Journal of Neurochemistry, 2005, **94**, 1739–1745 doi:10.1111/j.1471-4159.2005.03336.x

Runx1 expression defines a subpopulation of displaced amacrine cells in the developing mouse retina

Lee Stewart,* Mary Anne Potok,† Sally A. Camper† and Stefano Stifani*

**Center for Neuronal Survival, Montreal Neurological Institute, Montreal, Quebec, Canada*

†*Department of Human Genetics, University of Michigan Medical School, Ann Arbor, Michigan, USA*

Abstract

AML1/Runx1 (Runx1) is a mammalian transcription factor that plays critical roles in regulating the differentiation of a number of different cell types. In the present study, we have utilized mice expressing β-galactosidase (β-gal) under the control of the *Runx1* promoter to characterize the spatiotemporal expression pattern of *Runx1* during retinogenesis. Expression of β-gal was first detected at embryonic day 13.5 in post-mitotic cells located in the inner retina and overlapped with expression of the early amacrine and ganglion cell marker protein Islet1. During subsequent developmental stages, the number of β-gal-positive cells increased in a central-to-peripheral gradient until late embryogenesis but then decreased in the early post-natal retina. β-gal-positive cells were located primarily in the ganglion cell layer by late embryonic/early post-natal stages and were identified as a subpopulation of displaced amacrine cells by the continued expression of Islet1, as well as Pax6, and the coexpression of the amacrine cell subtype-specific markers choline acetyltransferase, calretinin and the 65-kDa isoform of glutamic acid decarboxylase. These findings identify *Runx1* as a novel marker for a restricted amacrine cell subtype and suggest a role for this gene in regulating the post-mitotic development of these cells.

Keywords: amacrine cells, choline acetyltransferase, ganglion cell layer, 65-kDa isoform of glutamic acid decarboxylase, retina, Runx1.

J. Neurochem. (2005) **94,** 1739–1745.

The differentiation of distinct neuronal subtypes at the correct time and place is a critical event during nervous system development. In that regard, the developing mammalian retina provides a relatively simple and well-defined experimental system to study the events that underlie the generation of different cell types from common progenitors. Six main classes of neurons and one type of glial cell are generated in the retina and the precise spatial and temporal pattern of retinogenesis is under the influence of both extrinsic and intrinsic cues (Marquardt and Gruss 2002). Although a number of transcription factors have been identified as important regulators of mammalian retinal development (Marquardt 2003), the mechanisms that regulate the acquisition of specific retinal neuron traits still remain poorly defined. In particular, little is known about the molecular characteristics of specific subtypes of each set of retinal neurons and how the differentiation of those individual subtypes is regulated.

The phylogenetically conserved *runt*/*Runx* gene family encodes a number of DNA-binding transcription factors that play important roles during development in both invertebrates and vertebrates (Wheeler *et al*. 2000; Coffman 2003; Otto *et al*. 2003). In particular, *Runx1* is required for definitive hematopoiesis in mice and mutations of its human homolog, *AML1*, are found in a high percentage of acute myeloid leukemias and other hematological disorders (Amman *et al*. 2001; Roumier *et al*. 2003). Recent studies have also identified a requirement for *Runx1* function during the post-mitotic development of selected populations of sensory and motor neurons in the murine hindbrain and cranial ganglia, implicating *Runx1* in the regulation of neuron subtype differentiation (Theriault *et al*. 2004). To determine if *Runx1* is involved in the development of additional

Received April 7, 2005; revision received May 20, 2005; accepted May 20, 2005.

Address correspondence and reprint requests to Dr Stefano Stifani, Center for Neuronal Survival, #F102, Montreal Neurological Institute, 3801 rue University, Montreal, Quebec, H3A 2B4, Canada.
E-mail: stefano.stifani@mcgill.ca

Abbreviations used: AMCA, Amino Methyl Coumarin Acetate; β-gal, β-galactosidase; ChAT, choline acetyltransferase; E, embryonic day; GAD65, 65-kDa isoform of glutamic acid decarboxylase; GAD67, 67-kDa isoform of glutamic acid decarboxylase; GCL, ganglion cell layer; IPL, inner plexiform layer; P, post-natal day.

neuronal cells, we have characterized its expression during retinal development. Here, we demonstrate for the first time that *Runx1* is transiently expressed in a subpopulation of displaced amacrine cells characterized by the expression of choline acetyltransferase (ChAT), the 65-kDa isoform of glutamic acid decarboxylase (GAD65) and the calcium-binding protein calretinin. These findings identify *Runx1* as a new marker of a specific subset of amacrine cells. Moreover, they suggest that *Runx1* may contribute during a defined temporal window to the mechanisms regulating the acquisition of a particular terminal phenotype by post-mitotic amacrine cells.

Experimental procedures

Tissue preparation

Runx1$^{lacZ/+}$ mice were generated and maintained as previously described (North *et al*. 1999). The recombined locus of *Runx1*$^{lacZ/+}$ mice encodes a fusion protein of the N-terminal 242 amino acids of Runx1 (containing a nuclear localization sequence) and β-galactosidase (β-gal). The expression of this nuclear fusion protein was shown to faithfully reproduce the expression pattern of *Runx1* transcripts (North *et al*. 1999; Theriault *et al*. 2004). For embryonic staging, the day of the appearance of the vaginal plug was considered as embryonic day 0.5 (E0.5). Embryos or pups were collected at stages E13.5 to post-natal day 5 (P5). Tail clippings were genotyped by both PCR analysis of genomic DNA (North *et al*. 1999) and incubation with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (2 μg/mL) in phosphate-buffered saline containing 1 M $MgCl_2$, 5 mM potassium ferrocyanide and 5 mM potassium ferricyanide. For embryonic stages, eyes were fixed *in situ* in 2% paraformaldehyde containing 10 mM sodium periodate and 70 mM L-lysine (PLP solution) for 2–4 h. Post-natal eyes were removed and immersion-fixed in 2% PLP solution for 4 h. After tissue freezing (Theriault *et al*. 2004), sections were cut on a cryostat, mounted on Superfrost™ slides, air-dried and stored at −20°C. All experimental procedures were carried out in accordance with the guidelines set forth by the Canadian Council for Animal Care.

Histochemical detection of β-galactosidase activity

Representative sections from each developmental stage examined (E13.5 to P5) were incubated in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactosidase overnight at 37°C as described previously (North *et al*. 1999) followed by counterstaining with Neutral Red (Sigma, St Louis, MO, USA).

Immunofluorescence

Tissue sections were first incubated in Image-iT™ FX Signal Enhancer (Molecular Probes, Eugene, OR, USA) for 30 min and then for 1 h in a blocking solution manufactured specifically for detecting mouse primary antibodies on mouse tissue (M.O.M. Kit; Vector Laboratories, Burlingame, CA, USA). The primary antibodies used to label different retinal cell types are listed in Table 1. Sections were incubated with primary antibodies at 4°C and then with the appropriate secondary antibodies for 20 min at room temperature (22°C). The following secondary antibodies were used for double-labeling studies: Cy3-conjugated goat anti-rabbit/mouse and FITC-conjugated goat anti-mouse/rabbit (1 : 200; Jackson ImmunoResearch, West Grove, PA, USA). Triple-labeling experiments were performed using the primary antibodies indicated in the text and the following secondary antibodies: Cy3-conjugated donkey anti-goat; FITC-conjugated donkey anti-rabbit and AMCA-conjugated donkey anti-mouse (1 : 40; Jackson ImmunoResearch). As controls, primary antibodies were omitted from the incubation steps, in which case no staining was observed.

In situ hybridization

Sense and antisense *Runx1* riboprobes were generated by first using a mouse *Runx1* cDNA (Image Clone ID 4037315) to amplify by PCR a ∼0.5-kb product corresponding to the start of the 3′ untranslated region. The following oligonucleotides were used for PCR: *Runx1*-3′-forward, 5′-TACTGAGCTGAGCGCCATCGC-CAT-3′ and *Runx1*-3′-reverse, 5′-GACCCAAAGCTGTAGCTGTC-TCT-3′ and the PCR product was cloned into pBluescript-SK. Riboprobes were generated in the presence of digoxigenin 11-UTP by transcription with T7 or T3 RNA polymerases, respectively. Wild-type E16.5 mouse embryos on a mixed C57/Bl6 × SJL background were fixed in 4% paraformaldehyde (2 h), embedded in paraffin using a Citadel tissue processor (Thermo Electron, Pittsburgh, PA, USA) and sectioned at 6 μm on a microtome. Tissue sections were then deparaffinized and washed in phosphate-buffered saline prior to treatment with 0.3% Triton X-100 followed by incubation in the presence of proteinase K (7.5 μg/mL), 100 mM glycine and then 0.25% acetic anhydride in 0.1 M triethanolamine. Sections were pre-hybridized at room temperature (1–2 h) in hybridization buffer containing 50% formamide, 5× saline sodium citrate buffer, 2% blocking powder (Roche Molecular Biochemicals, Indianapolis, IN, USA), 0.1% Triton X-100, 0.5% CHAPS, 5 mM EDTA, 50 μg/mL heparin and 1 mg/mL yeast tRNA and hybridized at 50°C overnight in the same buffer with ∼500 pg/μL riboprobe. After hybridization, sections were washed extensively and then incubated in a blocking solution containing 10% heat-inactivated sheep serum, 2% bovine serum albumin, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl and 0.1% Triton X-100 for 1 h. Sections were incubated with alkaline phosphatase-conjugated sheep anti-digoxigenin Fab fragments (1 : 400) in blocking solution (3 h). Antibody labeling was visualized with 4.5 μg/mL 4-nitroblue tetrazolium and 3.5 μg/mL 5-bromo-4-chloro-3-indoyl-phosphate.

Image acquisition

All images were captured with a DVC black and white camera mounted on an Axioskop fluorescence microscope (Zeiss, Toronto, ON, Canada). Grayscale images were digitally assigned to the appropriate red (Cy3), green (FITC) or blue (AMCA) channels using Northern Eclipse software (Empix, Mississauga, ON, Canada).

Results

Spatial and temporal *Runx1* expression in the developing retina

In *Runx1*$^{lacZ/+}$ embryos, retinal β-gal activity was first detected at E13.5 in only a few cells located in the inner region of the central retina, adjacent to the optic nerve (Fig. 1ai, inset; see also Figs 2b and c). From E14.5 to

Table 1. List of primary antibodies used in the current study

Antigen	Species	Target cells	Dilution	Supplier	Coexpression with β-gal**
β-gal	Rabbit	*Runx1*+	1:500	Cappel	N.A.
β-gal	Mouse	*Runx1*+	1:15	Hybridoma Bank (Dr. J. Sanes)*	N.A.
Brn3a	Mouse	Ganglion	1:100	Santa Cruz	-
Brn3b	Goat	Ganglion	1:500	Santa Cruz	-
Calbindin	Rabbit	Amacrine/Horizontal	1:200	Chemicon	-
Calretinin	Mouse	Amacrine	1:100	Chemicon	+
ChAT	Goat	Amacrine	1:10	Chemicon	+
Disabled1	Rabbit	Amacrine	1:200	Chemicon	-
GAD65	Mouse	Amacrine	1:200	Hybridoma Bank (Dr. D.I. Gottlieb)*	+
GAD67	Rabbit	Amacrine	1:200	Chemicon	-
Glycine transporter 1	Goat	Amacrine	1:500	Chemicon	-
Glutamine synthetase	Mouse	Muller glia	1:500	Sigma	-
Islet1	Mouse	Amacrine/Ganglion	1:25	Hybridoma Bank (Dr. T.M. Jessel)*	+
Ki67	Mouse	Progenitor	1:100	Pharmingen	-
Pax6	Mouse	Amacrine	1:5	Hybridoma Bank (Dr. A. Kawakami)*	+
Neurofilament-M	Mouse	Ganglion/Horizontal	1:100	Hybridoma Bank (Dr. T.M. Jessel)*	-
Nitric oxide synthase	Rabbit	Amacrine	1:200	Chemicon	-
Protein kinase C	Mouse	Bipolar	1:500	Sigma	-
Rhodopsin	Mouse	Rod photoreceptors	1:1000	Sigma	-
Syntaxin (HPC-1)	Mouse	Amacrine	1:200	Sigma	-
Tyrosine hydroxylase	Rabbit	Amacrine	1:500	Chemicon	-
Vesicular ACh transporter	Goat	Amacrine	1:500	Chemicon	+

*obtained from the Developmental Studies Hybridoma Bank, administered by the University of Iowa and the National Institute of Child Health and Human Development,
courtesy of the indicated investigators.
**indicates presence (+) or absence (-) of cells that coexpressed β-gal and a given retinal cell marker between stages E13.5 and P3.
N.A.; not applicable

E18.5, β-gal expression increased and progressed concentrically in a wave-like fashion until reaching the peripheral edges of the retina (Figs 1aii–iv). At E16.5–E17.5, β-gal activity appeared to reach a maximum and no further increase in the number of β-gal-positive cells was observed during the remaining embryonic period. At E13.5–E16.5, β-gal-positive cells were concentrated primarily in the inner portion of the retina with a few labeled cells located along the dorsal edge in the presumptive ganglion cell layer (GCL) (Figs 1ai–iii). By E18.5, the majority of β-gal-positive cells were near or within the GCL (Fig. 1aiv). In early post-natal retina (P1 and P3), the expression of β-gal in the GCL was significantly reduced (Figs 1av and vi) and was absent by P5 (data not shown). To confirm these observations, *in situ* hybridization studies were performed on wild-type retina. At E16.5, *Runx1* mRNA was detected in the inner region of the retina (Figs 1bvii and viii), in agreement with the β-gal expression pattern. No hybridization signals above background were observed with a control sense riboprobe (Figs 1bix and x). These combined observations show that *Runx1* is transiently expressed during retinal development and suggest that *Runx1* is expressed in a subpopulation of ganglion and/or displaced amacrine cells.

Expression of *Runx1* in a specific amacrine cell subpopulation

To determine whether *Runx1* is expressed in selected types of post-mitotic retinal neurons, we first compared the expression of β-gal with that of the proliferating cell marker protein Ki67. In the embryonic retina, β-gal expression did not overlap with Ki67 at either its onset at E13.5 (Fig. 2b) or at later stages (data not shown), suggesting that *Runx1* expression is initiated after retinal progenitors have exited the cell cycle. At E13.5, however, β-gal-positive cells already expressed Islet1 (Fig. 2c), an early marker of amacrine and ganglion cell differentiation (Galli-Resta *et al*. 1997).

To examine more directly the possibility that *Runx1* is expressed in a subset of ganglion or displaced amacrine cells, we focused our attention on early post-natal stages, when individual amacrine and ganglion cell types have begun to display specific immunological characteristics and the expression of β-gal is still detectable, although markedly decreased. In particular, rodent amacrine cells are born primarily during the latter half of embryonic development and undergo terminal differentiation during the early post-natal period (Voigt 1986; Cepko *et al*. 1996; Gábriel and Witkovsky 1998). At both E18 and P1, we found that the

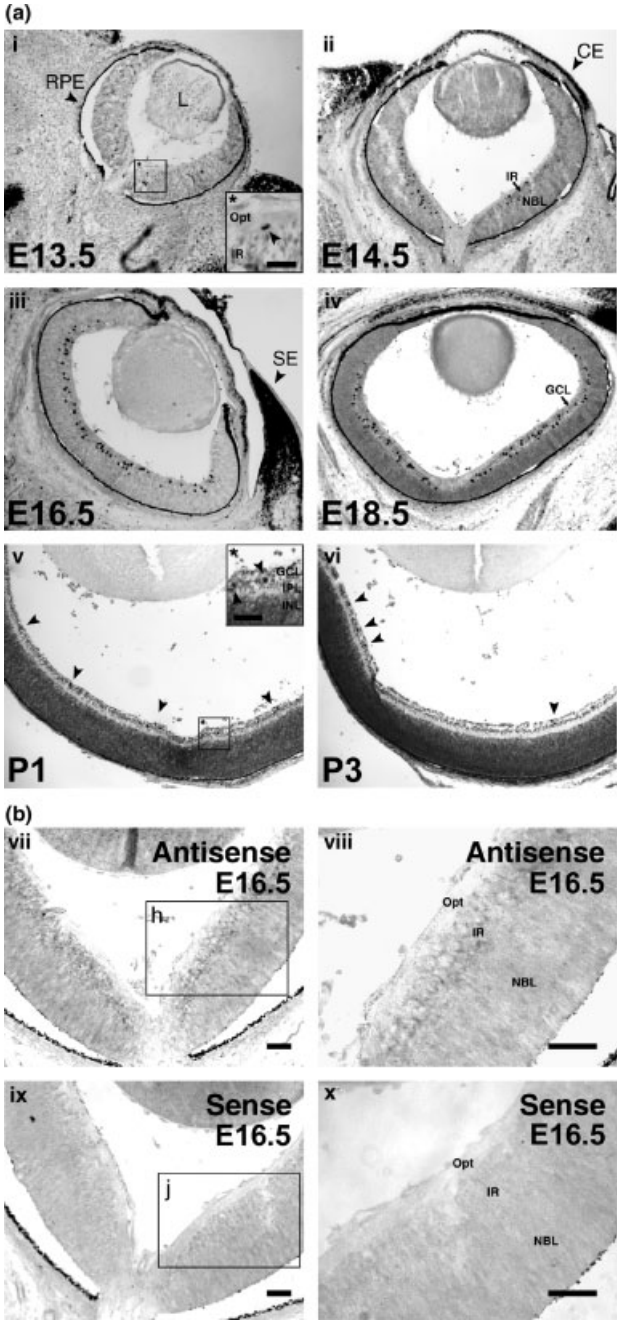

Fig. 1 Analysis of *Runx1* expression during retinal development. (a) Histochemical detection of β-galactosidase (β-gal) activity (dark nuclear staining) in neural retina of *Runx1*$^{lacZ/+}$ embryos or pups. (i) At embryonic day (E) 13.5, β-gal-positive cells were detectable in the central part of the inner retina, adjacent to the optic nerve (inset). (ii) At E14.5, there was an increase in the number of β-gal-positive cells in the inner retina. (iii) At E16.5, β-gal activity continued to increase and spread laterally towards the peripheral edge of the retina. At this stage, the number of β-gal-positive cells appeared to reach a maximum, showing no further qualitative increase at E18.5 (iv), at which point the majority of β-gal-positive cells were within the ganglion cell layer (GCL). Other areas of marked β-gal expression were the surface ectoderm (SE) and corneal epithelium (CE). (v and vi) In early post-natal [post-natal day (P) 1–P3] retina, β-gal expression in the GCL was noticeably reduced to only a few cells in the central retina and periphery (inset and arrowheads). No β-gal expression was observed at P5 (not shown). (b) *In situ* hybridization analysis of *Runx1* expression at E16.5 (dark non-nuclear staining). *Runx1* mRNA was localized to the inner region of the retina (vii and viii), in agreement with the expression of β-gal. No staining above background was observed in the inner region with a control sense riboprobe (ix and x). L, lens; IPL, inner plexiform layer; IR; inner retina; NBL, neuroblast layer; Opt, optic nerve; RPE, retinal pigmented epithelium. Magnification: 40× (a) and 100× (b); inset calibration bars, 50 μm.

β-gal-positive cells scattered throughout the GCL did not express the POU domain transcription factors Brn3a and Brn3b (also referred to as Pou4f1 and Pou4f2, respectively), markers of differentiating retinal ganglion cells (Liu *et al*. 2000) (Fig. 2f and data not shown). In agreement with this observation, β-gal expression did not overlap with other retinal ganglion cell markers, like neurofilament-M (Table 1), suggesting that *Runx1* is not expressed in ganglion cells. In contrast, virtually all β-gal-positive cells continued to express Islet1 (Fig. 2d) as well as Pax6 (Fig. 2e), a paired domain transcription factor that is involved in early retinal specification and whose expression persists in amacrine cells of the post-natal and adult retina (Walther and Gruss 1991; de Melo *et al*. 2003). No detectable overlap of β-gal expression and a number of markers of other retinal cell types was observed between E13.5 and P3 (Table 1).

At P3, β-gal-positive cells coexpressed multiple subtype-specific amacrine cell markers, including (i) the calcium-binding protein calretinin, which is expressed by cells in both the GCL and inner nuclear layer, along with densely labeled bands of processes in the inner plexiform layer (IPL) (Fig. 2g); (ii) GAD65, which is localized primarily in cells of the GCL that are adjacent to the developing IPL (Fig. 2h) and (iii) ChAT, which is expressed by mirror-symmetric cell populations in the GCL and inner nuclear layer (Fig. 2j). β-gal expression did not, however, overlap with that of the 67-kDa isoform of glutamic acid decarboxylase (GAD67) (Fig. 2i). In the post-natal retina, the GCL is largely GAD67-negative whereas the IPL and inner nuclear layer display strong GAD67 immunoreactivity (present study; de Melo *et al*. 2003). Triple-labeling experiments confirmed the presence of β-gal-positive/ChAT-positive/calretinin-positive (Fig. 2k, inset) as well as β-gal-positive/ChAT-positive/GAD65-positive cells (Fig. 2l, inset). We did not observe any overlap in the expression of β-gal and other markers of amacrine cell phenotypes (Table 1). Taken together, these results strongly suggest that *Runx1* expression during embryonic retinogenesis is correlated with selected amacrine cells that acquire a differentiated phenotype characterized by the post-natal expression of ChAT, GAD65 and calretinin.

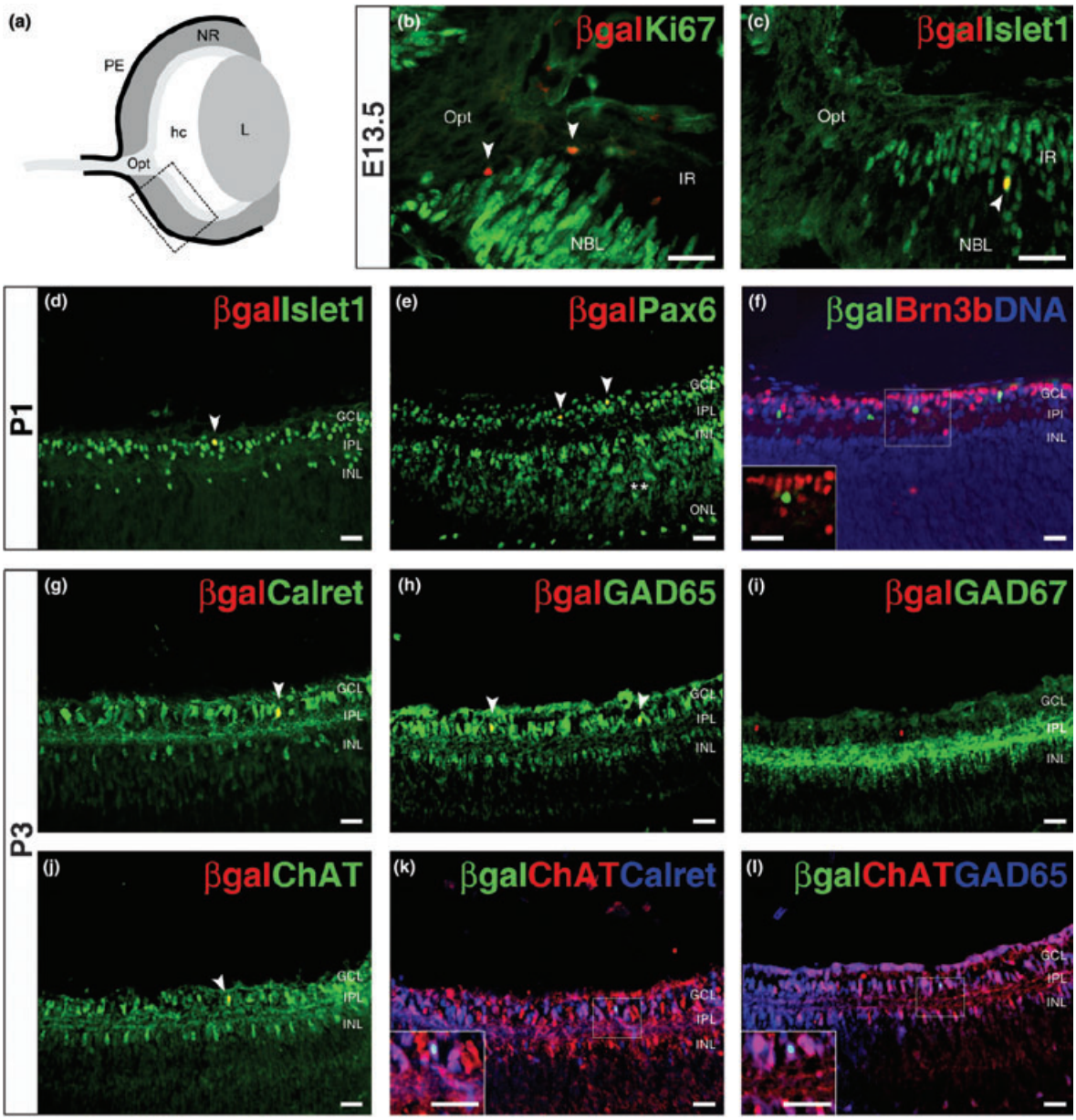

Fig. 2 Characterization of β-galactosidase (β-gal) expression in retinal neuron cell types of *Runx1*$^{lacZ/+}$ embryos or pups. (a) Schematic representation of a developing eye; the boxed area corresponds to the approximate field of view for panels (b–l). (b–f) Double-labeling analysis of the expression of β-gal (red channel in b–e and green channel in f) and specific marker proteins. β-gal expression began at embryonic day (E) 13.5 in the central retina and did not overlap with the mitotic cell marker Ki67 suggesting that *Runx1* is expressed in retinal cells that have exited the cell cycle (b, arrowheads point to cells expressing β-gal but not Ki67). In contrast, at E13.5 β-gal colocalized with Islet1, a marker of differentiating amacrine and ganglion cells (c, arrowhead points to β-gal-positive/Islet1-positive cell). Coexpression of β-gal and Islet1 continued throughout the remaining embryonic period and was also detected post-natally in the ganglion cell layer (GCL) (d, arrowhead), where β-gal was also coexpressed with Pax6 (e, arrowheads; asterisks indicate non-specific non-nuclear staining) but not with Brn3b (f, inset), strongly suggesting that β-gal-positive cells correspond to differentiating amacrine cells. Double- (g–j) or triple- (k and l) labeling analysis of β-gal expression in post-natal day (P) 3 retinas (red channel in g–j and green channel in k and l). β-gal-positive cells coexpressed the amacrine cell subtype-specific markers calretinin (g), 65-kDa isoform of glutamic acid decarboxylase (GAD65) (h) and choline acetyltransferase (ChAT) (j) but not the 67-kDa isoform of glutamic acid decarboxylase (GAD67) (i); arrowheads in (g, h and j) point to double-labeled cells. (k and l) Triple-labeling studies confirmed the presence of β-gal-positive/ChAT-positive/calretinin-positive (k, inset) and β-gal-positive/ChAT-positive/GAD65-positive (l, inset) cell populations. hc, hyaloid cavity; IR, inner retina; INL, inner nuclear layer; IPL, inner plexiform layer; L, lens; NBL, neuroblast layer; NR, neural retina; ONL, outer nuclear layer; Opt; optic nerve; PE, pigmented epithelium. Calibration bars, 50 μm.

Discussion

Runx1 is expressed during a defined developmental period in embryonic and post-natal retina

Runx1 is expressed in restricted regions of the murine nervous system and is involved in the post-mitotic development of specific populations of central and peripheral neurons. The present study extends our analysis of *Runx1* expression in murine hindbrain, sensory ganglia and olfactory structures (Theriault *et al*. 2004, 2005) to the developing retina. *Runx1* expression in the neural retina begins at E13.5 in the inner region of the central retina and increases in a central-to-peripheral gradient until approximately E16.5–E18.5 after which time no further increase in expression levels is observed. *Runx1* expression is significantly down-regulated in the early post-natal (P1–P3) retina where it is localized to cells in the GCL. Taken together, these findings reveal a developmental window of *Runx1* expression that overlaps with the differentiation of most retinal cell types. However, the spatial characteristics of *Runx1* expression suggest a role for this gene in the development of a subset of cells localized to the GCL. In addition, more cells express β-gal in the embryonic retina than post-natally, suggesting that *Runx1* may play a developmentally regulated role in the selection of a particular retinal neuron phenotype.

Runx1 is expressed in a selected subtype of displaced amacrine cells

As an immature IPL begins to form around E18.5, the majority of *Runx1*-expressing cells lay within the GCL. Ganglion cells account for approximately two-thirds of the neurons located in the GCL, the other third being displaced amacrine cells (Vaney and Young 1988; Marc and Jones 2002). In rodents, amacrine cells are born primarily during the latter half of embryonic development and undergo terminal differentiation during the early post-natal period (Cepko *et al*. 1996). At both embryonic and post-natal stages, we failed to detect expression of retinal ganglion cell markers in *Runx1*-expressing cells. In contrast, in P1 retina, *Runx1* is coexpressed with the transcription factors Islet1 and Pax6, which identify amacrine cells. By P3, *Runx1* expression is observed in a subset of ChAT-positive/GAD65-positive and ChAT-positive/calretinin-positive neurons in the GCL, both known phenotypes of displaced amacrine cells.

In the mammalian retina cholinergic amacrine cells have been shown to synthesize, store and release GABA as well as acetylcholine (Brecha *et al*. 1988; Kosaka *et al*. 1988) and most of the displaced GABAergic amacrines represent ON-cholinergic cells, whose dendrites extend and stratify within sublamina b of the IPL (Voigt 1986). Furthermore, calretinin is expressed in cholinergic as well as GABAergic amacrines of the mammalian retina (Goebel and Pourcho 1997; Völgyi *et al*. 1997; Gábriel and Witkovsky 1998). The available antibodies make it unfeasible to perform quadruple-labeling studies to directly demonstrate that β-gal, ChAT, GAD65 and calretinin are coexpressed simultaneously in selected amacrine cells. However, we have found that all β-gal-positive cells in the post-natal retina express ChAT and GAD65, strongly suggesting that *Runx1* is expressed in a population of amacrine cells that utilize the neurotransmitters acetylcholine and GABA along with the calcium-buffering protein calretinin. These observations are important for two main reasons: they provide the first evidence suggesting the existence in the post-natal mouse retina of a displaced amacrine cell subpopulation with such immunological characteristics and they identify *Runx1* as a new marker of this particular amacrine neuron subtype.

It is tempting to speculate that the functional role of ChAT-positive/GAD65-positive/calretinin-positive amacrine cells during adulthood may be similar to that described for starburst amacrine cells, namely modulating the directional selectivity of ganglion cells by releasing both excitatory (acetylcholine) and inhibitory (GABA) neurotransmitters (Famiglietti 1991; Vaney and Young 1988). It must be stressed, however, that in the adult mouse retina both GAD65 and GAD67 are expressed at high levels in the inner nuclear layer, whereas only GAD67 is present in the GCL (de Melo *et al*. 2003). Moreover, displaced cholinergic/GABAergic amacrine cells in the adult retina preferentially utilize GAD67 to synthesize GABA (Brandon and Criswell 1995). This pattern of expression is in contrast to what we observed in the early post-natal retina, namely that β-gal expression overlaps with GAD65 but not GAD67. These observations suggest that the existence of a β-gal-positive/ChAT-positive/GAD65-positive cell population might be transient and that the down-regulation of *Runx1* expression might be correlated with a conversion into a different phenotype (i.e. GAD67-positive) as retinal development progresses.

Summary and perspectives

The present results indicate that *Runx1* expression is established in the developing retina as early as E13.5 in a subpopulation of post-mitotic cells and ceases between P3 and P5. Based on their location and immunohistochemical properties, our data suggest that *Runx1*-expressing cells are a class of displaced cholinergic amacrine cells that also express the calcium-binding protein calretinin and GAD65. Thus, the Runx1 transcription factor may represent a novel marker for a selected population of displaced amacrine cells and may play an important role in the differentiation of this class of retinal interneurons.

Runx1 may be involved in transcriptional mechanisms that control the expression of sets of genes that define the phenotype of the Runx1-positive amacrine cells. In that regard, it is important to note that Runx1 is expressed in

cholinergic visceral motor neurons in the hindbrain (Theriault *et al*. 2004), suggesting a correlation between Runx1 activity and the cholinergic phenotype. Alternatively, Runx1 may be involved in mechanisms promoting the survival of selected amacrine cells during early stages of their differentiation. It is likely that Runx1 functions in developing amacrine cells involve DNA-binding-dependent transcriptional mechanisms. In the future, it will be important to elucidate the biological role of Runx1 during amacrine cell differentiation as well as the molecular mechanisms underlying its activity, including the identity of Runx1 target genes in the retina.

Acknowledgements

We thank Dr Nancy Speck for her generous contribution of the *Runx1*$^{lacZ/+}$ mouse line, F.M. Theriault for advice and Z. Dong and Y. Tang for assistance. This work was supported by grants from the Canadian Institutes of Health Research (MOP-42479 and MGC-14971) to SS; LS is the recipient of a J. Timmins Costello Postdoctoral Fellowship and SS is a Senior Scholar of the Fonds de la Recherche en Sante du Quebec.

References

Amman J. M., Nip J., Strom D. K., Lutterbach B., Harada H. and Lenny N. (2001) ETO, a target of t(8;21) in acute leukemia, makes distinct contacts with multiple histone deactylases and binds mSin3 through its oligomerization domain. *Mol. Cell Biol.* **21**, 6470–6483.

Brandon C. and Criswell M. H. (1995) Displaced starburst amacrine cells of the rabbit retina contain the 67-kDa isoform, but not the 65-kDa isoform, of glutamate decarboxylase. *Vis. Neurosci.* **12**, 1053–1061.

Brecha N., Johnson D., Peichl L. and Wassle H. (1988) Cholinergic amacrine cells of the rabbit retina contain glutamate decarboxylase and gamma-aminobutyrate immunoreactivity. *Proc. Natl Acad. Sci. USA* **85**, 6187–6191.

Cepko C. L., Austin C. P., Yang X., Alexiades M. and Ezzeddine D. (1996) Cell fate determination in the vertebrate retina. *Proc. Natl Acad. Sci. USA* **93**, 589–595.

Coffman J. A. (2003) Runx transcription factors and the developmental balance between cell proliferation and differentiation. *Cell Biol. Int.* **27**, 315–324.

Famiglietti E. V. (1991) Synaptic organization of starburst amacrine cells in rabbit retina: analysis of serial thin sections by electron microscopy and graphic reconstruction. *J. Comp. Neurol.* **309**, 40–70.

Gábriel R. and Witkovsky P. (1998) Cholinergic, but not the rod pathway-related glycinergic (AII) amacrine cells contain calretinin in the rat retina. *Neurosci. Lett.* **247**, 179–182.

Galli-Resta L., Resta G., Tan S.-S. and Reese B. (1997) Mosaics of islet-1 expressing amacrine cells assembled by short-range cellular interactions. *J. Neurosci.* **17**, 7831–7838.

Goebel D. J. and Pourcho R. G. (1997) Calretinin in the cat: colocalization with other calcium-binding proteins, GABA and glycine. *Vis. Neurosci.* **14**, 311–322.

Kosaka T., Tauchi M. and Dahl J. L. (1988) Cholinergic neurons containing GABA-like and/or glutamic acid decarboxylase-like immunoreactivities in various brain regions of the rat. *Exp. Brain Res.* **70**, 605–617.

Liu W., Khare S. L., Liang X., Peteres M. A., Liu X., Cepko C. and Xiang M. (2000) All Brn3 genes can promote retinal ganglion cell differentiation in the chick. *Development* **127**, 3237–3247.

Marc R. E. and Jones B. W. (2002) Molecular phenotyping of retinal ganglion cells. *J. Comp. Neurol.* **22**, 413–427.

Marquardt T. (2003) Transcriptional control of neuronal diversification in the retina. *Prog. Retinal Eye Res.* **22**, 567–577.

Marquardt T. and Gruss P. (2002) Generating neuronal diversity in the retina: one for nearly all. *Trends Neursci.* **25**, 32–38.

de Melo J., Du Qiu X. G., Cristante L. and Eisenstat D. D. (2003) Dlx1, Dlx2, Pax6, Brn3b, and Chx10 homeobox gene expression defines the retinal ganglion and inner nuclear layers of the developing and adult mouse retina. *J. Comp. Neurol.* **461**, 187–204.

North T., Gu T. L., Stacy T., Wang Q., Howard L., Binder M., Marin-Padilla M. and Speck N. A. (1999) Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. *Development* **126**, 2563–2575.

Otto F., Lubbert M. and Stock M. (2003) Upstream and downstream targets of RUNX proteins. *J. Cell. Biochem.* **89**, 9–18.

Roumier C., Fenaux P., Lafage M., Imbert M., Eclache V. and Preudhomme C. (2003) New mechanisms of AML1 gene alteration in hematological malignancies. *Leukemia* **17**, 9–16.

Theriault F. M., Roy P. and Stifani S. (2004) AML1/RUNX1 is important for the development of hindbrain cholinergic branchiovisceral motorneurons and selected cranial sensory neurons. *Proc. Natl Acad. Sci. USA* **101**, 10 343–10 348.

Theriault F. M., Nuthall H. N., Dong Z., Lo R., Barnabe-Heider F., Miller F. D. and Stifani S. (2005) Role for Runx1 in the proliferation and neuronal differentiation of selected progenitor cells in the mammalian nervous system. *J. Neurosci.* **25**, 2050–2061.

Vaney D. I. and Young H. M. (1988) GABA-like immunoreactivity in cholinergic amacrine cells of the rabbit retina. *Brain Res.* **438**, 369–373.

Voigt T. (1986) Cholinergic amacrine cells in the rat retina. *J. Comp. Neurol.* **248**, 19–35.

Völgyi B., Pollák E., Buzás P. and Gábriel R. (1997) Calretinin in neurochemically well-defined cell populations of rabbit retina. *Brain Res.* **763**, 79–86.

Walther C. and Gruss P. (1991) Pax-6, a murine paired-box gene, is expressed in the developing CNS. *Development* **113**, 1435–1449.

Wheeler J. C., Shigesada K., Gergen J. P. and Ito Y. (2000) Mechanisms of transcriptional regulation by Runt domain proteins. *Sem. Cell Dev. Biol.* **11**, 369–375.