The Chromatin Remodeler CHD7 Regulates Adult Neurogenesis via Activation of SoxC Transcription Factors

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SUMMARY

Chromatin factors that regulate neurogenesis in the central nervous system remain to be explored. Here, we demonstrate that the chromatin remodeler chromodomain-helicase-DNA-binding protein 7 (CHD7), a protein frequently mutated in human CHARGE syndrome, is a master regulator of neurogenesis in mammalian brain. CHD7 is selectively expressed in actively dividing neural stem cells (NSCs) and progenitors. Genetic inactivation of CHD7 in NSCs leads to a reduction of neuronal differentiation and aberrant dendritic development of newborn neurons. Strikingly, physical exercise can rescue the CHD7 mutant phenotype in the adult hippocampal dentate gyrus. We further show that in NSCs, CHD7 stimulates the expression of Sox4 and Sox11 genes via remodeling their promoters to an open chromatin state. Our study demonstrates an essential role of CHD7 in activation of the neuronal differentiation program in NSCs, thus providing insights into epigenetic regulation of stem cell differentiation and molecular mechanism of human CHARGE syndrome.

INTRODUCTION

Epigenetic regulations are essential for the maintenance of cell identity and the guidance of stepwise cell differentiation. Mutations in epigenetic regulators are linked to many human diseases, including cancer and mental retardation (Berdasco and Esteller, 2010; Jakovcevski and Akbarian 2012). As one family of chromatin regulators, ATP-dependent chromatin remodelers utilize the energy from ATP hydrolysis to slide nucleosomes, dissociate core histones, or relocate the entire histone octamers (Li et al., 2007). The dynamic change of nucleosome occupancy at gene promoters provides temporal control of transcription. Chromodomain-helicase-DNA-binding protein 7 (CHD7) belongs to the CHD family of chromatin remodelers. CHD proteins are involved in the regulation of multiple biological processes, including chromatin structure reorganization and gene expression (Hall and Georgel, 2007). Importantly, de novo heterozygous mutations of the CHD7 gene are the major cause of the human CHARGE syndrome, a genetic disease characterized by a complex constellation of birth defects (coloboma of the eye, heart defects, atresia of the choanae, severe retardation of growth and development, genital abnormalities, and ear abnormalities) (Vissers et al., 2004). It has been shown that CHD7 cooperates with another chromatin remodeling complex PBAF (polybromo- and BRG1-associated factor-containing complex) to regulate neural crest migration, implicating its role in the peripheral system (Bajpai et al., 2010). Interestingly, most of the CHARGE patients have mental retardation and olfactory anomalies ranging from absence to hypoplasia of the olfactory bulbs (OBs) (Blustajn et al., 2008; Vissers et al., 2004), suggesting that the corresponding neurogenic systems are impaired in those patients. A recent study identified CHD7 as a transcriptional cofactor of the essential NSC regulator Sox2 in NSCs using both proteomic and genomic approaches (Engelen et al., 2011), suggesting a role of CHD7 in neurogenesis. However, the function of CHD7 in mammalian neurogenesis and the molecular mechanism underlying its role in the human CHARGE syndrome remains largely unknown.

The subventricular zone (SVZ) of the lateral ventricle (LV) and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus are the major germinal zones of active neurogenesis during adulthood in the mammalian central nervous system (CNS) (Alvarez-Buylla and Garcia-Verdugo, 2002; Gage, 2000). Astrocyte-like type B cells in the adult SVZ are multipotent neural stem cells (NSCs) (Doetsch et al., 1999). These cells give rise to transit amplifying type C cells, which in turn differentiate into type A cells (neuroblasts) that migrate to the OB through the rostral migratory stream (RMS) (Alvarez-Buylla and Garcia-Verdugo, 2002). NSCs in the SGZ are also astrocyte-like cells with their cell bodies residing in the SGZ and their radial processes extending into the granular layer (GL) of the DG (Ming and Song, 2005). The SGZ NSCs mainly give rise to newborn granule cells that are integrated into GL. The adult neurogenic system has been used to study many human neurological-disease-related genes, in particular their roles in regulating NSC self-renewal, differentiation, and maturation of newborn neurons (Zhao et al., 2008). Molecularly, adult neurogenesis represents

a robust process involving sequential regulation of crucial transcription factors important for cell-fate commitment (Suh et al., 2009). Epigenetic regulation is proposed to be actively involved in regulation of this process (Ma et al., 2010). As a group of important epigenetic regulators, the roles of chromatin remodelers in adult neurogenesis remain largely unknown.

To gain insights into the mechanism of chromatin remodelers in regulating adult NSCs, we investigated the function of CHD7 using mouse genetic approaches. Here, we demonstrate that CHD7 expression is specifically enriched in active NSCs and progenitors in the SVZ and SGZ. A NSC-specific inactivation of CHD7 in adult mice leads to a dramatic decrease of neurogenesis. Strikingly, physical exercise can rescue the hippocampal neurogenesis defect in CHD7 mutants. We further show that loss of CHD7 in NSCs represses the expression of *Sox4* and *Sox11* genes by inducing chromatin condensation of their promoters. And, forced expression of Sox4 and Sox11 in CHD7 mutant NSCs can rescue the neuronal differentiation defect. In summary, our study reveals that the CHARGE syndrome protein CHD7 is a master regulator of governing the neurogenic potential of NSCs. Rescue of the CHD7 mutant phenotype via physical exercise indicates an alternative pathway to overcome CHD7 deficiency, demonstrating the advantage of using adult NSCs to study human brain disease causing genes. These results provide significant insights into the molecular mechanism of chromatin regulation of NSCs fate commitment, which also provide implication to molecular pathogenesis of CHARGE syndrome.
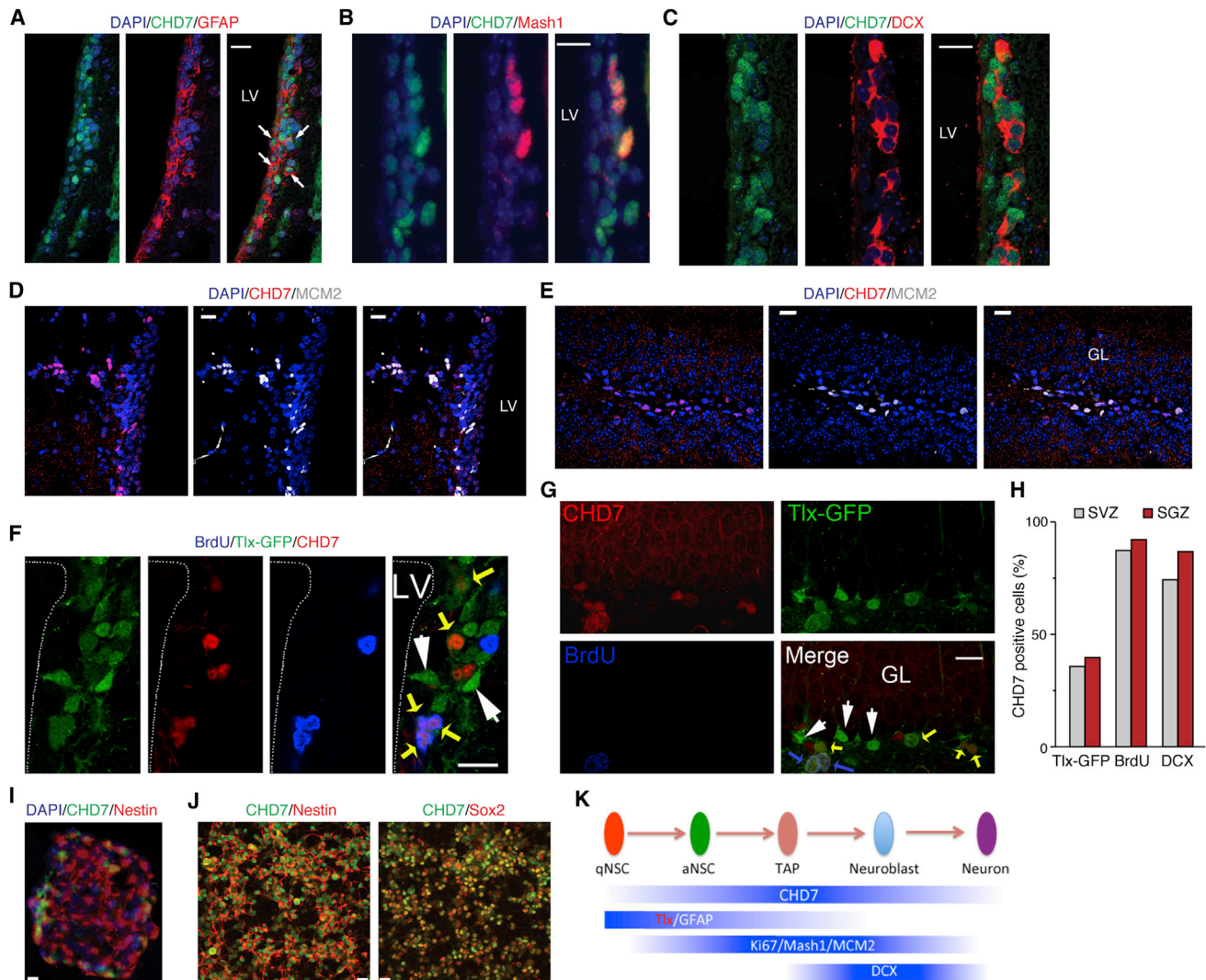
RESULTS

CHD7 Expression Is Enriched In Neurogenic Niches of the Adult Mouse Brain

We first examined the expression of CHD7 in adult mouse brain, particularly in adult NSCs, which to our knowledge has not been reported yet. Data from the Allen Brain Atlas show that the messenger RNA (mRNA) of the *CHD7* gene is highly expressed in adult neurogenic regions of the mouse brain, i.e., in the SVZ, RMS, and SGZ (Figure S1A available online). Consistent with the expression of the mRNA, immunostaining assays show that CHD7 protein is present in both the SVZ and SGZ cells (Figures S1B and S1C). To identify the CHD7-expressing cell population in the above-mentioned regions, we performed coimmunostainings of CHD7 with various cellular markers. Glial fibrillary acidic protein (GFAP) is a marker for astrocyte-like type B cells, namely, the NSCs in the SVZ (Doetsch et al., 1997), whereas Mash1 and DCX (doublecortin), respectively, mark type C cells, i.e., the transit amplifying cells (Parras et al., 2004), and type A cells, the neuroblasts. We found that some of the GFAP-positive cells express CHD7 (Figure 1A), indicating that CHD7 is expressed in a subpopulation of SVZ NSCs. Most of Mash1-positive or DCX-positive cells express CHD7 (Figures 1B and 1C), demonstrating that CHD7 is expressed in most of type C and A cells. We next analyzed the proliferation state of CHD7-expressing cells by costaining of CHD7 with a proliferation marker MCM2 (minichromosome maintenance complex component 2). In both the SVZ and SGZ, CHD7 colabels with MCM2 in most of the cells (Figures 1D and 1E), demonstrating that CHD7 is expressed in most fast-dividing cells. To further determine the expression of CHD7 in the adult NSCs, we made use of a trans-

genic mouse in which GFP expression is under the control of the Tlx BAC-based promoter. Tlx has been shown to be specifically expressed in NSCs, i.e., type B cells in the SVZ and type 1 cells in the SGZ (Liu et al., 2008; Niu et al., 2011). Our analyses demonstrate that the expression of Tlx-GFP recapitulates endogenous Tlx expression (Figures S1D–S1F). Adult Tlx-GFP mice were injected with bromodeoxyuridine (BrdU) 30 min before sacrifice in order to visualize dividing cells in neurogenic regions. Interestingly, most of Tlx-GFP^high cells in both the SVZ and SGZ were negative for BrdU (Figures 1F and 1G, white arrows), suggesting that they are in a quiescent state. In support of this finding, we observed that Tlx-GFP^high cells express NSC markers Nestin and GFAP, but not proliferation markers Ki67 and MCM2 (Figures S1G–S1I, arrows). Costaining of CHD7 with GFP and BrdU in these animals shows that some Tlx-GFP-positive cells express CHD7, and many of these colabeled cells were BrdU positive (Figures 1F and 1G, yellow arrows; Figure 1H). In contrast, most Tlx-GFP^high cells did not express CHD7 (Figures 1F and 1G, white arrows). Moreover, CHD7 is coexpressed with Nestin and Sox2 in both cultured neurospheres and monolayer NSCs (Figures 1I and 1J). These data reveal that NSCs start to express CHD7 upon exiting the quiescent state, and the expression of CHD7 persists in neural progenitors and neuroblasts (Figure 1K). This intriguingly selective expression pattern of this chromatin remodeler suggests that CHD7 is probably involved in a temporal regulatory program during NSC activation, lineage commitment, and progression.

Loss of CHD7 Leads to Reduction of Neurogenesis in the Adult SVZ

To investigate the function of CHD7 in adult neurogenesis, a CHD7 conditional knockout (KO) mouse line (CHD7^fl/fl) was established in which the exon 3 is flanked by two loxP sites (Figure S2A). We have previously generated a Tlx-CreERT2 mouse line using a BAC-mediated transgenic approach, where the Cre recombinase is only expressed in type B cells of the adult SVZ (Liu et al., 2008). The CHD7^fl/fl mice were crossed with Tlx-CreERT2 mice to achieve a tamoxifen (TMX)-inducible mutation of CHD7 in adult SVZ NSCs and their derivatives. Two weeks post-TMX induction (2 wpi), CHD7 was completely removed from the adult mouse SVZ as shown by immunohistochemistry (IHC) assay using a CHD7 antibody (Figure S2B). NSCs in the SVZ mainly give rise to neuroblasts that migrate to the OB and differentiate into mature neurons. To investigate the role of CHD7 in these processes, TMX-treated mice (Tlx-CreERT2; CHD7^fl/fl and the littermate control) were injected with BrdU and traced for 4 weeks. As shown in Figure 2A, there was a dramatic decrease of BrdU-positive cells in the OB of CHD7 mutants. The number of newborn neurons, as shown by costaining of BrdU and a mature neuronal marker NeuN, was significantly decreased (Figure 2B). Consistently, there were less DCX-positive neuroblasts in the OB of CHD7 mutants compared to the control (Figure 2C). Moreover, the CHD7^fl/fl mice were mated to another NSC-specific cre line, Nestin-CreERT2. As shown in Figure S2C, CHD7 is efficiently depleted in Nestin-CreERT2; CHD7^fl/fl mice upon TMX treatment. By applying the same BrdU tracing approach as illustrated in Figure 2A, we observed a similar mutant phenotype in these animals as in the Tlx-CreERT2; CHD7^fl/fl mice (data not shown). It has been

Figure 1. CHD7 Is Highly Expressed in the SVZ and the SGZ of the Adult Mouse Brain

(A) Confocal images of the mouse subventricular zone (SVZ) sections coimmunostained for CHD7 and GFAP. DNA is stained with DAPI (DAPI staining is in blue if not indicated). Arrows mark the CHD7 and GFAP double-positive cells. The scale bar represents 20 μm. (LV, lateral ventricle.)

(B and C) Coimmunostaining of CHD7 and Mash1 (B) or DCX (C) in the SVZ. Scale bar: 20 μm.

(D and E) Coimmunostaining of CHD7 and MCM2 in the SVZ (D) and the subgranule zone (SGZ) (E). Scale bar: 20 μm. (GL, granule layer.)

(F and G) Adult Tlx-GFP mice were injected with BrdU 30 min before being sacrificed. The SVZ (F) and SGZ (G) sections were coimmunostained for CHD7, GFP, and BrdU. Yellow and blue arrows mark Tlx-GFP$^+$/CHD7$^+$ and Tlx-GFP$^+$/BrdU$^+$ cells, respectively, whereas white arrows mark the GFP-high cells that are negative for both CHD7 and BrdU. Scale bar: 20 μm.

(H) Proportion of CHD7-positive cells among Tlx-GFP-, BrdU- (30 min pulse labeling), and DCX-positive cells. At least 1,000 cells were analyzed for each population in the SVZ and SGZ, respectively.

(I) Neurospheres were costained for CHD7 and Nestin. Note that only the Nestin-positive cells that are on the surface of neurospheres express CHD7. Scale bar: 20 μm.

(J) Monolayered NSCs were coimmunostained for CHD7 and Nestin (left panel), or Sox2 (right panel). Scale bar: 20 μm.

(K) A schematic illustration of CHD7 expression in adult neurogenic niches.

See also Figure S1.

proposed that SVZ NSCs are already predetermined to generate a certain subtype of neurons in the OB (Merkle et al., 2007). To analyze whether the decrease of neurogenesis is restricted to certain types of neurons, we performed BrdU costaining with markers (calretinin [CR], tyrosine hydroxylase [TH], and calbindin [CB]) representing different subtypes of interneurons in the OB. As shown in Figure 2D, no preferential loss of certain types of neurons was found in CHD7 mutants, indicating that there is a panneuronal phenotype. We thus assessed whether the self-renewal of NSCs is affected upon CHD7 inactivation. Immunostaining assays did not show any major change in expression of two NSC markers Nestin and Sox2 in the SVZ of CHD7 mutants (Figure S2D), suggesting that the number of NSCs was not altered. We then analyzed the cell proliferation in the
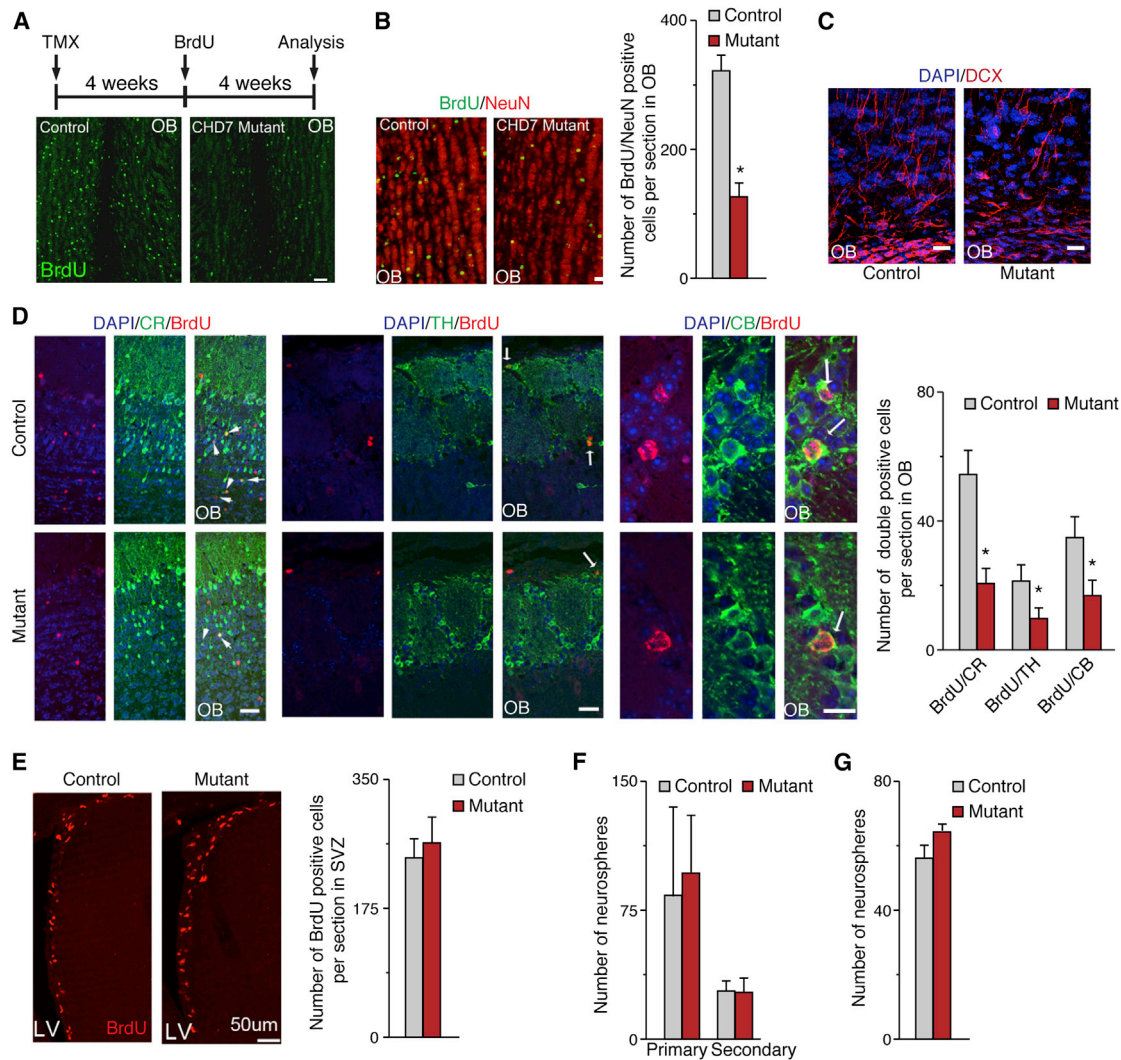
Figure 2. Loss of CHD7 Leads to a Decrease of Neurogenesis in the Adult SVZ

(A) A schematic diagram of the experimental design for experiments shown in (A), (B), (D), and the immunostaining of BrdU in the olfactory bulb (OB). Scale bar: 20 μm. Tamoxifen (TMX)-treated mice (Tlx-CreERT2; CHD7$^{fl/fl}$ and the littermate control) were injected with BrdU once per day for 5 consecutive days.

(B) Costaining of BrdU and NeuN in the OB. Scale bar: 20 μm. The right panel shows the quantification data of the number of BrdU/NeuN double-positive cells. Data are represented as mean ± SD (n = 5; * p < 0.01, Student's t test).

(C) DCX staining of the OB sections from the control and CHD7 mutants (Nestin-CreERT2; CHD7$^{fl/fl}$ and the littermate control, 1 month post-TMX injection). Scale bar: 20 μm.

(D) The OB sections were coimmunostained with BrdU and markers (Calretinin [CR], Tyrosine Hydroxylase [TH], and Calbindin [CB]) representing different subtypes of interneurons in the OB. Arrows mark double-positive cells. Scale bar: 20 μm. Quantification result is shown in the right panel. Value represents mean ± SD (n = 3, * p < 0.05; Student's t test).

(E) BrdU staining of the SVZ from the control and CHD7 mutant mice (Tlx-CreERT2; CHD7$^{fl/fl}$ and the littermate control, 1 month post-TMX injection) sacrificed 2 hr after BrdU injection. Quantification data are shown in the right panel. Value represents mean ± SD (n = 5).

(F) The SVZ cells (Tlx-CreERT2; CHD7$^{fl/fl}$ and the littermate control, 1 month post-TMX injection) were isolated for neurosphere assays. For the primary neurosphere formation assay, cells from one mouse were plated in four wells of one 24-well plate. For the secondary neurosphere formation, 200 cells dissociated from the primary neurospheres were seeded in a single well of one 96-well plate. The number of neurospheres was counted 7 days after seeding. Value represents mean ± SD (n = 4).

(G) Primary neurospheres obtained from (F) were passaged three times. Then 500 disassociated cells were plated into a single well of one 96-well plate and numbers of neurospheres were counted 7 days later. Value represents mean ± SD (n = 4).

See also Figure S2.

SVZ using a 2 hr BrdU-pulse labeling assay. As shown in Figure 2E, there was no significant difference of BrdU incorporation in the control and CHD7 mutants. Analysis with the proliferation marker Ki67 showed similar results (Figure S2E). We next monitored the self-renewal of NSCs using neurosphere formation assay. The number of primary, secondary, and long-term passaged neurospheres formed from the SVZ-derived cells did not significantly differ between the control and CHD7
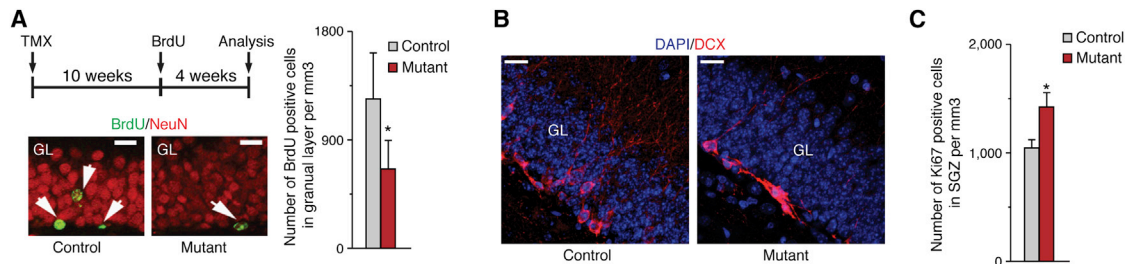
Figure 3. Loss of CHD7 in the Adult SGZ Leads to a Decrease of Hippocampal Neurogenesis
(A) The scheme of BrdU tracing experiment and the dentate gyrus (DG) sections were coimmunostained with BrdU and NeuN. Mice (Nestin-CreERT2; CHD7$^{fl/fl}$ and the littermate control) were injected with BrdU once per day for 10 consecutive days. Scale bar: 20 μm. The number of BrdU$^+$ cells in the GL was quantified as shown in the right panel. Value represents mean ± SD (n = 3, * p < 0.05; Student's t test).
(B) Confocal images of DCX staining in the GL of the control and CHD7 mutant mice (Nestin-CreERT2; CHD7$^{fl/fl}$ and the littermate control; 1 month post-TMX injection). Scale bar: 20 μm.
(C) Quantification of Ki67-postitive cells in the SGZ of the control and CHD7 mutants (Nestin-CreERT2; CHD7$^{fl/fl}$ and the littermate control; 1 month post-TMX injection). Value represents mean ± SD (n = 3, * p < 0.05; Student's t test).
See also Figure S3.

mutants (Figures 2F and 2G). Thus, loss of CHD7 impairs neurogenesis in the SVZ-OB without affecting the self-renewal of NSCs, which indicates that CHD7 is specifically required for the neuronal differentiation of NSCs. Indeed, the number of transit amplifying cells marked by Mash1 was slightly increased in CHD7 mutants (Figure S2E), indicating a possible blockage of these cells for further neuronal differentiation. Moreover, DCX immunostaining showed that the chain-like structure of migrating neuroblasts was not affected (Figure S2F), ruling out a major defect in the migration of neuroblasts. To further confirm the phenotype of CHD7 mutated NSCs observed in vivo, we established a SVZ NSC culture and tested the neuronal differentiation capacity of control and CHD7 mutant cells. In agreement with the in vivo finding, CHD7 mutant cells, both adult and fetal NSCs, generated significantly less Tuj1-positive neurons upon induction of differentiation (Figure S2G), which demonstrates an indispensable role of CHD7 during neuronal differentiation. In the SVZ, the majority of NSCs differentiate into neuronal lineage. The selective expression pattern of CHD7 in adult SVZ and the neuronal differentiation defect in CHD7 mutants suggest that CHD7, as a chromatin remodeler, plays a specific role for initiating the neuronal differentiation program on the chromatin level. These results also help to understand the olfaction defect that is frequently associated in the human CHARGE patient.

Loss of CHD7 Leads to Impairment of Neurogenesis in the Adult SGZ

The adult hippocampal neurogenic system has often been used to study functions of neurological disease genes linked to learning disability (Zhao et al., 2008). Many CHARGE patients have a learning disability (Bergman et al., 2011). The specific expression pattern of CHD7 in the adult SGZ suggests a potential role in regulating the hippocampal neurogenesis. We generated a Nestin-CreERT2; CHD7$^{fl/fl}$ mouse line, which allows us to ablate CHD7 in the SGZ NSCs upon TMX induction (Figure S3A). NSCs in the SGZ generate neuroblasts that give rise to granule cells in the GL. Using the same BrdU tracing approach as used for the SVZ-OB system, we observed that loss of CHD7 in the

SGZ NSCs results in a significant reduction of the number of BrdU-positive newborn neurons in the GL (Figure 3A). Moreover, DCX-positive neuroblasts in CHD7 mutants have shorter and less branched dendrites compared to the control (Figure 3B), suggesting a possible defect of dendritic development of newborn neurons. Both Sox2 and CCND2 have been shown to be essential for the maintenance of the SGZ NSCs (Favaro et al., 2009; Ferri et al., 2004; Suh et al., 2007). By analyzing the RNA extracted from the microdissected hippocampus, we did not observe significant alteration in the expression Sox2 and CCND2 genes in CHD7 mutants as compared to the control (Figure S3B). Intriguingly, we observed a slight increase of Ki67-positive cells and Tbr2-positive transit amplifying cells in the SGZ of CHD7 mutants (Figures 3C and S3C). The accumulation of these cells could be due to a possible blockage of further neuronal differentiation. Thus, like in the SVZ-OB system, depletion of CHD7 in the SGZ NSCs leads to reduction of adult hippocampal neurogenesis.

Ablating CHD7 in NSCs of the two adult neurogenic regions results in similar phenotype of neuronal differentiation defect, suggesting a general role of CHD7 in regulating neurogenesis. In support of this, we observed a similar phenotype by ablating CHD7 in radial glial cells during embryonic brain development (Figures S3D and S3E). Moreover, we analyzed whether loss of CHD7 in NSCs leads to an alternative fate of their progeny. There was no significant change of glial differentiation in all systems analyzed (SVZ, SGZ, and embryonic brain development) (Figures S3F–S3H). Interestingly, we observed an increase of cell death of newborn CHD7 mutant cells (Figures S3I and S3J), suggesting that CHD7 mutant cells may undergo apoptosis if they cannot be properly differentiated.

Physical Exercise Rescues the Defect of Neurogenesis in the SGZ of CHD7 Mutants

Epigenetic regulators are considered to be essential players of regulating cell responses to environmental stimuli (Ma et al., 2010). Interestingly, environmental stimuli can greatly affect the proliferation and survival of newborn cells in the adult CNS. For example, exposure of rodents to an enriched
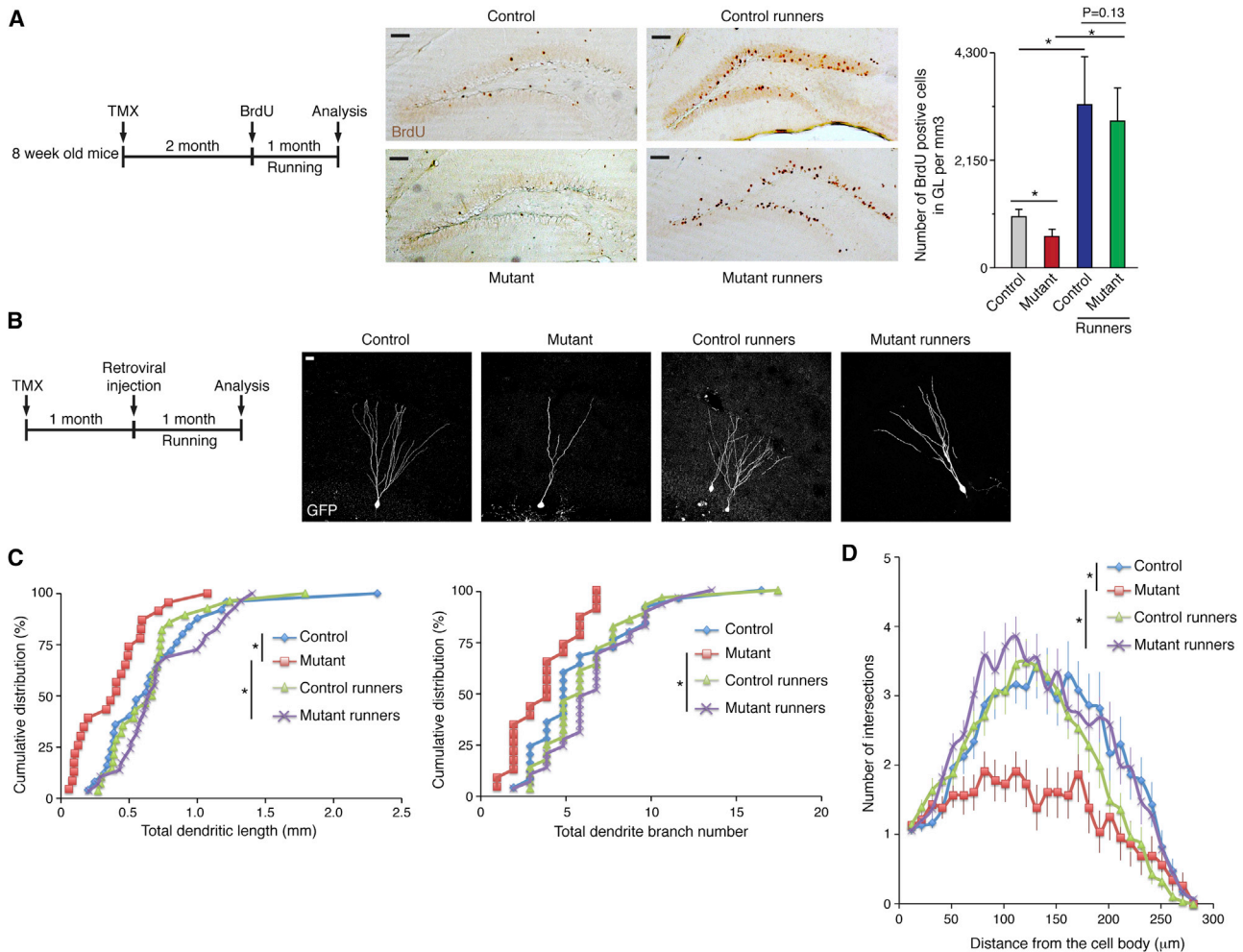
Figure 4. Voluntary Running Rescues the Hippocampal Neurogenesis Defect of CHD7 Mutants

(A) A schematic diagram of the experimental design and IHC staining of BrdU in the GL. Mice (Nestin-CreERT2; CHD7^{fl/fl} and the littermate control) were kept in running cages (only for the runners) and were injected with BrdU once per day for the first 12 consecutive days. Scale bar: 100 μm. The quantification of the number of BrdU^{+} cells is shown in the right panel. Value represents mean ± SD (nonrunners: n = 4; runners: n = 5, * p < 0.05; Student's t test).

(B) A schematic diagram of the experimental design and the representative images of reconstructed newborn neurons in GL with retrovirus-mediated expression of GFP. Scale bar: 20 μm.

(C) Summaries of dendrite properties of newborn neurons. The cumulative distribution plots of total dendrite length (left) and branch numbers (right) are shown. Each symbol represents a single GFP[+] neuron (4–5 mice per group; the number of traced neurons: control: n = 23; mutant: n = 23; control runners: n = 28; mutant runners: n = 29. * p < 0.05, Kolmogorov-Smirnov test).

(D) Sholl analysis of the dendritic complexity of GFP^{+} neurons. The data represent mean ± SEM (same groups of cells as (C) were analyzed; * p < 0.01, Student's t test).

See also Figure S4.

environment increases the survival of newborn neurons in the SGZ without affecting SVZ neurogenesis (Kempermann et al., 1997). Physical exercise, such as voluntary running, promotes SGZ neurogenesis by increasing cell proliferation and survival of the newborn granule neurons (van Praag et al., 1999). Given that CHD7 is expressed in active, but not quiescent, NSCs, and the chromatin remodeler identity of CHD7, we anticipated that CHD7 is maybe involved in regulating exercise-induced hippocampal neurogenesis. To test this, we divided the control and CHD7 mutant mice into nonrunner and runner groups. Running was performed as a voluntary exercise in a running wheel and newborn neurons were labeled with BrdU as illustrated in Fig-

ure 4A. First, we confirmed the reduction of BrdU-positive cells in the GL of CHD7 mutant nonrunners as compared to the control nonrunners (Figure 4A). As expected, one month of running significantly increased the number of BrdU-positive cells in the GL of the control runners as compared to the control nonrunners (Figure 4A). To our surprise, after running, numbers of BrdU-positive cells in CHD7 mutants were not significantly different from the control runners (Figure 4A). Similar results were obtained when older mice were used for this assay (Figure S4A). Moreover, there was no significant difference in the number of Ki67-positive cells between the control and mutant runners (Figure S4B), indicating that running attenuates the

accumulation of proliferating cells in CHD7 mutants (compared to Figure 3C).

The observation that CHD7-mutated neuroblasts have shorter and less branched dendrites indicates a defect of dendritic development (Figure 3B). We therefore monitored the dendritic development of newborn neurons in the control and CHD7 mutants. For this, retroviruses expressing GFP were stereotactically injected into the DG to label newborn cells. The animals were analyzed 4 weeks postretroviral injection, and GFP-positive newborn neurons were traced. Intriguingly, the total dendritic length of GFP-positive newborn neurons in the GL of CHD7 mutants was significantly shorter compared to the control (Figures 4B, 4C, and S4C). Although not statistically significant, GFP-positive neurons in CHD7 mutants have less total dendrite branch numbers as compared to the control (Figures 4B, 4C, and S4C). Sholl analysis further demonstrated a decrease in the dendritic complexity of CHD7 mutant neurons (Figure 4D). Thus, loss of CHD7 in the SGZ NSCs not only reduces the number of newborn neurons but also leads to the dendritic abnormality in newborn neurons. To test whether running can have any effect on the dendritic abnormality in CHD7 mutant neurons, we applied the same retroviral-labeling approach to control and CHD7 mutant running mice. Strikingly, all of these above-mentioned defects of dendritic development in newborn neurons of CHD7 mutants were completely rescued after running (Figures 4B–4D; Figure S4C). Together, these results suggest that physical exercise is capable of overcoming the neurogenic defect in the DG of CHD7 mutants, including both the number and the dendritic development of newborn neurons. Our data demonstrate that exercise-induced neurogenesis does not depend on CHD7, which suggests an alternative pathway can guide the neuronal differentiation in the absence of CHD7.

Sox4 and Sox11 Are Direct Target Genes of CHD7

All of our above-mentioned results suggest that CHD7 is important for activation of a neuronal differentiation program in NSCs. Next, we aimed to identify the direct target genes of CHD7 in NSCs that are responsible for its function in neurogenesis. For this, we took a computational approach by analyzing the expression profiling data from the Cancer Genome Atlas Project (TCGA) (Network, 2008) because we observed a heterogeneous expression of CHD7 in human brain tumors (data not shown). Because CHD7 occupancy at genes is correlated with active gene expression (Engelen et al., 2011; Schnetz et al., 2009), we focused on genes that are most positively correlated with CHD7 expression in the database. Interestingly, Sox4 and Sox11, two group C genes of the Sox gene family (Kuhlbrodt et al., 1998), are on top of the list of genes correlated most with CHD7 expression (Figure 5A). Several studies have shown that Sox4 and Sox11 are essential for neuronal property determination (Bergsland et al., 2006; Mu et al., 2012) The data from the Allen Brain Atlas demonstrate that the mRNAs of Sox4 and Sox11 genes are enriched in the two neurogenic regions of the adult mouse brain (Figure S5A). Thus, Sox4 and Sox11 genes could be candidate genes that are regulated by CHD7 during neurogenesis. Immunostaining results showed that the expression of Sox4 and Sox11 is indeed reduced in both the SVZ and SGZ of CHD7 mutants (Figures 5B and 5C). Consistently, the mRNAs of both Sox4 and Sox11 in the hippocampus were significantly downregulated in CHD7 mutants (Figure 5D). Thus, loss of CHD7 results in downregulation of two essential neurogenic fate determinants Sox4 and Sox11.

To further test whether Sox4 and Sox11 are direct target genes of CHD7, we used monolayer cultured NSCs, in which CHD7 is homogenously expressed (Figure 1J). We performed chromatin immunoprecipitation (ChIP) assays to monitor the binding of CHD7 to Sox4 and Sox11 genes. The results in Figure 5E show that CHD7 is associated with the promoters but not with the 3′ coding regions of the Sox4 and Sox11 genes. To unravel the mechanism of how CHD7 regulates the Sox4 and Sox11 genes, NSCs derived from CHD7$^{fl/fl}$ mice were infected with retroviruses encoding the Cre recombinase. As shown in Figure S5B, the mRNA of CHD7 is completely depleted 7 days postinfection. Given the fact that CHD7 is an active ATP-dependent nucleosome remodeler (Bouazoune and Kingston, 2012), we monitored the nucleosome occupancy at Sox4 and Sox11 promoters in the control and CHD7 mutant NSCs. The chromatin was extensively digested with micrococcal nuclease (MNase) to obtain mainly mononucleosome (Figure S5C); DNA was precipitated and quantified using primers encompassing various gene promoters. For the normalization, DNA was amplified with a primer flanking an intergenic region on the mouse chromosome 8, where CHD7 does not bind (Figure 5E). Importantly, quantitative real-time PCR analysis revealed that there were 2-fold more DNA of Sox4 and Sox11 promoters existing in the mononucleosomal form in CHD7 mutant NSCs as compared to the control, whereas promoters of several genes required for neurogenesis, including Pten, p21, Dlx1, and Nestin, were not affected (Figure 5F). These data suggest that depletion of CHD7 in NSCs leads to a compacted nucleosome organization at promoters of Sox4 and Sox11, which is refractory for gene expression. A recent study demonstrated that the genome-wide binding of CHD7 coordinates with H3K4 methylation and that the chromodomains of CHD7 recognizes H3K4 methylation (Schnetz et al. 2009). We thus performed ChIP assays to examine whether loss of CHD7 leads to changes of H3K4 methylation on promoters of the Sox4 and Sox11 genes. Consistent with the downregulation of Sox4 and Sox11 gene expression, the level of the active histone mark H3K4me3 on promoters of both Sox4 and Sox11 genes was decreased in CHD7 mutant NSCs, whereas the level of a repressive mark H3K27me3 was not changed (Figure S5D). Together, these results demonstrate that the association of CHD7 to the promoters of Sox4 and Sox 11 genes is essential for keeping these regions as open chromatin structure.

We next investigate whether downregulation of Sox4 and Sox11 is responsible for the neuronal differentiation defect in CHD7 mutants. For this, CHD7 mutant cells were infected with retroviruses encoding Sox4 and Sox11 together with GFP or GFP alone (Figure S5E), and 24 hr later, the cells were induced to differentiation. Importantly, overexpression of Sox4 or Sox11 in CHD7 mutant NSCs significantly increased the number of Tuj1-positive neurons among GFP-positive cells as compared to the transfection of GFP alone (Figure 5G), suggesting that overexpression of Sox4 or Sox11 was able to largely rescue the neuronal differentiation defect of CHD7 mutant NSCs. These data strongly support that Sox4 and Sox11 are important target genes of CHD7 in adult neurogenesis. A recent study showed that inactivation of Sox4 and Sox11 in adult mouse DG results
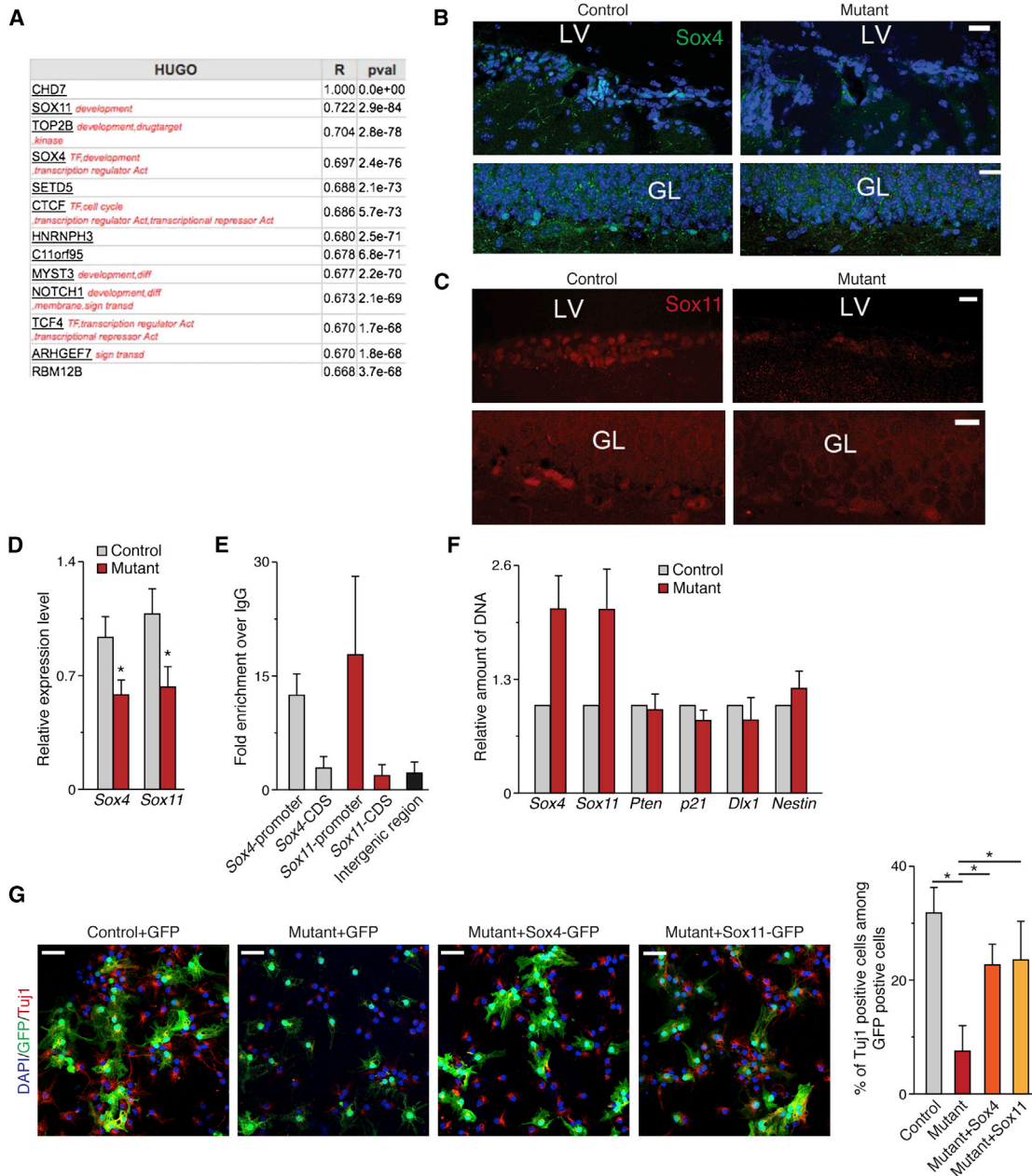
Figure 5. CHD7 Directly Regulates the Expression of Sox4 and Sox11

(A) List of the most positively correlated genes with CHD7 expression in 540 human GBMs from TCGA database.

(B and C) The SVZ and SGZ sections from mice (Nestin-CreERT2; CHD7$^{fl/fl}$ and the littermate control, 1 month post-TMX injection) were stained for Sox4 (B) or Sox11 (C). Scale bar: 20 μm.

(D) Quantitative PCR analysis of Sox4 and Sox11 expression in microdissected hippocampus tissue from mice (Nestin-CreERT2; CHD7$^{fl/fl}$ and the littermate control, 1 month post-TMX injection). Value represents mean ± SD (n = 4, * $p < 0.05$; Student's t test).

(E) ChIP analyses show enriched occupancy of CHD7 protein on promoter regions of *Sox4* and *Sox11* genes. As a negative control, no CHD7 occupancy was detected in one intergenic region on mouse chromosome 8. Value represents mean ± SD. Data were summarized from three independent experiments.

(F) The control and CHD7 mutant NSCs were digested with MNase to mostly mononucleosome. Ten nanograms of extracted DNA were amplified with primers for the individual promoter and normalized to the amount of DNA amplified from a primer for an intergenic region on mouse chromosome 8. Value represents mean ± SD. Data were summarized from three independent experiments.

(G) The control and CHD7 mutant NSCs were infected with retroviruses encoding GFP, Sox4-IRES-GFP, or Sox11-IRES-GFP and then induced to differentiation for 2 weeks. The cells were stained with antibodies against GFP and Tuj1. Scale bar: 20 μm. The numbers of Tuj1$^+$ cells among the total GFP$^+$ cells were counted, and the quantification data are shown on the right panel. Value represents mean ± SD (n = 5, * $p < 0.05$; Student's t test).

See also Figure S5.

in a similar phenotype as the CHD7 mutant, i.e., reduced neurogenesis due to the blockage of neuronal differentiation (Mu et al., 2012). These authors showed that Sox4 and Sox11 proteins are expressed in DCX-positive neuroblasts in the DG, but not in NSCs. Interestingly, both of Sox4 and Sox11 genes have been shown to be transcribed, but not translated, in adult NSCs (Beckervordersandforth et al., 2010). Our data showed that CHD7 is immediately expressed when NSCs become activated, which may be involved in the initiation of transcription of Sox4 and Sox11 genes in these cells. Taken together, our data suggest that in NSCs, CHD7 associates with promoters of the Sox4 and Sox11 genes to keep them as an open chromatin structure for permissive transcription, which is important for the activation of Sox4 and Sox11-dependent neuronal differentiation program.

DISCUSSION

There are two unique features of stem cells: long-term self-renewal and multiple lineage differentiation potential. For this, the expression of genes in stem cells should not only allow them to be kept in an undifferentiated status, but also allow them to continuously generate new progenies. Epigenetic regulations have been shown to be critical for stem cells to keep the differentiation program in a poised state. For instance in embryonic stem cells, promoters of many developmentally important transcription factors are modified as bivalent chromatin domain, which keeps them at low transcriptional level while leaving these genes poised for activation upon differentiation (Bernstein et al., 2006). In line with this, inactivation of epigenetic regulators often leads to aberrant differentiation of stem cell without affecting self-renewal. For example, loss of the H3K4 methyltransferase Mll1 or DNA methyltransferase DNMT3a in NSCs leads to reduction of postnatal neurogenesis, without affecting NSC proliferation (Lim et al., 2009; Wu et al., 2010). Multiple lineage specific genes expression was considered to be a hallmark of hematopoietic stem cells and epigenetic priming was shown to be important for this process (Walter et al., 2008). The lineage priming in NSCs was reported by Beckervordersandforth et al. (2010), when they discovered genes that are important for neuronal differentiaton like Sox4 and Sox11 are already transcribed at low level in NSCs. The mechanism behind the activation of Sox4 and Sox11 in NSCs was unclear. The CHD7/Sox4/Sox11 pathway we identified here provides an epigenetic mechanism of lineage priming in adult neurogenesis. CHD7 expression is associated with neurogenic cells that are undergoing lineage commitment, and inducible inactivation of CHD7 in the adult neurogenic niches lead to impaired neuronal differentiation. In the adult mammalian brain, NSCs can generate neurons very efficiently, both in the SVZ-OB and DG. The quiescent NSCs are uncommitted and they are negative for CHD7 expression; CHD7 expression appears upon NSC entering cell cycle, where it targets promoters of Sox4 and Sox11 to keep them as a transcriptional-permissive open chromatin state. This explains the low level expression of Sox4 and Sox11 in NSCs and neural progenitors (Beckervordersandforth et al., 2010). The protein expression of CHD7 and Sox4 and Sox11 overlaps in neuroblasts, indicating that CHD7 is probably required for the maintenance of Sox4 and Sox11 expression in these cells. The activation of Sox4 and Sox11 leads to sequential activation of the downstream neuronal differentiation and maturation pathway controlled by these factors (Bergsland et al., 2011). Notably, the Sox4 and Sox11 double KO mice showed developmental defects in many organs that are also affected in CHARGE patients (Schilham et al., 1996; Sock et al., 2004), suggesting the CHD7/Sox4/Sox11 regulation mechanism is probably a general pathway beyond the nervous system. Two very recent reports showed that several cell-cycle effectors regulate NSCs by controlling the expression of Sox2, suggesting a general regulation mechanism by targeting Sox factors in NSCs (Julian et al., 2013; Marqués-Torrejón et al., 2013).

The neuronal differentiation defect we observed in the CHD7 mutant provides insights into understanding human CHARGE syndrome. It was known that around 80% of the CHARGE patients have olfaction deficit, and most of them had anomalies of the OBs (Blustajn et al., 2008; Bergman et al., 2011). The mutant phenotype in mice observed in our study provides a cellular mechanism for this clinical syndrome, which is that the SVZ NSCs lacking CHD7 cannot efficiently differentiate into neurons. The impaired neuronal differentiation and dendritic development of newborn neurons in the hippocampus of adult CHD7 mutant provides a possible cellular mechanism of the learning and intellectual disability of 75% human CHARGE patients (Bergman et al., 2011). It is striking that running exercise leads to the rescue of neuronal differentiation defects in the DG of CHD7 mutants. These data suggest that exercise-induced neurogenesis is probably CHD7-independent, indicating an alternative pathway that can sufficiently drive neuronal differentiation in the absence of CHD7. On the other hand, this finding implicates that exercise might be beneficial for CHARGE patients, in particular, for the recovery of the hippocampal-related learning ability. However, whether newborn neurons in the GL of CHD7 mutants can be functionally integrated into the preexisting circuit needs to be further investigated. Our study demonstrates the potential of using mouse adult NSCs to study human brain disease-related gene mutations, which will not only help to unravel the molecular mechanism of different diseases but also provide possible treatment strategies. Intriguingly, mutations of CHD8, which can interact with CHD7 biochemically (Batsukh et al., 2010), were recently identified as one of the major genetic lesions in human autism patients (Neale et al., 2012; O'Roak et al., 2012; Talkowski et al., 2012). Human CHARGE patients also have autistic-like behavior (Hartshorne et al., 2005); thus, the function of CHD7 in the CNS identified in this study, in particular, the defect of dendritic development, may also provide insights into the understanding of the molecular basis of autism. Moreover, we observed that CHD7 is widely expressed in many tissues in the adult mouse, particularly in tissue-specific stem cell niches like hair follicles and bronchioles in the adult lung (data not shown). This suggests that CHD7 might be required for cell lineage commitment in many different tissues, although this needs to be further tested in different tissues using animal models.

EXPERIMENTAL PROCEDURES

Animals
Mice were housed according to standard conditions, and all animal experiments were conformed to the local and international guidelines for the use

of experimental animals. Tlx-CreERT2 mice have been described before (Liu et al., 2008, 2010). CHD7$^{fl/fl}$ mice were obtained from EUCOMM, and the neomycin selection cassette was removed by crossing with Flp deleter mice. Nestin-CreERT2 animals were generated as described elsewhere (Corsini et al., 2009). The Tlx-GFP reporter animal was obtained from the GENSAT project.

Immuonostaining, Confocal Imaging, and Analysis

Mice were perfused with 4% paraformaldehyde, and brains were postfixed overnight at 4°C. Coronal sections (7 μm for paraffin or 40 μm for vibratome) were prepared using a Microtome or Vibratome (Leica), respectively. Sections were blocked and peameablized in 5% normal swine serum in PBST (PBS+0.2% Triton X-100) for 1 hr before being incubated overnight at 4°C with primary antibodies. For immunohistochemistry, sections were incubated with biotinylated secondary antibodies (Vector Laboratories), amplified by a horseradish peroxidase system (ABC Kit, Vector Laboratories) and visualized using DAB staining (Sigma-Aldrich). Fluorescent images were captured using confocal laser-scanning microscopes (LSM780, LSM700, Zeiss; Leica SP5). To compare the control and CHD7 mutants, a series of sections containing at least 15 sections through the OB, SVZ, or DG were analyzed from each mouse. All experiments were carried out in a blind fashion to experimental conditions.

GFP-positive newborn granule cells were imaged with z stacks scanning with a 0.5 μm interval using a Leica SP5 confocal microscope. To analyze the dendritic structure of newborn neurons, reconstructions of the dendritic processes were made from the z stacks. The projection images were semiautomatically traced with National Institutes of Health ImageJ software using the NeuronJ plugin. The total dendritic length and branch number of each GFP-positive neuron were subsequently analyzed. The Sholl analysis for dendritic complexity was carried out by counting the number of traced dendrites that cross a series of concentric circles with 10 μm intervals from the cell soma.

Voluntary Running

Three to four tamoxifen-injected, age-matched mice were housed in a rat cage with one running wheel. During the first 12 days, animals were injected with BrdU once per day. The animals were sacrificed 18 days after the last BrdU injection.

Retroviral Preparation and Stereotactic Injection

The following retroviral vectors were used in this study: CAG-GFP; CAG-Sox4-IRES-GFP and CAG-Sox11-IRES-GFP. Mouse complementary DNAs of Sox4 and Sox11 (Life Technologies) were cloned into the vector CAG-IRES-GFP. High titers of retroviruses were generated by cotransfection of retroviral vectors and vectors expressing gal-pol and vsvg into HEK293T cells followed by ultracentrifugation of viral supernatant. To infect monolayer cultured NSCs, 1 μl of 1:20 diluted retroviruses were used to infect cells in a single well of one 24-well plate. For the stereotactic injection, mice were anesthetized and injected with 2 μl of CAG-GFP retroviruses into the left and right dentate gyrus. The coordinates from bregma were (in mm) −1.9 anterior/posterior, ±1.6 medial/lateral, and −1.9 dorsal/ventral from dura. For the runner groups, injected mice rested for 1 day before being put into the running cages. Animals were sacrificed 4 weeks postinjection. The brains were cut into 40 μm thick sections and stained with antibody against GFP.

Statistics

Statistical significance was determined by either two-tailed Student's t test or Kolmogorov-Smirnov test as indicated.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes five figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.05.002.

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