A Novel and Multivalent Role of Pax6 in Cerebellar Development

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Pax6 is a prominent gene in brain development. The deletion of *Pax6* results in devastated development of eye, olfactory bulb, and cortex. However, it has been reported that the *Pax6*-null *Sey* cerebellum only has minor defects involving granule cells despite *Pax6* being expressed throughout cerebellar development. The present work has uncovered a requirement of *Pax6* in the development of all rhombic lip (RL) lineages. A significant downregulation of *Tbr1* and *Tbr2* expression is found in the *Sey* cerebellum, these are cell-specific markers of cerebellar nuclear (CN) neurons and unipolar brush cells (UBCs), respectively. The examination of *Tbr1* and *Lmx1a* immunolabeling and Nissl staining confirmed the loss of CN neurons from the *Sey* cerebellum. CN neuron progenitors are produced in the mutant but there is an enhanced death of these neurons as shown by increased presence of caspase-3-positive cells. These data indicate that *Pax6* regulates the survival of CN neuron progenitors. Furthermore, the analysis of experimental mouse chimeras suggests a cell-extrinsic role of *Pax6* in CN neuron survival. For UBCs, using *Tbr2* immunolabeling, these cells are significantly reduced in the *Sey* cerebellum. The loss of UBCs in the mutant is due partly to cell death in the RL and also to the reduced production of progenitors from the RL. These results demonstrate a critical role for *Pax6* in regulating the generation and survival of UBCs. This and previous work from our laboratory demonstrate a seminal role of *Pax6* in the development of all cerebellar glutamatergic neurons.

Key words: *Atoh1*; *Pax6*; rhombic lips; *Tbr1* and cerebellar nuclear neurons; *Tbr2* and unipolar brush cells

Significance Statement

Pax6 is a key molecule in development. *Pax6* is best known as the master control gene in eye development with mutations causing aniridia in humans. *Pax6* also plays important developmental roles in the cortex and olfactory bulb. During cerebellar development, *Pax6* is robustly expressed in the germinal zone of all glutamatergic neurons [cerebellar nuclear (CN) neurons, granule cells, and unipolar brush cells (UBCs)]. Past work has not found abnormalities in the CN and UBC populations. Our study reveals that the *Pax6*-null mutation dramatically affects these cells and identifies *Pax6* as a key regulator of cell survival in CN neurons and of cell production in UBCs. The present study shows how *Pax6* is key to the development of glutamatergic cells in the cerebellum.

Introduction

The paired box transcription factor *Pax6* has been shown to have a fundamental role in the development of several CNS structures in which it is expressed during development. The absence of *Pax6*

results in the loss of progenitor and cell-type mis-specifications (Ericson et al., 1997; Toresson et al., 2000; Quinn et al., 2007). One brain region that has early and compartmental expression of *Pax6*, but has escaped the detection of a seminal role in development, is the cerebellum. However, in our recent work with *Pax6*, we have identified a transition of molecular identity in the *Pax6*-null cerebellum (Yeung et al., 2014), which prompted a re-examination of *Pax6* and early development of the cerebellum.

In the cerebellum, *Pax6* is expressed early on in the cells of the rhombic lip (RL) that give rise to neurons of the glutamatergic lineages, including the cerebellar nuclear (CN) neurons, granule cells (GCs), and unipolar brush cells (UBCs). Compared with the crucial control exerted by *Pax6* in other CNS regions, *Pax6* only seems to play a modulatory later-developmental role in cerebellar development. For example, the loss of *Pax6* has been reported to result in the aberrant organization of the external GC layer and foliation in the cerebellum (Engelkamp et al., 1999; Swanson et

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al., 2005; Swanson and Goldowitz, 2011). The influence of Pax6 on the CN neurons and UBCs, however, has not been reported.

We previously assessed the whole-genome transcriptomic profile of the cerebellum in the absence of Pax6 during embryonic development (Ha et al., 2012). Comparison of the transcriptome profiles from Pax6-null (*Sey*) mutant and wild-type cerebella revealed a significant decrease in the expression of *Tbr1* and *Tbr2* (*Eomes*) in the *Sey* cerebellum (Ha et al., 2012, 2015), transcription factors important for the development of CN neurons and UBCs, respectively (Englund et al., 2006; Fink et al., 2006). These data also suggested that a closer examination of cells in the glutamatergic lineage should be explored in the Pax6-null mutant cerebellum.

Here we report two dramatic phenotypes in the Pax6 knock-out mouse cerebellum: loss of glutamatergic CN neurons and UBCs. The loss of these cells seems largely attributable to enhanced cell death in RL-derived CN progenitors and enhanced cell death and decreased neurogenesis in the UBCs. Our data reveal a previously unreported requirement for Pax6 in the survival and generation of glutamatergic CN neurons and UBCs in the developing cerebellum. These findings support a revised view of the molecular program that underpins cerebellar development.

Materials and Methods

Mouse strains and husbandry. The Pax6 mutant strain Pax6^{Sey} (originally obtained from Robert Grainger and Marilyn Fisher, University of Virginia) was bred as heterozygous pairs, phenotyped for eye sizes and presence of cataracts, and genotyped as previously described (Swanson et al., 2005). Experimental Pax6^{Sey/+} embryos were generated by intercrossing Pax6^{Sey/+} mice.

The *Atoh1-lacZ* reporter strain *Atoh1*^{β-Gal} (obtained from Huda Zoghbi, Baylor College of Medicine) was genotyped by PCR according to the protocol previously described (Jensen et al., 2002). Experimental, double-heterozygous Pax6^{Sey/+}; *Atoh1*^{β-Gal/+} embryos were generated from the matings between heterozygous Pax6^{Sey/+} and *Atoh1*^{β-Gal/+} mice.

The morning that a vaginal plug was detected was designated as embryonic day (E) 0.5. All studies were conducted according to the protocols approved by Institutional Animal Care and Use Committee and Canadian Council on Animal Care at the University of Tennessee Health Science Center and the University of British Columbia.

Experimental mouse chimeras. Experimental mouse chimeras were generated as previously described (Goldowitz and Mullen, 1982; Goldowitz, 1989). The mutant component of the chimera was generated by a mating of heterozygous *Sey* mutants (Pax6^{Sey/+}), which yielded Pax6^{+/+}, Pax6^{Sey/+}, and Pax6^{Sey/Sey} embryos. To mark the wild-type cells of experimental chimeras, we used FVB-GFP mice [FVB.Cg-Tg(CAG-EGFP)B5Nagy/J; Jackson Laboratory; stock number: 003516]. Four-to-eight-cell embryos from Pax6^{Sey/+} matings (Pax6^{+/+}, Pax6^{Sey/+}, or Pax6^{Sey/Sey}) were cultured together with embryos from wild-type (GFP) matings overnight. After successful fusion, blastocysts were transplanted into the uterine horn of pseudopregnant host ICR females. Chimera embryos were collected on E18.5, and tail biopsies were taken for genotyping of the Pax6^{Sey} component using a mutagenically separated PCR technique as previously described (Swanson et al., 2005). Tissue was processed and sectioned as described below.

Percentage chimerism was estimated from expression of GFP fluorescence (wild-type cells) in various brain regions outside the cerebellum. For each chimeric brain, GFP expression from 13 to 16 coronal sections were analyzed and averaged.

CN neuron phenotype was assessed by counting Tbr1⁺ cells from 13 to 16 coronal sections across the full cerebellum, right and left sides inclusive. We determined the number of Tbr1⁺ CN neurons from the cerebellum of two wild-type Pax6^{+/+} <-> +/+ chimera, three heterozygous Pax6^{Sey/+} <-> +/+ chimera, three mutant Pax6^{Sey/Sey} <-> +/+ chimeras, and four mutant Pax6^{Sey/Sey} embryos. The total number of Tbr1⁺ CN neurons in each cerebellum was calculated, and averages were taken

for all groups of embryos. For the mutant chimeric cerebellum, the expected number of Tbr1⁺ cells was predicted based on the percentage chimerism (of the wild-type and mutant genotypes) multiplied by the average cell counts from wild-type and mutant cerebellum (see text). Statistical significance between the expected and observed Tbr1⁺ cells in the mutant chimeric cerebellum was determined by χ^2 test.

Tissue preparation and histology. All embryos were collected at every age from E11.5 to E18.5. Embryos harvested between E10.5 and E15.5 were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 1 h at 4°C. Embryos harvested at E16.5 or later were perfused with 4% paraformaldehyde in 0.1 M PB. The brain tissues were isolated and further fixed in 4% paraformaldehyde in 0.1 M PB for 1 h at room temperature. Fixed tissues were rinsed with PBS, followed by cryoprotection with 30% sucrose/PBS overnight at 4°C before embedding in optimal cutting temperature compound. Tissues were sectioned at 12 μ m for immunohistochemistry and cryosections were mounted on Superfrost slides (Thermo Fisher Scientific), air dried at room temperature, and stored at −80°C until used. In all cases, observations were based on ≥ 3 embryos per genotype per experiment.

Immunohistochemistry. Tissue sections were rehydrated to PBS. For bright-field immunohistochemistry, endogenous peroxidase activity was inhibited by treating the sections with 1% H₂O₂ in PBS followed by PBS-T (0.1 M PBS/0.1% Triton X-100) rinse. Sections were incubated at room temperature for 20 min with blocking solution (1% BSA and 5% normal serum in PBS-T) and subsequently incubated at room temperature overnight with primary antibodies in a humid chamber. Following PBS-T washes, the sections were incubated with biotinylated secondary antibodies (at 1:200; Vector Laboratories) and processed for PAP immunohistochemistry using the ABC Kit (Vector Laboratories) according to the manufacturer's protocol. Slides were dehydrated and coverslips were applied. For immunofluorescence, secondary antibodies labeled with fluorochrome were used to recognize the primary antibodies. The slides were coverslipped with FluorSave (Calbiochem, 345789). Primary antibodies used were as follows: chicken anti- β -gal (1:10,000; Abcam, Ab9361, RRID:AB_307210), rat anti-BrdU (1:300; Abcam, Ab6326, RRID:AB_305426), rabbit anti-active caspase-3 (1:500; Abcam, Ab13847, RRID:AB_443014), rabbit anti-LMX-1 (1:2000; Millipore, AB10533, RRID:AB_10805970), guinea pig anti-INSM1 (1:1000; a gift from Shiqi Jia, Max-Delbrück-Center for Molecular Medicine); rabbit anti-IRX3 (1:8000; a gift from Tom Jessell, Columbia University), rabbit anti-PAX6 (1:200; Covance, PRB-278P, RRID:AB_291612), rabbit anti-TBR1 (1:800; Abcam, Ab31940, RRID:AB_2200219), rabbit anti-TBR2 (1:800; Millipore, AB2283, RRID:AB_10806889).

Cell counts and BrdU analysis. We estimated the numbers of CN neurons and UBCs by counting cells positive for the appropriate cell marker (Tbr1 for CN neurons, Tbr2 for UBCs, and Lmx1a for both). Every 10th section across the whole E11.5–E16.5 cerebellum and the half E17.5 and E18.5 cerebellum were counted. The assessment of cell death in CN neuron progenitors was determined by counting anti-caspase-3-positive cells in the nuclear transitory zone (NTZ), subpial stream, and external germinal layer (EGL) of E12.5–E18.5 cerebella. The assessment of cell death in UBCs was determined by counting anti-caspase-3-positive cells in the RL (and immediately adjacent area) of E15.5–E18.5 cerebella.

To examine cell proliferation at the RL during UBC neurogenesis, timed pregnant females were injected intraperitoneally with BrdU (Sigma-Aldrich, B5002; 50 μ g/g of body weight) 1 h before the collection of embryos. To quantify the number of proliferating cells in the cerebellar RL, BrdU⁺ cells were counted in every 10th section across the whole E16.5 cerebellar RL.

To test the possibility of cell-fate change, cells produced during the period of CN neuron genesis (E10.5 and E11.5) were labeled with BrdU, and BrdU-labeled cells were analyzed with immunohistochemistry at E15.5. Timed pregnant females were injected intraperitoneally with BrdU (50 μ g/g of body weight) on E10.5 and E11.5, then embryos were collected on E15.5.

In all cases, observations were based on ≥ 3 cerebella per genotype. Statistical significance between wild-type and mutant was determined by two-tailed *t* test.

Figure 1. Transcription factors Pax6, Tbr1, and Lmx1a are expressed in the progenitors of CN neurons during cerebellar development. **A**, The whole-cerebellum transcription profile of *Pax6* and *Tbr1* during cerebellar development based upon CbGRITS data. *y*-axis $\log_2$ transformed and followed by a $2Z+8$ Z-score stabilized intensity value for microarray dataset. **B**, The cerebellum transcription profile of *Lmx1a* during cerebellar development based upon RIKEN FANTOM5 data. The *y*-axis shows expression level in tags per million (TPM). **C**, In the E13.5 cerebellum, immunohistochemistry reveals that Pax6 is expressed in the cells of the subplial stream, which houses newly generated CN neuron progenitors. **D**, **E**, Once the progenitors enter the NTZ, Pax6 is rapidly downregulated and now these CN neuron progenitors express Tbr1 (**D**) and Lmx1a (**E**). SS, Subplial stream. Error bars represent SE. Scale bars, 100 μ m.

Microscopy. Analysis and photomicroscopy was performed with a Zeiss Axiovert 200M microscope with the AxioCam/Axiovision hardware–software components (Carl Zeiss). Confocal microscopy was performed using an Olympus FV500 confocal laser scanning microscope and the Fluoview image capture and analysis software.

Results

Characterization of the absence of Tbr1⁺ CN neurons in the *Sey* cerebellum

Glutamatergic CN neurons are generated from the RL between E10.5 and E12.5 (Machold and Fishell, 2005; Fink et al., 2006). Pax6, Tbr1, and Lmx1a are expressed in glutamatergic CN neuron progenitors during development. Our time series cerebellar gene expression microarray dataset (available at cbgrits.org) reveals that during cerebellar development, transcription of *Pax6* is expressed throughout the course of embryonic and neonatal stages (Fig. 1A), while the expression of *Tbr1* peaks at E14 (Fig. 1A). Transcription levels of *Lmx1a* also peak at E13 and E14 [Fig. 1B; RIKEN Center for Life Science Technologies Arner et al., 2015]. Our immunohistochemical analysis shows that Pax6 is expressed in the newly generated CN neuron progenitors in the RL and expression persists as these cells migrate tangentially along the subplial stream (Fig. 1C). Between E13 and E15, CN neuron progenitors reach the NTZ, then downregulate Pax6 expression, and in turn initiate Tbr1 and Lmx1a expression (Fig. 1D,E).

Our *Pax6* transcriptome analysis reveals a significant reduction in *Tbr1* transcript in the *Sey* cerebellum during development (Ha et al., 2012, 2015). At E13.5, there is a 7.89-fold reduction in *Tbr1* transcript in the *Sey* cerebellum (Fig. 2A). Tbr1-immunolabeling in the E13.5 wild-type cerebellum detected expression in the CN neurons that entered the NTZ (Fig. 2B, left). By contrast, this Tbr1⁺ population is absent from the NTZ of the *Sey* cerebellum (Fig. 2B, right). Quantitative analysis of Tbr1⁺ cells in the NTZ indicated an elimination of Tbr1⁺ cells from the

E13.5 mutant cerebellum. At E15.5, when Tbr1⁺ cells are localized to the NTZ in wild-type cerebellum, 97.4% Tbr1⁺ cells are missing in the *Sey* cerebellum (Fig. 2C, red arrows; $p = 3.7 \times 10^{-5}$). By E18.5 in the wild-type cerebellum, the CN neurons have descended into the cerebellar core and Tbr1 expression is localized to the CN neurons at the medial level (i.e., fastigial nucleus; Fig. 2D). In contrast, Tbr1⁺ cells are not detected in the E18.5 *Sey* cerebellum (Fig. 2D). At all ages examined, there is no significant difference in the number and location of Tbr1⁺ cells in the cerebellum with one copy of Pax6 (i.e., *Pax6*^{+/Sey}) compared with the wild-type cerebellum (Fig. 2E).

GABAergic CN neurons that arise from ventricular zone (VZ) around the same time migrate radially to enter the NTZ and express *Irx3* (Morales and Hatten, 2006). To determine whether GABAergic CN neurons are also affected by the absence of Pax6, we examined *Irx3*⁺ cells in the wild-type and *Sey*-mutant cerebellum. In the E11.5 wild-type cerebellum, *Irx3*⁺ cells are seen at their origins in the VZ and migrating through the cerebellar core. By E13.5, the *Irx3*⁺ CN neurons reach the NTZ. By E15.5 the *Irx3*⁺ cells descend into the cerebellar core and are located more laterally than the glutamatergic Tbr1⁺ CN neurons. At these time points, the expression of *Irx3* is indistinguishable between the wild-type and *Sey*-mutant cerebellum (Fig. 2F). This finding indicates that the production and development of GABAergic CN neurons is not affected by the absence of Pax6.

Reduction of Lmx1a⁺ cells in the NTZ of the *Sey* cerebellum

We examined Lmx1a expression in *Sey* cerebellum to test whether the effect of the loss of Pax6 is a general elimination of glutamatergic CN neuron marker expression or is specific to elimination of Tbr1 expression in the CN neurons. Lmx1a⁺ cells in the *Sey* mutant is completely eliminated from the NTZ region at E13.5 (Fig. 3A) and reduced significantly at E15.5 ($p = 4.68 \times 10^{-4}$; Fig. 3B); similar to the aforementioned loss of Tbr1 expression in the absence of Pax6.

Figure 3. The lack of Pax6 in the *Sey* cerebellum results in the reduction of *Lmx1a*⁺ cells in the cerebellar nuclei. **A, B**, *Lmx1a* expression is observed in CN neuron progenitors that enter the NTZ of (**A**) E13.5 and (**B**) E15.5 wild-type cerebellum (white arrows). In the E13.5 and E15.5 *Sey* cerebellum, *Lmx1a*⁺ cells are largely absent (red arrows). The lateral aspects of the cerebellum highlight the c3 cells (**B**, bottom, white arrowheads) which are generated from the VZ and give rise to extracerebellar neurons. These cells also express *Lmx1a* and are not altered in the *Sey* mutant. Scale bars, 100 μ m.

←

Figure 2. The lack of Pax6 in the *Sey*-mutant cerebellum results in the absence of RL-derived *Tbr1*⁺ CN neurons but no apparent loss of VZ-derived *Ir3*⁺ CN neurons. **A**, The transcription profile from CbGrITS of *Tbr1* in the wild-type and *Sey* cerebellum at E13, E15, and E18. The *Sey* cerebellum has a significant reduction in *Tbr1* transcript at E13 and E15. y-axis log₂ transformed and followed by a 2Z + 8 Z-score stabilized intensity value for microarray dataset. **B–D**, Immunohistochemistry reveals that *Tbr1*⁺ cells are absent in the *Sey* cerebellum. Cells immunopositive for *Tbr1*⁺ are observed in the NTZ of the wild-type cerebellum (white arrows) at E13.5 (**B**), E15.5 (**C**), and E18.5 (**D**), but this *Tbr1*⁺ cell population is absent in the *Sey* cerebellum (red arrows). A few *Tbr1*⁺ cells are observed in the lateral cerebellum of the E15.5 *Sey* mutant (**C**, blue arrowhead). These cells comprise only 2.7% ($\pm$ 0.4%) of the total number of *Tbr1*⁺ cells in the wild-type cerebellum. **E**, Immunohistochemistry of *Tbr1* illustrates that the cerebellar expression of *Tbr1* in heterozygous (*Pax6*^{Sey/+}) at E13.5, E15.5, and E18.5 is not different from that of the wild-type littermates (**B–E**, compare white arrows). **F**, Immunohistochemistry of *Ir3* over developmental time reveals that these CN neuron populations are similar in the cerebellum of the wild-type (top) and *Sey* (bottom) mouse at E11.5, E13.5, and E15.5. At E11.5, the *Ir3*⁺ CN neurons are largely seen outside of the VZ. By E13.5, *Ir3*⁺ CN neurons have entered the NTZ. By E15.5, the *Ir3*⁺ CN neurons have started to colonize the cerebellar core. Error bars represent SE. Scale bars, 100 μ m.

In the E15.5 wild-type cerebellum, *Lmx1a* is also expressed in a set of cells dorsal to the NTZ, termed the c3 cells (Chizhikov et al., 2006). These c3 cells give rise to extracerebellar neurons and do not originate from the cerebellar RL (Millen et al., 2014). Expression of *Lmx1a* is unaffected in these cells in the *Sey* mutant (Fig. 3B, arrowheads), suggesting that *Lmx1a* expression is independent of Pax6. Thus, the effect of the *Pax6* null on *Lmx1a* expression aligns with a specific loss of CN neurons.

Cytoarchitecture of the *Sey* cerebellum indicates the loss of CN neurons

The altered expression of *Tbr1* and *Lmx1a* in the *Sey* cerebellum could suggest that either the glutamatergic CN neurons are present but fail to express the proper markers in the absence of Pax6 expression, or that CN neurons are deleted in the absence of Pax6 expression. To address this issue, we analyzed the cytoarchitecture of the wild-type and *Sey* cerebellum. In the wild-type cerebellum, nuclear neurons aggregate at the NTZ by E15.5 and can be readily visualized with cresyl violet staining (Fig. 4A, black arrow). In the *Sey* cerebellum, however, this nuclear mass is replaced by a fibrous and acellular matrix (Fig. 4B, red arrow). At E18.5, in the wild-type cerebellum, the CN cells from the NTZ occupy the deep portion of the cerebellum (Fig. 4C, black arrow). In the *Sey* cerebellum, however, there is no recognizable cellular aggregate in this region, suggesting the absence of CN neurons (Fig. 4D, red arrow).

Increased cell death in the CN neuron progenitors of *Sey* cerebellum

The absence of CN neurons in the *Sey* cerebellum could be due to the lack of generation of CN neuron progenitors from the RL. To address this possibility, we examined the earliest marker of CN neuron progenitors (i.e., *Atoh1*) in the *Sey* cerebellum to determine whether the CN neurons are initially generated in the mutant cerebellum. To do this, we crossed the *Atoh1*-reporter allele (*Atoh1*^{LacZ}) into the *Sey* mutant.

In the E11.5 wild-type cerebellum, CN neuron progenitors that arise from the RL and migrate along the subplial stream express *Atoh1* as indicated by β -gal-immunopositivity (Fig. 5A, arrows). Surprisingly, *Atoh1*⁺ cells are observed at the RL and subplial stream of the E11.5 *Sey* cerebellum (Fig. 5B, arrows). Quantitatively, the number of *Atoh1*⁺ cells in the *Sey* cerebellum is not significantly different from the wild type ($p = 0.31$). This finding indicates that CN neuron progenitors are produced from the RL even in the absence of Pax6.

As the production of CN neuron progenitors is unaffected in the *Sey* cerebellum, the loss of CN neurons found later in development could be a result of a change in cell fate or of enhanced cell death in the CN neuron progenitors. To determine whether

CN neuron progenitors have changed cell fate, we performed BrdU incorporation to mark the cells generated between E10.5 and E11.5, and analyzed the fate of these cells at E15.5. In the E15.5 wild-type cerebellum, the majority of the BrdU⁺ cells are found in the NTZ, with a few BrdU⁺ cells scattered above the VZ, and the EGL is devoid of BrdU⁺ cells. In contrast, the *Sey* cerebellum lacks BrdU⁺ cells in the NTZ and no ectopic BrdU⁺ cells are detected. BrdU-labeled cells are observed in the rest of the mutant cerebellum as in the wild-type cerebellum. These findings suggest that CN neuron progenitors have not undergone cell-fate change, but have disappeared from the *Sey* cerebellum.

The alternative explanation of cell death in the CN neuron progenitors can be explored using activated caspase-3 immunopositivity as a marker for apoptosis in the developing cerebellum. In the E12.5 cerebellum of normal and mutant embryos, the numbers of cells that are caspase-3⁺ are not significantly different. However, starting from E13.5, and more obvious by E15.5, the *Sey* cerebellum exhibits significantly increased numbers of caspase-3⁺ cells. The majority of caspase-3⁺ cells in the E13.5–E18.5 *Sey* cerebellum is localized to the anterior EGL, and few cells are found near the presumptive NTZ. At E15.5, the caspase-3⁺ cells are restricted to the anterior EGL (Fig. 5D, arrowheads). Caspase-3⁺ cells are rarely observed in the wild-type cerebellum at any of the examined ages (Fig. 5C; data not shown). Quantitatively, the number of caspase-3⁺ cells in the E13.5–E18.5 *Sey* cerebellum is 4–28-fold higher than in the wild-type cerebellum, and the differences between the *Sey* mutant and the wild type are significant (Fig. 5E). As caspase-3⁺ cells are found in the EGL, we sought to determine whether these dying cells are GCs or non-GCs (i.e., likely CN neuron progenitors) using a GC marker, *Insm1* (Duggan et al., 2008). At E13.5 and E15.5 in both wild-type and *Sey* cerebella, *Insm1* is robustly expressed in the cells of the EGL, while CN neurons in the NTZ are immunonegative for *Insm1* (Fig. 5F, G; data not shown). Double-labeling of *Insm1* and caspase-3 reveals that caspase-3 immunopositivity is present in both *Insm1*⁺ and *Insm1*[−] cells (Fig. 5H). However, in the E13.5 and E15.5 *Sey* cerebellum, 75.9 and 76.3%, respectively, of all caspase-3⁺ cells are negative for *Insm1* expression, indicating that the majority of the dying cells are likely not GCs (Fig. 5I).

Chimera analysis revealed that Pax6 is an extrinsic factor that controls the survival of CN neurons

Our data indicate a requirement of Pax6 in the survival of CN neuron progenitors. Pax6 may act as a cell-intrinsic or a cell-extrinsic factor for the survival of CN neurons. To address this issue, we examined experimental chimeras from wild-type and *Pax6*-null embryos (i.e., *Sey/Sey* ↔ *+/+*). The chimeric experiment affords the opportunity to determine how a wild-type environment impacts the survival of mutant cells and vice versa. If Pax6 acts as a cell-intrinsic factor for cell survival, we

Figure 4. Cytoarchitecture of the *Sey* cerebellum indicates the loss of CN neurons and UBCs. **A**, Cresyl violet staining reveals the aggregation of CN neurons at the NTZ in the E15.5 wild-type cerebellum (black arrow). **B**, In contrast, this nuclear mass is replaced by a fibrous and acellular matrix in the *Sey* cerebellum (red arrow). **C, D**, In the E18.5 cerebellum (**C**), the CN neurons have descended from the NTZ in the wild-type cerebellum (black arrow), while this nuclear mass is absent in the *Sey* cerebellum (**D**, red arrow). At the medial level, UBCs are found in the RL region of wild-type cerebellum (**C**, bounded by red dotted line), but the same region is devoid of cells in the *Sey* cerebellum (**D**, bounded by red dotted line). **E, F**, UBC progenitors (bounded by red dotted lines) are found in wild-type (**E**) and *Sey* (**F**) lateral cerebella. Scale bars, 100 μ m.

hypothesize that *Pax6*-null mutant cells do not give rise to CN neurons, and all CN neurons are wild type in the chimeric cerebellum. On the other hand, if Pax6 is an extrinsic factor for cell survival, *Pax6*-null cells can give rise to CN neurons.

The 25 experimental chimeras used in this analysis were generated from one embryo that came from a mating of heterozygous *Pax6*-null (*Sey/+*) mice and another embryo from a mating of wild-type mice (*+^{GFP}/+^{GFP}*). This combination should lead to approximately one-fourth of the chimeras being of *Sey/Sey* genotype. We examined the chimeric embryos at E18.5, and three embryos had a cellular contribution from *Pax6*-null mutant embryos, and each of these embryos exhibited craniofacial defects characteristic of the *Sey* mutant. The quantitative analysis of percentage chimerism (see Materials and Methods) estimated that these embryos comprised 0.6, 9.9, and 23% wild-type cells. To determine the phenotype of CN/Tbr1⁺ cell loss in these chimeras, we examined the wild-type, *Sey*, and chimeric cerebellum. In the wild-type cerebellum, counts of Tbr1⁺ cells in the CN region averaged 817 per whole cerebellum. This was compared with only 43 Tbr1⁺ cells in the CN region of the *Pax6*-null cerebellum. However, in the three chimeras, we counted 89, 167, and 178 Tbr1⁺ cells in the CN (Fig. 6A). These numbers of Tbr1⁺ cells in the CN trended with the percentage of chimerism.

In the cerebellar nuclear region of all three mutant chimeras, we found that Tbr1⁺ cells were both GFP-positive and GFP-negative (Fig. 6B–D). These data indicate that the Tbr1⁺ cells of the CN are of both wild-type and *Pax6*-null lineage. The cell-

Figure 5. CN neuron progenitors are generated in the *Sey*-mutant cerebellum but exhibit enhanced cell death. **A**, In the E11.5 wild-type (*Pax6*^{+/+}; *Atoh1*^{+/LacZ}) cerebellum, CN neuron progenitors express Atoh1 when the cells emerge from the RL and during migration along the subplial stream (arrows). **B**, A similar pattern of Atoh1-expressing cells is observed in the *Sey*-mutant (*Pax6*^{Sey/Sey}; *Atoh1*^{+/LacZ}) cerebellum (arrows). **C–E**, Cell death is assessed by immunolabeling for activated caspase-3 and quantified at E12.5–E18.5 in wild-type and *Sey* cerebella. **C, D**, The majority of cells undergoing cell death in the wild-type and *Sey* cerebella are observed at the anterior of the EGL (arrowheads). **D**, There is a dramatic increase in caspase 3⁺ cells in the mutant EGL. **E**, The *Sey* cerebellum shows significantly higher numbers of anti-caspase-3⁺ cells than that in the wild-type at E13.5 and later (**p* < 0.005). **F–H**, Double-labeling with *Insm1*, a GC-specific marker, and caspase-3 reveals that most of the dying cells in the *Sey* cerebellum are non-GCs. GCs robustly expressed *Insm1* in the wild-type (**F**) and *Sey* (**G**) EGL. Highlighted area in **G** is shown in **H** at higher magnification. **H**, In the *Sey* EGL, most caspase-3⁺ cells are *Insm1*-negative (blue arrows) and few cells coexpress *Insm1* and caspase-3 (yellow arrows). **I**, Proportion of caspase-3⁺ cells that are *Insm1*-positive (pink) or *Insm1*-negative (blue) in the E13.5 and E15.5 wild-type and *Sey* EGL. The *Sey* cerebellum exhibits a significantly increased number of total anti-caspase-3⁺ cells than the wild type at both E13.5 and E15.5 (**p* < 0.005). SS, Subplial stream. Error bars represent SE. Scale bars, 100 μ m.

intrinsic hypothesis predicts that cell genotype linearly correlates with cell phenotype. This is testable by calculating the expected number of Tbr1⁺ cells based on the percentage chimerism, and comparing this to the observed number of Tbr1⁺ CN cells (e.g., in a 50/50 chimera we would expect $(0.5 \times 817) + (0.5 \times 43) = 430$ Tbr1⁺ cells). A χ^2 test reveals that the actual numbers of Tbr1⁺ cell observed in these chimeric cerebella are significantly different from the expected numbers of Tbr1⁺ cells estimated (*P* value = 1.18×10^{-14} ; Fig. 6A). Thus, we rejected the null hypothesis and accept the alternative hypothesis that Pax6 acts as an extrinsic factor on the survival of CN neurons.

Characterization of the reduction of Tbr2⁺ UBCs in the Pax6-null cerebellum

The UBCs are RL derivatives generated at late embryonic times starting from E15, and the progenitors can be identified by Tbr2 expression (Fink et al., 2006). In the E15.5 wild-type cerebellum, the newly generated UBC precursors appear as a stream of Pax6⁺/Lmx1a⁺/Tbr2⁺ cells in the RL (Fig. 7A–C), and continue

to express these molecules as the UBCs migrate into the cerebellar core at E18.5 (Figs. 7E, 8B, white arrows).

Our Pax6 transcriptome data reveals a downregulation of *Tbr2* expression in the *Sey* cerebellum throughout embryonic development, with a threefold reduction at E18.5 (Fig. 7D; Ha et al., 2012). Immunolabeling for Tbr2 in the E18.5 *Sey* cerebellum reveals that Tbr2⁺ cells are absent from the medial cerebellar core (Fig. 7G), but some weak Tbr2-immunopositive cells are observed in the region of the RL at more lateral levels (Fig. 7H, white arrows). Quantitative analysis indicated an 86.9% loss of Tbr2⁺ cells from the E18.5 *Sey* cerebellum compared with the wild-type cerebellum (*p* = 7.71×10^{-5}).

Reduction of Lmx1a⁺ cells in the Sey cerebellum

We determined the expression of Lmx1a in the wild-type and Pax6-null cerebellum at E15.5–E18.5. In the E15.5 wild-type cerebellum, there are cells with strong Lmx1a staining in the area between the EGL and the cerebellar core (Fig. 8A, left, arrowheads). Such staining is absent in the *Sey* cerebellum (Fig. 8A,

right). However, there are *Lmx1a*-positive cells, such as the late-born GC progenitors, that have similar staining profiles in the wild-type and *Sey* RL. Likewise, the denser *Lmx1a* staining of cells in the choroid plexus is similar between the wild-type and *Sey* brain (Fig. 8A). The *Lmx1a* positivity in the *Sey*-mutant cerebellum supports the notion that *Lmx1a* expression is independent of Pax6.

At E18.5, *Lmx1a* in the wild-type cerebellum is expressed by the UBCs in the RL and those migrating into the cerebellar core (Fig. 8B, arrowheads). In the *Sey* cerebellum, similar to the effect on the aforementioned *Tbr2*⁺ expression, *Lmx1a*⁺ cells are absent at the medial level, but can be observed at more lateral levels (Fig. 8B, bottom right, arrowheads). For the whole cerebellum at E18.5, there is a 63.7% diminution of *Lmx1a*⁺ cells in the *Sey* cerebellum compared with the wild type ($p = 4.56 \times 10^{-3}$). These observations indicate a specific loss of *Lmx1a* in the UBC population of the *Sey* cerebellum.

Cytoarchitecture of the *Sey* cerebellum indicates the loss of UBCs

To determine whether the loss of *Tbr2* and *Lmx1* expression is a deficiency in gene expression or the loss of UBCs in the *Sey*, we examined the cytoarchitecture of the RL region with cresyl violet staining. Medially, there is a dense population of cells in the wild-type RL region where UBCs are normally found, and this same region is devoid of cells in the *Sey* cerebellum (Fig. 4C,D, regions bounded by red dotted lines). As found with anti-*Tbr2* and anti-*Lmx1a* immunostaining, cresyl violet staining revealed the presence of cells in the lateral RL regions of the *Sey* cerebellum (Fig. 4E,F, regions bounded by red dotted lines). These results confirm the loss of UBCs in the medial *Sey* RL.

Increased cell death and decreased cell production in the UBC germinal zone of the *Sey* cerebellum

The reduction of UBCs seen in the *Sey* cerebellum could be the result of an enhanced cell death and/or a reduced generation of UBCs. We compared the number of caspase-3⁺ cells between wild-type and *Sey* cerebella during the genesis of UBCs at E15.5–E18.5 (Fig. 9A). A moderate, and significant, increase in the number of caspase-3⁺ cells was found in the mutant RL at E16.5 (Fig. 9B).

To address whether a decrease in UBC production is contributing to the *Sey*-mutant UBC phenotype, we analyzed cell production at the RL during the period of UBC neurogenesis by acute BrdU labeling. Interestingly, we find that the area of the *Sey* RL is 56% smaller than the wild-type RL at E16.5 ($p = 1.44 \times 10^{-3}$; Fig. 9C,D). Furthermore, the total number of BrdU⁺ cells in the RL is reduced by 51% in the *Sey* mutant ($p = 1.39 \times 10^{-4}$; Fig. 9C,E). The reduction of BrdU⁺ cells in the mutant RL is not a result of changes in cell proliferation as the labeling indices are

Figure 6. Experimental chimera analysis demonstrates that Pax6 can be cell-extrinsic for CN neuron survival. Experimental chimeras were examined at E18.5. Three embryos (H, I, and O) with cellular contributions from the *Sey/Sey* genotype are examined for the phenotype of CN neuron survival. **A**, Quantitative analysis of the number of Tbr1⁺ cells in wild-type, *Sey*-mutant, and *Sey*-chimeric cerebella. Expected numbers of Tbr1⁺ cells for each chimera are calculated from the percentage chimerism and the Tbr1⁺ cell counts from wild-type and *Sey* cerebella. A χ^2 test reveals that the observed numbers of Tbr1⁺ cells in the chimeric cerebella are significantly different from the expected numbers. **B**, Cells positive for Tbr1 expression are localized to the medial part of the nuclear region (shown in box). Higher magnification of the area in the box is shown in **C** and **D**. **C**, Costaining with phenotype marker (Tbr1) and genotype marker (GFP) reveals that Tbr1⁺ cells in the chimeric cerebellum arise from both wild-type (GFP⁺ and Tbr1⁺; arrows) and *Sey/Sey*-mutant (GFP[−] and Tbr1⁺; arrowheads) genotypes. **D**, This image more clearly shows the mutant Tbr1⁺ cells (arrowheads) and wild-type Tbr1⁺ cells (arrows). CP, Choroid plexus. Error bars represent SE. Scale bars, 100 μ m.

similar between wild-type and *Sey* RL (Fig. 9F; in fact the *Sey* RL has a marginally higher labeling index than the wild-type RL). These data indicate that both enhanced cell death of UBCs and a markedly decreased pool of RL progenitors lead to the *Sey*-mutant UBC phenotype.

Discussion

Pax6 is a prominent gene involved in the development of multiple CNS regions and sensory organs (Walther and Gruss, 1991). The *Sey* cortex, which lacks the *Pax6* gene, exhibits a dramatic reduction in cortical neurogenesis and disruption of cortical lamination (Schmahel et al., 1993; Quinn et al., 2007). However, in the *Sey* cerebellum, there is only minor disorganization of the EGL with foliation defects and no apparent GC loss (Engelkamp et al., 1999; Swanson et al., 2005). This is surprising because Pax6 is richly expressed in the cells of the RL, which produces GCs, the most numerous neurons in the brain, as well as other neurons of the glutamatergic lineage. Pax6 is expressed sequentially by all RL

Figure 7. The loss of Pax6 results in the absence of Tbr2⁺ cells from the medial *Sey* cerebellum. **A–C**, In the normal E15.5 cerebellum, immunohistochemistry reveals that Pax6 (**A**), Lmx1a (**B**), and Tbr2 (**C**) label a stream of cells (within the dashed lines) found between the EGL and the interior face of the RL. **D**, The transcription level of *Tbr2* in the *Sey* cerebellum is significantly reduced at E15.5 and E18.5 as revealed by *Pax6* transcriptome analysis from CbGrITS. *y*-axis log₂ transformed and followed by a 2Z + 8 Z-score stabilized intensity value for microarray dataset. **E, F**, In the E18.5 wild-type cerebellum, expression of Tbr2 is observed in the UBCs moving out of the RL into the developing cerebellar core (arrows). **G**, In contrast, Tbr2⁺ cells are absent from the RL and cerebellar core of the *Sey* cerebellum at medial levels. **H**, Some Tbr2-positive cells are observed more laterally in the *Sey* cerebellum (arrows). CP, Choroid plexus. Error bars represent SE. Scale bars, 100 μ m.

derivatives: the glutamatergic CN neurons, GCs, and UBCs. The *Sey* phenotypes of CN neurons and UBCs have not been previously reported. Our findings identify major, and heretofore unreported, functions for Pax6 in the development of glutamatergic CN neurons and UBCs. This supports a more critical role for Pax6 in the molecular architecture of the developing cerebellum.

The role of Pax6 in glutamatergic CN neuron development

Expression of Pax6 marks the progenitors of glutamatergic CN neurons before the expression of cell-specific markers (e.g., Tbr1, Tbr2, and Lmx1a), which are expressed when the cells enter the NTZ (Fink et al., 2006; current study). In the *Sey* cerebellum, these downstream cell markers are absent and the NTZ is acellular, indicative of the loss of the CN neuron population. The finding of Atoh1⁺ CN neuron progenitors in the E11.5 *Sey* subplial stream indicates there is normal cell production. However, these Atoh1⁺ cells do not colonize the NTZ and we observed an increased number of caspase-3⁺ cells in the Insm1-negative, likely CN neuron, population of the *Sey* subplial stream/EGL. This finding indicates that the migration of CN neurons to the NTZ is disrupted in the *Sey* mutant and cell death seems to be a critical

factor for the loss of CN neurons. Interestingly, our study of *Sey* chimeras demonstrates a cell-extrinsic role in CN neuron survival in the mutant condition; that is, the presence of Pax6 wild-type cells rescues Pax6-null CN neurons.

Our observation that glutamatergic CN neurons exhibit cell death when Pax6 is removed points to a novel pathway mediated by Pax6 that promotes cell survival in the cerebellum. In other brain regions, Pax6 has been shown to regulate cell survival by controlling the expression of proapoptotic or antiapoptotic factors. For example, Pax6 is required for the survival of cortical radial glial cells by downregulating the expression of neurotrophin receptor p75^{NTR}, which is known for its proapoptotic activity (Nikoletopoulou et al., 2007). On the other hand, Pax6 promotes the survival of dopaminergic olfactory bulb neurons by upregulating the expression of crystalline α A, an antiapoptotic molecule that inhibits the activation of caspase-3 (Ninkovic et al., 2010). In the cerebellum, our *Pax6* transcriptome analysis identified a downregulation of *Bcl2l13* and *neurotrophin-3* (*Ntf3*) in the *Sey* mutant (Ha et al., 2012). *Bcl2l13* is an antiapoptotic molecule that protects mitochondrial membrane integrity, which in

Figure 8. The loss of Pax6 results in a reduction of Lmx1a⁺ cells in the *Sey* cerebellum. **A**, At E15.5, UBC progenitors with strong Lmx1a expression (left, white arrowheads) are missing in the *Sey* cerebellum (right). By contrast, cells with light Lmx1a expression, which are GC progenitors born about the same time as UBCs, have similar staining in cells of the wild-type and *Sey* RL. **B**, At E18.5, cells with Lmx1a expression in the wild-type cerebellum (left, white arrowheads) are absent from the *Sey* medial cerebellar core (right). Lmx1a⁺ cells are seen in the wild-type, lateral cerebellum (bottom left, white arrowheads), which are also observed in lateral portions of the E18.5 *Sey* cerebellum (bottom right, white arrowheads). CP, Choroid plexus. Scale bars, 100 μ m.

turn inhibits mitochondria-driven apoptosis (Jensen et al., 2014). Ntf3 is classically considered a prosurvival factor by binding the tyrosine kinase receptors (Maisonpierre et al., 1990; DiCicco-Bloom et al., 1993). Recently, the precursor form of Ntf3 has also been identified for its proapoptotic activity when binding to p75^{NTR} (Shen et al., 2013).

The role of Pax6 in UBC development

The absence of Pax6 also impacts the late-born UBCs. We found that during normal development, UBCs residing in the region between the RL and EGL express Pax6, Tbr2, and Lmx1a. In the Pax6-null cerebellum, Tbr2-expressing and Lmx1a-expressing UBCs are completely absent from the medial *Sey* cerebellum, although a few Tbr2⁺/Lmx1a⁺ cells are observed in the lateral RL. During the time of UBC genesis, we detected a significant increase in the number of caspase-3⁺ cells in the *Sey* RL region, where UBCs are generated and reside before their dispersion into the cerebellar core; indicating that some UBC progenitors undergo cell death in the Pax6-null cerebellum. However, the number of apoptotic cells in the *Sey* RL is insuff-

ficient to account for the missing Tbr2⁺ UBCs as cell death is only marginally increased at E16, and not different at E15, E17, and E18. In addition to cell death, we detected a deficit in cell production at the RL, the progenitor pool of RL lineages, in the *Sey* cerebellum. The E16 *Sey* RL is half the size of the wild-type RL and consists of 50% fewer proliferating cells, as demonstrated by BrdU analysis. Our data indicate that the major role for Pax6 in UBC development is the regulation of production with a minor role being in regulating cell death.

Revising the role of Pax6 in GC development

Previous studies have shown that Pax6 regulates the migration and differentiation, and not the survival or production, of GCs (Engelkamp et al., 1999; Swanson et al., 2005; Swanson and Goldowitz, 2011). Our caspase-3 immunolabeling, however, revealed that some Insm1⁺ GCs in the *Sey* EGL underwent cell death during development. The discrepancy may lie in the different tissue processing and staining that we used compared to processing and staining used by Engelkamp et al.

Figure 9. Enhanced cell death and decreased neurogenesis leads to the reduction of UBCs in the *Sey* cerebellum. **A**, Activated caspase-3 immunopositivity indicates the absence of apoptotic cells in the E16.5 wild-type cerebellum while caspase-3⁺ cells are found in the RL of E16.5 *Sey* cerebellum (arrowhead). **B**, Quantitative analysis of caspase-3 immunopositivity revealed a significant increase in cell death in the *Sey* cerebellar RL at E16.5 compared to the wild type (**p* < 0.005). **C–E**, The RL (**C**, area bounded by dotted lines) of E16.5 wild-type and *Sey* cerebella demonstrates the mutant RL is significantly smaller (**D**) and has significantly fewer BrdU⁺ cells (**E**) compared with the wild type (**p* < 0.005). **F**, Interestingly, the percentage of BrdU⁺ cells in the *Sey* RL is slightly higher than that in the wild type. Error bars represent SE. Scale bars, 100 μm.

Figure 10. A model molecular program underpinning the development of cerebellar glutamatergic neurons. Expression of Atoh1 in the RL is required to specify progenitor cells to RL lineages. Pax6 is subsequently expressed in all RL-derived glutamatergic neurons and regulates multiple developmental processes in these cell types. In the development of CN neurons, Pax6 regulates cell survival of progenitors. The present study identifies an enhanced cell death in the *Pax6*-null cerebellum that contributes to the loss of CN neurons. It is known that expression of Tbr1 regulates the migration of CN neurons and we find this is downstream of Pax6 function. In the development of UBCs, the survival and production of UBC progenitors require Pax6 function. Thus, in the *Pax6*-null mutant there is an enhanced cell death and reduction in UBC progenitor cells, which results in a reduction of UBCs. Tbr2 functions downstream of Pax6, and plays a role in UBCs migration. In the development of granule cells, the current findings suggest that Pax6 plays a role in cell survival and cell proliferation, in addition to cell differentiation and migration. Our current work also suggests an earlier function of Pax6 in regulating the replenishment of the RL progenitor pool.

The previous work found no effect of the *Pax6*-null mutation on GC proliferation in the EGL (Engelkamp et al., 1999). However, we do see a reduction in cell proliferation in the *Sey* RL at late embryogenesis. We have interpreted this as an effect on UBC genesis. However, we cannot exclude the possibility that it also impacts the generation of late-born GCs. In this manner, the *Sey* mutation may have an upstream impact on GC production.

A spatial role of Pax6 in the development of RL derivatives

We find that Pax6 has a global effect on the spatial organization of RL-derived cerebellar neurons in a lateral–medial fashion. We had an inkling of the spatial effect of the *Sey* from our analysis of *Pax6* experimental chimeras, where we found that *Pax6*-null GCs are largely excluded from the medial cerebellum (Swanson and Goldowitz, 2011). Now we also find that medially located Tbr1⁺ CN neurons are missing in the *Sey* mutant while the Irx3⁺ VZ-derived lateral CN populations are unaffected. In a similar manner, UBCs are largely absent from the *Sey* cerebellum with the exception of some Tbr2⁺ cells at the lateral cerebellar RL. These results suggest that there is a requirement of Pax6 function, direct or indirect, for the successful placement of cells in the medial cerebellum. The phenotypes could be due to a failure in the acquisition of a medial subtype identity or a failure in cell migration. In either case, it is now clear that novel functions of Pax6 in each of the glutamatergic neurons is both cell-type and spatially specific.

Pax6 and the molecular underpinnings of cerebellar development

RL precursors express Pax6 and Atoh1 (Yeung et al., 2014). The knock-out of Atoh1 results in the loss of Pax6 expression and eliminates all cerebellar glutamatergic neurons (Machold and Fishell, 2005; Englund et al., 2006; Fink et al., 2006). In contrast, our present work shows that the *Pax6* knock-out has no effect on the generation of Atoh1-expressing RL progenitors and eliminates only the CN neurons and medial UBCs. Thus, the extreme phenotype of the *Atoh1*-null cerebellum would place Atoh1 upstream of Pax6. In this model (Fig. 10), Atoh1 is necessary for the cell-fate specification of all RL-derived precursors (Yamada et al., 2014). In this study, we find that Pax6 regulates GC survival and proliferation. Furthermore, the present work illuminates the key function Pax6 plays in the development of CN neurons and UBCs. In glutamatergic CN neuron development, Pax6 is

upstream of *Tbr1* and *Tbr2*. In the *Pax6*-null cerebellum there is a complete loss of *Tbr1*⁺ CN neurons, while the *Tbr1*-knock-out only results in disorganized CN neurons without cell loss (Fink et al., 2006). Similarly, the *Tbr2* conditional knock-out has no apparent effect on CN neurons (R. Hevner, personal communication). For UBCs, Pax6 is similarly upstream of *Tbr2*, as illustrated by the conditional *Tbr2*-knock-out, which only demonstrates a mild migration defect (R. Hevner, personal communication) compared with the loss of the majority of UBCs in the *Sey* cerebellum.

Pax6 and the RL-lineage progenitor pool

The *Sey*-mutant RL progenitor pool, as manifested by BrdU incorporation, is dramatically reduced and this reduction is particularly evident by late embryogenesis. This finding suggests that Pax6 has a role in regulating the RL progenitor pool (Fig. 10). In fact, earlier work found that *Atoh1*⁺ cells rapidly emigrate from the RL and withdraw from the RL progenitor pool (Machold and Fishell, 2005). Based on our recent analysis of compartmentation in the RL, we speculated that another population of cells (those originating from the interior face of the RL; *Wls*⁺/*Pax6*⁺ cells) served to replenish the RL progenitor pool (Yeung et al., 2014). With Pax6 absent, a depletion of the neural progenitor pool has been described in the development of retina and cortex, as a result of precocious cell-cycle exit (Farhy et al., 2013) or shorter cell-cycle length (Estivill-Torrus et al., 2002). Interestingly from our CbGRiTS database (cbgrits.org), we find that the transcriptional levels of cyclins B1/2, which promote cell-cycle progression, and p57 (or *Cdkn1c*), which induce cell-cycle exit, are elevated in the *Sey* cerebellum, suggesting that both mechanisms may be perturbed in the cells of the RL progenitor pool. Further investigation in cell-cycle dynamics will elucidate the Pax6-dependent regulation in the RL progenitor pool.

This work reveals the novel and essential functions of Pax6 in neurogenesis of glutamatergic CN neurons and UBCs and, together with the revised Pax6 function in GC development, recognizes a more central role of Pax6 in generating the RL lineages. Our work brings to light the multivalent role of Pax6 in cerebellar development, which has commonalities to the role of Pax6 in the genesis of the eye and cortex. This new knowledge enhances our understanding of the molecules underpinning cerebellar development and, more importantly, the requirement of Pax6 in neurogenesis.

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