Early identification of retinal subtypes in the developing, pre-laminated chick retina using the transcription factors Prox1, Lim1, Ap2α, Pax6, Isl1, Isl2, Lim3 and Chx10

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In this study, antibodies toward the transcription factors Prox1, Lim1, Ap2α, Pax6, Isl1, Isl2, Lim3 and Chx10 were used to identify and distinguish between developing cell types in the pre-laminated chick retina. The spatio-temporal expression patterns were analysed from embryonic day 3 (E3) to E9, thus covering a time-span from the onset of retinal cell-fate determination to when retinal laminas can be distinguished. Most transcription factors were found at early stages of development, enabling us to trace various precursor cell populations throughout the lamination process. With time, each transcription factor expression became restricted to distinct laminas or sub-laminas of the maturing retina. These early emerging patterns were compared and found to be consistent with those of the hatched chick retina, where the outer nuclear layer label for Lim3, Isl1 and Isl2. In the inner nuclear layer, horizontal cells labeled for Prox1, Lim1, Isl1, Ap2α and Pax6, bipolar cell labeled for Lim3, Isl1 and Chx10 and amacrine cells labeled for Ap2α, Isl1 and Pax6. The ganglion cell layer labeled for Isl1, Pax6 and Isl2. The immunolabeling patterns of Lim3 and Isl2 have not previously been described in detail.

Transcription factors (TFs) have crucial roles in the establishment and maintenance of cellular phenotype. In the retina, which is a laminated structure with five main neuronal cell types (Kolb 2001, Masland 2001) each cell type expresses a different set of TFs that helps determine, define and control differentiation during development. In the mature retina TF expression patterns are separated within distinct laminas. The early developing retina, however, has a less strict organization and consists of both mitotic and post-mitotic cells whose identity cannot easily be defined on morphological criteria since retinal precursor cells appear morphologically homogenous.

Although different retinal cell types are born in a conserved sequential order, there is a considerable overlap in birth dates because the entire retinal cell population is produced during a relatively short time which in chicks only lasts for a few days (Kahn 1974, Altshuler 1991, Rapaport 2004). For this reason, it is also difficult to determine which cell type a precursor cell will ultimately become based solely on its time of birth. Furthermore, due to developmental translocations of undifferentiated precursor cells, neither their position nor their morphology provides conclusive evidence of their final fate. Thus, the use of markers such as antibodies directed against TFs expressed in differentiating cell populations is a powerful and indispensable tool in developmental biology since the identity of progenitor cells can be distinguished by their differential expression or co-expression of these factors early in development.

Our aims were to identify and distinguish between early precursors of retinal cell subtypes by investigating the spatio-temporal expression of several TFs known to play a role in early neuronal maturation and cell-fate determination, namely Prox1, Lim1, Ap2α, Pax6, Isl1, Isl2, Lim3 and Chx10. The immunoreactivity (IR) patterns of these TFs in the developing chick retina are described from embry-

onic day 3 (E3) to E9 and in the newly hatched chick retina (Figure 1).

Our results provide a detailed map over the transcription factor expression profiles during development in the pre-laminated retina. At early developmental stages several antibodies exhibited dynamic and changing immunoreactivity patterns. At later stages, lamina-specific IR patterns emerged and cell types could be identified by their presence at distinct laminar locations. Thus, we were able to trace the appearance, migration and developmental fate of retinal cells from the early onset of TF expression throughout the retinal lamination process. The onset of TF expression within the cell populations is in agreement with the sequence in which cell populations in the retina are born (Kahn 1974, La Vail 1991, Rapaport 2004), indicating that the studied TFs are important for the early and intrinsic specification of cell fate.

Materials and Methods

Animals

Fertilized White Leghorn eggs were obtained from Ova Production AB (Västerås, Sweden). After incubation at 37°C until the desired developmental stage (Hamburger 1951) embryos were killed by decapitation. The animal work followed the European community guidelines and the ARVO statements for use of animals in ophthalmic and vision research. Experiments were scrutinized in the local ethics committee (Uppsala djurförsöksetiska nämnd).

Immunohistochemistry

Eyes were dissected and processed for immunohistochemistry by fixation in 4% paraformaldehyde (15-20 minutes), wash in PBS (10 minutes) and cryoprotection in 30% sucrose (3-4 hours). These incubations were carried out at 4°C. The light fixation and cryoprotection was crucial for successful antibody staining. Eyes were embedded in Tissue-Tek (Sakura), frozen and transverse sections (10 μm thick, 12μm for st46) were cut in the temporal-nasal axis at the level of the lens and collected on Superfrost Plus glasses (Menzel-Gläser, Germany). Immunohistochemistry was carried out by re-hydration in PBS (15 minutes), blocking and permeabilization in 1% FCS and 0,1% Triton-X (30 minutes). Antibodies were diluted in block solution and reacted over night at 4°C (primary antibodies) or at room temperature for 2 hours (secondary antibodies). Between antibody reactions, sections were washed 3x5 minutes in PBS. Sections were mounted in Vectashield Hard-set (H-1400, Vector Laboratiories) and images were obtained from the central retina (opposite to the lens) in proximity to the optic stalk/optic nerve exit. Negative controls (without primary antibodies) were carried out under the same regimens.

Antibodies

Primary antibodies were directed towards Prox1 (1:4000, gift from Dr. M. Nakafuku), Lim1/2 (1:100, 4F2, Developmental studies hybridoma bank, DSHB), Ap2α (1:200, 3B5, DSHB), Pax-6 (1:200, PAX6, DSHB or 1:4000, AB5409, Chemicon), Isl1 (1:200, 40.2D6, DSHB), Isl2 (1:200, 51.4H9, DSHB), Lim3 (1:200, 67.4E12, DSHB), Chx-10 (1:4000 and 1:500, gift from Dr. J. Ericson and Dr. T. Furukawa). Secondary antibodies (1:200) were obtained from Vector Laboratories (Burlingame, CA) and Molecular Probes (Leiden, The Netherlands).

Image processing

Sections were analysed and fluorescence micrographs were acquired using a Zeiss Axioplan2 microscope with Axiovision software (3.0.6.1, Carl Zeiss Vision GmbH). Images were subsequently formatted, resized, rotated, enhanced and arranged for publication using Axiovision and Photoshop (v6.0.1, Adobe).

Figure 1. *See next page.* Spatial and temporal expression of the transcription factors Prox1, Lim1, Ap2α, Pax6, Isl1, Isl2, Lim3 and Chx10 from stage 20 through 35 and at stage 46. Micrographs of different stages of retinal development stained with antibodies directed towards the mentioned transcription factors reveal the dynamic patterns of immunoreactivity throughout the lamination process. Images were taken from the central part of the retina, in proximity to the optic stalk/ optic nerve exit. Abbreviated names of retinal layers are denoted as they emerge. E: embryonic day, St: stage according to Hamburger and Hamilton, DIC: Differential interference contrast, P0: postnatal day 0, pe: pigment epithelium, ne: neuroepithelium, (p-) gcl: (prospective-) ganglion cell layer, onl: outer nuclear layer, inl: inner nuclear layer, opl: outer plexiform layer, ipl: inner plexiform layer. Scale bar in bottom right corner is 20 μm and is valid for the entire figure.
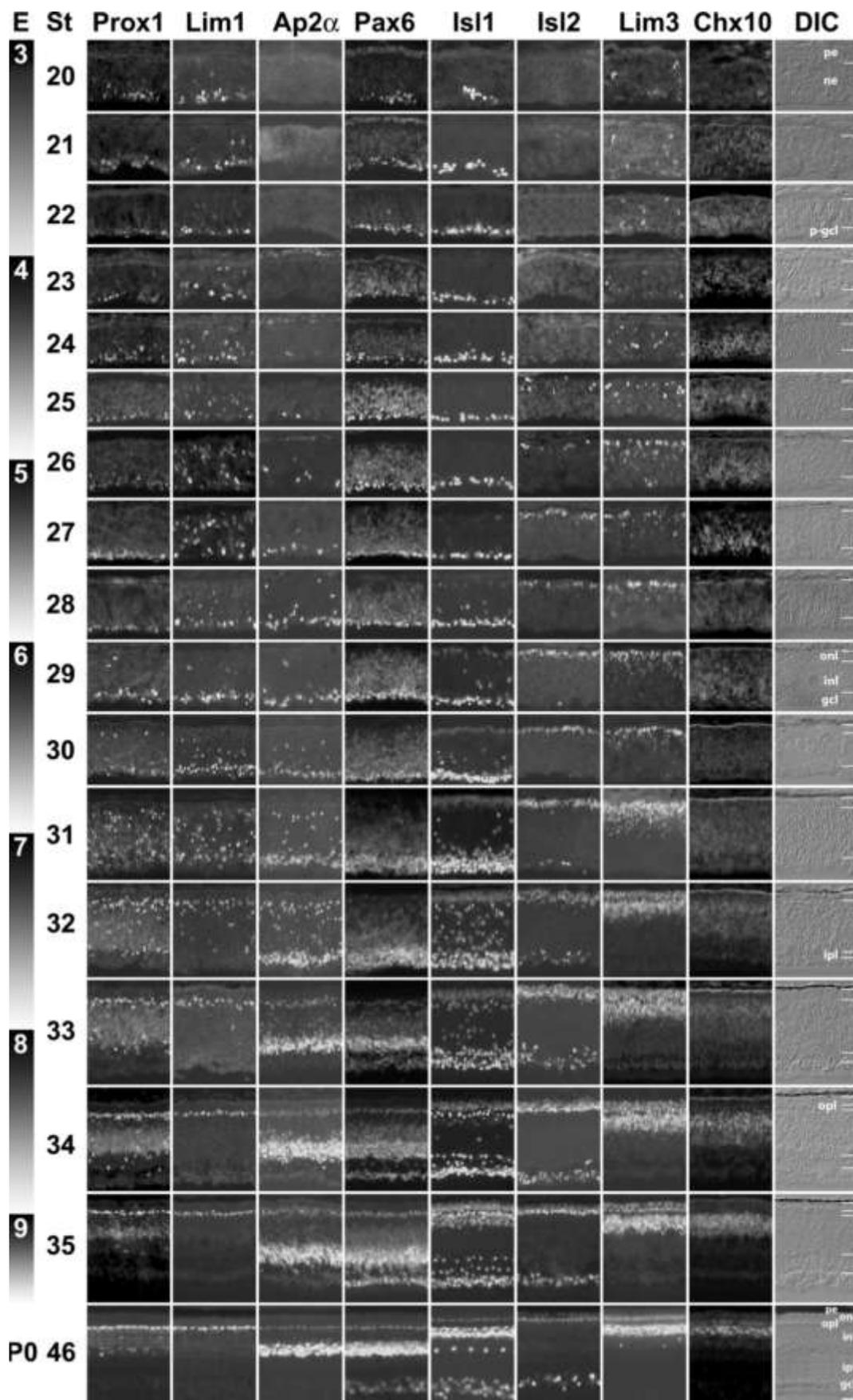
Results

Figure 1 shows the spatio-temporal immunoreactivity (IR) patterns of the TFs Prox1, Lim1, Ap2α, Pax6, Isl1, Isl2, Lim3 and Chx10 in chick retinas from Hamburger and Hamilton stage (st) 20 to st35, as well as in the newly hatched st46 retina. Control experiments without primary antibodies did not produce any IR (*not shown*).

Prox1

The spatio-temporal Prox1 IR pattern had three temporal phases. Prox1 positive (+) cells were first detected in the vitreal neuroepithelium (NE) at st20 where they remained until st29. Between st30 and st34 Prox1+ cells were scattered in all layers of the INL. Beyond st35, strong Prox1 IR was found in the horizontal cell layer and in scattered cells in the vitreal part of the INL. In addition, by st32 weaker Prox1+ IR appeared as a band in the middle of the INL. This band was undetectable at st46. Previous studies report Prox1 in retinal progenitor cells, horizontal cells, bipolar cells and AII-amacrine cells (Belecky-Adams 1997, Dyer 2003b).

Lim1

Lim1 IR was scattered at various positions of the NE during early development and was later restricted to the horizontal cell layer. Immunoreactivity was first detected at st20. At st28-30 the majority of Lim1+ cells aligned underneath the prospective ganglion cell layer. From st33 Lim1+ cells formed a single line of cells in the horizontal cell layer. The Lim1 IR pattern was consistent with the reported bi-directional migration of horizontal cells (Edqvist 2004).

Ap2α

Ap2α IR, first detected at st24, was located in the vitreal NE at early stages and later detected as a thick band in the amacrine cell layer as well as in the horizontal cell layer. The total IR increased considerably during st31-33, with scattering of cells and thickening of the amacrine cell layer. In the st46 retina IR was found in the horizontal cell layer and in amacrine cells of the INL. Previous studies report Ap2α in amacrine cells and horizontal cells (Bisgrove 1999, Edqvist 2004).

Pax6

Pax6 IR was detected strongly in individual cells as well being detected as a diffuse gradient. Immunoreactivity was first detected at st19 (*not shown*) in a vitreal-to-ventricular gradient with the strongest IR on the vitreal side of the NE. Between st20 and st32 strong IR was detected in individual cells in the vitreal retina. Beginning at st31 IR thickened considerably in the innermost inner nuclear layer (similar in timing and location as Ap2α). Between st25 and st33 a vitreal-ventricular Pax6 gradient was present again. Between st33 and st35, four bands with varying intensities were detected: one in the ganglion cell layer, one in the innermost inner nuclear layer, one weaker band located in the middle of the inner nuclear layer (similar to the weak Prox1 band) and one in the horizontal cell layer. At st46 all immunoreactive bands except the weak inner nuclear layer-band were still detectable. Pax6 is known to have a fundamental function in eye development and has previously been reported in retinal precursor cells, ganglion cells, amacrine cells and horizontal cells (Belecky-Adams 1997, Callaerts 1997, Marquardt 2001).

Isl1

Isl1 IR was restricted to vitreally located cells from st19 (not shown) until st28. The first Isl1+ cells outside the prospective ganglion cell layer were detected at st28. At st30, IR thickened in the ganglion cell layer and another band of IR was detected in the outer nuclear layer. Between st31 and st33, Isl1+ cells separated into a ganglion cell layer-population, an innermost inner nuclear layer population, and into a population of cells scattered in the middle of the inner nuclear layer. By st35, IR was detected in the outer nuclear layer and ganglion cell layer, in cells located on the internal rim of the inner nuclear layer, in the horizontal cell layer, in the bipolar cell layer and also in cells in the inner plexiform layer. At st46, the IR was similar to st35 but no immunoreactive cells were detected in the inner plexiform layer. The Isl1+ cells in the amacrine cell layer are likely cholinergic amacrine cells, and those in the inner plexiform layer are likely displaced amacrine cells en route to the ganglion cell layer, respectively (Galvez 1977, Sugihara 1983, Galli-Resta 1997).

Isl2

Isl2 IR was detected in two cell populations with distinct expression patterns. Immunoreactivity was first detected at st25 in the ventricular NE. The
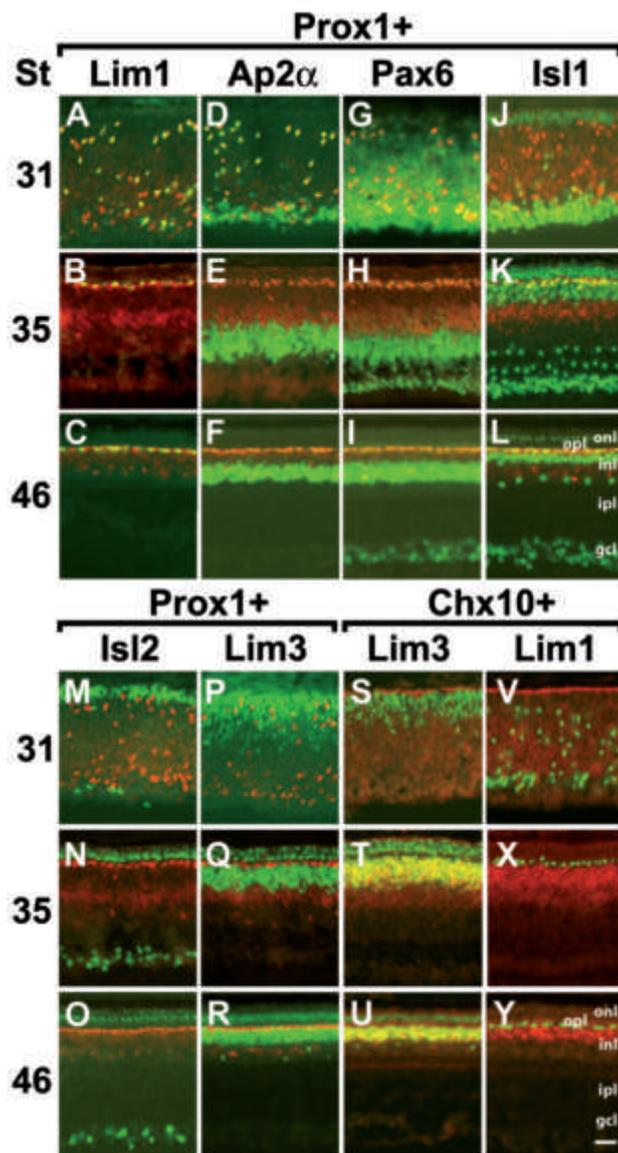
Figure 2. Co-expression of TFs in the central retina at stages 31, 35 and 46. Co-labeling for Prox1 (red, A-R) and other TFs (green) reveal how Lim1 (A-C), Ap2α (D-F), Pax6 (G-I), and Isl1 (J-L) are co-expressed in horizontal cells. Except for a band of co-expression in the middle of the INL at st35 (H), no other cell populations are double positive for Prox1 and Pax6. Co-labeling for Prox1 with Isl2 (green, M-O) and Lim3 (green, P-R) show no overlapping expression patterns. Co-labeling for Chx10 (red, S-Y) with Lim3 (green, S-U) and Lim1 (green, V-Y) show a high degree of co-expression in the bipolar cell population (Lim3) but none in the horizontal cell population (Lim1). St: stage according to Hamburger and Hamilton, onl: outer nuclear layer, opl: outer plexiform layer, inl: inner nuclear layer; ipl: inner plexiform layer, gcl: ganglion cell layer. Scale bar in Y is 20μm and is valid for A-Y.

number of IR cells increased with time and eventually the majority (maybe all) cells in the outer nuclear layer were Isl2+. Beginning at st31, IR was detected in the ganglion cell layer and by st46 two distinct Isl2+ populations were found, one in the outer nuclear layer and the other in the ganglion cell layer.

Lim3

At early stages, Lim3 IR was scattered throughout the NE and was later restricted to two bands in ventricular retina with occasional IR detected outside these bands at st46. The first Lim3+ cells were detected at st20 as scattered cells at all levels of the NE. By st26 the majority of Lim3+ cells were located on the ventricular side of the NE and by st28, IR was completely restricted to the ventricular NE. By st31, IR intensity increased and by st32, two separate populations could be distinguished in the outer nuclear layer and in the bipolar cell layer. At st46, IR was also detected in scattered cells in the internal inner nuclear layer.

Chx10

Chx10 IR was detected during two phases: an early phase characterized by scattered cells throughout the NE, and a late phase characterized by IR in the bipolar cell layer. Chx10 IR was diffusely distributed across the NE between st20 and st29 and was undetectable between st30 and st32. At st33, IR was detected in the outer region of the inner nuclear layer. By st34-35, the IR grew stronger and by st46 IR was only detected in the bipolar cell layer. Chx10 is known to be expressed in retinal precursor cells and in bipolar cells (Chen 2000, Dyer 2003a, Green 2003). Noteworthy is that Chx10 IR was detected in bipolar cells after the onset of Lim3 IR, but prior to Isl1 IR.

Double labeling analysis

To verify and distinguish between cell types expressing different combinations of TFs, we analyzed co-expression of selected TFs in st31, 35 and 46 retinas (Figure 2). The analysis revealed that most of the Prox1+ horizontal cell population was also Lim1+ (Figure 2A-C). Prox1 and Ap2α were co-expressed in scattered cells across the retina at st31 (Figure 2D), and in cells of the horizontal cell layer at st35 and at st46. Co-expression of Prox1 and Ap2α in the amacrine cell layer could not be detected (Figure 2D-F). Prox1 and Pax6 were co-expressed in the horizontal cell layer at st35 and st46 (Figure 2H-I) and in the weak band in the middle of the inner nuclear layer at st35 (Figure 2H). Co-expression of Prox1 and Pax6 was not detected in the amacrine cell- or ganglion cell layer at st35

Timing of transcription factor appearance in retinal neurons
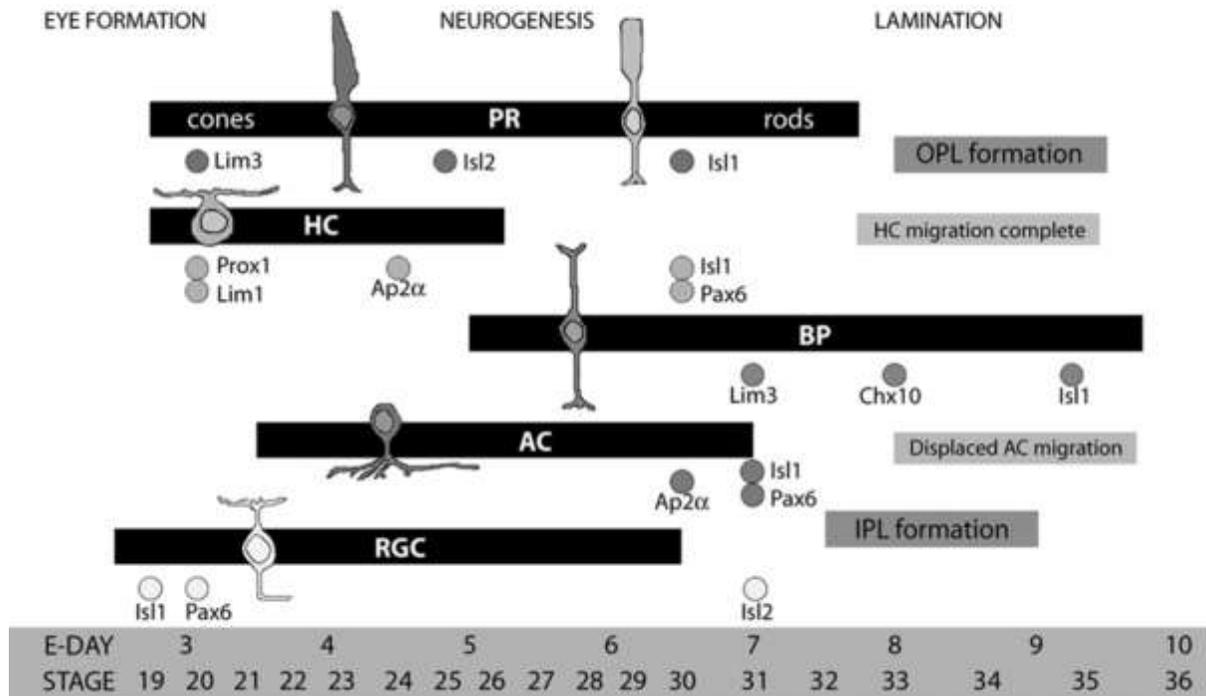
Figure 3. Schematic diagram of the onset of transcription factor expression in different cell types. The earliest detectable time of TF onset in a cell population is indicated as dots underneath the black bars. These stages were determined for each cell type when it was possible to draw conclusions on cellular phenotype based on laminar location, time of birth relative to other populations, co-expression of other TFs (or lack thereof), and/ or migratory behavior. The black bars represents the stages during which the various cell types are generated according to Kahn (1974). Note that sometimes not all cells within a certain cell population are necessarily labeled, for instance Lim1+ horizontal cells, Isl1+ amacrine cells and Isl2+ cells in the ganglion cell layer. PR: photoreceptors, HC: horizontal cells, BP: bipolar cells, AC: amacrine cells, RGC: retinal ganglion cells, OPL: outer plexiform layer, IPL: inner plexiform layer.

and st46 (Figure 2G-I). Prox1 and Isl1 were co-expressed in putative migrating horizontal cells at st31 and in the horizontal cell layer at st35 and st46, while co-expression elsewhere was not detected (Figure 2J-L). Neither Lim3 nor Isl2 were co-expressed with Prox1 in any of the examined stages (Figure 2M-O and P-R). Chx10 co-expressed with the majority of Lim3+ cells in the inner nuclear layer at st35 and st46, but not with the Lim3+ cells in the outer nuclear layer (Figure 2S-U). Neither Lim1 and Chx10 (Figure 2V-Y), nor Pax6 and Lim3 (*data not shown*) co-expression could be detected at any of the examined stages.

Discussion

This work has provided a detailed TF- expression map in the developing chick retina where TFs that define all retinal cell types can be compared directly across all developmental stages, from the earliest stages of neurogenesis until retinal lamination is completed. The spatio-temporal IR expression patterns of Prox1, Lim1, Ap2α, Pax6, Isl1, Isl2, Lim3 and Chx10 were examined. Previously reported in situ and IR patterns for Prox1, Chx10, Pax6, Lim1, Ap2α and Isl1 in chick, mouse and zebrafish retinas are consistent with the results in this study (Belecky-Adams 1997, Galli-Resta 1997, Bisgrove 1999, Vogel-Hopker 2000, Edqvist 2004, Shkumatava 2004). In addition, we have by co-expression analysis described how various cell populations can be subdivided into subtypes already at early developmental stages based on differential expression profiles. Furthermore, we have described the labeling patterns for two novel markers in the developing retina: Lim3 and Isl2. Lim3 is here of particular interest since it is found in putative bipolar cells prior to the expression of Chx10, which currently is the earliest known marker for bipolar cells (Rowan 2004).

The TF expression in different cell populations, as well as the stage for onset of expression, is summarized in Figure 3. To identify the earliest time at which different cell types expressed different TFs, and because the same TF expression sometimes occurs in two or more cell types, we used different identification criteria at different time points. For instance, prior to st27 Isl1 appears to be exclusively expressed in ganglion cells but after st27, Isl1+ cells are also likely to be amacrine- and horizontal cells. Thus, Isl1+ horizontal cells were distinguished from Isl1+ amacrine cells at st30 by the fact that they were migrating. Amacrine cells were identified at st31 as the inner nuclear layer and ganglion cell layer began to separate, and Isl1 expressing bipolar cells were identified at st35 as they appeared in the inner nuclear layer. Differential co-expression patterns at different stages also helped in the identification process (Figure 2). It should, however, be noted that the Isl1 antibody used in this study recognizes both the Isl1 and Isl2 TFs (Tsuchida 1994), and therefore it is possible that Isl1-IR detected in the outer nuclear layer is a result from cross-reactivity with the Isl2-epitope. In addition, previous studies on Ap2α and Ap2β detected only Ap2β in horizontal cells (Bisgrove 1999), and therefore the Ap2α antibody used in this study might have cross-reacted with the Ap2β-epitope in horizontal cells.

Previous studies on the role of TFs in retinal development showed that combinations of homeobox and basic helix-loop-helix TFs are instrumental in generating both large cell populations, as well as subtypes within populations, and that the fate-determining and fate-biasing actions of TFs are exerted in both retinal precursor cells and post-mitotic neurons (Fuhrmann 2000, Hobert 2000, Hatakeyama 2001, Vetter 2001, Inoue 2002, Shirasaki 2002, Marquardt 2003, Akagi 2004). The models put forward to describe retinal development state that distinct pools of proliferating retinal precursor cells exist, and while these pools can be biased to produce certain cell types, they are in principle able to produce any type of post-mitotic retinal neuron depending on the state of competence (Turner 1990, Alexiades 1997, Cepko 1999, Adler 2000, Dyer 2001, Livesey 2001). Competence of retinal precursor cells is intrinsically defined but is likely to be modulated by extrinsic factors (Fuhrmann 2000, Livesey 2001). Some also hypothesise that newborn retinal neurons are in principle born uncommitted,

and depend mainly on extrinsic modulation for their final determination (Wetts 1988, Turner 1990, Adler 1999, Adler 2000).

In this study, the time points for the earliest markers to be expressed in different populations correlate with the temporal birth sequence for the retinal cell classes (Kahn 1974, La Vail 1991, Rapaport 2004). For example: ganglion cells express Isl1 at st19 (*not shown*), horizontal cells Prox1 and Lim1 at st20, cone photoreceptors express Lim3 at st20, amacrine cells Ap2α at st30, and bipolar cells express Lim3 at st31 (Figure 3). Our observation that TF expression onset is concomitant with birth-date implies that cell fate is determined in precursor cells before or shortly after the cell's last mitosis. Furthermore, it reveals that cell type determinants are likely to be early, intrinsic and highly specific rather than late, general and extrinsic, as suggested by others (Wetts 1988, Turner 1990, Belecky-Adams 1996, Adler 1999, Adler 2000). Our results also indicate that precursor cell identity for all retinal neural cell types can be identified early, supporting a model whereby cell fate of post-mitotic neurons is, at least in part, intrinsically determined around the time of the last mitosis, indicating that the expression of specific TF signatures substantially limits the developmental repertoire of precursor cells.

Furthermore, by double labeling, we observed the existence of subtypes within the retinal cell populations based on differential labeling patterns. For instance, horizontal cells can be subdivided based on Prox1, Lim1, Ap2α and Isl1 expression patterns (manuscript in preparation), bipolar cells can be divided based on Chx10, Lim3 and Isl1, and amacrine cells on Ap2α and Isl1. Thus, since certain TF alone or in combination are expressed in distinct fractions of cells within a larger population, this indicates that not only the cell type, but also the subtype, is molecularly defined earlier than previously hypothesized. Knocking out population-defining TFs such as Prox1 or Chx10 during development can have detrimental effects, as shown in mice deficient for Prox1 and Chx10 that lack their entire horizontal- or bipolar cell populations, respectively (Dyer 2003b, Green 2003, Rutherford 2004). Perturbation of other TFs such as Barlh2 and NeuroAB have yielded subtype-specific losses in the amacrine cell population (Mo 2004, Ohkawara 2004). Further studies will show whether perturbation of any TFs reported herein,

such as e.g. Lim1, Lim3 and Isl2 will loose entire cell populations or only fractions thereof.

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