were selected based on coverage of the gene of interest and inclusion of candidate regulatory regions. The ideal BAC covered the entire gene (transcribed genomic region) up to, but not including, the promoter regions of neighbouring genes. If multiple suitable BACs were available, priority was given to the one that included the longest upstream sequence for the chosen gene, to maximize inclusion of relevant regulatory regions. Greater detail for each MaxiPromoter can be found by searching the parental BAC name (Table 1) on the University of California Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu/, accessed Feb. 4, 2019). For each gene, the parental BAC was retrofitted, targeted into a mouse C57BL/6N embryonic stem cell line, and the resulting line was used to successfully derive ten mouse strains, which were assigned a JAX name and stock number (Table 1).
Figure 1A depicts in more detail our HuGX two-step BAC retrofitting process (Schmouth et al. 2013). First, Hprt homology arms were inserted into the BAC vector backbone, to allow for homologous recombination targeting immediately 5’ the Hprt gene on the mouse X Chromosome. Second, an icre (Shimshek et al. 2002) fused to ERT2 (Feil et al. 1997) cassette with a stop codon was inserted at the ATG encoding start codon of the human gene. Thus, the resulting MaxiPromoter construct contained a reporter gene under the influence of the human regulatory regions of the specified gene. Importantly, no exogenous polyadenylation (poly(A)) signal was added, so that ideally splicing would continue to the natural 3’ untranslated region and poly(A) signal. The new MaxiPromoter alleles are named, for example B6.Cg-Hprt<sup>tm340(Ple253-icre/ERT2)Ems</sup>, indicating the insertion is at the Hprt mouse gene, is targeted mutation (tm) number 340 generated by the Simpson laboratory (Ems), and contains (shown in brackets) the Pleaides promoter (Ple) number 253 driving icre/ERT2 (Table 1).

Figure 1B depicts the resulting MaxiPromoter strains for histological expression analysis. First, the MaxiPromoter-icre/ERT2 strains were crossed to mice that carried the cre-reporter allele Gt(ROSA)26Sortm14(CAG-tdTomato)Hze, for brevity called ROSA-stop-tdTomato because it contains the ubiquitous ROSA promoter driving a loxP/stop/loxP-tdTomato. Then the resulting adult offspring carrying both alleles were fed tamoxifen food, or no-tamoxifen control food. Tamoxifen results in the iCre/ERT2 protein, which is normally sequestered in the cytoplasm, entering the nucleus to remove the stop, allowing ROSA driven tdTomato expression. Thus, only cells that expressed icre driven by the restricted MaxiPromoter at the time of adult tamoxifen exposure showed tdTomato expression.
It was important to demonstrate that our icre-reporting system for histological analysis of inducible alleles was able to report on wide spread expression throughout the target tissues, and thus would properly reflect the expression of the restricted promoters. Therefore, we crossed mice carrying the UBC-cre/ERT2 ubiquitously expressing inducible allele to the cre-reporter strain ROSA-stop-tdTomato, and adult offspring carrying both alleles were fed tamoxifen, or no-tamoxifen, food. Figure S2A shows, for example, that with tamoxifen, tdTomato epifluorescence was observed in many cells throughout all layers of the retina, and the endothelial and stromal layers of the cornea. However, with no tamoxifen, only occasional positive cells, or groups of cells, were observed in the retina and cornea.

17 MiniPromoter Strains – icre/f3/ERT2/f3 (inducible-first constitutive-ready)

The 17 MiniPromoter strains included 14 MiniPromoters selected for therapeutically interesting regions and cell types of the brain and eye as described in Table 2 and below for selected lines, plus three controls. The MiniPromoters chosen for further restricted expression were all tested here for the first time in the mouse genome driving icre. They were a combination of ten unaltered previously successful designs (Portales-Casamar et al. 2010; de Leeuw et al. 2014; de Leeuw et al. 2016), one new cut down of a previous design (de Leeuw et al. 2014; de Leeuw et al. 2016), and two designs taken directly from the literature (Visel et al. 2007; Ariza-Cosano et al. 2012; de Leeuw et al. 2016). Table S1 details the design sources and any previous publication, including previous MiniPromoter characterization, for each promoter.

The ten unaltered MiniPromoters were designed previously (Portales-Casamar et al. 2010; de Leeuw et al. 2014; de Leeuw et al. 2016). Briefly, candidate regulatory regions were selected
based on sequence conservation, experimental data provided in the UCSC Genome Browser, and a manual review of the scientific literature. Also included in this set were two controls: Promoterless, with nothing cloned into the promoter location; and CAGGS, a ubiquitous promoter (de Leeuw et al. 2016).

The cut down MiniPromoter Ple265 (PCP2) was designed based on Ple155 (PCP2), which we had previously shown able to deliver lacZ and/or EmGFP expression in bipolar ON cells of the mouse retina as well as Purkinje cells of the cerebellum (de Leeuw et al. 2014; de Leeuw et al. 2016). The original Ple155 was designed by concatenating two cis-regulatory human regions (Prom1, 986 bp on the sense strand of PCP2, and Prom2, 666 bp on the antisense strand of the PCP2) associated with the two known transcription start sites (TSSs) of the two isoforms of the PCP2 gene (NM_174895 and NM_001271830), respectively. For the new Ple265 design, we hypothesized that the observed eye expression could be independently driven by one of these two promoter regions. We exploited the availability of genome-wide cap-analysis of gene expression (CAGE) data for >800 human cell samples from the FANTOM5 consortium to assess this hypothesis (Arner et al. 2015). We found that the Prom1 cis-regulatory region harboured two sub-regions of active TSSs, associated with distinct expression in the testis and in the eye. A similar analysis of the Prom2 cis-regulatory region highlighted a single region of active TSSs associated with expression in the brain, mainly the cerebellum. To test if one of these cis-regulatory regions could drive specific expression in the eye only, we designed a new MiniPromoter, Ple265, which is 60% the size of Ple155 and exclusively composed of the Prom1 sequence.
The two literature designs were chosen from the VISTA enhancer project, the main goal of which is to identify distant-acting noncoding regulatory elements that drive developmental gene expression in a region-specific manner (Visel et al. 2007). Generally, VISTA enhancers are identified using extreme evolutionary sequence conservation and/or chromatin immunoprecipitation sequencing (ChIP-seq) data, and then tested in combination with a mouse minimal promoter, \textit{Hspa1a} (aka Hsp68) (Visel et al. 2007). We selected two such VISTA enhancers, one each for the forebrain and midbrain. Also included in this set was one control; the VISTA minimal promoter alone (\textit{Hspa1a}).

Originally 18 MiniPromoters were selected and cloned into our plasmid backbone (except Promoterless), targeted into a mouse C57BL/6N embryonic stem cell line, and the resulting line was used to successfully derive the 17 mouse strains, which were assigned a JAX name and stock number (Table 2).

Figure 1C depicts in more detail the MiniPromoter cloning process. First we generated a plasmid backbone (pEMS2001) that contained: a mouse 5′ \textit{Hprt} homology arm (Portales-Casamar et al. 2010); a multiple cloning site for introduction of MiniPromoters; \textsc{icre} fused to ERT2, with f3 sites flanking the ERT2 (Schlake and Bode 1994; Feil \textit{et al.} 1997; Shimshek \textit{et al.} 2002); woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) mut6 version, which abolishes WPRE internal transcriptional activities (Zanta-Boussif \textit{et al.} 2009); an SV40 poly(A) signal; a human \textit{HPRT} complimentary sequence (Portales-Casamar \textit{et al.} 2010); and a mouse 3′ \textit{Hprt} homology arm (Portales-Casamar \textit{et al.} 2010). The expression cassette was also flanked by insulators (Sakurai \textit{et al.} 2010); 5′ of the MiniPromoter and 3′ of the poly(A) signal.
The insulators were designed to minimize ‘site of insertion’ genomic effects on the relatively small MiniPromoters, compared to the BAC MaxiPromoters. MiniPromoters were cloned 5’ of the reporter cassette.

For the new icre/f3/ERT2/f3 allele (tm#a), one f3 site was introduced into the spacer between cre and the ERT2 domain, without interrupting the reading frame or changing the spacer length. Another f3 was placed 3’ of the ERT2 stop codon so that recombination between the two f3 sites by Flp recombinase will delete the ERT2 domain and bring a new stop codon to the icre open reading frame, resulting in the constitutive allele (tm#b). The new MiniPromoter alleles are named, for example B6.Cg-\(Hprt^{\text{tm366a(Ple67-icre/ERT2)Ems}}\), indicating the insertion is at the \(Hprt\) mouse gene, is targeted mutation (tm) number 366 generated by the Simpson laboratory (Ems), is the inducible-first constitutive-ready allele (a), and contains (shown in brackets) the Pleaides promoter (Ple) number 67 driving icre/ERT2 (Table 2).

Figure 1D depicts the resulting MiniPromoter tm#a strains for histological expression analysis. MiniPromoter-icre/f3/ERT2/f3 strains were crossed to ROSA-stop-tdTomato and the resulting adult offspring carrying both alleles were fed tamoxifen, or no-tamoxifen control food. TdTomato expression was then observed in cells that expressed icre driven by the MiniPromoter at the time of adult tamoxifen exposure. Figure 1E depicts the production of MiniPromoter constitutive tm#b strains. The MiniPromoter-icre/f3/ERT2/f3 strains were crossed to mice that carried the germline flp-deleter allele Tg(CAG-flpo)1Afst/Mmucd, for brevity called CAG-flpo because it contains the ubiquitous CAG promoter driving a codon optimized flippase recombinase (flpo) (Raymond and Soriano 2007), resulting in the
recombination of the frt-f3 sites to remove the ERT2. Figure 1F depicts the resulting
MiniPromoter tm#b strains for histological expression analysis. MiniPromoter-icre strains were
crossed to ROSA-stop-tdTomato resulting in the recombination of the loxP sites to remove the
stop. TdTomato expression was then observed in all the cells that had expressed icre driven by
the MiniPromoter during either development and/or adulthood.

Again, we demonstrate that our icre-reporting system, used for histological analysis of both
inducible and constitutive expression, was able to report on wide spread expression throughout
the target tissues. For inducible expression, the same UBC-cre/ERT2 controls were used as
detailed for MaxiPromoters above (Figure S2A). For constitutive expression, we crossed mice
carrying the ACTB-cre ubiquitously expressing allele to the cre-reporter strain ROSA-stop-
tdTomato, and analyzed adult offspring carrying both alleles. Figure S2B shows, for example,
that tdTomato epifluorescence was observed in many cells throughout most layers of the
retina, and in the epithelial, endothelial, and stromal layers of the cornea. We also analyzed
adult mice carrying only the ROSA-stop-tdTomato allele. Figure S2C shows that without a cre
allele, no tdTomato epifluorescence was observed.

All 27 icre strains showed expression by RT-PCR

Each MaxiPromoter and MiniPromoter strain should be producing icre transcripts dependent
upon the respective promoter, but independent of icre protein status (conditional or
constitutive). Therefore, for each of the twenty-seven strains, two to three brain regions, or the
eye, were chosen based on where expression was expected, harvested from N2 male mice, and
assessed by semi quantitative RT-PCR (Table 3). All tissues were positive, with the band
intensity being consistent across tissues for a single strain, but with a range of intensities by strain. MaxiPromoter strains showed variable strong, weak, and very weak RT-PCR bands, whereas all MiniPromoter mice showed strong bands. Of particular note was the unexpected strong transcription observed for the MiniPromoter control strain bearing no promoter (none, aka Promoterless). This was presumably due to the influence of the genomic Hprt locus, despite the presence of insulators flanking the MiniPromoter inserts (Figure 1C). Using this RT-PCR data, a subset of strains was chosen for further breeding and tdTomato histological analysis. Two of the ten MaxiPromoter strains were chosen, one strong and one weak expresser (Figures 2 and 3). Four of the 17 MiniPromoter strains were chosen, and tested both as the inducible tm#a and constitutive tm#b alleles (Figures 4-7).

**Ple272 CLDN5 MaxiPromoter expressed in the endothelial cells of the blood-retina and blood-brain barrier**

Claudin 5 (CLDN5) is an integral membrane protein and component of tight junctions (Nitta *et al.* 2003). Tight junctions develop between endothelial cells of the blood vessels in the central nervous system, and play a key role in establishing the blood-brain barrier. Several diseases are associated with *CLDN5*, including velocardiofacial syndrome (Sirotkin *et al.* 1997) and gray platelet syndrome (Nurden *et al.* 2008). *CLDN5* was chosen for its expression in the endothelial cells of the blood-retina and blood-brain barrier.

In this inducible strain, the *CLDN5* MaxiPromoter expressed as expected for the endogenous gene in all tissues examined (Table 4). This included in the target blood vessels of the retina and brain (Figure 2). Expression was lower in female mice as expected due to X-chromosome
inactivation. Co-labeling with anti-CLDN5 confirmed that expression localized to the endothelial cells of the blood vessels of the retina and brain. Mice carrying this inducible allele, who were fed a no-tamoxifen control diet, unexpectedly showed lower but still detectable levels of iCre activity, which importantly remained restricted to the blood vessels of the retina and brain. This iCre “leakiness” (tamoxifen-independent Cre activity) rendered the strain constitutive at a low level and inducible to a high level (Murray et al. 2012). Finally, positive expression was also observed in the spinal cord and heart (Table 4), which was expected as blood vessels in the spinal cord and cardiomyocytes are known to express CLDN5 (Sanford et al. 2005; Paul et al. 2013).

Ple274 CRH MaxiPromoter expressed in multiple endogenous brain regions, but showed only partial cellular overlap

Corticotropin-releasing hormone (CRH) is a neuropeptide that orchestrates the neuroendocrine, autonomic, and behavioral stress responses (Bale and Vale 2004). To this end, CRH possesses a dual mode of action; it acts as a secretagogue controlling the activity of the hypothalamic-pituitary-adrenal axis, and as a neuromodulator at extra hypothalamic sites. Several diseases are associated with CRH, including post-traumatic stress disorder (Hockings et al. 1993; Geracioti et al. 2008) and postpartum depression (McCoy et al. 2003; Meltzer-Brody et al. 2011). CRH was chosen particularly for its expression in the paraventricular hypothalamic nucleus (PvH), which is involved in neurohormonal mechanisms (Kovacs 2013; Rigas et al. 2018).
In this inducible strain, the CRH MaxiPromoter showed a distribution of expressing neurons, which largely reflected the pattern of endogenous CRH (Merchenthaler et al. 1982; Keegan et al. 1994) (Table 4). Major CRH expression domains that contained tdTomato positive neurons included the piriform cortex (Pir), neocortex (Ctx), hippocampus, PvH, medial geniculate nucleus (MGM), Barrington nucleus, and inferior olive (Figure 3A). However, structures showed rather sparse labelling, which was lower in female mice as expected due to X-chromosome inactivation. Of note, some CRH-expressing brain regions such as the olfactory bulb, central amygdala, or bed nucleus of the stria terminals, showed only isolated tdTomato positive neurons. To investigate the co-expression of tdTomato with endogenous CRH expression at the cellular level we performed double in situ hybridization (ISH) (Figure 3B). The double ISH revealed that, albeit the tdTomato positive neurons were generally located within the correct brain region, only a subset of neurons in a given structure co-expressed endogenous Crh mRNA. As expected, mice carrying this inducible allele, who were fed a no-tamoxifen control diet did not show any tdTomato expression.

**Ple155 PCP2 MiniPromoter expressed primarily in the retinal bipolar ON neurons**

Purkinje cell protein (PCP2, aka. L7) may function as a modulator for G protein-mediated cell signaling (Zhang et al. 2002; Willard et al. 2006). PCP2 is involved in the maturation of cerebellar Purkinje cell neurons in the brain, but is also well recognized for its specificity to bipolar ON cell neurons of the retina in the eye (Vandaele et al. 1991). It was chosen for its expression in both these cell types, with the long-term goal of separating these expressions. We have previously demonstrated that the single MiniPromoter Ple155 (PCP2, 1,652 bp) showed
restricted expression in both the Purkinje and bipolar cells in X-chromosome knock-in mice driving lacZ (de Leeuw et al. 2014) and in rAAV driving icre and EmGFP (de Leeuw et al. 2016).

In the inducible strain (tm347a), the PCP2 MiniPromoter showed endogenous-like expression in the retina, but was unexpectedly non-specific in the brain (Table 4). In the retina, this included expression primarily in bipolar cells, but with low levels of expression in the ganglion cell layer (GCL) and in amacrine cells (Figure 4A). Expression was lower in female mice as expected due to X-chromosome inactivation. Co-labeling with anti-PCP2 confirmed that expression was primarily localized to the bipolar ON cells of the retina. Mice carrying this inducible allele, who were fed a no-tamoxifen control diet, were mainly negative, with only a few cells expressing.

In the constitutive strain (tm347b), the PCP2 MiniPromoter was not specific and expressed throughout the retina and cornea, and remained non-specific in the brain (Table 4). In the retina, expression was primarily in the inner nuclear layer (INL, bipolar and amacrine cells), and in the GCL and the Müller glia, in the cornea expression was observed in all three layers of the cornea (epithelial, stromal, and endothelial) (Figure 4B). Expression was lower in female mice as expected due to X-chromosome inactivation.

**Ple265 PCP2, a smaller MiniPromoter, expressed primarily in the retinal bipolar ON neurons**

To test if a smaller promoter could drive specific expression in only the bipolar ON neuronal cells of the retina, we designed Ple265 (PCP2, 986 bp), which is a 60% cut down of Ple155. Based on the bioinformatic design, Ple265 was expected to capture the bipolar expression pattern of the eye, but eliminate the brain expression. However, our results show that smaller Ple265 PCP2 promoter maintained the same expression pattern as the larger Ple155 (PCP2).
In the inducible strain (tm348a), the PCP2 MiniPromoter showed endogenous-like expression in the retina, but was unexpectedly positive and non-specific in the brain (Table 4). In the retina, expression was primarily in the bipolar cells, but with low levels in the GCL and amacrine cells (Figure 5A). Co-labeling with anti-PCP2 confirmed that retinal expression was primarily localized to the bipolar ON cells. Mice carrying this inducible allele, who were fed a no-tamoxifen control diet, were mainly negative, with only a few cells expressing.

In the constitutive strain (tm348b), the PCP2 MiniPromoter was not specific and expressed throughout the retina and cornea, and was non-specific in the brain (Table 4, Figure 5B). Expression was lower in female mice as expected due to X-chromosome inactivation.

**Ple198 SLC6A4 MiniPromoter expressed in the retinal ganglion and amacrine cells**

Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 (SLC6A4, aka SERT) is a transmembrane protein located on synaptic vesicles, and is involved in the transport of the neurotransmitter serotonin from synaptic spaces into presynaptic neurons (Lebrand et al. 1998). SLC6A4 has been demonstrated to express primarily in the raphe nuclei of the brain. In the developing retina, *in-situ* hybridization studies have demonstrated that SLC6A4 is transcribed transiently in retinal ganglion cells (Upton et al. 1999; Garcia-Frigola and Herrera 2010). Furthermore, a SLC6A4-cre mouse strain showed recombination in retinal ganglion cells (Assali et al. 2017). Several diseases are associated with SLC6A4, including obsessive-compulsive disorder (Sinopoli et al. 2017), anxiety (Arias et al. 2012), and post-traumatic stress disorder, as well as depression-susceptibility in people experiencing emotional trauma (Kuzelova et al. 2010). SLC6A4 was originally chosen for the raphe nuclei, and we have previously demonstrated
MiniPromoter Ple198 (SLC6A4, 2,826 bp) showed, an expected restricted expression during development in the thalamus with rAAV driving icre (de Leeuw et al. 2016).

In the inducible strain (tm351a), the SLC6A4 MiniPromoter expressed specifically in the retina and cornea, but non-specifically in the brain and thus did not capture the expected raphe expression (Table 4). In the retina, this included expression in widely spaced cells in the GCL and, and in the cornea in the stromal cell layer (Figure 6A). Overall, expression was lower in female mice as expected due to X-chromosome inactivation. Co-labeling with anti-POU domain, class 4, transcription factor 1 (POU4F1, also known as BRN3A) confirmed expression in retinal ganglion cells in the GCL, and labeling with anti-glutamate decarboxylase 65/67 (GAD65/67) confirmed expression in GABAergic amacrine cells in the INL. However, anti-Calretinin and anti-tyrosine hydroxylase (TH) failed to label tdTomato-expressing cells (data not shown). As expected, mice carrying this inducible allele, who were fed a no-tamoxifen control diet did not show any tdTomato expression.

In this constitutive strain (tm351b), the SLC6A4 MiniPromoter was not specific and expressed throughout multiple layers of the retina and cornea, and non-specifically in the brain, again not capturing the raphe expression (Table 4). In the retina, this included expression primarily in the INL, but also in the GCL and Müller glia, and in the cornea, this included the epithelial, stromal, and endothelial cell layers (Figure 6B). Overall, expression was lower in female mice as expected due to X-chromosome inactivation.

As Ple198 (SLC6A4) expression was detected in POU4F1 immuno-reactive retinal ganglion cells, it could be that this MiniPromoter recapitulates the endogenous expression in this cell-type,
but not in a developmental context. Additionally, serotonin uptake has been described in GABAergic amacrine cells in the mammalian retina (Osborne and Beaton 1986; Wassle and Chun 1988; Menger and Wassle 2000), although it is currently unclear if this uptake is conducted by SLC6A4. As tdTomato expression was detected in GAD65/67 immuno-reactive amacrine cells, Ple198 (SLC6A4) could be recapitulating the endogenous expression in these cells. However, serotonin accumulating amacrines have been found to co-label with calretinin in *Xenopus laevis* (Gabriel 2000) and 90% of serotonin accumulating cells have been found to co-label with TH in the mouse retina (Trakhtenberg *et al.* 2017), but antibodies to either of these markers failed to co-label with tdTomato in this strain. Therefore, we can conclude expression is in a subset of GABAergic amacrines, but were not able to demonstrate that these were serotonin accumulating.

**Ple264 NR2E1 MiniPromoter expressed primarily in the retinal Müller glia cells**

Nuclear receptor subfamily 2, group E, member 1 (NR2E1) is a transcriptional regulator that has been demonstrated to play a critical role in brain and eye development (Stenman *et al.* 2003; Miyawaki *et al.* 2004; Christie *et al.* 2006; Corso-Díaz and Simpson 2015). NR2E1 is important in regulation of the processes that underlie neurogenesis, spatial learning, anxiety-like behaviour, and aggression (Young *et al.* 2002; Abrahams *et al.* 2005; O'Leary *et al.* 2018). In addition, NR2E1 has been implicated in schizophrenia, bipolar disorder, metastatic castration-resistant prostate cancer, and post-stroke neurogenesis (Abdolmaleky *et al.* 2018; Jia *et al.* 2018; Nampoothiri *et al.* 2018). *NR2E1* was originally chosen for a neurogenic pattern in the brain and for the Müller glia of the retina (Miyawaki *et al.* 2004; Schmouth *et al.* 2012a; Corso-Díaz and Simpson 2015). We have previously demonstrated MiniPromoter Ple264 (NR2E1, 3,026 bp)
shows restricted expression in the Müller glia of the retina in rAAV (de Leeuw et al. 2016). Also, based on that work, we did not expect to capture the endogenous brain expression of NR2E1 with this MiniPromoter.

In the inducible strain (tm346a), the NR2E1 MiniPromoter expressed as expected in the retina and the brain (non-specifically) (Table 4). In the retina, this included endogenous-like expression mainly in the Müller glia (Figure 7A). Expression was lower in female mice as expected due to X-chromosome inactivation. Co labeling with anti-Sox9 confirmed the expression was primarily localized to the Müller glia. As expected, mice carrying this inducible allele, who were fed a no-tamoxifen control diet did not show any tdTomato expression.

In the constitutive strain (tm346b), the NR2E1 MiniPromoter was not specific and expressed in an additional cell type in the retina, in the cornea, and as expected in the brain (non-specifically) (Table 4). In the retina, this included expression in both amacrine and Müller glia, and in the cornea in the stromal and endothelial cell layers (Figure 7B). Expression was lower in female mice as expected due to X-chromosome inactivation.

**Discussion**

The first problem this work addressed was the need for more cre recombinase expressing transgenic mouse strains. We have substantially added to the cre-driver collection by developing 27 strains for cre expression in the brain and eye. These strains include 10 MaxiPromoters driving icre/ERT2, 14 MiniPromoters driving icre/f3/ERT2/f3 (from which 14 more constitutive alleles can be derived), and three controls driving icre/f3/ERT2/f3 (from which three more constitutive alleles can be derived). All strains were characterized by RT-PCR,
and two MaxiPromoter and four MiniPromoter strains were also chosen for detailed characterization. Since both the inducible and constitutive alleles were characterized for the MiniPromoter strains, a total of 10 new cre alleles were characterized by crossing to cre-reporter mice followed by extensive histology.

We found the HuGX methodology, which docks constructs on the X Chromosome at the same site just 5′ of the Hprt gene (Schmouth et al. 2012b; Schmouth et al. 2013), to be rapid. MaxiPromoters (BACs) retrofitting with the homology arms (3 months) is substantially more challenging than MiniPromoters (plasmids) cloning (2 weeks), but from construct to germline is possible in 20 weeks. In both cases the failure rate is low (9% (1/11) for MaxiPromoters, and 5% (1/19) for MiniPromoters), and occurred when, despite four or more correctly targeted ESC clones, all the chimeras failed to pass the targeted allele through the germline. Thus, this is a viable, rapid, methodology to make cre-driver strains.

The X-chromosome location of cre, resulting from the HuGX methodology, presents both a challenge and an opportunity. The challenge is related to fact that there are published examples where the sex of the parent carrying cre has been shown to broaden undesirably the deletion phenotype, secondary to expression in either the maternal and/or paternal germline (Hayashi et al. 2003; Kobayashi and Hensch 2013). With the Hprt knock-in strategy, when hemizygous males are desired for study, the cre allele must be brought through the maternal parent, without the option of shifting to the paternal if problems arise. However, using cell specific promoters to drive cre, as in this study, should reduce or eliminate this challenge. The unique opportunity is that when the cre is brought through either the maternal or paternal
parent, X-chromosome inactivation mosaic heterozygous females are obtained; mosaicism can be a useful analytical tool, as exemplified by this study of expression and others (Sakata et al. 2016; Renthal et al. 2018).

The most impressive endogenous-like expression for the MaxiPromoters was the CLDN5 inducible strain, which showed reproducible strong expression in the blood vessel endothelial cells of the brain, eye, and spinal as expected (Nitta et al. 2003; Sanford et al. 2005; Paul et al. 2013). This strain also showed some iCre leakiness, but even this expression remained restricted to the blood vessels. Cre protein leakiness is a known concern associated with tamoxifen inducible alleles (Murray et al. 2012), and may be due to incomplete cre sequestering when a large amount of cre protein is produced. We conclude the BAC employed in this work contained all the necessary regulatory regions for endogenous expression of this gene, which is further supported by the successful MiniPromoters we have previously developed from this gene (Portales-Casamar et al. 2010; de Leeuw et al. 2014; de Leeuw et al. 2016). This strain may be of particular interest as expression of proteins to modify, or temporarily open, the blood brain barrier is of great interest for therapeutic accessibility to the brain. This cre-driver strain could be used to target the CLDN5-positive endothelial cells by both experiments employing breeding to a conditional allele, or by intravenous delivery of a cre-dependent virus.

The most impressive endogenous-like expression amongst the MiniPromoters was the NR2E1 inducible strain, which showed reproducible strong expression in the Müller glia of the retina (Miyawaki et al. 2004; Schmouth et al. 2012a; Corso-Díaz and Simpson 2015). Based on
previous studies in rAAV (de Leeuw et al. 2016), the MiniPromoter worked as expected, containing all the necessary regulatory regions for expression in Müller glia. Also, as predicted by the rAAV studies, we observed a low level expression throughout the brain and not the endogenous neurogenic brain expression expected for this gene. This strain may be of particular use in that Müller glia are an important target in retinal therapy, since they provide essential components to the neurons of the retina and thus may be able to widely deliver a neuroprotective therapeutic (Ueki et al. 2015). This cre-driver strain could be used to target the Müller glia by both experiments employing breeding where the conditional allele expression is itself restricted, or by direct-eye delivery of a cre-dependent virus.

We were delighted with the functionality of the new inducible-first, constitutive-ready allele (icre/f3/ERT2/f3). With this allele, one targeted ESC line having gone germline allows the generation of two cre-driver strains. The initial strain carries a tamoxifen inducible conditional ice/ERT2 allele, and the second, after rearrangement by the Flp recombinase to remove ERT2, a constitutively active icre allele. We tested this allele by generating four pairs of MiniPromoter strains; inducible and constitutive and showed the allele functioned as expected with no deleterious effect of the amino acids added by the presence of the f3 sites. In our work, which exclusively focused on adult mice, the inducible allele was the most informative, and the constitutive allele showed substantially broader and stronger expression as expected of a historical marker capturing both the embryonic and adult expression.

The second problem this work addressed was to restrict expression further, even beyond that of the endogenous gene, to specific cell types in the brain and eye for spatial and temporal
control of cre-dependent genes. As highlighted above, there were some very successful strains produced by the HuGX strategy, but we also want to discuss examples of the challenges. Based on our previous overall success using human BACs to delineate non-coding regulatory regions of human brain genes (Schmouth et al. 2013), we were surprised by the result with the CRH gene. The CRH MaxiPromoter showed expression in the target regions of the brain, but only a subset of neurons in a given structure co-expressed endogenous Crh mRNA. This observation, however, is in line with previous reports demonstrating a significant amount of ectopic and missing expression observed in different Crh-cre driver and reporter strains (Keegan et al. 1994). Due to the partial overlap in correct expression observed, we expect that the missing expression is likely due to some regulatory element(s) that have not been captured in the selected BAC, or from the human regulatory regions present in the BAC being non-functional in the mouse. This strain represents an example of how even with large BACs the HuGX method can fail to reproduce fully the endogenous expression. In contrast, other groups have used 3' knock-in at the endogenous mouse locus to produce successful Crh cre-drivers (Taniguchi et al. 2011; Krashes et al. 2014).

For the MiniPromoter strategy to narrow expression we generated three controls strains, two with ubiquitous promoters (Hspa1a and CAGGS) and one promoterless (none), which were all analyzed by RT-PCR for cre transcription (Table 3). Of these, the unexpected and concerning result was the strong and widespread transcription obtained when no promoter was cloned into our MiniPromoter expression cassette, especially given the presence of flanking insulators. This was particularly surprising in that we (Portales-Casamar et al. 2010; de Leeuw et al. 2014), and others (Farhadi et al. 2003; Palais et al. 2009; Samuel et al. 2009), have successfully used
this location 5’ of the ubiquitous \( \text{Hprt} \) gene for restricted expression of small promoters before, even without insulators. We were therefore delighted to find that when a specific promoter was placed into the current expression cassette, restricted expression could be obtained (Figures 4-7, and Table 4). Thus, we suspect the addition of insulators was of little value in our experiments, and the neutrality of the \( \text{Hprt} \) genomic region may not be as complete as previously thought (Farhadi \textit{et al.} 2003; Palais \textit{et al.} 2009; Schmouth \textit{et al.} 2013; de Leeuw \textit{et al.} 2014).

Another example of lack of locus neutrality may be the \( \text{PCP2} \) (Ple155) inducible MiniPromoter strain, which drove strong and reproducible expression specifically in the bipolar ON cells of the retina, as expected (de Leeuw \textit{et al.} 2016). However, based on our previous results, and those of others, with Ple155 (\( \text{PCP2} \)) driving icre expression in rAAV (de Leeuw \textit{et al.} 2016; Chan \textit{et al.} 2017), we expected the \( \text{PCP2} \) (Ple155) inducible MiniPromoter strain to also drive specific expression in the Purkinje cells of the cerebellum. What we observed instead was low-level expression throughout the brain. We expect that this finding may be due to the ubiquitous \( \text{Hprt} \) locus influencing the expression of the promoter to become ubiquitous in the brain. Regardless, this strain may be of particular interest in that bipolar ON cells are an important target in retinal therapy for stationary night blindness and melanoma associated retinopathy (Scalabrino \textit{et al.} 2015; Zeitz \textit{et al.} 2015; Martemyanov and Sampath 2017), and may have additional utility in optogenetic strategies (Mace \textit{et al.} 2015). This cre-driver strain could be used to target the bipolar ON cells by both experiments employing breeding where the conditional allele expression is itself restricted, or by direct-eye delivery of a cre-dependent virus.
In this evolving field, what do we see as the best way forward for further expanding the cre-driver collection and for cell-type restricted strains? Certainly, CRISPR has now made knocking in cre at any mouse gene at least as fast and easy as docking 5′ of *Hprt*. Methods such as synthesized guide RNAs, short homology sequences, purified Cas9 protein, and direct introduction into mouse zygotes, have already enabled researchers to develop new constitutive and inducible cre-driver mice (Feng *et al.* 2016; Hasegawa *et al.* 2016; Ackermann *et al.* 2017; Mohsen *et al.* 2017; Daigle *et al.* 2018). However, with this strategy the possibility remains of generating a deleterious allele in the very gene whose normal expression is to be captured. In this regard, it is probably best to avoid knock in at the ATG, which typically produces a null allele and heterozygous mouse, in favour of knock-in at the 3′ end of the gene, but even this may produce a hypomorphic allele affecting gene expression. Nevertheless, these endogenous-locus strategies are powerful and may very accurately capture the full complexity of the mouse gene expression. Combining them with the new inducible-first constitutive-ready allele developed here would add even further improvement. Still, the locus 5′ of the *Hprt* remains a viable option for a variety of situations including: when mutation of the endogenous gene is undesirable, when a BAC is likely to contain all the necessary regulatory regions (MaxiPromoter), when the purpose is to capture only a portion of the natural endogenous expression (MiniPromoter), and when a human (or any non-mouse) gene is being used and studied. Thus, even today, multiple strategies for the generation of cre-driver strains are warranted.
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### Tables

| Chosen For          | Gene    | Ple | Parental BAC (RP11-) | BAC Size (bp) | Retrofitted BAC (bEMS) | Targeted ESC line (mEMS) | Strain Name (B6.Cg-) | JAX Stock # |
|---------------------|---------|-----|----------------------|--------------|------------------------|-------------------------|-----------------------|-------------|
| Thalamus            | SLTRK6  | 252 | 398A22               | 197,372      | 168                    | 5708*                   | Hprt<sup>tm</sup>340(Ple252-icre/ERT2)Ems | 023685      |
| Basal Ganglia       | AGTR1   | 270 | 487M24               | 231,190      | 237                    | 5930§                   | Hprt<sup>tm</sup>343(Ple270-icre/ERT2)Ems | 023688      |
| Blood Brain Barrier | CLDN5   | 272 | 16C10                | 197,627      | 191                    | 5806*                   | Hprt<sup>tm</sup>332(Ple272-icre/ERT2)Ems | 023677      |
| PvH                 | CRH     | 274 | 1006F7               | 205,667      | 192                    | 5758*                   | Hprt<sup>tm</sup>353(Ple274-icre/ERT2)Ems | 023698      |
| Cortex              | HTR1B   | 277 | 990K4                | 196,504      | 228                    | 5794*                   | Hprt<sup>tm</sup>335(Ple277-icre/ERT2)Ems | 023680      |
| Striatum            | KCNA4   | 278 | 63H13                | 191,783      | 240                    | 6120§                   | Hprt<sup>tm</sup>357(Ple276-icre/ERT2)Ems | 023702      |
| Hippocampus         | NEUROD6 | 281 | 1087H14              | 209,931      | 193                    | 5724*                   | Hprt<sup>tm</sup>333(Ple281-icre/ERT2)Ems | 023678      |
| Hippocampus         | NPY2R   | 283 | 937K11               | 188,618      | 241                    | 5899§                   | Hprt<sup>tm</sup>354(Ple281-icre/ERT2)Ems | 023699      |
| Neural Stem Cells   | SOX3    | 286 | 1019M14              | 208,479      | 244                    | 5931§                   | Hprt<sup>tm</sup>355(Ple286-icre/ERT2)Ems | 023700      |
| Hypoxia             | SPRY1   | 287 | 1126D8               | 209,197      | 248                    | 6107*                   | Hprt<sup>tm</sup>358(Ple287-icre/ERT2)Ems | 023703      |

**Table 1.** Ten novel Hprt targeted human MaxiPromoter strains generated.

*<sup>*</sup>, mEMS4857 was the parental ESC line; §, mEMS4855 was the parental ESC line; BAC, bacterial artificial chromosome; bEMS, BAC Elizabeth M. Simpson; ESC, embryonic stem cell; JAX, The Jackson Laboratory; mEMS, mammalian cell line Elizabeth M. Simpson; Ple, Pleiades promoter; PvH, paraventricular hypothalamic nucleus.
| Chosen For                  | Gene/ Genomic Region | Ple | MiniP Size (bp) | Plasmid name (pEMS) | Targeted ESC line (mEMS) | Strain Name (B6.Cg-) | JAX Stock # |
|----------------------------|----------------------|-----|----------------|---------------------|--------------------------|----------------------|-------------|
| Raphe Nuclei               | FEV                  | 67  | 2,202          | 2011                | 6017*                    | Hprt<sup>tm366oa</sup>Ple<sup>667icre/ERT2</sup>Ems | 023711      |
| Gabaergic Neurons          | GPR88                | 94  | 3,049          | 2014                | 6029*                    | Hprt<sup>tm590ap</sup>Ple<sup>94-icre/ERT2</sup>Ems | 023704      |
| Purkinje, Bipolar          | PCP2                 | 155 | 1,652          | 2004                | 5985*                    | Hprt<sup>tm347oa</sup>Ple<sup>155-icre/ERT2</sup>Ems | 023692      |
| Raphe Nuclei               | SLC6A4               | 198 | 2,826          | 2015                | 6044*                    | Hprt<sup>tm310oa</sup>Ple<sup>198-icre/ERT2</sup>Ems | 023696      |
| Thalamus                   | TNNT1                | 232 | 1,209          | 2010                | 6006*                    | Hprt<sup>tm350oa</sup>Ple<sup>232-icre/ERT2</sup>Ems | 023695      |
| Cortex                     | C8ORF46              | 251 | 2,453          | 2002                | 5937*                    | Hprt<sup>tm367oa</sup>Ple<sup>251-icre/ERT2</sup>Ems | 023712      |
| Substantia Nigra           | PITX3                | 253 | 2,484          | 2003                | 5929*                    | Hprt<sup>tm360oa</sup>Ple<sup>253-icre/ERT2</sup>Ems | 023705      |
| Neurogenic, Müller Glia    | NR2E1                | 264 | 3,026          | 2006                | 6004§                    | Hprt<sup>tm46oa</sup>Ple<sup>264-icre/ERT2</sup>Ems | 023691      |
| Bipolar                    | PCP2                 | 265 | 986            | 2005                | 5991*                    | Hprt<sup>tm348oa</sup>Ple<sup>265-icre/ERT2</sup>Ems | 023693      |
| Glia, Astrocytes           | S100B                | 266 | 2,982          | 2007                | 5950*                    | Hprt<sup>tm365oa</sup>Ple<sup>266-icre/ERT2</sup>Ems | 023710      |
| Glia, Oligodendroglia      | UGT8                 | 267 | 3,014          | 2009                | 5970*                    | Hprt<sup>tm361oa</sup>Ple<sup>267-icre/ERT2</sup>Ems | 023706      |
| Glia, Oligodendroglia      | OLIG1                | 304 | 2,596          | 2018                | 6089*                    | Hprt<sup>tm362oa</sup>Ple<sup>304-icre/ERT2</sup>Ems | 023707      |
| Forebrain                  | hs671↑               | N/AP| 2,129          | 2016                | 6068*                    | Hprt<sup>tm349oa</sup>hs<sup>671-icre/ERT2</sup>Ems | 023694      |
| Midbrain                   | hs1218↑              | N/AP| 2,338          | 2017                | 6070*                    | Hprt<sup>tm363oa</sup>hs<sup>1218-icre/ERT2</sup>Ems | 023708      |
| Minimal Promoter Control   | Hspa1a↑              | N/AP| 878            | 2012                | 6051*                    | Hprt<sup>tm352oa</sup>Hspa1a<sup>-icre/ERT2</sup>Ems | 023697      |
| Ubiquitous Control         | CAGGS                | N/AP| 1,723          | 2013                | 6096*                    | Hprt<sup>tm364oa</sup>CAGGS<sup>-icre/ERT2</sup>Ems | 023709      |
| Promoterless Control       | (none)               | N/AP| 0              | 2001                | 5895*                    | Hprt<sup>tm345oa</sup> icre/ERT2<sup>Ems</sup> | 023690      |

Table 2. 17 novel Hprt targeted MiniPromoter strains generated.

*, mEMS4855 was the parental ESC line; §, mEMS4857 was the parental ESC line; †, genomic regions from the VISA Enhancer Project ([https://enhancer.lbl.gov/](https://enhancer.lbl.gov/)); ‡, synonym Hsp68; ESC, embryonic stem cell; JAX, The Jackson Laboratory; mEMS, mammalian cell lines Elizabeth M. Simpson; MiniP, MiniPromoter; N/AP, not applicable; pEMS, plasmid Elizabeth M. Simpson; Ple, Pleiades promoter.
| Ple | Gene/Genomic Region | Regions Tested | RT-PCR Results | Chosen for Detailed Characterization |
|-----|---------------------|----------------|----------------|--------------------------------------|
| 252 | SLITRK6             | Telenceph (w/o hipp), Hipp, Diencephalon | Weak | No |
| 270 | AGTR1               | Telenceph (w/o hipp), Hipp, Diencephalon | Weak | No |
| 272 | CLDN5               | Olfactory Bulb, CB | Strong | Yes; see Fig 2, Table 4 |
| 274 | CRH                 | Olfactory Bulb, Telenceph (w/o hipp), Hindbrain (w/o CB) | Very weak | Yes; see Fig 3, Table 4 |
| 277 | HTR1B               | Telenceph (w/o hipp), CB | Very weak | No |
| 278 | KCNA4               | Olfactory Bulb, Telencephalon, Diencephalon | Strong | No |
| 281 | NEUROD6             | Cortex, Hipp | Strong | No |
| 283 | NPY2R               | Telenceph (w/o hipp), Hipp, Diencephalon | Strong | No |
| 286 | SOX3                | Olfactory Bulb, Telencephalon, Hipp | Strong | No |
| 287 | SPRY1               | Olfactory Bulb, Telencephalon, Hipp | Strong | No |
| 67  | FEV                 | Diencephalon, Midbrain, Hindbrain (w/o CB) | Strong | No |
| 94  | GPR88               | Telenceph (w/o hipp), Hipp, Diencephalon | Strong | No |
| 155 | PCP2                | Eyes, Diencephalon, CB | Strong | Yes; see Fig 4, Table 4 |
| 198 | SLC6A4              | Olfactory Bulb, Diencephalon, Hindbrain (w/o CB) | Strong | Yes; see Fig 6, Table 4 |
| 232 | TNNT1               | Hipp, Diencephalon, Hindbrain (w/o CB) | Strong | No |
| 251 | CBORF46             | Olfactory Bulb, Telenceph (w/o hipp), Hipp | Strong | No |
| 253 | PITX3               | Diencephalon, Midbrain, Hindbrain (w/o CB) | Strong | No |
| 264 | NR2E1               | Olfactory Bulb, Telenceph (w/o hipp), Hipp | Strong | Yes; see Fig 5, Table 4 |
| 265 | PCP2                | Eyes, Diencephalon, CB | Strong | Yes; see Fig 7, Table 4 |
| 266 | S100B               | Midbrain, Hindbrain (w/o CB), CB | Strong | No |
| 267 | UGT8                | Diencephalon, Midbrain, Hindbrain (w/o CB) | Strong | No |
| 304 | OLIG1               | Telenceph (w/o hipp), Diencephalon, Midbrain | Strong | No |
| N/AP| hs671               | Eyes, Telenceph (w/o hipp), Hipp | Strong | No |
| N/AP| hs1218              | Telenceph (w/o hipp), Diencephalon, Midbrain | Strong | No |
| N/AP| Hspa1α*             | Telenceph (w/o hipp), Hipp, Diencephalon | Strong | No |
| N/AP| CAGGS               | Telenceph (w/o hipp), Hipp, Diencephalon | Strong | No |
| N/AP| (none)              | Telenceph (w/o hipp), Diencephalon, Hindbrain (w/o CB) | Strong | No |

Table 3. Summary of semi-quantitative RT-PCR results for the 27 strains developed.

Two to three key regions of interest were chosen for each strain and RNA prepared from N2 male mice. RT-PCR bands were scored as either: strong, weak, or very weak. MaxiPromoters are described first, followed by MiniPromoters below the line. *, synonym Hsp68; CB, cerebellum; Hipp, hippocampus N/AP, not applicable; Ple, Pleiades promoter; Telenceph, telencephalon; w/o, without.
| Ple | Gene | Allele | Retina | Cornea | Brain | Spinal Cord | Heart |
|-----|------|--------|--------|--------|-------|-------------|-------|
| 272 | CLDN5| tm332  | Positive: endothelial cells | Negative | Positive: endothelial cells | Positive | Positive |
| 274 | CRH  | tm353  | Negative | Negative | Positive: Ctx, MGM, Pir, PvH | Positive | Negative |
| 155 | PCP2 | tm347a | Positive: primarily in bipolar cells, low level in amacrine and GCL | Negative | Positive: low level non-specific* in the CB, Ctx, Hth, Thal | Negative | Negative |
| 155 | PCP2 | tm347b | Positive: primarily in INL, low level in GCL and Müller glia | Positive: subset of endothelial, epithelial, stroma | Positive: high level non-specific in the CB, Ctx, Hipp, Hth, OB, Stri, Thal | Negative | Positive |
| 265 | PCP2 | tm348a | Positive: primarily in bipolar cells, low level in amacrine and GCL | Negative | Positive: low level non-specific in the BS, Ctx, OB | Negative | Negative |
| 265 | PCP2 | tm348b | Positive: primarily in INL, low level in GCL and Müller glia | Positive: subset of endothelial, epithelial, stroma | Positive: high level non-specific in the CB, Ctx, Hipp, Hth, OB, Stri, Thal | Negative | Positive |
| 198 | SLC6A4| tm351a | Positive: widely spaced in the GCL and INL | Positive: stroma | Positive: low level non-specific in the blood vessels | Positive | Positive |
| 198 | SLC6A4| tm351b | Positive: primarily in INL, low level in GCL and Müller glia | Positive: endothelial, epithelial, stroma | Positive: high level non-specific in the blood vessels, Ctx, Hipp, Hth, OB, Stri, Thal | Positive | Positive |
| 264 | NR2E1| tm346a | Positive: primarily in Müller glia | Negative | Positive: low level non-specific in the blood vessels, Ctx, Hipp, Hth, OB, Stri, Thal | Positive | Positive |
| 264 | NR2E1| tm346b | Positive: primarily in Müller glia, low levels in the INL | Positive: endothelial, stroma | Positive: high level non-specific in the blood vessels, Ctx, Hipp, Hth, OB, Stri, Thal | Positive | Positive |

Table 4. Summary of cre-reporter expression pattern for ten strains.

**tm#a** (conditional) strains were crossed to a tdTomato cre reporter strain and expression induced in adult mice with tamoxifen. **Tm#b** (constitutive) strains were first generated by crossing tm#a strains to a flp germline-deleter strain to remove the ERT2 portion of the allele, and then the resulting tm#b was crossed to a tdTomato cre reporter strain. For both tm#a and tm#b, tdTomato expression was visualized from adult mouse tissue by epifluorescence.
MaxiPromoters are described first, followed by MiniPromoters below the line. *, neuronal and glial brain expression; CB, cerebellum; Ctx, cortex; GCL, ganglion cell layer; Hth, hypothalamus; Hipp, hippocampus; INL, inner nuclear layer; MGM, medial geniculate; OB, olfactory bulb; Pir, piriform cortex; Ple, Pleiades promoter; PvN, paraventricular nucleus; Stri, striatum; Thal, thalamus.
Figure Legends

Fig.1 MaxiPromoters (inducible) and MiniPromoters (inducible-first constitutive-ready).

A) MaxiPromoter inducible alleles were constructed by first inserting into a selected human bacterial artificial chromosome (BAC), *Hprt* homology arms and human *HPRT* coding sequence in the vector backbone, and then icre (improved cre)/ERT2 (mutant estrogen receptor) fusion cassette at the ATG encoding start codon of the human gene. Homologous recombination of the modified BAC at the mouse *Hprt*\textsuperscript{b-m3} deleted allele resulted in insertion of the MaxiPromoter driving icre/ERT2 immediately 5′ of *Hprt* and ‘correction’ of the *Hprt* deletion (*Hprt*\textsuperscript{tm#}).

B) Icre expression was histologically characterized by crossing the MaxiPromoter strain to the ROSA-stop-tdTomato strain and adult offspring carrying both alleles being fed tamoxifen-containing food so that the iCre/ERT2 protein recombined the loxP sites, removed the stop, and allowed ROSA driven tdTomato expression.

C) MiniPromoter inducible-first constitutive-ready alleles (tm#a) were constructed in plasmids with insulators (I) surrounding the expression cassette, a MiniPromoter driving icre/ERT2 with f3 sites surrounding ERT2 (icre/f3/ERT2/f3), WPRE transcriptional stabilization element, SV40 poly(A), and *Hprt* homology arms and human *HPRT* coding sequence. As in A), homologous recombination of the plasmid at the mouse *Hprt*\textsuperscript{b-m3} deleted allele resulted in insertion of the MiniPromoter driving icre/ERT2 immediately 5′ of *Hprt* and ‘correction’ of the *Hprt* deletion (*Hprt*\textsuperscript{tm#c}).

D) As in B), icre expression was characterized by crossing the MiniPromoter strain (tm#a) to the ROSA-stop-tdTomato strain and adult offspring being fed tamoxifen. The iCre/ERT2 protein recombined the loxP sites, which allowed ROSA driven tdTomato expression.

E) MiniPromoter constitutive alleles (tm#b) were generated by crossing the MiniPromoter tm#a strain to the CAG-flpo
deleter strain, which recombined the f3 sites in the germline and removed ERT2 \((Hprt^{tm#b})\). F

Similar to B) and D), icre expression was characterized by crossing the MiniPromoter strain \((tm#b)\) to the ROSA-stop-tdTomato strain, but with this constitutive allele, testing required no tamoxifen. The iCre protein recombined the loxP sites, which allowed ROSA driven tdTomato expression.
Fig. 2 MaxiPromoter Ple272-icre/ERT2 (CLDN5) (inducible) expressed in the endothelial cells of the blood-retina and blood-brain barrier.

Expression in the MaxiPromoter Ple272-icre/ERT2 strain was examined using tdTomato epifluorescence (red). Adult mice that carried both the icre/ERT2 and ROSA-Stop-tdTomato alleles were fed either tamoxifen or no tamoxifen. A) Retina. First panel: tamoxifen fed male and female mice stained in the retinal blood vessels, with female staining slightly less as expected due to X-chromosome inactivation; no-tamoxifen male showed unexpected blood vessel staining due to icre “leakiness” (tamoxifen-independent cre activity). Hoechst nuclear stain (blue). Second panel: data shown without Hoechst. Third panel: tamoxifen fed male mouse co-stained with anti-CLDN5 antibody (green) demonstrated co-labeling with tdTomato (merge, yellow) and thus endogenous-like expression in the endothelial cells of the blood vessels (white arrow).

B) Brain. First panel: tamoxifen fed male and female mice stained in blood vessels of the brain (hippocampal region shown), with female staining less as expected due to X-chromosome inactivation; no-tamoxifen male showed some blood vessel staining due to icre leakiness. Hoechst nuclear stain (blue). Second panel: data shown without Hoechst. Third panel: tamoxifen fed male mouse co-stained with anti-CLDN5 antibody (green) demonstrated co-labeling with tdTomato (merge, yellow) and thus endogenous-like expression in the endothelial cells of the blood vessels (white arrow). Scale bars represent 100 µm.
Fig. 3 MaxiPromoter Ple274-icre/ERT2 (CRH) (inducible) expressed in the appropriate brain regions, but with partial cellular overlap.

Expression in the MaxiPromoter Ple274-icre/ERT2 strain was examined using tdTomato epifluorescence (red). Adult mice that carried both the icre/ERT2 and ROSA-Stop-tdTomato alleles and were fed either tamoxifen or no tamoxifen (shown with Hoechst nuclear stain (blue)). A) Tamoxifen-fed male (first column) and female (second column) mice showed tdTomato positive neurons in brain regions representing major CRH expression domains including the piriform cortex (Pir), the paraventricular nucleus of the hypothalamus (PvN), the medial division of the medial geniculate (MGM), and the neocortex (Ctx). Female mice stained less as expected due to X-chromosome inactivation. Mice carrying this inducible allele, who were fed a no-tamoxifen control diet (third column), as expected did not show any tdTomato expression. B) Tamoxifen fed male mouse was assessed for co-expression by double in situ hybridization. Depicted are bright field photomicrographs of coronal brain sections. Black arrows indicate cells expressing only Crh mRNA (silver grains). Red arrowheads indicate cells only expressing tdTomato mRNA (red staining). White arrowheads indicate cells co-expressing Crh and tdTomato. Scale bars represent 100 µm.
Fig. 4 MiniPromoter Ple155-icre/f3/ERT2/f3 (PCP2) (inducible) expressed in the bipolar ON cells of the retina.

Expression in the MiniPromoter Ple155-icre/f3/ERT2/f3 strain was examined both as an A) inducible allele, and B) constitutive allele using tdTomato epifluorescence (red). **A** Adult mice that carried both the inducible icre/f3/ERT2/f3 and ROSA-Stop-tdTomato alleles were fed either tamoxifen or no tamoxifen. First panel: tamoxifen fed male and female mice stained primarily in the bipolar cells of retina, with female staining slightly less as expected due to X-chromosome inactivation; no-tamoxifen male showed only occasional positive staining. Hoechst nuclear stain (blue). Second panel: data shown without Hoechst. Third panel: tamoxifen male mouse co-stained with anti-PCP2 antibody (green) demonstrating co-labeling with tdTomato (merge, yellow), and thus endogenous-like expression in the bipolar ON cells of the retina (white arrow). **B** Adult mice that carried both the constitutive icre and ROSA-Stop-tdTomato alleles. First panel, columns 1 and 2: male and female mice stained throughout the retina (ganglion cell layer, inner nuclear layer, Müller glia), with female staining slightly less as expected due to X-chromosome inactivation. Second panel, columns 1 and 2: data shown without Hoechst. First and second panel, column 3: male and female mice stained throughout all three layers of the cornea (epithelial, stromal, and endothelial). Scale bars represent 100 µm.
**Fig. 5** MiniPromoter Ple265-icre/f3/ERT2/f3 (*PCP2*) (inducible) expressed in the bipolar ON cells of the retina.

Expression in the MiniPromoter Ple265-icre/f3/ERT2/f3 strain was examined both as an A) inducible allele, and B) constitutive allele using tdTomato epifluorescence (red). **A)** Adult mice that carried both the inducible icre/f3/ERT2/f3 and ROSA-Stop-tdTomato alleles were fed either tamoxifen or no tamoxifen. First panel: tamoxifen fed male mice stained primarily in the bipolar cells of retina; no-tamoxifen male showed only occasional positive staining. Hoechst nuclear stain (blue). Second panel: data shown without Hoechst. Third panel: tamoxifen male mouse co-stained with anti-PCP2 antibody (green) demonstrating co-labelling with tdTomato (merge, yellow), and thus endogenous-like expression in the bipolar ON cells of the retina (white arrow). **B)** Adult mice that carried both the constitutive icre and ROSA-Stop-tdTomato alleles. First panel, columns 1 and 2: male and female mice stained throughout the retina (ganglion cell layer, inner nuclear layer, Müller glia), with female staining slightly less as expected due to X-chromosome inactivation. Second panel, columns 1 and 2: data shown without Hoechst. First and second panel, column 3: male and female mice stained throughout all three layers of the cornea (epithelial, stromal, and endothelial), with female staining less as expected due to X-chromosome inactivation. Scale bars represent 100 µm.
Figure 1.

MaxiPromoter Strains

A) MaxiP

5' HA Intragenic Human Gene Hprt 3' HA

B) x ROSA-stop-tdTomato

When Fed Tamoxifen Food

MiniPromoter Strains

C) MiniP

5' HA lcre ERT2 W fRT OA 3' HA

D) x ROSA-stop-tdTomato

When Fed Tamoxifen Food

E) x CAG-flpo

F) x ROSA-stop-tdTomato
A) MaxiPromoter Ple272-icre/ERT2 (CLDN5) (inducible) Retina

Male X<sup>0</sup>Y Tamoxifen  Female X<sup>0</sup>X Tamoxifen  Male X<sup>0</sup>Y No Tamoxifen

Hoechst (blue)  tdTomato (red)

B) MaxiPromoter Ple272-icre/ERT2 (CLDN5) (inducible) Brain

Male X<sup>0</sup>Y Tamoxifen  Female X<sup>0</sup>X Tamoxifen  Male X<sup>0</sup>Y No Tamoxifen

Hoechst (blue)  tdTomato (red)

Figure 2.
A) MaxiPromoter Ple274-icre/ERT2 (CRH) (inducible) Fluorescence

B) MaxiPromoter Ple274-icre/ERT2 (CRH) (inducible) In Situ Hybridization

Figure 3.
Figure 4.
A) MiniPromoter Ple265-icre/ERT2 (PCP2) (inducible)

Male X<sup>ky</sup>Y Tamoxifen

Male X<sup>ky</sup>Y No Tamoxifen

Retina

Hoechst (blue)  tdTomato (red)

tdTomato (red)

Male X<sup>ky</sup>Y Tamoxifen

Retina

Hoechst (blue)  tdTomato (red)

Anti-PCP2 (green)

Merge (yellow)

B) MiniPromoter Ple265-icre (PCP2) (constitutive)

Male X<sup>ky</sup>Y

Female X<sup>ky</sup>X

Male X<sup>ky</sup>Y

Retina

Hoechst (blue)  tdTomato (red)

tdTomato (red)

Cornea

Female X<sup>ky</sup>X

Hoechst (blue)  tdTomato (red)
Figure 6.
Figure 7.
Supplemental Materials

A) Generating females for breeding and males for RT-PCR analysis

B6/J-Hprt<sup>+/+</sup>  X  B6/NTac-Hprt<sup>tmIndy(Prek-creERT2)Emx1/Y</sup> Chimera

B6-Hprt<sup>tmIndy(Prek-creERT2)Emx1/Y</sup>  X  B6/J-Hprt<sup>+/Y</sup>

B6-Hprt<sup>tmIndy(Prek-creERT2)Emx1/Y</sup>



B) Generating B6129F1 inducible cre tm<sup>a</sup> females and males for histology

B6-Hprt<sup>tmIndy(Prek-creERT2)Emx1/Y</sup>  X  129-Hprt<sup>+/Y</sup>, ROSA-stop-tdTomato

B6129F1-Hprt<sup>tmIndy(Prek-creERT2)Emx1/Y</sup>, ROSA-tdTomato (cre conditional)

B6129F1-Hprt<sup>tmIndy(Prek-creERT2)Emx1/Y</sup>, ROSA-tdTomato (cre conditional)



C) Generating B6129F1 constitutive cre tm<sup>b</sup> females and males for histology

B6-Hprt<sup>tmIndy(Prek-creERT2)Emx1/Y</sup>  X  B6/J-Hprt<sup>+/Y</sup>, CAG-flpo

B6-Hprt<sup>tmIndy(Prek-creERT2)Emx1/Y</sup>, CAG-flpo  X  129-Hprt<sup>+/Y</sup>, ROSA-stop-tdTomato

B6129F1-Hprt<sup>tmIndy(Prek-creERT2)Emx1/Y</sup>, ROSA-tdTomato (cre conditional)

B6129F1-Hprt<sup>tmIndy(Prek-creERT2)Emx1/Y</sup>, ROSA-tdTomato (cre conditional)
Fig. S1 Typical breeding strategy for MaxiPromoter and MiniPromoter mice.

A) Generating B6 females for breeding and males for RT-PCR analysis. Male chimeras carrying cells from the targeted B6/NTac embryonic stem cell line, for either a MaxiPromoter or MiniPromoter inserted at the Hprt gene on the X Chromosome, were bred to female B6/J to generate germline heterozygous female mice (B6-Hprt<sup>tm#a(Ple#-ice/ERT)Ems/+</sup>). These heterozygous females were then bred to B6/J males to generate both more heterozygous female mice for further breeding and hemizygous male mice (B6-Hprt<sup>tm#a(Ple#-ice/ERT2)Ems/Y</sup>) for RT-PCR analysis. B) Generating B6129F1 inducible cre tm#a females and males for histology. Heterozygous female MaxiPromoter or MiniPromoter mice were bred to 129-ROSA-stop-tdTomato males to generate both B6129F1 heterozygous female and hemizygous male inducible mice, conditionally expressing tdTomato once fed tamoxifen food. C) Generating B6129F1 constitutive cre tm#b females and males for histology. Heterozygous female MiniPromoter mice were bred to B6/J-CAG-flpo males to generate heterozygous female constitutive cre mice (B6-Hprt<sup>tm#b(Ple#-ice)Ems/+</sup>), these females were then bred to 129-ROSA-stop-tdTomato males to generate both B6129F1 heterozygous female and hemizygous male constitutive cre mice, conditionally expressing tdTomato for histology. #, stands for all numbers in the collection; 129, 129S1/SvImJ; B6/J, C57BL/6J; B6/NTac, C57BL/6NTac; B6, mixed genetic background of B6/NTac and B6/J; tm, targeted mutation; Ple, Pleiades promoter. Agouti, A<sup>w-J</sup> from B6/NTac embryonic stem cells or A<sup>w</sup> from 129; black, a from B6/J.
A) UBC-cre/ERT2 (inducible)

Tamoxifen

Retina

Hoechst (blue)  tdTomato (red)

tdTomato (red)

No Tamoxifen

Cornea

Hoechst (blue)  tdTomato (red)

B) ACTB-cre (constitutive)

C) ROSA-stop-tdTomato

Retina

Hoechst (blue)  tdTomato (red)

tdTomato (red)

Cornea

Hoechst (blue)  tdTomato (red)
Fig. S2 Control strains showed the typical expression of cre.

A) The UBC-cre/ERT2 strain was used as a control for the tamoxifen-inducible strains. Adult mice that carried both the UBC-cre/ERT2 and ROSA-stop-tdTomato alleles were fed either tamoxifen food or no tamoxifen control food, and cre expression was examined using tdTomato epifluorescence (red). First panel: tamoxifen fed mouse stained throughout most layers of the retina; no-tamoxifen mouse also stained at a low level in the retina due to cre “leakiness”. Hoechst nuclear stain (blue). Second panel: data shown without Hoechst. Third panel: tamoxifen fed mouse stained in the stromal and endothelial layers of the cornea; no-tamoxifen mouse also stained at a low level in the cornea due to cre “leakiness”. B) The ACTB-cre strain was used as a control for the constitutive strains. Adult mice carried both the ACTB-cre and ROSA-stop-tdTomato alleles. First panel: mouse stained throughout most layers of the retina. Second panel: data shown without Hoechst. Third panel: mouse stained in all three layers of the cornea (epithelial, stromal, and endothelial). C) The ROSA-stop-tdTomato strain, not having been crossed to any cre strain, was used as a negative control for the constitutive strains. First panel: as expected, no tdTomato expression was observed in the retina. Second panel: data shown without Hoechst. Third panel: as expected, no tdTomato expression was observed in the cornea. Scale bars represent 100 µm.
| Ple | Gene/ Genomic Region | Size (bp) | Design Type | Design Source | Previously Published (PMID) |
|-----|----------------------|-----------|-------------|---------------|-----------------------------|
| 67  | FEV                  | 2,202     | Unaltered   | KI mouse, rAAV| 20807748, 24761428, 27164903 |
| 94  | GPR88                | 3,049     | Unaltered   | rAAV          | 27164903                   |
| 155 | PCP2                 | 1,652     | Unaltered   | KI mouse, rAAV| 24761428, 27164903         |
| 198 | SLC6A4               | 2,826     | Unaltered   | rAAV          | 27164903                   |
| 232 | TNNT1                | 1,209     | Unaltered   | KI mouse      | 24761428                   |
| 251 | C8ORF46              | 2,453     | Unaltered   | rAAV          | 27164903                   |
| 253 | PITX3                | 2,484     | Unaltered   | rAAV          | 27164903                   |
| 264 | NR2E1                | 3,026     | Unaltered   | rAAV          | 27164903                   |
| 265 | PCP2                 | 986       | Cut Down    | Ple155        | N/AP                       |
| 266 | S100B                | 2,982     | Unaltered   | rAAV          | 27164903                   |
| 267 | UGT8                 | 3,014     | Unaltered   | rAAV          | 27164903                   |
| 304 | OLG1                 | 2,596     | Unaltered   | rAAV          | 27164903                   |
| N/AP| hs671                | 2,129     | Literature  | VISTA RI mouse (DPYD) | 17130149, 23253453, 27164903 |
| N/AP| hs1218               | 2,338     | Literature  | VISTA RI mouse (OTX2) | 17130149, 27164903        |
| N/AP| Hspa1a*              | 878       | Literature  | VISTA control | 17130149                   |
| N/AP| CAGGS                | 1,723     | Unaltered   | rAAV          | 27164903                   |

**Table S1. Design details for the 15 MiniPromoters plus one control promoter.**

*, synonym Hsp68; KI, knock in; N/AP, not applicable; Ple, Pleiades promoter; PMID, PubMed ID number; rAAV, recombinant adeno-associated virus; RI, random insertion; VISTA, VISTA Enhancer Project (https://enhancer.lbl.gov/).
Fig. 6 MiniPromoter Ple198-icre/f3/ERT2/f3 (SLC6A4) (inducible) expressed in retinal ganglion and amacrine cells.

Expression in the MiniPromoter Ple198-icre/f3/ERT2/f3 strain was examined both as an A) inducible allele, and B) constitutive allele using tdTomato epifluorescence (red). A) Adult mice that carried both the inducible icre/f3/ERT2/f3 and ROSA-Stop-tdTomato alleles were fed either tamoxifen or no tamoxifen. First panel: tamoxifen fed male and female mice stained in the ganglion cell layer and inner nuclear layer of the retina, with female overall staining slightly less as expected due to X-chromosome inactivation; no-tamoxifen male, as expected, did not show any tdTomato expression. Hoechst nuclear stain (blue). Second panel: data shown without Hoechst. Third panel: tamoxifen fed male and female mice stained in the stromal layer of the cornea, with female overall staining slightly less as expected due to X-chromosome inactivation; no-tamoxifen male, as expected, did not show any tdTomato expression. Fourth panel: tamoxifen male mouse co-stained with anti-POU4F1 (also known as BRN3A; green, columns 1 and 2) and anti-GAD65/67 (green, columns 3 and 4) demonstrating co-labeling with tdTomato (merge, yellow), and thus expression in the retinal ganglion and GABAergic amacrine cells of the retina (white arrows). B) Adult mice that carried both the constitutive icre and ROSA-Stop-tdTomato alleles. First panel, columns 1 and 2: male and female mice stained throughout the retina (ganglion cell layer, inner nuclear layer, Müller glia), with female staining slightly less as expected due to X-chromosome inactivation. Second panel, columns 1 and 2: data shown without Hoechst. First and second panel, column 3: male and female mice stained throughout all three layers of the cornea (epithelial, stromal, and endothelial), with female staining less as expected due to X-chromosome inactivation. Scale bars represent 100 μm.
Fig. 7 MiniPromoter Ple264-icre/f3/ERT2/f3 (NR2E1) (inducible) expressed in the Müller glia cells of the retina.

Expression in the MiniPromoter Ple264-icre/f3/ERT2/f3 strain was examined both as an A) inducible allele, and B) constitutive allele using tdTomato epifluorescence (red). A) Adult mice that carried both the inducible icre/f3/ERT2/f3 and ROSA-Stop-tdTomato alleles were fed either tamoxifen or no tamoxifen. First panel: tamoxifen fed male and female mice stained in the Müller glia of the retina, with female staining less as expected due to X-chromosome inactivation; no-tamoxifen male, as expected, did not show any tdTomato expression. Hoechst nuclear stain (blue). Second panel: data shown without Hoechst. Third panel: tamoxifen male mouse co-stained with anti-SOX9 (green) demonstrating co-labelling with tdTomato (merge, yellow), and thus endogenous-like expression in the Müller glia cells of the retina (white arrow).

B) Adult mice that carried both the constitutive icre and ROSA-Stop-tdTomato alleles. First panel, columns 1 and 2: male and female mice stained in the amacrine cells and Müller glia of the retina, with female staining less as expected due to X-chromosome inactivation. Second panel, columns 1 and 2: data shown without Hoechst. First and second panel, column 3: male and female mice stained in the stromal and endothelial cell layers of the cornea, with female overall staining slightly less as expected due to X-chromosome inactivation. Scale bars represent 100 µm.