The BAF Complex Interacts with Pax6 in Adult Neural Progenitors to Establish a Neurogenic Cross-Regulatory Transcriptional Network

Jovica Ninkovic,[1,4] Andrea Steiner-Mezzadri,[1] Melanie Jawerka,[4] Umut Akinci,[5] Giacomo Masserdotti,[1,4]
Stefania Petricca,[1] Judith Fischer,[1] Alexander von Holst,[7] Johanes Beckers,[3,8] Chichung D. Lie,[2,9] David Petrik,[10]
Erik Miller,[12] Jiong Tang,[12] Jiang Wu,[11] Veronique Lefebvre,[13] Jeroen Demmers,[6] Amelia Eisch,[10] Daniel Metzger,[14]
Gerald Crabtree,[12] Martin Irmler,[3] Raymond Poot,[5] and Magdalena Götz[1,4,15,*]

[1]Institute for Stem Cell Research
[2]Institute of Developmental Genetics
[3]Institute of Experimental Genetics
Helmholtz Centre Munich German Research Center for Environmental Health (GmbH), 85764 Neuherberg, Germany
[4]Physiological Genomics, Medical Faculty, University of Munich, Schillerstrasse 46, 80633 Munich, Germany
[5]Department of Cell Biology
[6]Proteomics Center
Erasmus MC, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands
[7]Department of Anatomy and Cell Biology, Heidelberg University, Im Neuenheimer Feld 307 and Interdisciplinary Center Neurosciences (IZN),
69120 Heidelberg, Germany
[8]Center of Life and Food Sciences Weihenstephan, Technical University Munich, 85354 Freising, Germany
[9]Institute of Biochemistry, Emil Fischer Center, University Erlangen-Nürnberg, 91054 Erlangen, Germany
[10]Department of Psychiatry
[11]Department of Physiology and Developmental Biology
UT Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390-9070, USA
[12]Howard Hughes Medical Institute, Beckman Center B211, 279 Campus Drive, Stanford University School of Medicine, Stanford,
CA 94305-5323, USA
[13]Department of Cell Biology (NC10), Cleveland Clinic Lerner Research Institute, 9500 Euclid Avenue, Cleveland, OH 44195, USA
[14]Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), INSERM U964 / CNRS UMR 7104 / Université de Strasbourg,
67404 Illkirch, CEDEX, France
[15]Munich Cluster for Systems Neurology (EXC 1010 SyNergy), Ludwig Maximilian University of Munich, 80539 Munich, Germany
*Correspondence: magdalena.goetz@helmholtz-muenchen.de
http://dx.doi.org/10.1016/j.stem.2013.07.002

SUMMARY

Numerous transcriptional regulators of neurogenesis have been identified in the developing and adult brain, but how neurogenic fate is programmed at the epigenetic level remains poorly defined. Here, we report that the transcription factor Pax6 directly interacts with the Brg1-containing BAF complex in adult neural progenitors. Deletion of either Brg1 or Pax6 in the subependymal zone (SEZ) causes the progeny of adult neural stem cells to convert to the ependymal lineage within the SEZ while migrating neuroblasts convert to different glial lineages en route to or in the olfactory bulb (OB). Genome-wide analyses reveal that the majority of genes downregulated in the Brg1 null SEZ and OB contain Pax6 binding sites and are also downregulated in Pax6 null SEZ and OB. Downstream of the Pax6-BAF complex, we find that Sox11, Nfib, and Pou3f4 form a transcriptional cross-regulatory network that drives neurogenesis and can convert postnatal glia into neurons. Taken together, elements of our work identify a tripartite effector network activated by Pax6-BAF that programs neuronal fate.

INTRODUCTION

Cell fate specification includes multiple steps in restricting progenitor potential and directing the expression of genes that elicit a lineage-specific program. According to the "master regulator concept," a single gene expressed in competent tissue is capable of inducing the expression of the entire lineage-specific transcriptional cascade, resulting in final and complete fate commitment (Baker, 2001). However, the molecular mechanisms by which these master regulators work are not clear. It is unknown for most lineages if master regulators directly control multiple genes executing lineage decision and differentiation of cells further along the lineage or only control a few downstream effector genes, as, e.g., proposed by the concept of terminal selector genes (Hobert, 2011). The transcription factor Pax5, a master regulator of B cell fate in the hematopoietic system, promotes B cell differentiation and maintenance of B cell fate (Medvedovic et al., 2011), exploiting the epigenetic machinery to either shut down expression of genes of other lineages or activate genes of the B cell lineage (McManus et al., 2011). Thus, Pax5 directly regulates many effector genes and stabilizes the
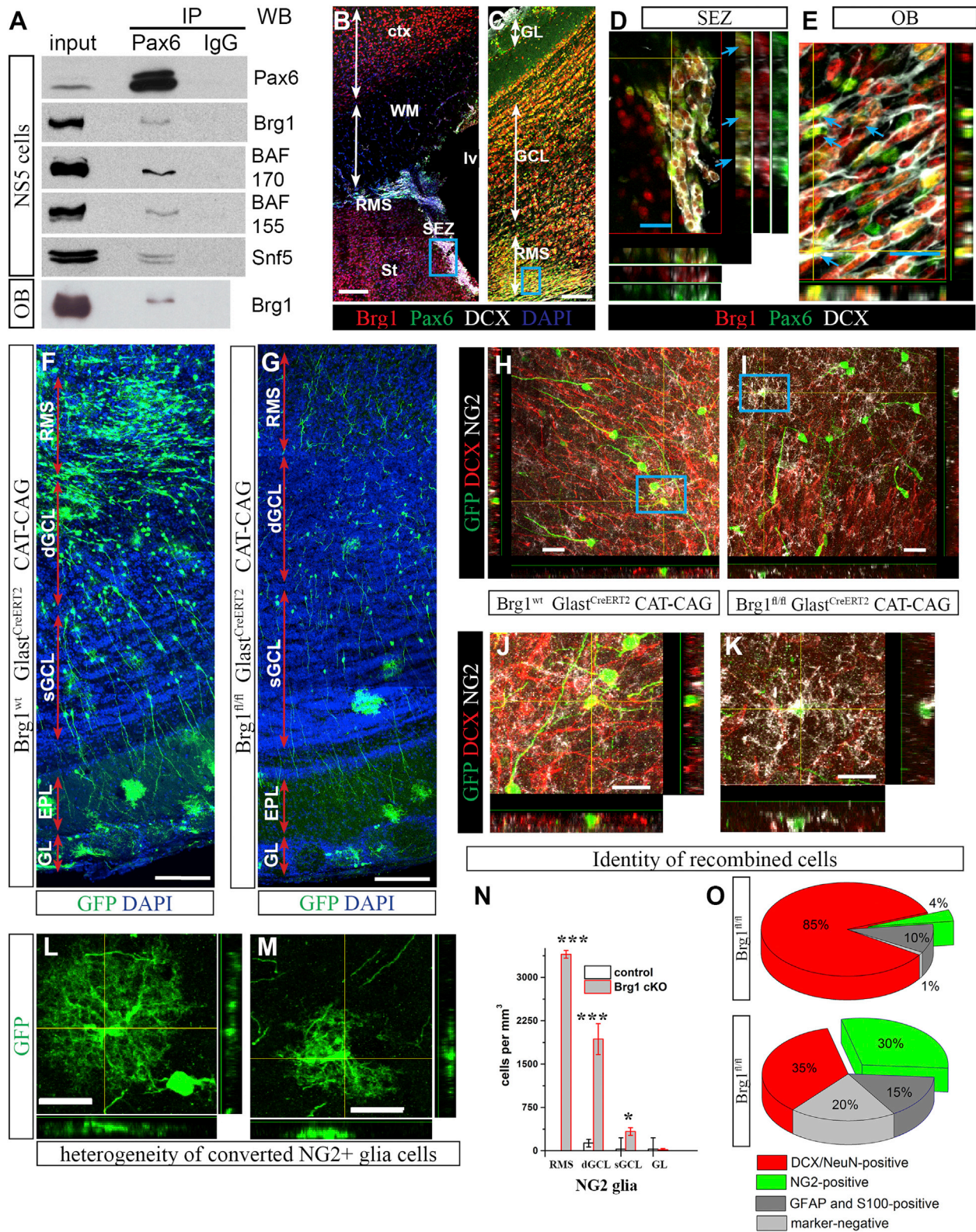
Figure 1. Loss of Brg1 Function Renders Neuronal Progenitors into Glial Cells

(A) Western blot depicting the direct interaction of Pax6 and BAF complex.

(B–E) Micrographs depicting coexpression of Pax6 and Brg1 in DCX+ neuroblasts (blue arrow in D and E). (D) and (E) are magnifications of area boxed in (B) and (C), respectively.

(legend continued on next page)

lineage decision using the epigenetic machinery. Other master regulators have relatively weak activation capacity themselves (e.g., Hnf3b and Sry transcription factors) but have the capacity to open chromatin-enabling binding and transactivation by other transcription factors executing the lineage decision (Zaret and Carroll, 2011).

These mechanisms remain largely open for neural fate decisions. Key transcriptional regulators of neurogenic fate have been identified in the developing and adult brain, such as Pax6, Neurog2, Ascl1, SoxC, and Dlx2 (Bergsland et al., 2011; Hack et al., 2005; Heins et al., 2002; Mu et al., 2012; Nieto et al., 2001; Petryniak et al., 2007). However, it is still largely unknown how these transcription factors act at the molecular level to direct neuronal fate decisions. While ChIP experiments and mouse mutant analysis for Pax6, Ascl1, and SoxC (Sox4 and Sox11) revealed binding and regulation of genes involved in proliferation as well as neurogenesis (Asami et al., 2011; Bergsland et al., 2011; Castro et al., 2011), the key molecular targets allowing these factors to orchestrate neurogenesis and neuronal reprogramming have not yet been identified.

In direct reprogramming, it appears that the number of factors necessary for fate conversion is inversely correlated to the distance of their lineage relation. If cells share a common lineage, such as glia with neurons, they can be converted with one factor (Davis et al., 1987; Heinrich et al., 2010; Heins et al., 2002), while turning more distinctly related cells, such as fibroblasts, into neurons requires several factors (Vierbuchen et al., 2010; Yoo et al., 2011). This may reflect the state of the chromatin in the initial cell and reinforces the importance of transcription factors in chromatin restructuring and opening of new binding sites for fate conversion (Siersbæk et al., 2011; Zaret and Carroll, 2011). However, virtually nothing is known about the interaction of key neurogenic factors with the chromatin-modifying machinery.

Several chromatin remodeling factors have been found to be important during neural development, including members of the Brm-associated factor (BAF), CHD, and ISWI complexes (Engelen et al., 2011; Ho et al., 2009; Yip et al., 2012). For example, conditional deletion of the ATPase subunits Snf2l or Brg1 of the ISWI or BAF chromatin remodeling complexes reduced neural stem cell (NSC) proliferation and self-renewal in the developing forebrain (Lessard et al., 2007; Matsumoto et al., 2006; Yip et al., 2012). These chromatin-remodeling complexes appear necessary to regulate expression of proproliferative genes, such as Foxg1 (Yip et al., 2012), and Notch and Sonic Hedgehog pathway genes (Lessard et al., 2007). Interestingly, whether the BAF complex regulates proliferation or neuronal differentiation (Wu et al., 2007) depends on its subunit composition (Ho et al., 2009). In mammals, BAF complexes contain the ATPase subunits Brg1 or Brahma, which are mutually exclusive and essential for remodeling activity, and comprise up to 12

Brg1/BAF subunits. Depending on specific BAF complex subunits, this complex is involved in NSC maintenance or neuronal differentiation (Ho et al., 2009; Singhal et al., 2010). However, it is not known how these specific complexes are targeted to and regulate the respective and distinct targets and to which extent they may interact with specific transcription factors.

To shed light on the molecular mechanisms underlying the function of key neurogenic master regulators in fate specification and conversion, we chose to search for Pax6 interactors. Pax6 is not only a master regulator in eye development (Baker, 2001) and in neurogenesis (Brill et al., 2008; Götz et al., 1998; Hack et al., 2005; Heins et al., 2002; Stoykova and Gruss, 1994), but it is also sufficient to reprogram glia into neurons (Berninger et al., 2007; Buffo et al., 2005; Heins et al., 2002). Understanding how Pax6 exerts its neurogenic function is therefore of crucial interest to reveal the basic principles of endogenous and enforced neurogenesis.

RESULTS

Transcription Factor Pax6 Interacts with BAF Chromatin Remodeling Complex in Neurogenic Progenitors

In order to understand the mechanisms underlying Pax6-mediated neurogenesis, we purified Pax6-containing complexes from NSCs expressing Pax6 (Figure S1A available online) and used mass spectrometry to examine their composition. Pax6 complexes were purified by either Pax6 antibody (Pax6-IP, Figure 1A) or FLAG antibody from NSCs stably expressing FLAG-tagged Pax6 (FLAG-Pax6-IP, Figure S1B). In either case, multiple subunits of the BAF complex were present in the Pax6 samples. The interaction of Pax6 with the BAF complex was confirmed by western blot (WB) detection of the BAF complex in Pax6 immunoprecipitations (Figure 1A). Thus, Pax6 physically interacts with Brg1-containing BAF chromatin remodeling complexes in NSCs. To examine this in the brain, we prepared nuclear extracts from the core of the adult mouse olfactory bulb (OB), which is enriched in Pax6+ neuroblasts (Hack et al., 2005; Brill et al., 2008). Immunoprecipitation with Pax6 antibody followed by WB for Brg1 (Figure 1A) confirmed the interaction of Brg1-containing complexes with Pax6 in the OB.

To examine colocalization of Pax6 and Brg1 at the cellular level, we performed immunostaining for Brg1 in the adult brain, which showed a broad expression of Brg1 in neurons and astrocytes throughout the brain, while surprisingly weak immunoreactivity for Brg1 was detectable in white matter (WM) where mostly oligodendrocytes and their precursors reside (Figure 1B; Figure S1C). In the subependymal zone (SEZ), the region of the adult brain generating OB interneurons in Pax6-dependent manner (Hack et al., 2005; Brill et al., 2008), Brg1-immunoreactive nuclei were detected in GFAP+ astrocytes and NSCs as well as in Ascl1+

(F and G) Representative micrographs of the olfactory bulbs of Brg1 cKO (G) and corresponding control (F) 28 days after recombination.

(H–K) Micrographs depicting the immunoreactivity of recombined cells for DCX and NG2. (J) and (K) are magnifications of area boxed in (H) and (I), respectively.

(L and M) Micrographs depicting the diverse morphology of NG2+ cells generated from the Brg1-deficient neural progenitors.

(N) Histogram depicting the total number of NG2+ cells in OB 28 days after recombination. Data are shown as mean ± SEM and n(animals) ≥ 5. *p ≤ 0.05; ***p ≤ 0.005.

(O) Pie charts illustrating the identity of recombined cells in the OB 28 days after recombination. Data are shown as mean and n(animals) ≥ 5.

Scale bars: 100 μm in (B), (C), (F), and (G); 50 μm in (L)–(M); 20 μm in (D), (E), and (H)–(K). Abbreviations: ctx, cerebral cortex; cc, corpus callosum; RMS, rostral migratory stream; SEZ, subependymal zone; St, striatum; lv, lateral ventricle; GL, glomerular layer; GCL, granule cell layer; EPl, external plexiform layer; dGCL, deep granule cell layer; sGCL, superficial granule cell layer. See also Figures S1 and S2.

transient amplifying progenitors (TAPs) and Doublecortin (DCX)+ neuroblasts (Figures S1D and S1E and data not shown). Importantly, we also observed the expression of BAF53a and BAF45a (Figures S1F and S1G; but not BAF45b and BAF53b, Figures S1H and S1I), subunits characterizing the neural-progenitor-specific BAF complexes (Lessard et al., 2007). Conversely, Pax6 immunoreactivity is largely restricted to neuroblasts in the SEZ and rostral migratory stream (RMS), where it colocalizes with Brg1 (Figures 1D and 1E). Together with the immunoprecipitation experiments, these data suggest that Pax6 interacts with the neural-progenitor-specific, Brg1-containing BAF chromatin remodeling complex in neuroblasts in vivo.

Loss of Brg1 in Adult NSCs Converts OB Neurogenesis into Gliogenesis

To test the functional relevance of the observed Pax6-BAF interaction, we ablated Brg1 in the Brg1^{fl} mouse line (Indra et al., 2005; Matsumoto et al., 2006) by tamoxifen (TM) inducible Cre-based excision in Glast^{CreERT2} mice, mediating genomic recombination in astrocytes and NSCs (Mori et al., 2006; Ninkovic et al., 2007). These mice were also crossed with the CAG-CAT-GFP reporter line (Nakamura et al., 2006), allowing visualization of the recombined cells. By 9 days post TM administration (9 dpt), 95% of reporter+ cells in Glast^{CreERT2}/Brg1$^{fl/fl}$ mice (further referred as Brg1 cKO) were no longer Brg1-immunopositive, while virtually all reporter+ cells were Brg1+ in Glast^{CreERT2}/Brg1$^{fl/+}$ or Glast^{CreERT2}/Brg1$^{+/+}$ mice (further referred as Brg1 controls) (Figures S2A–S2C).

While no altered neurogenesis was detectable in the SEZ, RMS, or OB of Brg1 cKO mice 9 dpt (Figure S2D), 28 dpt the number of recombined cells was significantly reduced in the OB (Figures 1F and 1G) compared to Brg1 controls. As this may be due to cell death, we examined activated caspase3, an indicator of programmed cell death. Indeed, the number of activated caspase3+ cells was significantly increased in the OB and RMS, but not in the SEZ, of Brg1 cKO mice compared to controls (Figure S2E and data not shown). This suggests that cell death is initiated at later stages in the neuroblasts when they migrate along the RMS. We also observed an increase in GFP+ cells located just beside the RMS (Figure S2F–S2H) with a morphology reminiscent of oligodendrocyte progenitor cells (OPCs). Staining for the transcription factor Olig2 (data not shown) and proteoglycan NG2 labeling of OPCs (Dimou et al., 2008) confirmed the strikingly higher number of GFP+ OPCs in Brg1 cKO (Figures 1I and 1K–1O; 2,000× increase) while virtually no GFP+ OPCs were detectable in the OB of control animals (Figures 1H and 1J). Notably, GFP+ OPCs were present only at the end of the RMS, in the core of the OB, and in the deep granule cell layer (GCL) (Figure 1N). The newly generated OPCs displayed a variety of morphologies with different levels of cellular complexity (Figures 1K–1M) indicative of different stages in the oligodendrocyte lineage. However, even 2 months after TM, no GFP+ cells had matured into oligodendrocytes immunoreactive for GST-π. Interestingly, other glial cells, like GFAP+ astrocytes (Figure 1O and Figures S2J–S2K′) and marker-negative cells (Figures S2L and S2L′—cells were immunoprobed for more than 20 antigens indicative of astroglial, neuronal, oligodendroglial, endothelial, and microglial lineage as well as fibroblasts; see Experimental Procedures), were also increased in Brg1 cKO OB,

reflecting a broader conversion toward gliogenesis and some cells failing to adopt any coherent cell identity after loss of Brg1 (Figure 1O and Figure S2L). Conversely, cells of the neuronal lineage were strongly reduced in number to less than one-third (Figure 1O and Figure S2M), with the only exception being neurons in the superficial GCL (Figure S2M), indicating that this neuronal sublineage is selectively spared by Brg1 depletion and accounts for almost half of the remaining neurons. Ergo, inducible, cell-specific deletion of Brg1 in vivo converts adult OB neurogenesis to gliogenesis starting in the RMS and is accompanied by increased cell death.

Glast^{CreERT2}-mediated recombination is not restricted to the SEZ and RMS, the origin of adult OB neurogenesis, but also depletes Brg1 in astrocytes throughout the brain. Therefore, it is possible that some astrocytes in the OB and RMS may be converted to OPCs. While no GFP+ OPCs were detected outside the OB (data not shown), it remains possible that specifically astrocytes in the OB would be more easily converted to OPCs. To examine the fate of cells originating in the SEZ directly, we injected dsRed-expressing MLV-based retroviral vectors 9 dpt into the SEZ of Glast^{CreERT2}//Brg1$^{fl/fl}$//CAT-CAG-GFP animals in order to label Brg1 cKO progenitors (dsRed+/GFP+) and their WT cellular counterparts (not recombined and hence GFP−, but dsRed+) already in the SEZ (Figures S3A and S3B). When we analyzed the identity of labeled cells in the OB 7 days later, most of the dsRed+, GFP− control cells (more than 90%) were DCX+ neuroblasts, as is normally the case (Figure S3B). However, only a minority (40%) of the Brg1-deficient cells (dsRed+, GFP+) originating in the SEZ had acquired a neuroblast identity (Figure S3B) and a significant proportion expressed Olig2 (Figure S3B). As this proportion was similar to the GFP+, dsRed− (38% for DCX and 36% for Olig2), we conclude that there is no major additional source of cells contributing to the OPCs in the OB of Brg1 cKO mice. Thus, most cells from the SEZ fail to complete their neurogenic fate upon Brg1 deletion and convert to gliogenesis.

Viral vector injection causes an injury in the SEZ, which may affect the lineage progression. To address this possibility and verify the above findings, we deleted Brg1 with the Nestin-CreER^{T2} line (Lagace et al., 2007) mediating recombination exclusively in nestin+ cells of the SEZ, but not in the nestin-negative parenchymal glia. Consistent with our findings with the Glast^{CreERT2} mice shown above, mice that lack Brg1 in nestin+ cells and their progeny had significantly fewer GFP+ cells reaching the OB and acquiring a neuronal identity compared to control mice 30 dpt (Figures S3C and S3D). Similar to the Glast^{CreERT2}-mediated deletion of Brg1, these cells expressed Olig2. Taken together, multiple independent experimental approaches confirm that Brg1 is an essential component of SEZ-derived neurogenesis, and that the absence of Brg1 causes the conversion of neuroblasts in the RMS and OB to the glial lineages.

Niche-Dependent Gliogenesis Elicited by Loss of Brg1

Because our fate mapping experiments demonstrated that the reporter+ glia in the OB originate from the SEZ, we examined cells in the SEZ of Brg1 cKO mice. However, even 28 dpt (17 days after loss of Brg1 protein), numbers of DCX+ neuroblasts among the recombined (GFP+) cells in the SEZ

did not significantly differ between Brg1 cKO and control mice (Figures S3E and S3F), suggesting that conversion to gliogenesis occurs only when neuronal cells exit the SEZ. This strikingly late fate conversion may be due to a powerful role of the niche environment or other intrinsic mechanisms sufficient to stabilize neuronal fate (Beckervordersandforth et al., 2010; Colak et al., 2008; Lim et al., 2006). To distinguish between these two possibilities, we isolated progenitors from the SEZ of Brg1 cKO or control mice 9 dpt and cultured them in a primary SEZ culture system maintaining neurogenesis (Costa et al., 2011). Consistent with the in vivo analysis of neurogenesis in the SEZ (Figure S3E), we did not observe any difference in cell composition of the SEZ in Brg1 cKO and respective controls after isolating cells 7 dpt and analyzing them 4 hr after plating (Figure S3G). However, cells lacking Brg1 were not able to proceed further along the neurogenic lineage also after 7 days in vitro, because only 9% of them were DCX+ neuroblasts in contrast to 60% of control cells (Figures 2A–2D). Intriguingly, Brg1-deficient cells now differentiated in CD24+ ependyma-like cells in addition to NG2+ OPCs (Figures 2C and 2D and Figure S3H).

These observations prompted us to ask if ependymal cells are generated also in the SEZ in vivo after Brg1 deletion. To address this question, we used the recently developed Split-Cre technology that specifically mediates recombination in SEZ NSCs using two halves of Cre driven by GFAP and P2 prominin promoters simultaneously active in NSCs (Beckervordersandforth et al., 2010). Stereotactic injection of two lentiviruses encoding each half of Cre under the respective GFAP or P2 promoter into Brg1$^{fl/fl}$//CAG-CAT-GFP or Brg1$^{fl/+}$//CAG-CAT-GFP mice allows us to selectively delete Brg1 in NSCs and follow their progeny by the GFP reporter. The majority of GFP+ cells were neuroblasts 60 days after virus injection into control mice with very few ependymal cells labeled (Figure S3I–S3K), while the progeny of Brg1-deficient NSCs contained 30% ependymal cells (Figure S3I–S3K). Taken together, these data imply that the local niche not only influences the selection of glial fate subtype after Brg1 deletion, but also contributes to maintenance of some cells as neuroblasts even in the absence of Brg1, because virtually all cells convert to gliogenesis outside this neurogenic niche in vitro.

Mode of Fate Conversion Determined by Continuous Single-Cell Live Imaging In Vitro

Because Brg1-deficient cells in vitro largely convert into the same cell types as in vivo, we used single-cell continuous live imaging for 7 days in vitro followed by postimaging immunostaining to discriminate whether the conversion of Brg1 cKO cells from neurogenesis to gliogenesis occurs by selective cell death, selective proliferation, or a true fate conversion (Movie S1, Movie S2, and Movie S3; Figures 2E–2I). In agreement with the population-based analysis, 93% of lineage trees in controls contained only neuronal cells, and only a small fraction of trees, derived from the NSCs, had generated both neurons and glia (Figures 2E and 2G). In contrast, when we observed Brg1 cKO cells, most of the trees contained either NG2 glia or ependyma-like cells, but only a minority had generated neurons only (Figure 2F). Interestingly, cells giving rise to ependyma-like cells were the only lineage that initially performed a series

of fast symmetric proliferative divisions (Figure 2F). Other than this, cell cycle length did not differ between control and Brg1 cKO cells (Figure 2G), nor could we observe any difference in regard to cell death that was rather rare in both control and Brg1 cKO cells (Figures 2E, 2F, and 2H). Thus, Brg1 cKO cells convert to glial lineages by fate change rather than selective cell death or proliferation. Because most of the control and Brg1 cKO cells at the start of these cultures are neuroblasts (63% ± 4%, Figure S2D), and no selective cell death of Brg1 cKO cells was observed, these experiments demonstrate that many Brg1 cKO neuroblasts directly convert to glial lineages.

Loss of Brg1 Leads to Downregulation of Pax6 Targets in Adult SEZ and OB

Given the surprisingly specific defects in neurogenesis after deletion of Brg1, we used genome-wide expression analysis to identify genes involved in this phenotype. We isolated the SEZ and core of the OB (containing neuroblasts entering the OB) from Brg1 cKO and control mice 10 dpt, just 1 day after loss of Brg1 protein. Genome-wide expression profiling (GST 1.1 gene array, Affymetrix, USA) revealed 244 significantly regulated genes (change in the expression level >1.2 or <0.8 and p < 0.05) in the OB (Table S1 available online) and 136 genes in the SEZ (Table S2 and Figure S4A), and qPCR on independent samples confirmed the reliability of this analysis (Figure 3A). Importantly, most of the significant gene ontology (GO) terms were related to neurogenesis, synaptic transmission, axonogenesis, and cell migration, consistent with defective neurogenesis (Figures S4B and S4C). The other major terms were related to DNA and RNA metabolism and cell replication and cell cycle, suggestive of defects in cell cycle regulation in Brg1 cKO SEZ and OB and possibly linked to the very specific cell division pattern of ependymal cell generation we observed using live imaging (Figure 2F). Interestingly, 64% of the downregulated genes in SEZ (Figure S4E) are expressed in NSCs and their progeny, as identified in our previous transcriptome analysis (Beckervordersandforth et al., 2010 and Figures S4D–S4F). Most intriguingly, 89.79% of the downregulated genes had predicted Pax6 binding sites, a significant enrichment in regard to the expected frequency (63% expected, Genomatix Matinspector) (Figure 3B). This suggests that most genes downregulated in Brg1 cKO may be regulated by Pax6 in conjunction with a Brg1-containing BAF complex.

Deletion of Pax6 Phenocopies Brg1 Deletion and Converts Adult SEZ Neurogenesis to Gliogenesis

In order to test the above suggestion directly in vivo, we crossed the floxed Pax6 mice (Ashery-Padan et al., 2000) with Glast^{CreERT2} mice to delete Pax6 in the same manner as we deleted Brg1. While Pax6 protein was more stable than Brg1 and disappeared only 21 dpt (Figures S5A–S5C), the phenotype emerging thereafter was remarkably similar to the phenotype observed in Brg1 cKO mice. As in the Brg1 cKO mice, we observed fewer numbers of GFP+ cells reaching the OB in the Pax6 cKO mice compared to controls (Figures 3C and 3D), and most of them no longer differentiated along the neuronal lineage (DCX+/ NeuN+ 45%, compared to 82% in controls, Figures 3E and 3G–3H), but rather converted to glial identities (Figures 3F and 3G–3H) in ratios similar to the Brg1 cKO cells
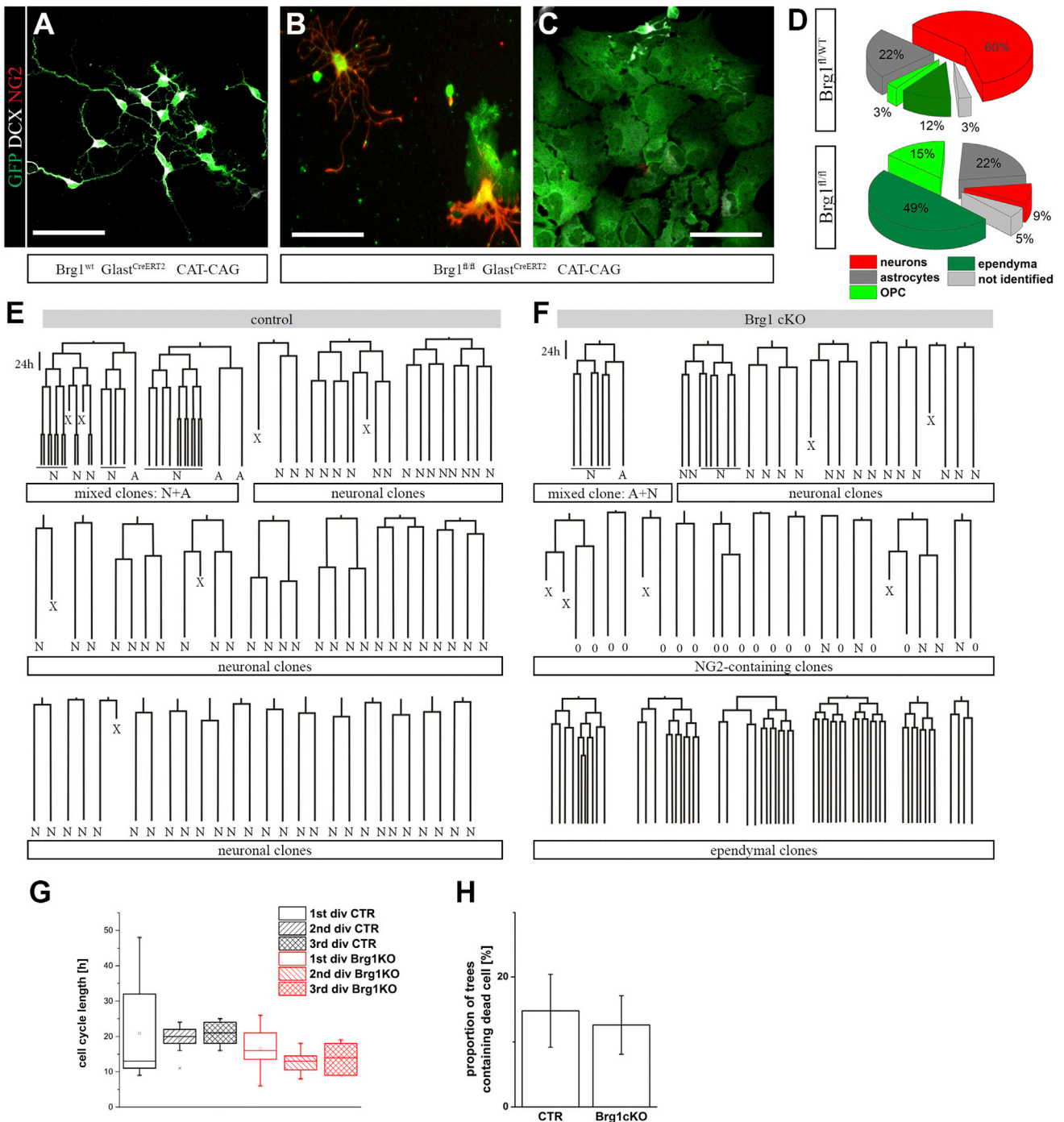
Figure 2. Loss of Brg1 Results in Full Conversion of Neural Progenitors to Gliogenesis In Vitro

(A–C) Micrographs depicting the immunoreactivity of GFP+ progeny of NSCs derived from either Brg1 cKO or age matched controls (10 days after TM induction) after 7 days in vitro.

(D) Pie charts depicting the composition of the NSC progeny following Brg1 depletion or controls after 7 days in vitro. Data are shown as mean and n(animals analyzed) $\geq$ 7.

(E and F) Representative trees illustrating the predominant behavior of the Brg1 KO or control neural progenitors in vitro.

(G) Box-chart showing the cell cycle length (the time between two divisions) of control and Brg1 cKO progenitors.

(H) Histogram depicting the proportion of clones containing at least one dead cell. Data are shown as mean (three experiments) ± SEM.

Scale bars: 100 μm in (A), (B), and (C). See also Figure S3, Movie S1, Movie S2, and Movie S3.
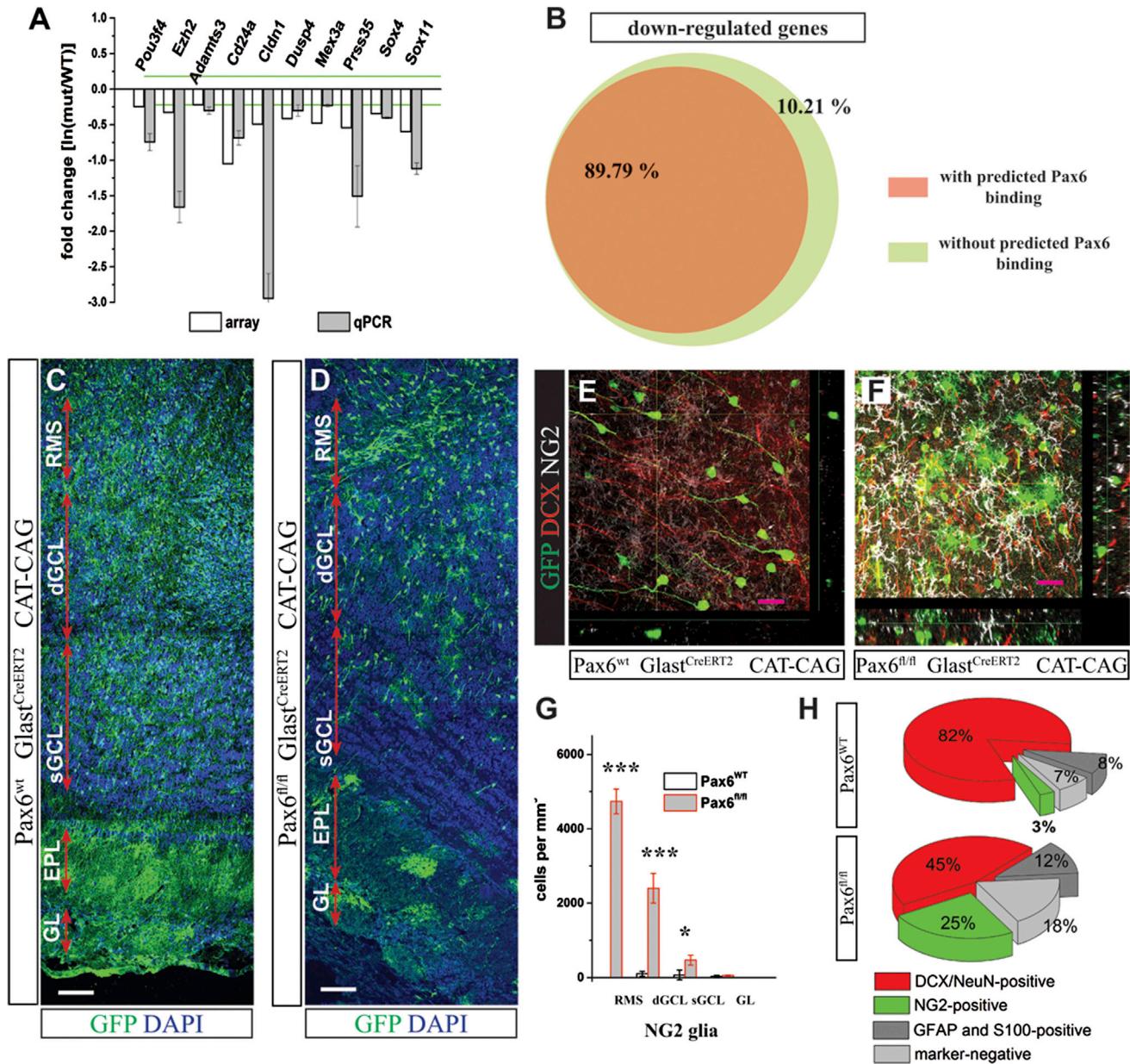
Figure 3. Brg1 Deletion Results in Downregulation of Pax6 Target Genes and Is Phenocopied by Pax6 Deletion

(A) Histogram depicting the comparison of a specific gene set misregulated after Brg1 deletion and measured by microarray and qPCR on independent samples. Data are shown as mean and n(animals) ≥ 3.

(B) Venn diagram depicting predicted Pax6 binding sites in the promoters of genes deregulated in Brg1 KO.

(C and D) Representative micrographs of the OB of controls (C) and Pax6 cKO (D) 60 days after recombination.

(E and F) Micrographs depicting the immunoreactivity of recombined, GFP+ cells.

(G) Histograms depicting the total number of NG2+ cells in OB 60 days after recombination. Data are shown as mean ± SEM and n(animals) ≥ 7. *p ≤ 0.05; ***p ≤ 0.005.

(H) Pie charts illustrating the identity of recombined cells in the 60 days after recombination. Data are shown as mean and n(animals) ≥ 3.

Scale bars: 100 μm in (C) and (D); 20 μm in (E) and (F). For abbreviations see Figure 1. See also Figure S4.

(Figures 1H–1O). Thus, consistent with the interaction of Pax6 with Brg1-containing BAF complex in vitro and in vivo, deletion of either of these proteins results in the same phenotype with severe defects in OB neurogenesis, implying a key role of this transcriptional complex in regulating neurogenesis.

Pax6 and Catalytically Active Brg1 Are Both Essential for Forced Neurogenesis

The above findings prompted us to examine to which extent Pax6-induced neurogenesis would also require the presence of Brg1. Toward this end, we prepared neurosphere cells from
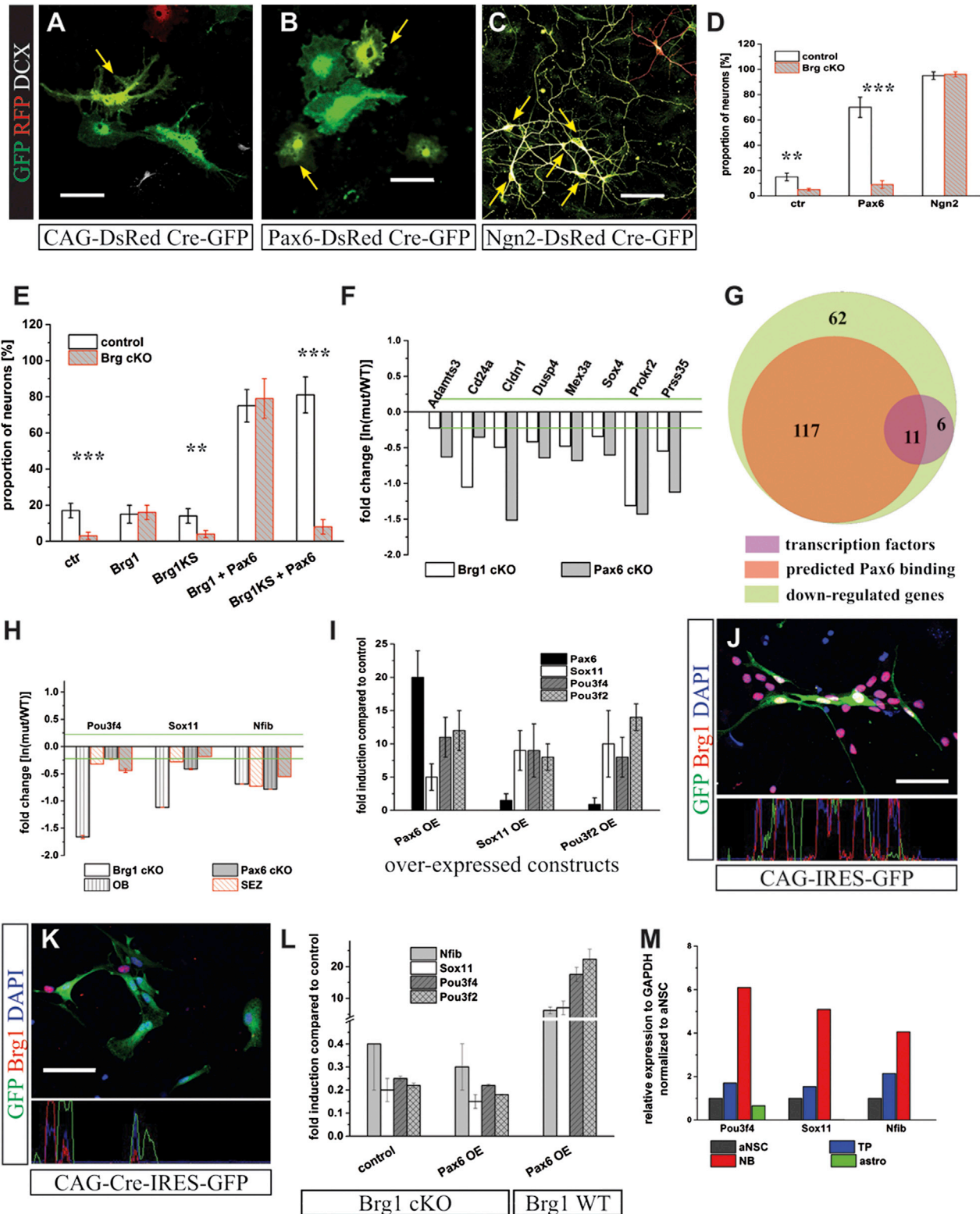
Figure 4. Pax6 Requires Catalytically Active Brg1 for Its Neurogenic Function

(A–C) Micrographs depicting immunoreactivity for neuronal marker DCX of Brg1 cKO cells (green) derived from Brg1$^{fl/fl}$ neurospheres after overexpression of control vector (A), Pax6 (B), and Ngn2 (C) 7 days after transduction.

(D and E) Histograms depicting the proportion of neurons generated from neurosphere cells 7 days after expression of Pax6, Ngn2, Brg1, and Brg1KS (ATPase deficient form).

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the adult SEZ of Brg1 fl/fl animals and transduced them in vitro with viral vectors encoding Cre to obtain Brg1-deficient cells (Figures 4A–4D). Two days after transduction, Brg1 protein was virtually absent from the Cre-transduced cells, while 95% of control virus transduced cells were still Brg1+ (data not shown). Importantly, Cre-transduced, Brg1 cKO cells largely failed to generate any DCX+ neurons (Figures 4B and 4D), in agreement with the above observed results that Brg1 is necessary for endogenous neurogenesis. Moreover, Pax6 over-expression did not induce neurogenesis in Brg1 cKO cells (cotransduced with CreIRESGFP virus), in contrast to the control situation where Pax6 transduction induced about 70% DCX+ neurons (Figure 4D). Importantly, the full-length form of Brg1, but not the ATPase deficient form (Brg1KS), restored both endogenous and Pax6-induced neurogenesis (Figure 4E), demonstrating that Brg1 needs to be catalytically active to mediate neurogenesis. The requirement of Brg1 for neuro-genesis is rather specific for Pax6, as transduction with the neurogenic factor Ngn2 resulted in very efficient induction of neurogenesis to over 90% even in the absence of Brg1 (Figures 4C and 4D). Thus, Pax6 and Brg1 are not only required for endogenous OB neurogenesis, but also, neurogenesis forced by Pax6 overexpression requires the presence of catalytically active Brg1 and hence chromatin remodeling activity.

Identification of a Neurogenic Transcriptional Network Downstream of Pax6 and Brg1

Given the similarity of the phenotypes after deletion of either Pax6 or Brg1 in adult NSCs of the SEZ and functional interaction in forced neurogenesis, we next asked to which extent this is also reflected at the transcriptional level. First, we examined randomly selected genes, found to be downregulated in the core of the OB or the SEZ of Brg1 cKO mice, in the OB and SEZ of Pax6 cKO mice. Consistent with the similarity at the phenotypic level, 90% of these genes downregulated after loss of Brg1 were also downregulated after loss of Pax6 (Figure 4F and data not shown), in agreement with the observation that the majority of these genes possess Pax6-binding sites. This provided us with an opportunity to search for genes implement-ing neurogenesis. We therefore searched for transcriptional regulators downregulated upon Brg1 depletion with a common regulatory motif including Pax6. This was the case for 11 of the 17 downregulated transcription factors (Figure 4G; FrameWorker, Genomatix, Germany) and only for 4 of these a specific consensus DNA binding sequence was described (Sox11, Sox4, Pou3f4, and Nfib). Indeed, all these are expressed in the SEZ and RMS (Figures S5G–S5J) and their expression is downregulated in both Brg1 cKO and Pax6 cKO OB (Figure 4H).

This reduction was relevant at protein levels; for example, Nfib was present in virtually all neuroblasts in the RMS, but upon loss of Pax6 (GFP+ cells in Pax6 cKO 60 dpt) only 17% of recom-bined, GFP+ neuroblasts expressed Nfib (Figure S5K–S5M).

Pax6 was also sufficient to induce Sox11 and Pou3f2/4 expression within 24 hrs in neurosphere-derived cells (Figure 4I) in a Brg1-dependent manner (Figures 4J–4L). Interestingly, the expression of Sox11, Nfib, and Pou3f4 was highest in neuro-blasts expressing the highest levels of Pax6 (Figure 4M). Thus, the regulation and expression of these transcription factors is consistent with a role downstream of the Pax6-Brg1 complex in neuroblasts.

Most importantly, these downstream transcription factors were also predicted to cross-regulate each other, thus poten-tially forming a self-sustaining cross-regulatory network critical for neurogenic fate maintenance in the adult brain. To test this we first examined if each of these factors could indeed induce/increase expression of the respective others. Indeed, Sox11 overexpression in adult neurosphere-derived cells increased mRNA for Nfib, and Pou3f4 and Pou3f2 overexpression increased Sox11, Pou3f4, and Nfib, but none of these increased Pax6 mRNA levels (Figure 4I), consistent with the concept of a downstream cross-regulatory transcriptional network. Chro-matin-immunoprecipitation (ChIP) followed by quantitative PCR (qPCR) further demonstrated not only Pax6 binding in the promoter regions of Sox11, Nfib, and Pou3f4, but also binding of the respective other members of this network (Figures 5A–5C). Moreover, ChIP-seq experiments identified Brg1 bind-ing in the promoters of Sox11, Pou3f4, and Nfib (Figure 5D) in NSCs isolated from E10.5 embryos. These data further support the concept that Pax6 binds in close interaction with a Brg1-con-taining BAF complex in the promoter regions of each member of this cross-regulatory network.

As a further test for Sox11, Pou3f4, and Nfib acting as a cross-regulatory neurogenic network initiated by Pax6 interacting with Brg1-containing chromatin remodeling complexes, we exam-ined to which extent neuronal specification and differentiation genes downregulated after Brg1 depletion contain a regulatory motif composed of SoxC, Pou3f4, and Nfib binding sites. Inter-estingly, a total of 65% of genes downregulated in the Brg1 cKO have binding sites for Nfib, Sox11, and Pou3f4 with conserved distance and orientation (25%) or a regulatory module containing at least two of them (40%, Figure S6A). Sox11, Nfib, and Pou3f4 themselves are among these regulatory-module-containing genes (Figure S6A), further supporting the validity of this motif analysis because these bindings have been confirmed by ChIP-qPCR (Figures 5A–5C). Other than these, however, the set of SoxC/Pou3f4/Nfib binding genes does not comprise

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(F) Histogram depicting downregulation of genes following Brg1 or Pax6 deletion. Gene expression in the mutant and WT is normalized to the GAPDH.

(G) Venn diagram depicting transcription factors downregulated in Brg1 cKO and harboring Pax6 binding sites in their promoters.

(H) Histogram showing the downregulation of Pou3f4, Sox11, and Nfib in the OB and SEZ following Brg1 and Pax6 deletion.

(I) Histogram depicting the Pax6-mediated induction of the expression of Pou3f4 and Sox11 in neurosphere cells 24 hr after transfection.

(J and K) Micrographs depicting deletion of Brg1 in neurosphere cells 36 hr after nucleofection of corresponding plasmid.

(L) Histogram depicting the Pax6-mediated induction of the expression of Pou3f4, Sox11, and Nfib in control or Brg1 cKO neurosphere cells 24 hr after Pax6 nucleofection.

(M) Histogram depicting the relative expression of Pou3f4, Sox11, and Nfib in the FACS-purified populations enriched in NSCs and their progeny.

Scale bars: 100 μm in (A)–(C), (J), and (K). Data in (D), (E), (H), (I), and (L) are shown as mean ± SEM and n(animals) ≥ 3 and in (M) as mean n(animals) ≥ 3. **p ≤ 0.01; ***p ≤ 0.005.
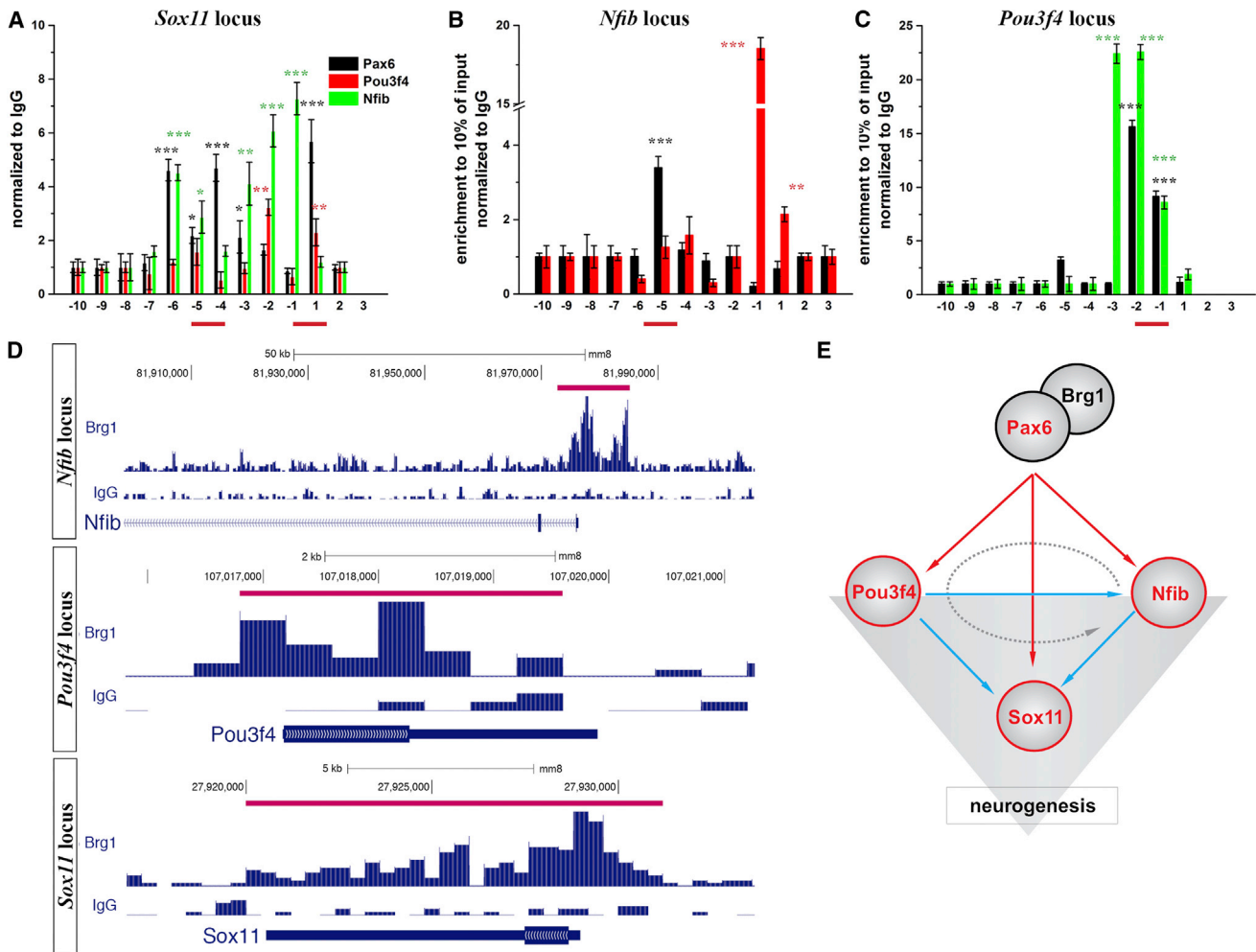
Figure 5. Identification of the Core Regulatory Network Downstream of Pax6-Brg1 Complex Necessary for Neurogenesis

(A–C) Histograms depicting ChIP signals of Pax6, Pou3f4, and Nfib. x axis indicates the position in the promoters in kb starting from the TSS and the underlined position contained the predicted Pax6 binding site. Data are shown as mean ± SEM and n(experiments) ≥ 5.

(D) Genome browser snapshot containing the Brg1 ChIP-seq signals.

(E) Scheme depicting cross-regulatory loop regulated by Pax6-Brg1 complex.

See also Figures S5 and S6, Table S1, and Table S2.

further regulatory transcription factors, but rather comprise effector molecules involved in neuronal migration and differentiation or interaction to extracellular matrix. These data therefore suggest that Sox11, Pou3f4, and Nfib act as a cross-regulatory transcriptional network downstream of Pax6/Brg1-containing complexes regulating a multitude of effector genes involved in neuronal differentiation and migration, thereby implementing and stabilizing the initial fate (Figure 5E).

The Function of BAF-Pax6 Complex Is Necessary to Maintain the Core Network in the Neuroblasts

The above concept suggests an effector network stabilizing neuronal fate in neuroblasts or late stages of TAPs, when Pax6 and the downstream effectors are detectable by immunostaining. Moreover, upon deletion of either Brg1 or Pax6, conversion to gliogenesis occurred in the RMS and core of the OB, i.e., regions mostly composed of neuroblasts. However, we had

also observed ependymal cells in the SEZ emerging after Brg1 deletion in NSCs, consistent with a possible direct conversion of NSCs into this fate upon Brg1 deletion. To further test fate conversion after Brg1 deletion at later stages in the lineage, we used MLV-based retroviruses to transduce only fast dividing TAPs and neuroblasts with Cre as previously shown (Colak et al., 2008). Transduced cells were analyzed 21 days after stereotactic virus injection, allowing sufficient time for the transduced progenitors to differentiate in the OB. While 90% of control-virus-infected cells had arrived in the OB and differentiated into DCX or NeuN+ neurons with the typical morphology of granule cells (Figure 6A, 6B, and 6E–6G and Figure S6B), cells transduced with the Cre-containing virus were mostly located outside the OB next to the RMS in the cortical WM or the striatum (Figure 6C, 6E, and 6F). Accordingly, most Cre-transduced cells were Olig2+ or NG2+ glia located outside the SEZ and the RMS (Figure 6F, 6H, and 6I) and only a few, mostly located in the

superficial GCL, a neuronal population spared by Brg1 deletion described above, expressed DCX (Figure 6F and data not shown). Interestingly, Olig2 expression already started in cells within the RMS (Figure 6H and 6I), suggesting that the fate conversion upon Brg1 deletion starts in the RMS in agreement with transcriptional changes in neuroblast genes (Figure 3A and Figure S4). Interestingly, a significant (40%) proportion of the Cre-transduced Brg1 cKO cells remained in the SEZ even 21 days after the transduction. These were nonproliferative (Ki67−, data not shown) astrocytes (GFAP+) or CD24+ ependymal cells at the ventricular lumen (Figure 6K–6M). Importantly, the latter were never observed among control transduced cells (Figure 6M), indicating that Brg1 deletion in fast proliferating cells integrating the MLV virus results in conversion to ependymal cells in the SEZ, i.e., the reverse of ependymal cell-neuroblast conversion after stroke injury (Carlén et al., 2009). Taken together, these experiments indicate that the Pax6-BAF complex is required at later stages in the NSC lineage for neuroblast fate maintenance, and its absence in these progenitors results in conversion to the glial lineage as they fail to upregulate the cross-regulatory effector network.

A Minimal Neurogenic Network Is Sufficient for Forced Neurogenesis and Is Independent of Brg1

If Sox11, Nfib, and Pou3f4 can indeed function as a neurogenic effector network as suggested by the above loss-of-function experiments, these factors should also be sufficient to replace Pax6 in forced neurogenesis in gain-of-function experiments and act independent of Brg1, as expected for factors downstream of Pax6 and Brg1. We tested these predictions first in adult neurosphere-derived cells, which already express Nfib (Figures S7A and S7B), by introducing Sox11 and/or Pou3f4/Pou3f2. Indeed, transfection with Sox11 increased the proportion of transduced cells differentiating into neurons 6-fold (30% DCX+ cells; 5% after transfection with control dsRed plasmid, see also Haslinger et al., 2009; Mu et al., 2012), and Pou3f2 (as well as Pou3f4, data not shown) was even more efficient with an increase of about 12-fold more neurons (about 60% DCX+ cells; Figure 7A). Cotransfection of both Sox11 and Pou3f2 elicited neurogenesis in 75% of transduced cells, a proportion not significantly different from the neurogenesis elicited by Pax6 overexpression (Figure 4D). Moreover, Sox11 and Pou3f2 could still induce and enhance neurogenesis in the absence of Brg1 after cotransduction of Cre into Brg1fl/fl neurosphere cells (Figure 7A), in pronounced difference to Pax6 (Figure 4D). However, their function was critically dependent upon the presence of the other members of the core regulatory network, because genetic deletion of both Sox4 and Sox11 simultaneously or knockdown of Pou3f2, Pou3f4, or Nfib (Figures S7C and S7D) significantly reduced neurogenesis of neurosphere cells (Figure 7B). Likewise, deletion of Sox4 and Sox11 or knockdown of Pou3f2 interferes with forced neurogenesis upon transduction with Pax6, Sox11, or Pou3f2 (Figure 7C). These data further substantiate the concept of the cross-regulatory effector network, because the other two members (endogenously expressed Nfib or overexpressed Sox11 or Pou3f2) were not sufficient to instruct neurogenesis in the absence of the third member. We therefore conclude that the cross-regulatory transcriptional network of SoxC, Pou3f, and Nfib is sufficient and necessary

to achieve equal levels of neurogenesis in the absence of Brg1, consistent with its function downstream of this initiator complex.

Lastly, we examined to which extent this network may have a broader relevance for reprogramming postnatal glial cells, which would not generate neurons endogenously. Mixed glial cultures from the postnatal cerebral cortex were cultured for 7 days and infected with MLV-based viral vectors encoding for Pax6 or Sox11 or Pou3f2 (because again, Nfib was found to be expressed in these cells endogenously), and we examined the transduced cells 7 days later. As expected, virtually no DCX+ neurons were observed among control-virus-infected cells (Figures 7D and 7F), while Pax6 was sufficient to instruct neurogenesis in 40% of all transduced cells (Figures 7E and 7F). Strikingly, the combination of Sox11 and Pou3f2 was at least as efficient in instructing the majority of glial cells toward neurogenesis (Figure 7F), demonstrating that these factors are indeed able to instruct neuronal differentiation also in glial cells.

DISCUSSION

Here, we demonstrate a role of chromatin remodeling in conjunction with a specific neurogenic fate determinant, Pax6, in neuronal fate commitment in the adult mouse brain in vivo. We showed a physical interaction between Pax6 and a Brg1-containing BAF complex and the functional requirement of either factor for adult neurogenesis. If only one of these is missing, neuroblasts originating in the SEZ differentiate into glial cells in the SEZ, en route to the OB, or even after completing the normal migration and reaching the OB. Intriguingly, the conversion occurs largely to NG2 glia in the OB and parenchymal regions surrounding the RMS, while many ependymal cells are generated within the SEZ. This is of particular interest in light of signals inhibiting oligodendrogliogenesis in this region (Colak et al., 2008) and in regard to the finding that even fast proliferating cells infected with the MLV-Cre virus can differentiate into ependymal cells when not able to complete their neurogenic fate.

Importantly the loss of Brg1 affects with a striking specificity Pax6-mediated neurogenesis. The specificity of this function is particularly intriguing, as Brm, the other ATPase subunit of BAF complexes, is expressed at even higher levels than Brg1 in neuroblasts (Figures S7E and S7F), but is obviously not able to compensate for the loss of Brg1. Thus, not only individual BAF subunits (Lessard et al., 2007; Yoo et al., 2009) convey specificity to a BAF complex, but also the respective ATPase subunit. This concept is further substantiated by specific defects upon Brg1 deletion in glial cell differentiation (Limpert et al., 2013; Weider et al., 2012). Interestingly, Brg1 plays an important role in oligodendrocyte differentiation in early postnatal development and interacts with the transcription factor Olig2 in this context (Yu et al., 2013), but its virtual absence in Olig2+ cells in the adult forebrain suggests an intriguing difference between the molecular mechanisms acting at these different stages.

Indeed, the specific function of Pax6-Brg1/BAF complex in neurogenesis also appears to be specific for the adult SEZ lineage because the phenotypes caused by Brg1 or Pax6 deletion in the developing telencephalon are rather different from each other (Götz et al., 1998; Heins et al., 2002; Lessard et al., 2007; Matsumoto et al., 2006). Despite the coexpression of Pax6 and Brg1 and neurogenic function of Pax6 in this region
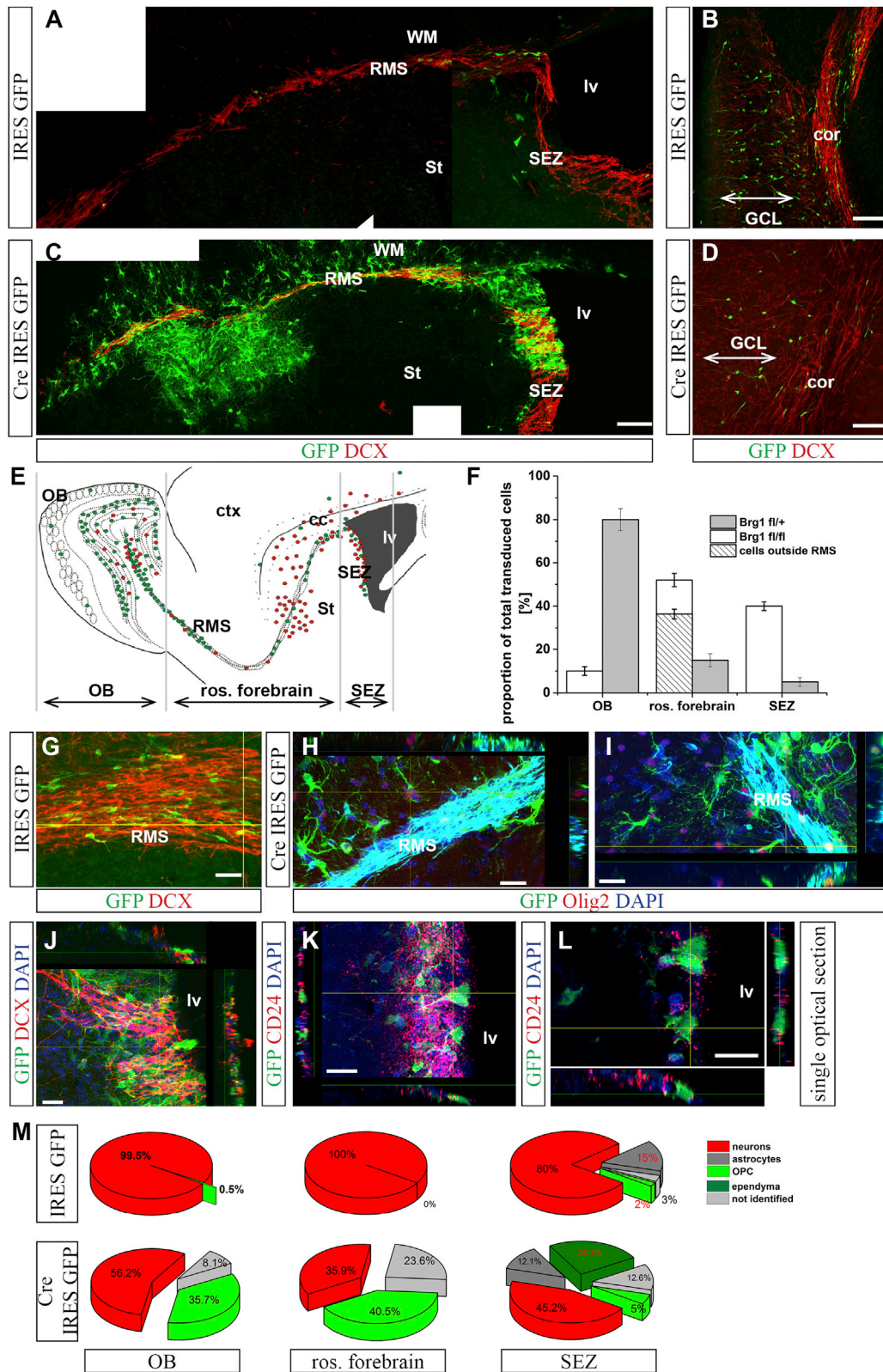
(Götz et al., 1998; Heins et al., 2002), deletion of Brg1 at early developmental stages does not affect neurogenic fate acquisition, but rather affects stem and progenitor cell proliferation (Matsumoto et al., 2006). This raises the intriguing suggestion that this interaction may have specific functions in adult neurogenesis that are not required in the developing brain. We therefore propose that mechanisms for neuronal fate stabilization are particularly relevant in niches where gliogenesis is more prevalent. This concept is further substantiated by the relevance of these factors in reprogramming glial cells into neurons.

Maintenance of Neurogenic Fate in the Adult Brain by a Cross-Regulatory Neurogenic Network

One of the major differences between the developing and adult brain is the prevalent neurogenesis in the former while gliogenesis largely dominates in the latter. Indeed, transplantation of many NSCs into the adult brain parenchyma results in their conversion to gliogenesis, whereas they readily generate neurons in the developing brain (for review see Ninkovic and Götz, 2013). Thus, neuroblasts face particular challenges in the adult brain to not convert to gliogenesis. Moreover, while the stem cells in the developing telencephalon, the radial glial cells, express high protein levels of neurogenic factors such as Pax6, adult NSCs express these only at lower mRNA levels and need to reach high protein levels when progressing further along the lineage (Beckervordersandforth et al., 2010; Feng et al., 2013). Indeed, our analysis shows that these two challenges can be overcome by Pax6 interacting with a Brg1/BAF chromatin remodeling complex and activating a cross-regulatory neurogenic transcriptional network required for stabilizing neurogenic fate in the adult brain. First, we have shown that lack of either Pax6 or Brg1 results in conversion of SEZ-derived cells toward different glial cells, depending on their local environment as described above. But even after isolation in vitro, SEZ cells lacking Brg1 or Pax6 can no longer complete a neurogenic fate, suggesting that their intrinsic neurogenic factors inherited from NSCs are not sufficient to prompt further progress along the neurogenic lineage outside this niche. This demonstrates the critical role of Pax6-Brg1/BAF complex activity in increasing the levels of neurogenic fate determinants. Notably, this requires a catalytically active form of Brg1, demonstrating that chromatin remodeling is indeed critical for this step.

Here we discovered the molecular basis of Pax6/Brg1 complex-mediated neurogenic fate maintenance by enhancing the expression of neurogenic transcription factors forming a cross-regulatory network. These transcription factors cross-regulate each other and regulate neuronal effector genes. Importantly, each member of this cross-regulatory network consisting of SoxC, Pou3f, and Nfib factors is critical for neurogenesis. Previous work has already shown that SoxC factors are necessary for neurogenesis (Mu et al., 2012), and their regulation depends also

on a chromatin remodeler interacting with Sox2 and Chd7 (Feng et al., 2013). Interestingly, Chd7 is also downregulated upon Pax6 or Brg1 deletion, further supporting the concept that Pax6/BAF complex acts as an upstream regulator of these. In addition we demonstrate here that also lowering the levels of Pou3f or Nfib interferes with neurogenesis, demonstrating the key role of each member of the cross-regulatory network to achieve full neurogenesis. This cross-regulatory network then activates genes mediating neuronal differentiation because a large proportion of neuronal differentiation genes possess a motif for either all three or at least two of these factors, such as DCX, Tubb2b, CD24, and Prokr2.

Thus, our data suggest the following sequence of events (Figure S6C). Initiating neurogenic fate is mediated by factors such as Pax6 that allow for altering the chromatin state via recruitment of a chromatin remodeling complex, such as the BAF complex. This Pax6-BAF complex then activates a cross-regulatory transcriptional effector network sufficient to maintain the high expression of genes involved in neuronal differentiation, thereby executing the lineage decision. At this later stage, lineage commitment can occur independent of Brg1 as demonstrated by the expression of SoxC and Pou3f in cells lacking Brg1. Notably, genes controlled by the cross-regulatory network have a Pax6 binding site in addition to the Sox-Nfib-Pou3f regulatory motif, compatible with the idea that Pax6-Brg1 complex might be important for making these loci accessible for high-level expression that can then, once Nfib, Pou3f, and SoxC are expressed at sufficiently high levels, be exerted independent of the initial role of Pax6 and Brg1 (Figure S6C). Indeed, the deletion of Brg1 in more committed TAPs and neuroblasts resulted in the switch to the glia cells, adopting either NG2 glia or ependymal glial fates. Consistent with this model, reduction of some members of this cross-regulatory network results in defects of adult SEZ neurogenesis as detailed above. Ultimately, this work has unraveled a highly specific interaction of Pax6 with a chromatin remodeling complex, consistent with the role of pioneer transcription factors (Zaret and Carroll, 2011), and has further elucidated the molecular logic of neurogenesis and neuronal fate stabilization by illuminating a cross-regulatory effector network.

EXPERIMENTAL PROCEDURES

Animal Experiments

Viral Constructs Virus Production and Stereotactic Injections

A CAG-dsRed vector (Heinrich et al., 2010) was used for fate mapping experiments, and Split-Cre constructs for the stem-cell-specific recombination (Beckervordersandforth et al., 2010) and viral particles were produced as previously described (Beckervordersandforth et al., 2010; Hack et al.,

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Figure 6. Brg1 Function Is Necessary in the TAPs and Neuroblasts

(A–D) Composite images of cells transduced with control (A and B) and Cre-expressing virus (C and D) 21 days after injections of the virus in the SEZ of Brg1 floxed animals. (B) and (D) are images of cells settled in the OB.

(E) Scheme depicting the position of control-virus- (green dots) and Cre-virus- (red dots) transduced cells 21 days after stereotactic injections.

(F) Histogram depicting the distribution of transduced cells 21 days after injection. Data are shown as mean (three animals) ± SEM.

(G–M) Micrographs and pies (M) depicting the identity of transduced cells after 21 days.

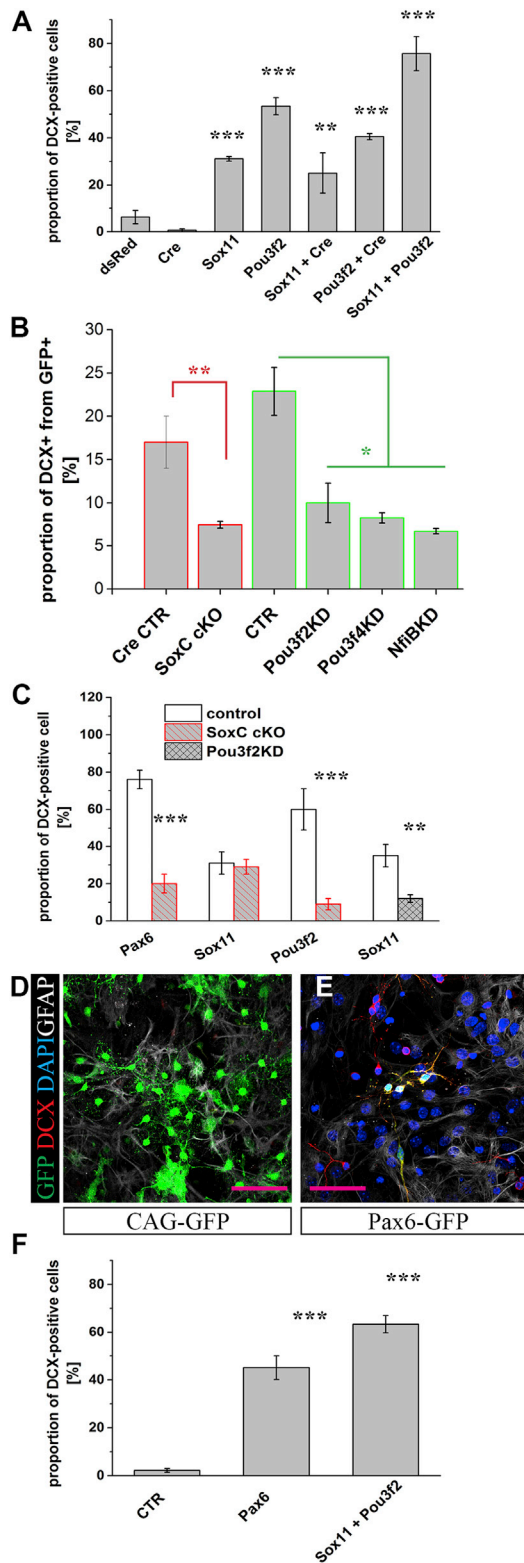Scale bars: 100 μm in (A)–(D) and 20 μm in (G)–(L). Abbreviations: cor, core of the OB; and see Figure 1.

Figure 7. Cross-Regulatory Loop Genes Induce Neurogenesis from Glia Cells Independent of Pax6-Brg1 Complex

(A and B) Histograms depicting the proportion of neurons generated after forced expression (A) or loss of function (B) of cross-regulatory loop genes in cells derived from neurospheres after 7 day in vitro.

2005). The stereotactic injections were done as previously described (Beckervordersandforth et al., 2010).

Histology and Immunostaining
Histological analysis was done according to the manufacturer protocols for the specific antibodies. The detailed protocols are presented in the Supplemental Experimental Procedures.

FACS and mRNA Profiling
FACS of the particular neuronal populations and the mRNA profiling was performed as previously described (Beckervordersandforth et al., 2010; Fischer et al., 2011).

ChIP Assays
The qChIP experiments were performed as described elsewhere (Cuddapah et al., 2009; Lee et al., 2006) with minor modifications described in the Supplemental Experimental Procedures.

Quantitative Analysis
Quantifications (proportions of reporter+ cells among particular cell types) were done using ImageJ software to analyze confocal z stacks ensuring that each cell would be counted separately and only once. At least 10 (per animal) corresponding sections in compared animals were sampled and analyzed at different medio-lateral positions to cover the entire extension of the OB or SEZ, and the number of animals is indicated for every experiment. All results are presented as mean and standard error of the mean. Statistical analysis was performed in Microcal Origin 7.5 using ANOVA and Mann-Whitney U test to test for significance.

Detailed experimental procedures are available in the Supplemental Experimental Procedures on line.

ACCESSION NUMBERS

Array data have been submitted to Gene Expression Omnibus (GEO) under the accession number GSE39362.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes seven figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.07.002.

(C) Histogram depicting the proportion of neurons generated after forced expression of Pax6 or core regulatory network members in SoxC- and Pou3f2-deficient neurosphere cells after 7 days in vitro.
(D and E) Micrographs depicting immunoreactivity of cells derived from glia cultures enriched for OPCs after overexpression of Pax6 (E) and control vector (D) for 7 days in vitro. (F) Histogram depicting the proportion of neurons generated from the postnatal glia after the overexpression of Pax6 or its downstream targets.
Data in (A)–(C) and (F) are shown as mean ± SEM and n(independent experiments) ≥ 7. **p ≤ 0.01; ***p ≤ 0.005. Scale bars: 100 μm. See also Figure S7.

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