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TECHNIQUES

In Vivo Evidence for Unbiased Ikaros Retinal Lineages Using an Ikaros-Cre Mouse Line Driving Clonal Recombination

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Background: We showed previously that the transcription factor Ikaros is expressed in early but not late retinal progenitors cells (RPCs), and is necessary and sufficient for the production of early-born neurons. Preliminary evidence using retinal explant cultures qualitatively suggested that Ikaros-positive RPCs might share a common lineage with Ikaros-negative RPCs. Results: To explore further this question in vivo in a quantitative manner, we generated BAC transgenic mouse lines expressing Cre recombinase under the regulatory elements of the *Ikaros* gene, and crossed them with Cre reporter lines. Different transgenic lines labeled a variable number of RPCs, resulting in either dense or sparse radial arrays of reporter-positive progenies. Analysis of over 800 isolated cell arrays, which are most likely clones, confirmed that Ikaros-expressing RPCs generate both early- and late-born cell types in the same lineage, and that the overall cell composition of the arrays closely resembles that of the population of the mature retina. Interestingly, another sparse line did not label arrays, but appeared to specifically reflect Ikaros postmitotic expression in amacrine and ganglion cells. Conclusions: These mouse lines confirm the unbiased potential of the Ikaros lineage in vivo and provide novel tools for clonal lineage tracing and single neuron tracking in the retina. *Developmental Dynamics 241:1973–1985, 2012.* © 2012 Wiley Periodicals, Inc.

Key words: lineage; competence; temporal identity; cell fate; cell specification; development

Key Findings

- The Ikaros retinal lineage gives rise to both early- and late-born cell types and is confirmed unbiased in vivo
- One Ikaros-Cre mouse line allows sparse recombination in retinal progenitors and is validated as a tool for in vivo lineage tracing
- A distinct Ikaros-Cre mouse line allows sparse recombination specifically in some postmitotic early-born retinal cell types

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INTRODUCTION

The different cell types that make up a mature retina are produced in a fixed, yet overlapping chronological order. Generally, retinal ganglion, horizontal, cone, and amacrine cells are produced during early phases, whereas rod, bipolar and Müller cells are produced during late phases of retinogenesis. Exactly how retinal progenitor cells (RPCs) change over time to generate the right cell type on schedule remains unclear, but cell intrinsic mechanisms appear to play a major part (reviewed in Agathocleous and Harris, 2009). Cell mixing experiments have shown that the differentiation potential of RPCs remains unchanged when they are placed in a temporally inappropriate environment (Watanabe and Raff, 1990;

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Altshuler and Cepko, 1992; Lillien and Cepko, 1992; Belliveau and Cepko, 1999). Moreover, when RPCs are plated at clonal density, they generate clones that are of similar size and composition as clones that develop in situ, and the general order of cell type production in the population is reproduced (Cayouette et al., 2003). Although feedback inhibition signals from the environment can clearly influence the proportion of specific cell types produced (Reh and Tully, 1986; Reh, 1992; Waid and McLoon, 1998; Wang et al., 2005), even in clonal cultures, these results are consistent with a model in which RPCs do not require specific instructive environmental signals for proper cell fate specification, but rather depend on cell intrinsic cues acting to bias their developmental potential over time (Cayouette et al., 2006; Gomes et al., 2011).

Recently, we identified the zinc finger transcription factor Ikaros as a regulator of temporal competence in RPCs (Elliott et al., 2008). Ikaros is expressed in early, but not late-stage, RPCs and mice lacking Ikaros generate fewer early-born cell types such as ganglion, horizontal, and amacrine cells, whereas they have normal numbers of late-born cell types. Importantly, misexpression of Ikaros in late-stage RPCs is sufficient to confer early temporal competence, allowing generation of early-born cell types at an inappropriate stage without affecting proliferation. Together, these results suggest a model in which Ikaros functions as a permissive factor in RPCs to bias generation of early-born cell types. One question arising from this model is whether the Ikaros-positive RPC pool observed at early stages of retinogenesis actually gives rise to the Ikaros-negative RPC pool observed at later stages. An alternative model is that a population of Ikaros-negative RPCs might exist at early stages of retinal development, but only amplifies at late-stages of retinogenesis to contribute to the generation of late-born cell types.

To get preliminary insight into this question, we initially used retinal explant cultures to show that retrovirus-infected clones can bear both Ikaros-positive and Ikaros-negative RPCs (Elliott et al., 2008). Here, to further challenge this prediction in vivo, we generated BAC transgenic mouse lines that express Cre recombinase under the regulatory elements of the *Ikaros* locus, and crossed them to Cre reporter mouse lines. Analysis of reporter gene expression revealed that the Ikaros lineage is made up of both early- and late-born retinal cell types, supporting a model in which Ikaros-positive RPCs give rise to Ikaros-negative RPCs. Fortuitously, we found that some BAC transgenic lines generated during the course of this study produced very sparse Cre-mediated deletions in RPCs, leading to isolated radial arrays of reporter-positive cells in the mature retina. We present evidence that one mouse line can be used for in vivo clonal gene inactivation and lineage tracing in the developing retina. Analysis of the composition of over 800 clones confirmed quantitatively that the Ikaros lineage is unbiased, reflecting the proportions of different cell types described in the whole retina. Conversely, another mouse line did not show reporter expression in RPCs, but only labeled sparse post-mitotic retinal ganglion and amacrine cells, likely reflecting the influence of post-mitotic Ikaros regulatory sequences in these early-born cell types. Thus, we anticipate that these mouse lines will become invaluable tools in the field to genetically tag and modify single isolated RPCs and their progeny, or single neurons in the retina.

RESULTS

Generation of Ikaros-Cre BAC Transgenic Lines

To study the Ikaros lineage in vivo, we introduced Cre recombinase in the *Ikaros* gene in the context of cloned genomic DNA and used pronuclear injection of fertilized embryos to generate transgenic mouse lines. Since regulatory sequences directing *Ikaros* expression in the retina are unidentified, we reasoned that a bacterial artificial chromosome (BAC) carrying the whole gene as well as large flanking genomic regions would likely recapitulate most of Ikaros endogenous expression pattern. Therefore, we selected the RP23-373H2 clone, which carries 203 kb of chromosomal DNA, and where the *Ikaros* gene is flanked by more than 60 kb of intergenic DNA on each side (Fig. 1A). Bacterial recombination was used to insert a cassette carrying the Cre recombinase followed by an IRES-Venus reporter at the start codon in exon 2 (Fig. 1A). Correct recombinants were screened for a restriction enzyme polymorphism expected to create a new BamHI band (Fig. 1B), and were further verified with other restriction enzymes and sequencing. Following pronuclear injection, 11 independent mouse founders corresponding to as many distinct genomic integrations of the modified BAC were recovered by running a Southern blot using a Cre probe (Fig. 1C). Two founders never gave progeny, and consequently nine stable Ikaros-Cre lines were recovered (thereafter referred to as IKCre lines). Transgenic animals were identified by a PCR-based genotyping strategy (Fig. 1D) and, as expected, Cre expression in the retina was detected by RT-PCR (Fig. 1E).

To verify that Cre recombinase is functional, we first crossed the nine different BAC transgenic lines with the ROSA 26 (R26) reporter line (Soriano, 1999), which triggers expression of β-Gal after Cre-mediated excision of a stop signal, allowing the permanent labeling of Cre-positive cells and their progeny. While non-transgenic controls never showed β-Gal activity, all nine transgenic lines had β-Gal-positive cells in the retina at P12–P15 (Fig. 2A), but the cell number and arrangement varied from one line to another. Four lines had radial arrays of positive cells (lines A, B, F, and I), suggesting Cre expression in dividing progenitor cells. While positive cells in two lines (lines A and B) encompassed the majority of the retina, two lines (lines F and I) appeared less penetrant, with sparsely distributed arrays. Line B very rarely transmitted the transgene and was eventually lost. Five other lines did not label radial arrays, but instead labeled isolated cells, suggestive of a later post-mitotic expression of Cre (e.g., lines D, G, and H). Based on this initial survey, we decided to keep and investigate further one line for each type of expression profile. Therefore, the experiments below focus on three

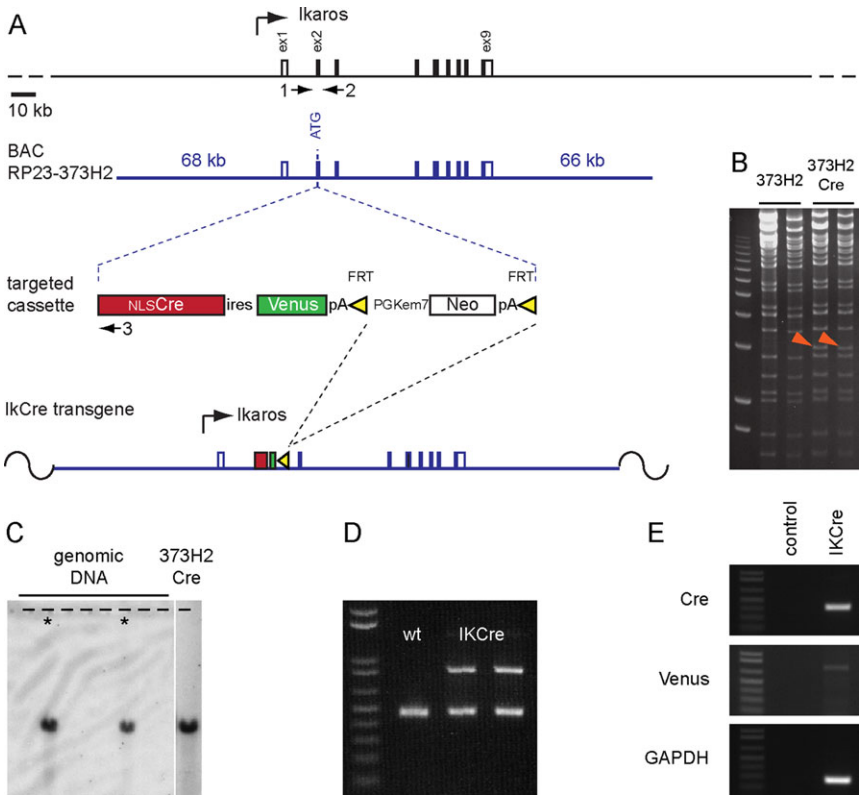

Fig. 1.

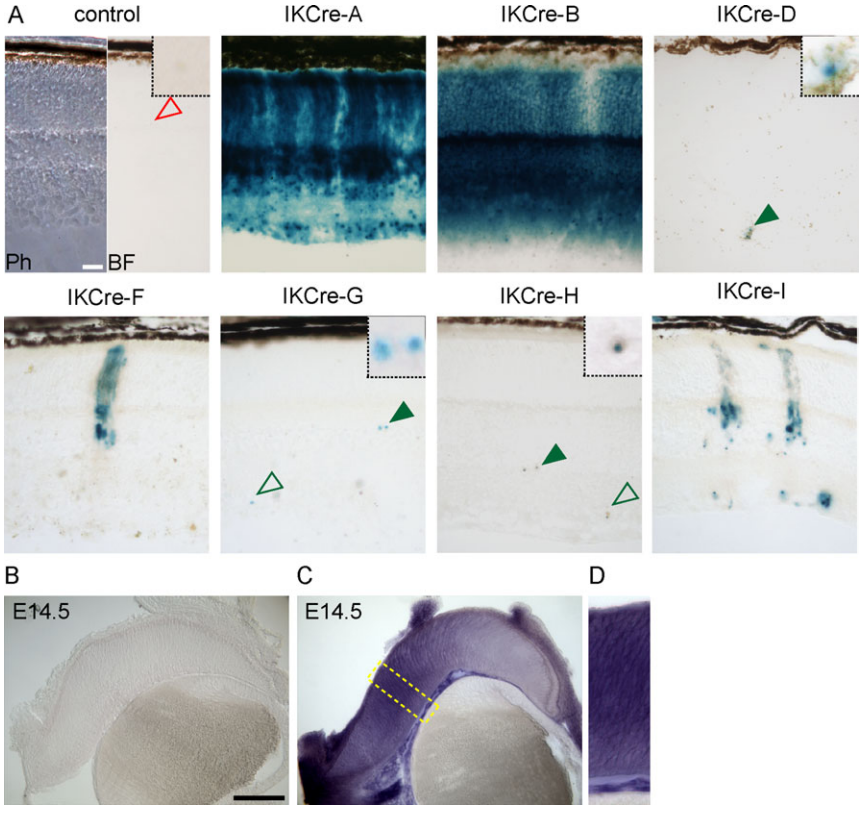

Fig. 2.

lines that are currently breeding in our colony: lines A and F, displaying dense versus scarce arrays,

Fig. 1. Generation of the Ikaros-Cre (IKCre) mouse transgenics. **A**: The Ikaros gene structure is depicted at top. The BAC genomic clone RP23-373H2 (blue) encompasses the whole Ikaros gene as well as large flanking genomic regions on both sides. Cre-IRES-Venus insertion in the BAC clone was achieved by bacterial recombination and necessitated the concomitant incorporation of a selection cassette (PGKem7-Neo). However, the latter was flanked by FRT sites, as shown, and was deleted to obtain the final IKCre transgene (373H2 Cre; bottom). **B**: Bacterial clones carrying correct insertion of the targeted cassette at the ATG of Ikaros were initially selected based on a BamHI restriction enzyme polymorphism expected to create a new band (arrowheads). **C**: Following pronuclear injection of the modified 373H2 Cre BAC, founder transgenic mice were identified by Southern Blot using a Cre probe. Founders showed a band (asterisks) while negative animals, not carrying the Cre gene, gave no signal (other lanes). As positive control, the modified BAC DNA was loaded on the same gel (373H2 Cre; right). **D**: PCR strategy to genotype IKCre transgenic animals. Primer 1 and 2 (see A for binding homology) amplify a control band in all genomic DNA samples, whereas in transgenic samples only, primers 1 and 3 additionally amplify a second larger band in a 3-primers reaction. **E**: RT-PCR on E14.5 retinas showed that both Cre and Venus transcripts are produced from the BAC transgene in the IKCre-A line (lane IKCre). Control lane: Without RT enzyme to exclude amplification from genomic DNA or contamination.

Fig. 2. Cre reporter expression in the different BAC transgenic lines. **A**: Initial survey of Cre activity for all IKCre transgenic lines in the retina. Transverse sections from seven different IKCre lines crossed to the R26 reporter line are shown at P12–P15. β-Gal activity was revealed using X-Gal substrate (blue-green) and pictures were captured in brightfield (BF). Four lines (A, B, F, and I) displayed radial arrays of cells spanning several retinal layers. The five other lines showed isolated cells as illustrated here for lines D, G, and H (lines C and E are not shown). Solid arrowheads indicate isolated X-Gal-positive cells magnified in the insets, whereas open arrowheads indicate additional positive cells in the field. The control panel corresponds to a retina negative for IKCre transgene, but carrying the R26-β-Gal reporter. The left half of the panel is seen in phase contrast (Ph). No X-Gal-positive cell is found in control retinas (red arrowhead). In all panels, the retinal pigment epithelium (RPE) at the top appears brown and does not express β-Gal. **B–D**: Ikaros in situ hybridization on E14.5 wild-type eyes. A half-retina section is showed at the optic nerve level. Ikaros expression appears ubiquitous in the retina (C), whereas no signal is detected with a sense probe control (B). A magnified view of the boxed region in C is shown in D. Scale bars = 50 μm.

respectively, and line G, displaying isolated cells.

Although weak *Venus* transcripts were produced by the IKCre transgene (Fig. 1E), we could not detect Venus protein expression despite repeated attempts using a variety of approaches. This could stem from the low expression levels of *Ikaros* mRNA in the developing retina (Elliott et al., 2008) (see Discussion section). Similarly, low expression from the transgene likely translates into inefficient and/or infrequent Cre-mediated deletion, and thus probably accounts for the small subset of Ikaros-positive cells labeled in some lines. This conclusion is supported by our concomitant inability to detect the Cre protein directly using immunohistochemistry.

We next wanted to verify the specificity of Cre expression in the transgenic lines compared to endogenous Ikaros. To do this, we first performed wholemount in situ hybridization for Ikaros on E11.5 whole embryos and E14.5 eyes. Although Ikaros was prominently expressed in the liver at E11.5, as previously reported (Georgopoulos et al., 1992), it was also detected apparently ubiquitously at lower levels in the whole embryo (not shown). In the eye, we consistently found that Ikaros is expressed ubiquitously (Fig. 2C, D), as we reported previously (Elliott et al., 2008). Consistent with widespread Ikaros expression in embryonic tissue, Cre-mediated recombination did not display any clear tissue-specificity (see Discussion section).

Ikaros-Cre Mouse Lines Active in Early Retinal Progenitors

To characterize Cre activity more thoroughly in the retina, we reasoned that a fluorescent reporter would be more versatile than β-Gal, by revealing cell morphologies more clearly and allowing fluorescent co-stainings with cell-type-specific markers. We, therefore, crossed the IKCre transgenic lines A, F, and G to the R26-YFP reporter line (Srinivas et al., 2001), which triggers expression of YFP upon Cre-mediated excision of a stop signal.

Eyes from the mouse lines generated were collected at different stages of development spanning the entire period of retinogenesis through adult for each of the three IKCre lines. The retinas were sectioned, stained for YFP, and examined under an epifluorescence or confocal microscope. In both lines A and F, we found YFP-positive cells in the optic cup around E10.5, although their number was much greater in line A than line F (Fig. 3A, F). As expected, the YFP-positive cells in early embryonic retinas had typical RPC morphology (Fig. 3G). At progressively older embryonic stages and through the postnatal period, YFP-positive cells were still observed in the progenitor layer, while some also appeared in the differentiating neuronal layers (Fig. 3C,D and H,I). In the adult retina, YFP-positive cells were observed in all three layers (Fig. 3E and J). Overall, this expression pattern indicates that both lines A and F express Cre in early progenitor cells, although with different efficiencies. Line A is closer to the apparently ubiquitous Ikaros expression in RPCs at early stages (Elliott et al., 2008), whereas the low subset of Ikaros-positive RPCs achieving Cre-mediated deletion of the reporter in line F leads to sparse arrays of YFP progenies. These expression patterns match what was observed with the β-Gal reporter in these lines.

An Ikaros-Cre Mouse Line Active in Early Born Retinal Neurons

In the IKCre-G mouse line, we found few, if any, YFP-positive cells at embryonic stages (Fig. 3K–M). During postnatal stages of retinal development, however, a few positive cells were found in the neuronal layers, sometimes in small clumps (Fig. 3N), but more often in isolation. By P21, YFP-positive cells were observed in the inner nuclear layer (INL) and ganglion cell layer (GCL), but were absent from the outer nuclear layer (ONL), which contains the photoreceptors cells that make up more than 70% of all retinal cell types (Fig. 3O). The late onset of expression, together with the absence of radial cell arrays and photoreceptor labeling suggest that Cre is not activated in early progenitor cells in the IKCre-G line, but rather post-mitotically, in a subset of defined cell types. Interestingly, expression of Ikaros protein was previously reported in differentiated retinal ganglion cells (RGCs), amacrine and horizontal cells, which are all early-born neuronal cell types, whereas it was absent in early-born cone photoreceptors or any late-born cell types (Elliott et al., 2008). Based on the hypothesis that distinct regulatory elements at the Ikaros locus could drive progenitor versus neuronal expression of the gene, we next sought to determine whether Cre-mediated post-mitotic deletion occurs specifically in early-born neurons in the IKCre-G line.

A majority of labeled cells in both the INL and GCL were positive for the amacrine markers syntaxin or Pax6 (Fig. 4A, B), and many in the GCL expressed Brn3 (Fig. 4C, E), a marker of most RGCs (Wang et al., 2002; Badea et al., 2009a; Nadal-Nicolas et al., 2009). Thus, Cre-mediated deletion in the IKCre-G line occurs in RGCs and amacrine cells, which are known to express the Ikaros protein. Although some YFP-positive horizontal cells were found, they were rare. This may simply reflect the low abundance of horizontal cells in the retina, or that the IKCre-G line does not fully recapitulate endogenous Ikaros expression in this cell type. Much like the sparse array distribution observed with the IKCre-F line, the neurons observed in the IKCre-G line were sparsely labeled, with many regions containing single isolated amacrine and RGC, which enabled visualization of their morphology (Fig. 4A and F).

Of note, we found that many positive cells in the mature IKCre-G line were microglia, which are not generated from RPCs, but are thought to derive from the bone marrow (Prinz and Mildner, 2011). These cells were easily recognized by their distinct planar morphology, small cell bodies located in the outer or inner plexiform layer, and expression of the lectin RCA (Fig. 4G). Since RCA-positive YFP cells were not observed in the early embryonic retina, it is possible that IKCre only labels late-arriving bone-marrow-derived and not yolk-sac-derived microglia.

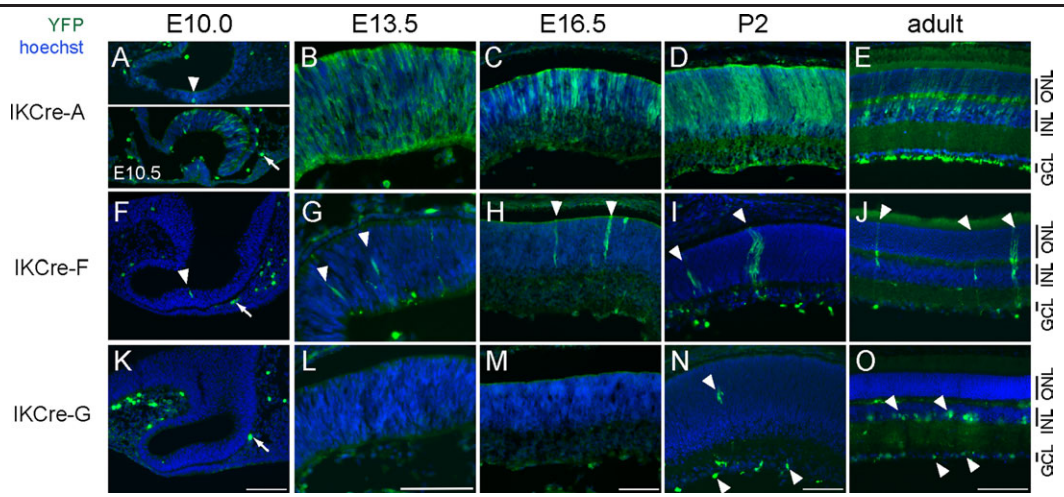

Fig. 3. Time-course of retinal YFP reporter expression in the IKCre mouse lines. Transverse retinal sections were collected at different stages of development (as indicated) and stained for YFP (green) in the IKCre mouse line A (**A–E**), line F (**F–J**), and line G (**K–O**). In the IKCre-A line, YFP-positive cells are found in the progenitor layer as early as E10.0 (A; top, arrowhead) and in radially-oriented arrays spanning all layers at progressively later stages of development and in adults (B–E). In the IKCre-F line, YFP-positive cells are detected from E10.0 in the progenitor layer (F, arrowhead) and in isolated radial arrays at progressively older ages and in adults (G–J, arrowheads). In the IKCre-G line, almost no positive cells are detected in the retinal neuroepithelium at embryonic stages (K–M), whereas some YFP-positive cells can be found at postnatal stages (N; arrowheads). In adults, YFP cells are found in the basal half of the inner nuclear layer (INL) and in the ganglion cell layer (GCL), but they do not form radial arrays (O; arrowheads). Green staining outside the retina most prominent at E10.0 (A, F, K, arrows) represents background and is also found in non-transgenic controls (not shown). Hoechst nuclear dye is shown in blue. ONL, outer nuclear layer. Scale bars = 100 μm.

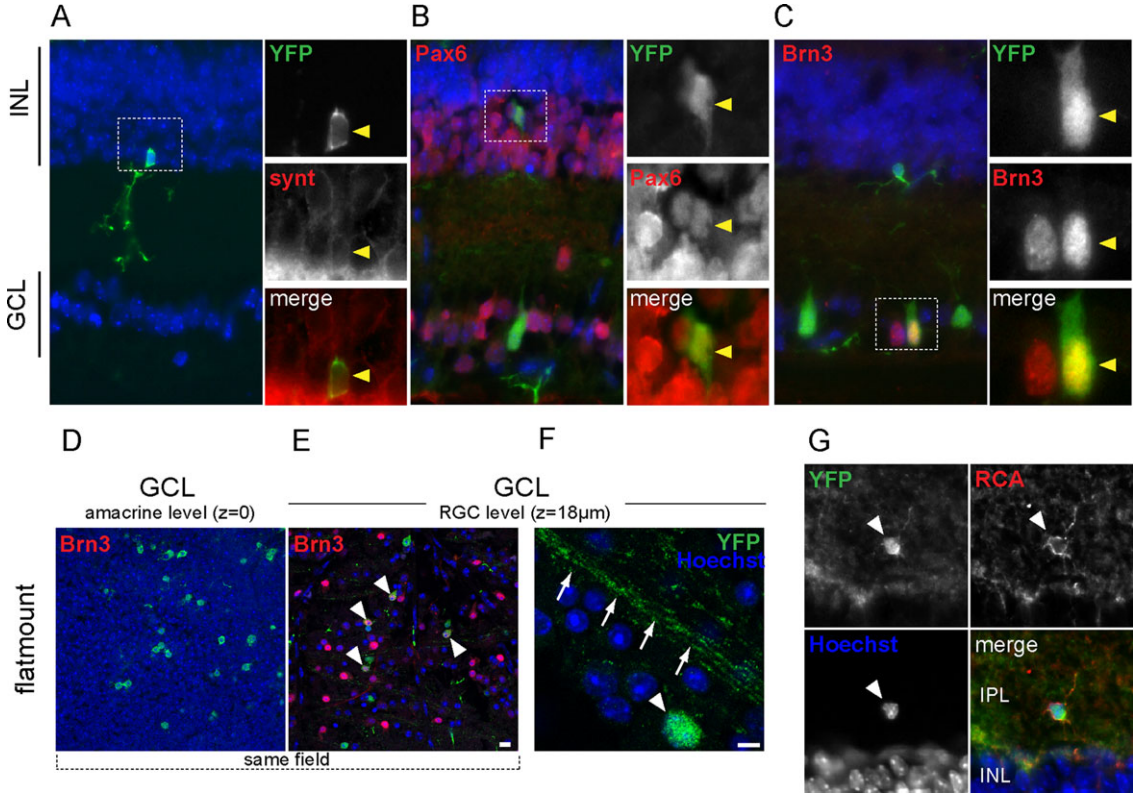

Fig. 4. The IKCre-G line mediates post-mitotic recombination in isolated amacrine and ganglion cells. P14 retinal sections from the IKCre-G line were stained for YFP (green) and the amacrine cell markers syntaxin (A) or Pax6 (B), the ganglion cell marker Brn3 (C–E), or the microglia marker RCA (G). **A**: Example of a syntaxin-positive amacrine cell with its cell body located in the inner nuclear layer (INL) and its processes in the inner plexiform layer. **B**: Example of a Pax6-positive amacrine cell with its cell body in the INL. **C**: Retinal ganglion cells are distinguished from displaced amacrine cells in the GCL by the expression of Brn3. In A to C, separate channels for the boxed region are showed on the right, with arrowheads pointing to the cell of interest. **D–F**: Confocal z-stack through the GCL on a retinal flat-mount showing the Brn3-negative displaced amacrine cells (D) and the Brn3-positive ganglion cells (E) in the same field, but at different z positions. F: Ganglion cell axons travelling towards the optic nerve are also labeled by the YFP reporter (arrows; arrowhead: cell body). **G**: Some YFP-positive cells found in the inner plexiform layer (IPL, arrowhead) express the microglial cell marker RCA, but do not belong to retinal lineage. Scale bars = 10 μm.

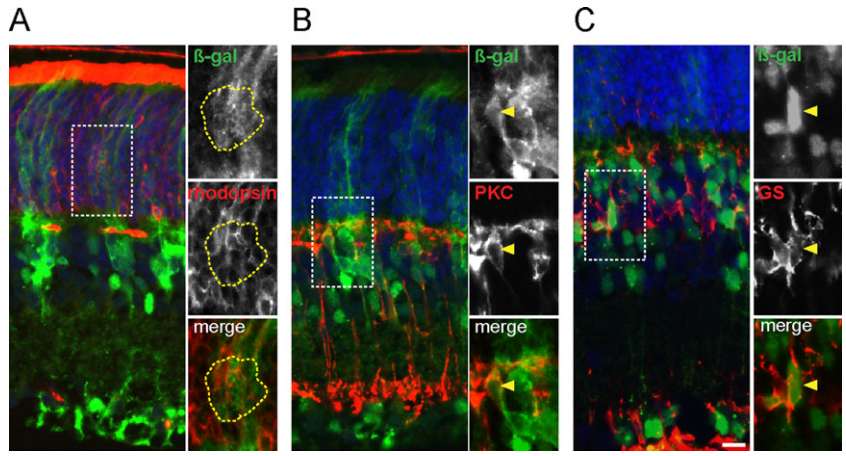

Fig. 5. The IKCre-A lineage contains late-born retinal cell types. P13 retinal sections from the IKCre-A line crossed to the R26 reporter were stained for the β-Gal reporter (green) and the rod photoreceptor marker rhodopsin (**A**), the bipolar cell marker protein kinase Cα (PKC; **B**), and the Müller cell marker glutamine synthase (GS; **C**). IKCre-A lineages commonly comprised cells positive for all three markers. Separate channels for the boxed region are showed on the right, with dashed circles or arrowheads indicating the cell(s) of interest. Hoechst nuclear dye is shown in blue. Scale bar = 10 μm.

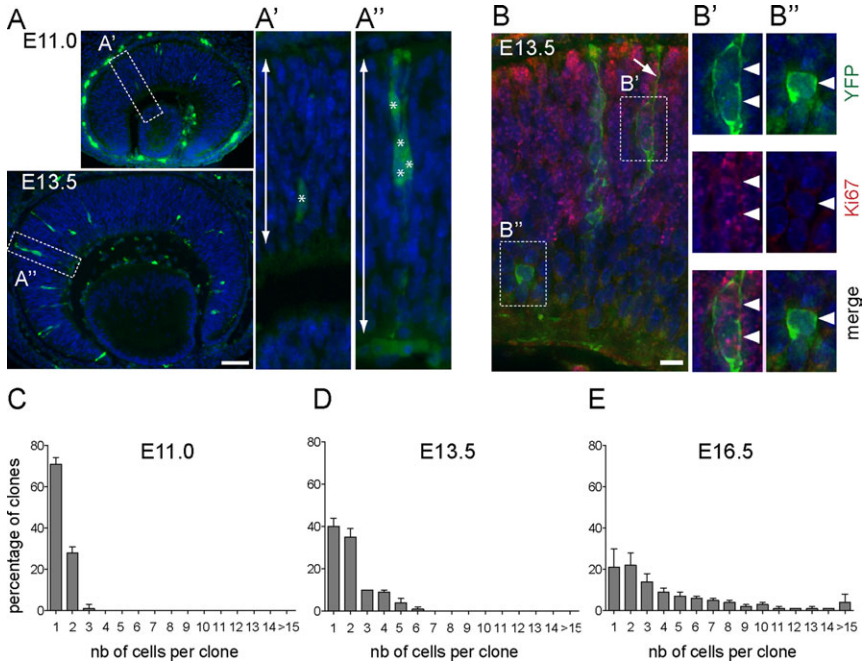

Fig. 6. Radially-oriented cell arrays in the mature IKCre-F retina originate from single progenitors. **A**: Retinal sections at E11.0 and E13.5 stained for the YFP reporter (green) show groups of 1 to 5 cells (asterisks) likely to represent small clones. **A'** and **A"** are magnified views of the corresponding boxed regions in **A**. The double arrows indicate the width of the retina. Positive cells seen outside the retina are non-specific staining caused by the secondary antibody. **B**: Three putative clones at E13.5. A two-progenitor array is found on the right. Both cells display typical neuroepithelial morphology with basal and apical (arrow) processes, and stain for the proliferation marker Ki67 (**B'**; arrowhead). By contrast, on the left, a single cell in the basal layer has a more rounded morphology and is negative for Ki67 (**B"**), likely representing a clone of a single postmitotic neuron. **B'** and **B"** represent separate channel views (with hoechst) for the corresponding boxed regions in **B**. **C–E**: The number of cells per clone increases as development proceeds, from mostly 1–2 cells at E11.0 to a progressively wider range of 1 to >15 cells at E13.5 and E16.5. The graphs represent the proportion of clones of each size in retinas of 3 different embryos (mean ± s.d.). The total number of arrays analyzed is 90 (E11.0), 292 (E13.5), and 571 (E16.5). Scale bars = 100 μm (A) and 10 μm (B).

The Ikaros Retinal Lineage Contains Late-Born Cell Types

Our observations that lines A and F contain cells in all layers suggested that Ikaros-expressing RPCs are multipotent and give rise to both early- and late-born cell types. To confirm reporter expression in late-born cells, we stained adult retinas from the IKCre-A line with markers of rods, bipolars, and Müller glia, which were all found to co-express the Cre reporter (Fig. 5). These results, along with our clonal analysis in the IKCre-F line (see below), indicate that although Ikaros expression is downregulated in late RPCs, the early-set Ikaros lineages give rise to both early- and late-born cell types.

An Ikaros-Cre Mouse Line as a Tool for Clone-Restricted Genetic Modification In Vivo

Our observation that the IKCre-F line undergoes sparse recombination in RPCs suggested that this particular line could be used for clonal gene inactivation and lineage tracing in vivo. To explore this possibility, we first asked whether cell arrays observed in the IKCre-F line could represent clones originating from single RPCs. We thus studied the expression pattern of the reporter around the time when the first RPCs are found. Careful examination of early stage retinas revealed several isolated RPCs expressing YFP from E10.0 (Fig. 3F). Retinas between E11.0 and E13.5 typically had many small groups of 1 to 5 cells (Fig. 6A), with some arrays progressively containing basally-located cells in the emerging differentiated cell layers. Positive cells with elongated morphology characteristic of RPCs expressed the proliferation marker Ki67 (Fig. 6B). If these isolated progenitors actually generated the cell arrays observed in the adult retina, we predicted a steady increase in the number of cells per array over time. Indeed, a clear shift towards larger size was observed at progressively older ages (Fig. 6C–E). Together, these results indicate that, in the IKCre-F line, single RPCs are labeled from E10.0 and expand to form radially-oriented arrays of cells in the adult retina.

Since these observations are consistent with the notion that the majority of radial arrays are clones, we will refer to them as such from here.

Next, we investigated the nature and composition of labeled lineages in the mature retina. Generally, four main conditions should be met for a mouse line to be useful for clonal gene inactivation and lineage tracing. First, the clones should show no spatial bias in their distribution. Consistently, we found that cell arrays appeared equally distributed along the circumferential axis of the eye. Second, the clones should be sufficiently isolated from each other. Although the density varied across the retina and some regions were too densely packed for analysis, other regions with isolated clones were commonly found (Fig. 7A, B). Third, the reporter labeling should provide sufficient information regarding cell morphology and allow co-staining for cell-specific markers. As shown in Figure 7A, cell morphology and cell body position within the retinal layers were easily visualized. As an example, Figure 8 illustrates the morphology-based analysis of one large clone containing 45 photoreceptors, 2 bipolar cells, 2 Müller glia, 3 amacrine cells, and 2 cells in the GCL. We also used markers of horizontals (Lim-1), amacrines (Pax6), and RGCs (Brn3), which made the identification of these cell types more reliable (Fig. 7C–E). Finally, the fourth condition for validation of the IKCre-F line as a tool for lineage tracing is that clone composition should be unbiased and mirror cell type proportions observed in the whole retina, an expected outcome if equipotent RPCs randomly undergo Cre-mediated recombination from E10.0. In order to obtain quantitative confirmation by cell type in the IKCre-F line, we analyzed in detail the composition of 820 clones from 5 different adult retinas. As expected, clone size varied from one to about 70 cells, indicating heterogeneity in the proliferation capacity of RPCs randomly labeled from E10.0 (Fig. 7F). Since the largest clones observed here did not contain more cells than the clones obtained by retroviral vectors analysis (Turner et al., 1990), this result reinforced our conclusion that the arrays are actual clones that arise from a single cell. Most interestingly, when we compared the proportion of the different cell types generated in the clones with that of previously published retroviral lineage tracing studies (Turner et al., 1990), or with proportions of the different cell types observed in the entire retinal cell population (Turner et al., 1990; Jeon et al., 1998), we found a general similarity both in terms of proportion of cells in the clones (Fig. 7G and Table 1), and as a proportion of clones containing at least one of each cell type (Fig. 7H; Turner et al., 1990). These results suggest that the composition of the clonal population in the IKCre-F line is unbiased and generally representative of the global retinal cell population.

Single cells represented about 25% of the counted arrays. The composition of these one-cell clones was largely dominated by amacrines, RGCs, and horizontal cells, at the expense of photoreceptors (Fig. 7G). This was expected since early RPCs that are about to exit the cell cycle while starting to express Cre would, indeed, preferentially generate early-born cell types. Alternatively, we cannot fully exclude that Cre expression in the IKCre-F line is not limited to RPCs, but is also found in some early-born postmitotic neurons, as seen in the IKCre-G line. However, this should result in a striking overrepresentation of RGCs and amacrine cells that we do not observe in our analysis of all clones, which generally match described proportions for the entire retina (Fig. 7G and Table 1). In addition, horizontal cells are over-represented in IKCre-F single cell arrays (Fig. 7G), but were only very rarely labeled in the IKCre-G post-mitotic line, further suggesting that Cre expression in the IKCre-F line is mostly limited to RPCs. The presence of many single-cell clones containing late-born cell types, however, suggests that some cells might recombine after cell cycle exit, or that Cre expression begins at later stages of development in some RPCs. Further experiments will be required to establish how late new Cre-mediated recombination events occur, and thus define a time window of RPC competence corresponding to the lineages observed in adults.

Unfortunately, we could not provide specific information about cone photoreceptors in this analysis since the cone markers available to us only reveal the apical outer segment, making co-labeling with cytoplasmic YFP from the Cre reporter very challenging.

Altogether, very sparse Cre-mediated recombination combined with unbiased early lineage labeling identifies the IKCre-F transgenic line as a useful tool for in vivo clonal gene inactivation and lineage tracing in the retina. Additionally, the analysis of clone composition in the IKCre-F line extends the more qualitative observation made in the non-clonal IKCre-A line that Ikaros lineages commonly include late-born cell types, suggesting that early Ikaros-expressing RPCs contribute to all cells in the retina.

DISCUSSION

In this study, we generated transgenic mice expressing Cre recombinase under the control of 203 kb of genomic DNA at the *Ikaros* locus. We used these animals to show quantitatively and in vivo that the Ikaros lineage is unbiased and contributes to both early- and late-born cell type production. In addition, we show that two alternative mouse lines generated here can be used as tools for in vivo clonal genetic modifications/lineage tracing in RPCs, or tracking of early-born neuronal cell types in the retina. We discuss below the significance and implications of these findings.

Cre Recombination in RPCs

Although the Ikaros protein is found in virtually all neuroepithelial progenitor cells forming the prospective rat retina (Elliott et al., 2008), the IKCre transgenic lines we generated do not achieve reporter expression in all mouse RPCs. Instead, some lines exhibit widespread, yet non-ubiquitous, Cre-reporter expression (IKCre-A and B), while others only label RPCs very sparsely (IKCre-F and I). Inter-line variability probably stems

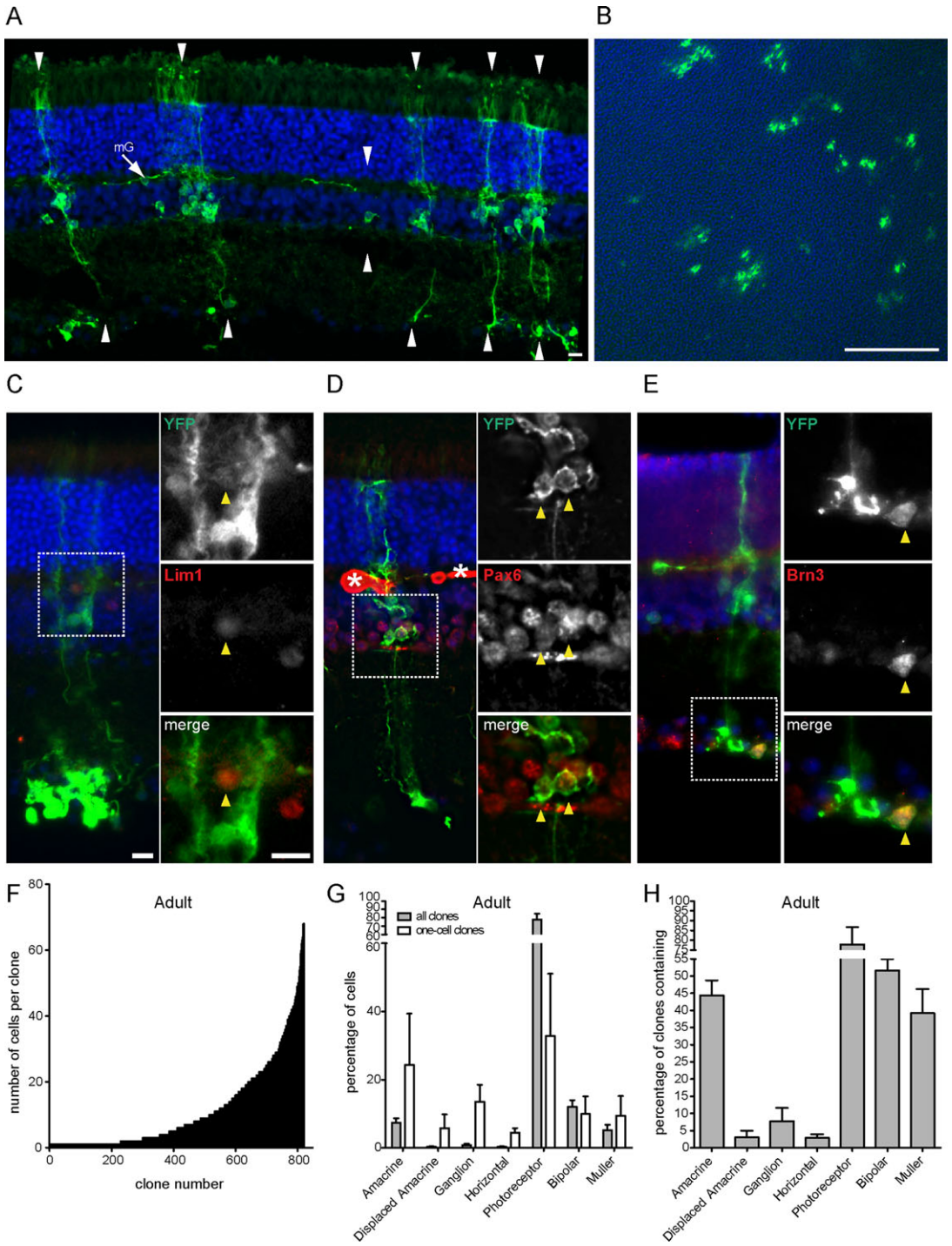

Fig. 7. In vivo clonal analysis in the adult IKCre-F retina. **A**: Section showing five different YFP-positive clones (between arrowheads) in the same field. The clones comprise a variable number of different cell types. Some microglial cells (mG) are also seen in this image (see text). **B**: Flat-mount view of a portion of the retina showing scattered YFP-positive clones. A single confocal layer is shown en face. **C–E**: Cell-type-specific markers used to distinguish horizontal (C, Lim1), amacrine (D, Pax6), and ganglion cells (E, Brn3) from the other cell types, which are readily identified by morphology and position of their cell bodies within the retinal layers. Separate channels for the boxed region are showed on the right, with arrowheads indicating the cells of interest. Asterisk in D points to non-specific staining in blood vessels. **F**: Representation of the number of cells per clone from a total of 820 analyzed from 5 different transgenic animals. All clones are individually plotted in X and sorted by increasing size. The distribution of clone sizes varies greatly from as few as 1 to as many as 69 cells per clone. **G**: The proportion of each cell type in all clones (grey) versus only one-cell clones (white). **H**: The proportion of clones containing at least one cell of each type. Results in G and H are shown as mean ± s.d. Hoechst nuclear dye is seen in blue. Scale bar = 10 μm (A, C–E) and 100 μm (B).

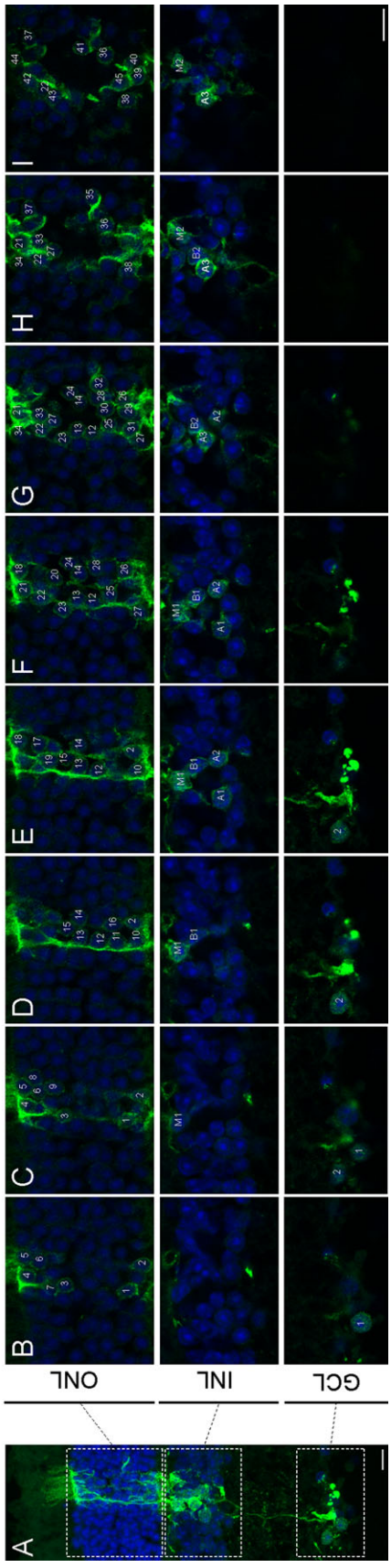

Fig. 8. Illustration of the morphology-based analysis of one large clone in the IKCre-F retina. **A**: Confocal projection of the clone in a section from an adult IKCre-F retina stained for YFP reporter (green) and the nuclear dye Hoechst (blue). **B–I**: Consecutive confocal slices at 1-µm intervals through the entire clone. The outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL) regions boxed in A are shown separately. YFP-positive cells are found in all three layers in this clone. Each cell is numbered. The clone consists of 45 photoreceptors in the ONL (numbers only), 3 amacrines (A1–3), and 2 bipolars (B1–2) in the INL, and 2 cells in the GCL (numbers only). Scale bar = 10 µm.

from the number of BAC copies integrated in the transgenic array and/or from the chromosomal surroundings at the integration site, which differ for each transgenic founder mouse.

A possible explanation to account for incomplete penetrance is suggested by the indirect detection method of Cre expression, combined with the low level of expression of the *Ikaros* mRNA in RPCs seen by in situ hybridization and PCR (Elliott et al., 2008). Although it cannot be ruled out that some regulatory modules are missing from the BAC clone, it is likely that Cre from the transgenic BAC is weakly transcribed, resulting in low protein amounts that only achieve recombination in a subset of Ikaros-positive RPCs. This conclusion is supported by our failure to detect the Venus protein from the transgene, whereas both *Cre* and *Venus* transcripts are detected by RT-PCR. As such, low transcription efficiency from the BAC would inconsistently produce enough Cre for recombination, but along with the typical decrease in translation efficiency associated to IRES sequences, would consistently fail to generate sufficient levels of Venus protein for detection. Once activated via permanent stop sequence excision, however, β-gal or YFP reporters used in this study are expressed from the potent R26 locus, which is unrelated to *Ikaros* regulation.

Ikaros appears to show very little cell specificity in embryonic stages, being expressed ubiquitously at low levels in most tissues while displaying higher expression in the liver, thymus, and striatum, for example (www.genepaint.org, and not shown). Consequently, it is difficult to evaluate and validate the specificity of expression of the Cre since we suspect a fully penetrant lineage labeling of all Ikaros-expressing cells would encompass most, if not all, adult cells. Moreover, all Ikaros antibodies tested so far only allow immunohistochemical detection in rat tissue, preventing the direct comparison of Cre and endogenous Ikaros expression patterns. It is notable, however, that albeit variably penetrant, our transgenic lines do show little embryonic tissue specificity, much like what we observe by in situ hybridization for *Ikaros*. Furthermore, subsequent post-mitotic retinal cell type

TABLE 1. Proportion of Mouse Retinal Cell Types Found Across Different Studies (Mean ± s.d.)[a]

Reference	Amacrine	Displaced amacrine	Ganglion	Horizontal	Photoreceptor	Bipolar	Müller
IKCre-F (this study)	**7.4**% ± 1.3	**0.26**% ± 0.16	**0.77**% ± 0.44	**0.28**% ± 0.14	**77.8**% ± 7.0	**12.0**% ± 2.0	**5.2**% ± 1.6
Turner et al. (1990) (E12.5)	**3.2**% ± 1.0	**0.3**% ± 0.14 [d]		**0.1**% ± 0.1	**85.8**% ± 3.7	**9.6**% ± 2.0	**0.8**% ± 0.8
Young et al. (1985)[b]	**8.4**%	**2.7**% [d]		**0.3**%	**74.5**%	**10.3**%	**2.7**%
Jeon et al. (1998)[c]	**6.5**%	**0.8**%	**0.6**%	**0.5**%	**74.9**%	**6.9**%	**2.6**%

[a]The current study and Turner et al. (1990) measured proportions among randomly labeled clones, whereas numbers from the other studies reflect whole retina analysis.
[b]As cited in Turner et al. (1990).
[c]Proportions were calculated from the three retinas counted by first averaging the number of cells per mm^2 for all quadrants in each of the retinal layers. Subsequently, the proportion of cell type per layer as reported in the study was applied to obtain the number of each cell type per mm^2, and ultimately their respective proportion in the whole retina.
[d]Includes both displaced amacrines and ganglion cells. For all studies, proportions of unidentified cells were not included.

restriction exhibited in the IKCre-G line is compatible with known Ikaros-specific expression in RGCs and amacrine cells. Importantly, unsolved specificity issues do not preclude the use of the IKCre-F line as a clonal Cre-driver in the early retina.

A critical question only touched on by our previous work concerned the potential specificity of Ikaros-expressing RPCs. While late RPCs do not express Ikaros, they could derive from the Ikaros lineage, or alternatively, represent distinct lineages that amplify late from a scarce and elusive population of early Ikaros-negative RPCs. In the first case, the Ikaros RPCs are likely unbiased in their potential, and their progeny would encompass all retinal cell types. In the second case, they would exhibit a degree of bias in competence that should be reflected in the lineage composition. Previous evidence in favor of unbiased Ikaros lineages was obtained by infecting early rat retinal explants with a GFP retrovirus, and showing that, 5 days later, clones could be composed of both Ikaros-positive and Ikaros-negative RPCs (Elliott et al., 2008). All progenitors at the time of infection were assumed to be Ikaros-positive. This current work was started in part as a way to validate this conclusion in vivo, and extend its significance by adding a quantitative type of analysis. One intuitive way to proceed would be to look for Ikaros-negative RPCs at early stages of retinogenesis, the progeny of which would appear negative upon Cre-mediated lineage labeling. The inability of our IKCre transgenics to achieve consistent Cre recombination in all Ikaros-positive RPCs, however, precluded such an approach. If low *Ikaros* expression is at the root of incomplete penetrance issues, even an Ikaros-Cre knock-in mouse produced by ES cell recombination might generate escaper RPCs that express Ikaros but fail to undergo Cre-mediated recombination. Therefore, instead of relying on a potentially false negative result, we feel a strong conclusion is best reached backwards, from the careful analysis of Ikaros-derived cell types in the mature retina. In this work, our qualitative observations in the IKCre-A line and clonal analysis in the IKCre-F line similarly indicate that early Ikaros-expressing RPCs have the potential to generate all cell types of the retina in largely appropriate proportions. Although Müller cells appear slightly over-represented in our clonal analysis compared to previously published study (see Table 1), this variation most likely stems from differences in the methodology used to estimate the proportion of Müller cells and from the mouse genetic background used in the different studies. Overall, these results support a model in which transient Ikaros expression acts as a permissive temporal competence signal to bias early-born neuron production rather than defining a limited range of cell fates.

Lineage tracing in the mouse retina proved a powerful tool and established the existence of multipotent progenitors able to give rise to all retinal cell types in radially-arranged arrays (Price et al., 1987; Turner et al., 1990). These seminal studies relied on retroviral infection of RPCs in vivo from E12.5, and typically generated clones that widely varied in size. Technical difficulties associated with in utero surgical injections, however, might have limited its widespread use for lineage tracing from early RPCs. Subsequently, a couple of studies cleverly exploited chimerism or X inactivation in mouse to confirm such conclusions and refine some observations like tangential dispersion (Williams and Goldowitz, 1992; Reese and Tan, 1998). Rather than labeling sparse clones, however, these latter genetic approaches differentially labeled one half of RPCs versus the other. Consequently, it is unclear how frequently the arrays obtained represented true clones.

We propose here that due to its very low rate of recombination, the IKCre-F line can be used to genetically label and track single RPCs from E10.0, making it relatively easy to analyze a large number of RPC lineages in vivo. Although it is inherently difficult to unambiguously demonstrate that all cell arrays in the IKCre-F line are actual clones, the observation of isolated Cre-recombined RPCs at E10.5, the progressive increase in array size over time, and the finding that the largest arrays observed here do not contain more cells than those observed by retroviral lineage tracing strongly suggest that most, if not all, cell arrays observed in the IKCre-F line are actual clones that derived

from a single RPC. Although the classical retroviral lineage studies in the retina generated groundbreaking discoveries and have been extensively cited, conclusions on the potential of early RPCs were based on the analysis of 93 clones generated from virus infection at E12.5 and 222 clones at E13.5 (Turner et al., 1990). Considering the significance of this data for the field, the analysis of a larger number of clones from an earlier time point would be desirable, and in particular mandatory to test various models of retinogenesis. For example, a large number of clones will be required to test in vivo a stochastic model of retinal cell fate specification that we recently proposed from the analysis of lineages in culture (Gomes et al., 2011). This project further asks for lineage data at several intermediate stages of retinogenesis, and these results will be best compared to adult clone composition in the context of a unique and consistent mouse line.

There are currently other mouse lines available to achieve sparse Cre-mediated recombination in the retina (Badea et al., 2009b; Yun et al., 2009; Brzezinski et al., 2011). In these lines, a drug such as tamoxifen is used to activate Cre recombinase activity at a specific time, and limiting dilutions can be applied to restrict recombination to a subset of RPCs. Such pharmacological approaches, however, have notoriously variable efficiencies, and finding the right drug dosage to achieve clonal recombination is often not straightforward. Another recently developed strategy for clonal analysis in the retina combines the conditional expression of the tumor virus A (TVA) receptor in a specific population of cells and retroviruses expressing the avian leukosis virus EnvA protein to specifically infect subsets of the Cre-recombined cells, thus achieving clonal lineage labeling even from non-clonal Cre-driver lines (Beier et al., 2011). This method, however, involves the use of several mouse lines as well as concommitant retroviral injections, which are inherently difficult and similarly subject to experimental variations. The IKCre-F line described here offers an alternative genetic tool for lineage analysis in the retina without the need for pharmacological induction or in utero retroviral injections.

Notably, the use of Cre recombinase makes the IKCre-F line versatile, as recombination can turn on a reporter (as in this study), but can also inactivate or over-express a gene of interest in a lineage-specific manner, thus allowing a clonal type of conditional genetics in the mouse retina. Additionally, most conditional Cre-driver lines currently available in the retina achieve recombination either in specific neuronal populations or in a widespread manner in RPCs. Because the clonal Cre line described here is unbiased in the laminar (tangential) dimension, but instead active in radial arrays, it is bound to be a useful addition to the mouse retina genetic toolbox. We anticipate the IKCre-F line could prove useful in a number of situations. For example, it could be used to (1) assess the cell-autonomous function of a particular gene in neurogenesis, or (2) look at the morphology and behavior of mutant cells amid wild-type neighbors if a mutated gene disrupts retina lamination, thus obscuring its mechanism of action. Next, (3) this line opens up the possibility to address whether neurons born from a common lineage make preferential connections or form functional units in the retina, a recent area of interest in the developing cortex (Yu et al., 2009; Li et al., 2012), or (4) whether the large number of neuronal subtypes arise randomly in the retina, or if single lineages exhibit a bias towards certain subtypes, an as yet unanswered question.

Cre Recombination in Postmitotic Neurons

Five IKCre lines out of nine generated in this study expressed the reporter in isolated cells in the adult retina. Although superficially this pattern is very different from radial arrays, the fact that recombination appears limited to amacrine and RGC strongly suggests that Cre is expressed under the influence of a different set of Ikaros enhancer(s) in the IKCre-G line. Indeed, these neurons represent the most abundant cell types in which Ikaros protein is found post-mitotically (Elliott et al., 2008). Given that many other types, including the most numerous photoreceptors, express neither the Ikaros protein nor the Cre-reporter, two points can be made: (1) accurate post-mitotic Ikaros expression might rely on a devoted set of CIS regulatory sequences that are at least partly present on the BAC transgene; (2) in such a post-mitotic context, Cre expression from the BAC transgene clearly exhibits an Ikaros cell-specificity that could not be verified in RPCs-expressing lines due to ubiquitous Ikaros expression in the embryonic retina (see above). It remains unclear, however, why horizontal cells that were previously shown to express Ikaros failed to consistently undergo Cre-mediated recombination in the IKCre-G line. Of note, while amacrine cells and RGCs are born during embryogenesis, Cre-reporter in the IKCre-G line is rarely detectable before birth. This could represent the delay necessary for Cre-mediated deletion of the stop signal at the Rosa locus in differentiating cells, or the inherent nature of Ikaros regulation in mature postmitotic neurons.

Much like sparse recombination in IKCre-F allows to track and genetically modify isolated RPCs, sparse recombination in the IKCre-G line could be exploited to visualize the morphology of single amacrine and RGCs cells. While the cytoplasmic YFP reporter used in this study is not optimal to outline neuron processes, several better-suited reporters could be used, including a recent highly efficient alkaline-phosphatase at the R26 locus (Badea et al., 2009a). This could be used to address in vivo the function of a gene in neuronal polarization, neurite branching, or axon navigation towards the optic nerve and the brain, in the case of RGCs. Potentially, this line could also be interesting to selectively study the connections that amacrine cells and RGCs establish together in the inner plexiform layer.

Conclusions

In conclusion, we have generated transgenic mice that allowed us to confirm in vivo the unbiased nature of the Ikaros lineage, but also add to the genetic toolbox available to study neural development. Although we have highlighted a few potential uses for these mouse lines, we expect that many more will be uncovered once

they become available to the research community.

EXPERIMENTAL PROCEDURES

Generation of the IKCre BAC transgenic mice, genotyping, and RT-PCR

The Ikaros-containing RP23-373H2 mouse BAC clone was obtained from BACPAC Resources Center (CHORI, Oakland, CA). BAC modifications were done using bacterial recombination using the SW102 and SW105 strains provided by Recombineering at NCI-Frederick (Frederick, MD). Two initial rounds of recombination were used to replace the loxP and the lox511 sites found in the BAC vector backbone (pBACe3.6) by Ampicillin and Zeomycin resistance, respectively. This was done to avoid in vivo deletions or inversions between different copies of the Cre-expressing transgene once it is integrated into the genome as an array. Then, a targeting vector with the insertion cassette flanked by genomic regions homologous to Ikaros on both sides of the ATG in exon 2 was assembled using conventional cloning. The insertion cassette consisted of nlsCre-iresVenus-SV40 polyA FRT-PGKem7:Neo-bGHpA-FRT. The iresVenus sequence came from the pQCXIX plasmid (Clonetech, Mountain View, CA), and the FRT-PGKem7-Neo-bGHpA-FRT was cloned from the PL451 plasmid (NCI-Frederick) first modified to remove the loxP site. Finally, a third round of bacterial recombination allowed placing the insertion cassette at the Ikaros start codon on the BAC. Recombinants clones were selected by the kanamycin resistance conferred by the em7-neo part of the insert. Correct recombination was first ensured by the occurrence of a BamHI restriction polymorphism, followed by multiple nested PCRs and sequencing. Subsequently, one targeted clone was transferred into the SW105 bacterial strain, where the FLP recombinase was induced with arabinose in order to delete the PGKem7-Neo-bGHpA selection cassette. Therefore, the final BAC transgene carried only an nlsCre-ires-Venus-FRT insertion.

The targeted RP23-373H2-IKCre BAC was linearized with the PI-SceI homing endonuclease cutting once into the vector backbone, and then purified by sepharose column chromatography. After pronuclear injection into fertilized oocytes, the transgenic founders were identified via Southern blot using tail DNA and a probe against the Cre portion of the insert. cDNA for RT-PCR to detect Cre and Venus transcripts was prepared using Qiagen RNAeasy kit (Qiagen, Valencia, CA) and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA).

Retina Preparation for Clonal Analysis

Adult eyes were collected and incubated in 2.5% dispase in HBSS for 75 min to facilitate further dissection. Retinas were then isolated and flattened out before being fixed on a slide and embedded. This procedure allowed to maximize the number of useful sections that were perfectly transverse, and to avoid introducing a regional bias in the arrays analyzed. For clone size analysis at embryonic stages, whole heads were sectioned, and only sections taken at the level of the optic nerve were used for counting.. In all cases, 25-μm sections were collected, and only cell clusters separated by 4 or more rows of negative nuclei were counted as clones. Since tangential spread of differentiating neurons is limited in the retina, this method made it unlikely to mistakenly aggregate cells of two or more clones. Only GCL cells positive for Brn3 were counted as RGCs, with all others considered to be displaced amacrines. Cone photoreceptors were not singled out, hence photoreceptor data encompass both rods and cones.

Histology, Immunostaining, and In Situ Hybridization

Eyes were collected, fixed in 4% paraformaldehyde, sucrose protected, and sectioned with a cryostat. Whole retinas or sections were stained as previously described (Elliott et al., 2008), using the following antibodies: rabbit anti-Egfp (1:1,000; Invitrogen), mouse anti-syntaxin (1:500; Sigma, St. Louis, MO), goat anti-Brn3 (1:400; Santa Cruz Biotechnology, Canta Cruz, CA), rabbit anti-β-Gal (1:1,000; Cappel, Cochranville, PA), mouse anti-rhodopsin 4D2 (1/100; kind gift from R. Molday, UBC), mouse anti-Protein kinase C (1/100; Santa Cruz Biotechnology), mouse anti-Glutamine synthase (1:100; Chemicon, Temecula, CA), mouse anti-Ki67 (1/200; BD Biosciences, San Jose, CA), rabbit anti-Lim1 (1:2,000; gift from Jessell Lab, New York, NY), mouse anti-Pax6 (1/100; DSHB, Iowa City IA). The Biotinylated Ricinus Communis Agglutinin I (RCA I, RCA120) (1/1000; Vector Labs, Burlingame, CA) was used to detect microglia. In some instances, X-Gal substrate (Invitrogen) was used to detect β -Gal activity. For in situ hybridization, E14.5 heads were fixed overnight in PFA and dehydrated in methanol. Whole mount in situ hybridization was then performed on whole heads or isolated eyes using established procedures and an *Ikaros* antisense probe for the full coding sequence with the corresponding sense probe as a negative control.

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REFERENCES

Agathocleous M, Harris WA. 2009. From progenitors to differentiated cells in the vertebrate retina. Annu Rev Cell Dev Biol 25:45–69.

Altshuler D, Cepko C. 1992. A temporally regulated, diffusible activity is required for rod photoreceptor development in vitro. Development 114:947–957.

Badea TC, Cahill H, Ecker J, Hattar S, Nathans J. 2009a. Distinct roles of transcription factors brn3a and brn3b in controlling the development, morphology, and function of retinal ganglion cells. Neuron 61:852–864.

Badea TC, Hua ZL, Smallwood PM, Williams J, Rotolo T, Ye X, Nathans J. 2009b. New mouse lines for the analysis of neuronal morphology using CreER(T)/loxP-directed sparse labeling. PLoS One 4:e7859.

Beier KT, Samson ME, Matsuda T, Cepko CL. 2011. Conditional expression of the TVA receptor allows clonal analysis of descendents from Cre-expressing progenitor cells. Dev Biol 353:309–320.

Belliveau MJ, Cepko CL. 1999. Extrinsic and intrinsic factors control the genesis of amacrine and cone cells in the rat retina. Development 126:555–566.

Brzezinski JAt, Kim EJ, Johnson JE, Reh TA. 2011. Ascl1 expression defines a subpopulation of lineage-restricted progenitors in the mammalian retina. Development 138:3519–3531.

Cayouette M, Barres BA, Raff M. 2003. Importance of intrinsic mechanisms in cell fate decisions in the developing rat retina. Neuron 40:897–904.

Cayouette M, Poggi L, Harris WA. 2006. Lineage in the vertebrate retina. Trends Neurosci 29:563–570.

Elliott J, Jolicoeur C, Ramamurthy V, Cayouette M. 2008. Ikaros confers early temporal competence to mouse retinal progenitor cells. Neuron 60:26–39.

Georgopoulos K, Moore DD, Derfler B. 1992. Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment. Science 258:808–812.

Gomes FL, Zhang G, Carbonell F, Correa JA, Harris WA, Simons BD, Cayouette M. 2011. Reconstruction of rat retinal progenitor cell lineages in vitro reveals a surprising degree of stochasticity in cell fate decisions. Development 138:227–235.

Jeon CJ, Strettoi E, Masland RH. 1998. The major cell populations of the mouse retina. J Neurosci 18:8936–8946.

Li Y, Lu H, Cheng P, Ge S, Xu H, Shi SH, Dan Y. 2012. Clonally related visual cortical neurons show similar stimulus feature selectivity. Nature 486:118–121.

Lillien L, Cepko C. 1992. Control of proliferation in the retina: temporal changes in responsiveness to FGF and TGF alpha. Development 115:253–266.

Nadal-Nicolas FM, Jimenez-Lopez M, Sobrado-Calvo P, Nieto-Lopez L, Canovas-Martinez I, Salinas-Navarro M, Vidal-Sanz M, Agudo M. 2009. Brn3a as a marker of retinal ganglion cells: qualitative and quantitative time course studies in naive and optic nerve-injured retinas. Invest Ophthalmol Vis Sci 50:3860–3868.

Price J, Turner D, Cepko C. 1987. Lineage analysis in the vertebrate nervous system by retrovirus- mediated gene transfer. Proc Natl Acad Sci USA 84:156–160.

Prinz M, Mildner A. 2011. Microglia in the CNS: immigrants from another world. Glia 59:177–187.

Reese BE, Tan SS. 1998. Clonal boundary analysis in the developing retina using X-inactivation transgenic mosaic mice. Semin Cell Dev Biol 9:285–292.

Reh TA. 1992. Cellular interactions determine neuronal phenotypes in rodent retinal cultures. J Neurobiol 23:1067–1083.

Reh TA, Tully T. 1986. Regulation of tyrosine hydroxylase-containing amacrine cell number in larval frog retina. Dev Biol 114:463–469.

Soriano P. 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet 21:70–71.

Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, Costantini F. 2001. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol 1:4.

Turner DL, Snyder EY, Cepko CL. 1990. Lineage-independent determination of cell type in the embryonic mouse retina. Neuron 4:833–845.

Waid DK, McLoon SC. 1998. Ganglion cells influence the fate of dividing retinal cells in culture. Development 125: 1059–1066.

Wang SW, Mu X, Bowers WJ, Kim DS, Plas DJ, Crair MC, Federoff HJ, Gan L, Klein WH. 2002. Brn3b/Brn3c double knockout mice reveal an unsuspected role for Brn3c in retinal ganglion cell axon outgrowth. Development 129: 467–477.

Wang Y, Dakubo GD, Thurig S, Mazerolle CJ, Wallace VA. 2005. Retinal ganglion cell-derived sonic hedgehog locally controls proliferation and the timing of RGC development in the embryonic mouse retina. Development 132:5103–5113.

Watanabe T, Raff MC. 1990. Rod photoreceptor development in vitro: intrinsic properties of proliferating neuroepithelial cells change as development proceeds in the rat retina. Neuron 4: 461–467.

Williams RW, Goldowitz D. 1992. Structure of clonal and polyclonal cell arrays in chimeric mouse retina. Proc Natl Acad Sci USA 89:1184–1188.

Yu YC, Bultje RS, Wang X, Shi SH. 2009. Specific synapses develop preferentially among sister excitatory neurons in the neocortex. Nature 458:501–504.

Yun S, Saijoh Y, Hirokawa KE, Kopinke D, Murtaugh LC, Monuki ES, Levine EM. 2009. Lhx2 links the intrinsic and extrinsic factors that control optic cup formation. Development 136: 3895–3906.