Sequential regulatory loops as key gatekeepers for neuronal reprogramming in human cells

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Direct conversion of somatic cells into neurons holds great promise for regenerative medicine. However, neuronal conversion is relatively inefficient in human cells compared to mouse cells. It has been unclear what might be the key barriers to reprogramming in human cells. We recently elucidated an RNA program mediated by the poly pyrimidine tract binding protein PTB to convert mouse embryonic fibroblasts (MEFs) into functional neurons. In human adult fibroblasts (HAFs), however, we unexpectedly found that invoking the documented PTB–REST–miR-124 loop generates only immature neurons. We now report that the functionality requires sequential inactivation of PTB and the PTB paralog nPTB in HAFs. Inactivation of nPTB triggers another self-enforcing loop essential for neuronal maturation, which comprises nPTB, the transcription factor BRN2, and miR-9. These findings suggest that two separate gatekeepers control neuronal conversion and maturation and consecutively overcoming these gatekeepers enables deterministic reprogramming of HAFs into functional neurons.

Neurogenesis is known to take a series of cellular programming events from initial morphogenesis to subsequent maturation to form distinct cell subtypes in the nervous system1,2. These sequential events are likely induced by specific morphogenetic signals in combination with step-wise activation or repression of cell-autonomous gene expression programs, as indicated by expression waves of lineage-specific transcription factors3 and their distinct functional requirements4. While most of our knowledge about neurogenesis in mammals is derived from genetically tractable systems, particularly mice, we know relatively little about the parallel process in humans. Mice and humans are clearly different in brain size and the time required for neural development, which may thus involve distinct regulatory strategies5, and understanding such critical differences is vital for using mice as models to study neural development and neurodegenerative diseases in humans6,7.

Recent breakthroughs in regenerative medicine provide new approaches to studying specific human diseases by using patient-derived cells. Through reprogramming8–10, for example, human fibroblasts can be converted to induced pluripotent stem cells, which can be redifferentiated to specific cell types, such as neurons, for functional studies11,12. Alternatively, fibroblasts can be directly transdifferentiated into neurons with a set of neuron-specific transcription factors13–17. Many studies have demonstrated the feasibility of such transdifferentiation approaches in mouse and human embryonic fibroblasts, but it is a general consensus that human cells are much harder to convert, especially those from aged patients18. Even though embryonic fibroblasts, but it is a general consensus that human cells are much harder to convert, especially those from aged patients18. 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molecule inhibitors, but all induced neurons were immature, indicating that PTB knockdown is sufficient to activate a full neuronal program in mouse, but not human, cells. We now uncover a second loop that promotes neuronal maturation in human cells. It consists of the PTB paralog nPTB (which is encoded by PTBP2; we use nPTB as a gene alias); another neuron-specific miRNA, mir-9; and the transcription activator BRN2 (which is encoded by POU3F2; we use BRN2 as a gene alias). In this loop, BRN2 transcriptionally activates mir-9, which in turn post-transcriptionally diminishes nPTB. These findings highlight the intertwined regulatory loops that are sequentially required for cell fate switch to the neuronal lineage.

RESULTS

Initial barrier to neuronal conversion in human cells

The PTB–miR-124–REST loop is self-sustaining once triggered by initial PTB knockdown. Because the loop is conserved in mammals, we applied the same strategy to two primary HAF lines, derived from a 42-year-old male and an 86-year-old female. Both lines showed the expression of fibroblast markers, such as fibronectin, FSP1 and vimentin, without detectable contamination by neural cells, as indicated by the lack of a series of markers for neurons, neural crest progenitors or neural crest derivatives (Supplementary Fig. 1a). PTB knockdown potently withdrew these cells from cycling, as shown by markedly reduced 5-ethyl-2'-deoxyuridine (EdU) signals and increased cells in G2 (Fig. 1a and Supplementary Fig. 1b), with 9–15% of the seeding cells showing bipolar neuronal morphology and positive staining for the pan-neuronal marker Tuj1 (Fig. 1b). This conversion rate is similar to that in mouse embryonic fibroblasts (MEFs)28.

To increase the conversion efficiency, we tested a set of small molecules that have been reported to enhance neuronal differentiation29–33. None of these compounds alone was able to turn HAFs into Tuj1-positive cells (Supplementary Fig. 1c), but when individual compounds were combined with PTB knockdown, we detected various levels of enhancement (Fig. 1c). Initially, the induced neuron-like cells did not look healthy (Supplementary Fig. 1d). We therefore titrated individual compounds and tested various combinations (Fig. 1c), eventually establishing a three-small-molecule (3SM) combination (Fig. 1c) optimal for converting HAFs to Tuj1-positive cells in long-term culture.
We monitored the conversion kinetics, and after conditioning the cells by PTB knockdown for 3 d, the 3SM cocktail was able to boost the number of Tuj1-positive cells as early as 3 h, reaching a plateau in ~1 d, while the fibroblast marker fibronectin gradually declined to nearly undetectable over a period of ~4 d, resulting in highly efficient conversion (~90%) of initial HAFs to Tuj1-positive cells (Fig. 1d,e). In control shRNA-treated cells, while 3SM treatment for 3 h had no effect (Supplementary Fig. 1e), continuous treatment with 3SM alone induced some transient effects on cellular restructuring, as indicated by vaulted nuclei (Supplementary Fig. 1e). In contrast, PTB shRNA (shPTB) rapidly induced a persistent switch to neuronal morphology, 3SM treatment for 3 h enhanced the morphogenetic induction, and the continuous presence of 3SM yielded even stronger enhancement (Supplementary Fig. 1e). These data established that the 3SM cocktail alone was insufficient, but its combination with shPTB potently induced a cell fate switch. The dramatic reduction in cycling cells coupled with negative staining for Nestin and Sox2 (both neural progenitor markers) strongly argue against the possibility of neural stem cell–like intermediates during this conversion process (Fig. 1a and Supplementary Fig. 1f). Of note, if we applied the same protocol optimized for HAFs to MEFs, cells died rapidly (data not shown), indicating critical differences in kinetic requirement between mouse and human cells.

**A second human-specific barrier to neuronal maturation**

To our surprise, we detected little expression of markers for mature neurons, such as MAP2, neurofilament (NF) and NeuN, in shPTB/3SM-treated cells, indicating that the converted neuron-like cells from HAFs were arrested at an immature stage (Fig. 2a and Supplementary Fig. 2a). As expected, we failed to record any neuronal activity—for example, Ca^{2+} influx in response to membrane depolarization or the induction of action potentials upon current injection—even on

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**Figure 2 Generation of functional neurons from HAFs by sequential knockdown of PTB and nPTB. (a)** PTB knockdown-induced neuron-like cells from HAFs showed extensive Tuj1 (red) but rare MAP2 (green) expression. *n* = 3 independent biological repeats. Nuclei were stained with DAPI (blue). (b,c) Time-course analysis of nPTB expression by western blotting in shPTB-treated MEFs (b) and HAFs (c). shluc, control shRNA against luciferase. PTB and nPTB were examined by western blotting. β-actin served as a loading control; *n* = 2 independent experiments. Uncropped versions of all western blots are shown in Supplementary Figure 7. (d) The reprogramming strategy by sequential knockdown of PTB and nPTB. HAFs were first infected by an inducible lentivirus containing the expression cassettes for shPTB and red fluorescence protein (RFP) in combination with an shnPTB-expressing lentivirus. After hygromycin selection, cells were treated with the three small molecule inhibitors (3SM). shnPTB expression was induced by the addition of doxycycline (Dox) to the medium at different time points. FGF2, fibroblast growth factor 2; NTF, neurotrophic factors. See Online Methods and Supplementary Figure 2. (e) Representative images of converted neuron-like cells from HAFs upon nPTB knockdown at different time points. Top, cells treated with Dox on different days; Tuj1 (green) staining at day 14. Bottom, cells treated with Dox from days 2–9; MAP2 (green) staining at day 14. RFP signal (insets) indicates successful shnPTB lentiviral infection. Selected from two biological repeats showing similar results. (f) Top, representative trace of whole-cell currents under voltage clamp recording mode. Typical voltage-dependent Na+ currents were reversibly blocked by the sodium channel inhibitor TTX (middle); these partially recovered after washing out drug (bottom). 10 of 10 examined cells showed the recorded activity. (g) Representative trace of repeated action potentials induced by current injection. 8 of 11 examined cells showed the recorded activity. (h) GABA<sub>a</sub> receptor–mediated current responses elicited by focal application of GABA (blue), which could be completely blocked with the GABA<sub>a</sub> receptor–specific blocker PiTX. 10 of 10 examined cells showed the recorded activity. (i–k) Spontaneous postsynaptic currents (PSCs) recorded on RFP-marked neurons cultured with GFP-marked glia (l). Inset shows fast-kinetic PSCs. EPSCs were blocked by NBOX + APV (j). Inset highlights GABA-induced inhibitory PSCs (IPSCs), blocked with PiTX (k). 9 of 11 examined cells showed the recorded activity. All scale bars, 20 µm.
prolonged culture of the converted cells. This indicates a second barrier to neuronal maturation: maturation appears to be automatically invoked in MEFs, as we showed previously, but not in HAFs.

To search for the missing regulator(s), we performed RNA-seq on converted neuron-like cells in comparison with beginning HAFs. We noted the induction of a large panel of REST target genes. However, unlike in MEFs, we observed a sustained increase in mRNA for the RNA binding protein nPTB, a PTB paralog in the nervous system, in PTB-depleted HAFs (Supplementary Fig. 2b). As nPTB is known to be induced for several days during initial neuronal induction, followed by graduate decline when neuroblasts are differentiated into functional neurons or during cortical development, we hypothesized that dynamic regulation of nPTB might be a key difference between murine and human cells during transdifferentiation. To test this hypothesis, we monitored nPTB expression after PTB knockdown in both MEFs and HAFs. In contrast to MEFs and consistent with the RNA-seq data, we found that the induced nPTB protein showed persistent expression in neuron-like cells from HAFs (Fig. 2b,c). This suggests a human-specific barrier to neuronal maturation.

Overcoming both barriers by sequential PTB/nPTB knockdown

To test the two-barrier hypothesis, we generated an inducible knockdown cell line by infecting HAFs with four validated lentiviral shRNA pools against nPTB (Supplementary Fig. 2c,d). Curiously, we observed cell death if PTB and nPTB were co-depleted in both MEFs and HAFs (data not shown), indicating that a sequential process is critical for neuronal differentiation despite the likely timing difference between mouse and human cells. We therefore tested PTB knockdown with shRNA, followed by selection with hygromycin for 3 d and then depletion of nPTB (indicated by RFP expression from the shnPTB expression unit) upon doxycycline addition at different time points (Fig. 2d).

After seeding PTB knockdown cells in differentiation medium for 2 d or beyond, nPTB knockdown generated healthy, MAP2-positive (stained at day 14) cells (Fig. 2e). The resulting neuron-like cells remained healthy in culture for 3 months, the longest time we tested.

We next determined whether such neuron-like cells produced by sequential PTB and nPTB knockdowns possessed neuron-specific membrane properties. We detected fast-activating and inactivating inward Na+ currents and outward K+ currents after doxycycline treatment for 4 weeks followed by co-culturing with GFP-marked glial cells for another 2 weeks (Fig. 2f). The Na+ currents could be blocked by the voltage-gated sodium channel-specific inhibitor tetrodotoxin (TTX), which could be partially relieved after washing out the inhibitor, indicative of an active binding and passive disassociation process (Fig. 2f).

We also observed repeated action potentials upon current injection (Fig 2g). The cells also expressed functional GABA_A-receptors,
as based on their response to GABA, which could be specifically blocked by picrotoxin (PTX), a GABA_A receptor–specific inhibitor (Fig. 2h). We also detected spontaneous postsynaptic currents of various amplitude and frequencies, which could be sequentially blocked by inhibitors against the excitatory (NBQX (2,3-dihydroxy-6-nitro-7-sulamoyl-benzo[f]quinoxaline-2,3-dione) + APV (dL-2-amino-5-phosphonovaleric acid)) and inhibitory (NBQX + APV + PTX) receptors (Fig. 2i–k). Collectively, these data demonstrate the functionality of induced neurons from hSFCs by sequential PTB and nPTB knockdown in the presence of glial cells.

nPTB regulates BRN2 during neuronal maturation

The above data suggest that nPTB knockdown is sufficient to overcome a critical barrier to neuronal maturation, indicating the activation of a new regulatory program. To understand the mechanism, we examined the expression of key transcription factors previously shown to generate functional neurons in human cells. This revealed their induction, with one exception, by PTB knockdown (Fig. 3a), consistent with these genes being targets for REST in HAFs (Supplementary Fig. 3a). The exception was BRN2, a cortex-specific transcription factor, which remained repressed in PTB knockdown HAFs (Fig. 3a). Notably, sequential knockdown of PTB and nPTB potently induced BRN2 expression (Fig. 3b).

We next determined the potential contribution of activated BRN2 to neuronal maturation. We first tested various combinations of transcription factors in combined with PTB knockdown, finding that the inclusion of BRN2 was critical for efficient MAP2 expression (Supplementary Fig. 3b) and for invoking Ca^{2+} influx

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**Figure 4** BRN2 is essential for neuronal maturation in hNPCs and activation of mature-neuron-specific miRNAs.

(a) Immunofluorescence analysis of neurons derived from hNPCs upon BRN2 knockdown. Nuclei were stained with DAPI (blue), n = 3 independent biological repeats. Scale bar, 20 μm. (b) Expression of SOX2 (white; infrared fluorescence), but not MAP2 (green), in BRN2-depleted hNPCs. Nuclei were stained with DAPI (blue), n = 3 independent biological repeats. Scale bar, 20 μm. (c) Representative BRN2 ChIP-seq signals at MIR124-3 and MIR9-2 loci before or after hNPC differentiation. Two anti-BRN2 antibodies (mouse monoclonal IgG B-2 and goat IgG C-20) were used for constructing ChIP-seq libraries. (d) BRN2 knockdown HAFs (Fig. 3a). BRN2 knockdown is sufficient to overcome a critical barrier to neuronal maturation, indicating the activation of a new regulatory program. To understand the mechanism, we examined the expression of key transcription factors previously shown to generate functional neurons in human cells. This revealed their induction, with one exception, by PTB knockdown (Fig. 3a), consistent with these genes being targets for REST in HAFs (Supplementary Fig. 3a). The exception was BRN2, a cortex-specific transcription factor, which remained repressed in PTB knockdown HAFs (Fig. 3a). Notably, sequential knockdown of PTB and nPTB potently induced BRN2 expression (Fig. 3b).

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hPTBP2 or mPTBP2, luciferase reporter luciferase (RL) relative to Firfly luciferase (FL) in independent experiments. For miR-9 sponge: Kd, knockdown. Bottom quantification (expression. GAPDH served as loading control; neurons induced by sequential knockdown of PTB and nPTB by PTB knockdown coupled with BRN2 overexpression were similar as based on several key electrophysiological properties (Supplementary Table 1). Collectively, these data demonstrate that PTB knockdown plus BRN2 expression is sufficient to produce functional neurons from HAFs.

BRN2 is essential for hNPC differentiation

We next wished to establish functions of BRN2 in neuronal maturation in a different physiological context: namely, differentiation of human neural progenitor cells (hNPCs) to cortical neurons upon withdrawal of basic fibroblast growth factor from culture medium. These hNPCs showed positive expression of neuron stem cell–specific markers, such as nestin, Sox2, and BRN2 (Supplementary Fig. 4a). Upon BRN2 knockdown, we found that, while early neuronal differentiation events were not affected, as evidenced by normal TuJ1 expression (Fig. 4a), the expression of MAP2, a marker associated with more mature neurons, was severely compromised (Fig. 4b and Supplementary Fig. 4b,c). BRN2 is similarly required for the expression of the mature neuron marker NeuN in PTB/nPTB sequential knockdown cells (Supplementary Fig. 4d). Inward Na+ currents (Fig. 4c) were rarely detected in BRN2-depleted hNPCs (Supplementary Fig. 4e). These data demonstrate that BRN2 is required for hNPC differentiation into functional neurons.

BRN2 activates neuron-specific miRNAs

To understand how BRN2 mediates neuronal maturation, we further took advantage of the hNPC system to perform BRN2 chromatin immunoprecipitation and sequencing (ChIP-seq) with two independent antibodies (C-20 and B-2) before and after hNPC differentiation (Supplementary Fig. 4f,g). We detected ~5,000 BRN2 binding peaks in 3,987 genes (Supplementary Table 2). Gene ontology analysis of all expressed genes indicated that BRN2

(Supplementary Fig. 3c). We then engineered an inducible polycis-tronic unit in a stable HAF line to express both shPTB and BRN2 (Fig. 3c). Doxycycline treatment induced rapid PTBP1 knockdown and gradual BRN2 expression, and after 2 weeks of such treatment, we detected multiple markers for mature neurons, including MAP2, NCAM, vGLUT1 and NeuN (Fig. 3c).

To demonstrate the functionality of these neurons, after 1 week of culture with GFP-marked glial cells, we recorded TTX-sensitive sodium currents (Fig. 3d), current–triggered repeated action potentials (Fig. 3e), spontaneous occasion action potentials (Fig. 3f), and functional AMPA and NMDA currents characterized by using specific inhibitors (Fig. 3g). After culturing with GFP-marked glial cells for 3 to 4 weeks (Fig. 3h), we detected spontaneous postsynaptic activities of variable amplitudes and frequencies (Fig. 3i–k). Neurons induced by sequential knockdown of PTB and nPTB or by PTB knockdown coupled with BRN2 overexpression were similar as based on several key electrophysiological properties (Supplementary Table 1). Collectively, these data demonstrate that PTB knockdown plus BRN2 expression is sufficient to produce functional neurons from HAFs.

Figure 5 Specific miRNAs repress nPTB during neuronal differentiation. (a) miR-9 and miR-124 target sites in the 3’ UTR of nPTB. Illustrated are deduced base pairing potentials, calculated free energies, and specific mutations introduced (green). (b) or miR-9 (c) mimics in HAFs. GAPDH and β-actin (ACTB) served as controls. For miR-124: box 1 versus 2: υ = 3, t = 3.614, P = 0.0364; box 1 versus 3: υ = 3, t = 5.328, P = 0.012. For miR-9: box 1 versus 2: υ = 4, t = 6.124, P = 0.00360; box 1 versus 3: υ = 3, t = 6.861, P = 0.00634. n = 3 independent experiments. (d,e) Expression of nPTB protein (top) and quantified changes (bottom) in response to overexpression of miR-124 (d) or miR-9 (e) sponge in differentiated hNPCs (n = 3 independent experiments). GAPDH served as loading control. (f) Top, nPTB expression in response to the overexpression of miR-124 or miR-9 sponge in HAF-derived neurons induced by shPTB plus BRN2 expression. GAPDH served as loading control; Kd, knockdown. Bottom quantification (n = 3 independent experiments). For miR-9 sponge: υ = 4, t = 5.503, P = 0.0053; for miR-124 sponge: υ = 4, t = 4.910, P = 0.0080. (g) Luciferase reporter activities (Renilla luciferase (RL) relative to Firefly luciferase (FL) control) in response to cotransfected WT or mutant miRNA mimics in HEK293T cells. hPTBP2 or mPTBP2, luciferase reporter containing the human or mouse nPTB 3’ UTR, respectively; NC, nonspecific control mimic. For box 1 versus 2: υ = 4, t = 4.197, P = 0.01373; box 2 versus 3: υ = 4, t = 10.512, P = 0.00046; box 2 versus 4: υ = 4, t = 6.409, P = 0.00306; box 5 versus 6: υ = 4, t = 5.827, P = 0.00432; box 7 versus 8: υ = 3, t = 9.095, P = 0.00281; box 9 versus 10: υ = 4, t = 4.057, P = 0.01539. n = 3 independent experiments. (b) and (d) and (h) Map2 and NeuN expression in HAF-derived neurons upon PTB knockdown or sequential PTBP1 and PTB knockdown plus the ectopic expression of an shRNA-resistant Flag-tagged nPTB (n = 2 independent experiments). CDSM, coding sequence mutation. All data are mean ± s.d. Two-tailed unpaired Student’s t-test (*P < 0.05, **P < 0.01, ***P < 0.001).
regulates a large repertoire of genes critical for neurogenesis and maturation (Supplementary Fig. 4.h,i).

Among BRN2 targets, we noted that BRN2 directly bound the MIR124-3 gene on chromosome 20 and the MIR9-2 gene on chromosome 5 (Fig. 4d). In contrast to the MIR124-3 locus, which showed unaltered BRN2 binding before and after hNPC differentiation, BRN2 on multiple locations in the MIR9-2 locus became detectable only in differentiated NPCs (Fig. 4d), which we further validated by ChIP followed by quantitative PCR (qPCR) (Fig. 4e). By monitoring mature miR-124 and miR-9 with mir-21 as a negative control in shPTB-treated HAFs, we again detected the induction of mir-9, but not mir-124, in response to BRN2 overexpression for 6 d, although both miRNAs were induced after prolonged BRN2 expression for 12 d (Fig. 4f). These data suggest that BRN2 binds and regulates MIR124 expression in hNPCs, but such modest levels of mir-124 in hNPCs appears insufficient to trigger neuronal differentiation. In contrast, BRN2-regulated mir-9 seems to drive neuronal differentiation, consistent with the burst induction of the mir-9–2 precursor between E12.5 and E13.5, when BRN2 begins to express in the mouse brain39-41.

We also confirmed BRN2 binding to both MIR9 and MIR124 loci in HAFs treated with shPTB and shnPTB (Fig. 4g). MIR9 showed progressive induction in response to sequential knockdown of PTB and nPTB in HAFs (Fig. 4h). In contrast, MIR124 was highly induced in response to PTB knockdown, but, for unknown reasons, it became repressed in HAFs treated with shPTB and shnPTB (Fig. 4h). In any case, these data suggest that MIR124 induction may responsible for neuronal conversion and subsequent MIR9 activation may account for progression of induced neuron-like cells to functional neurons.

The nPTB–BRN2–mir-9 loop for neuronal maturation

The induction of mir-9 by BRN2 provides a clue to a new regulatory axis for neuronal maturation, as we noted potential target sites for both mir-9 and mir-124 in the 3’ UTR of nPTB with typical base-pairing potentials in prospective seed regions (Fig. 5a). We thus asked whether these two neuron-specific miRNAs were able to diminish the expression of endogenous nPTB. A previously study showed that mir-124 potentially inhibits PTB with relatively minor effects on nPTB42. In PTB-downregulated HAFs, we found that both mir-124 and mir-9 mimicked downregulation of nPTB at both mRNA and protein levels (Fig. 5b,c). As expected, shBRN2 prevented nPTB downregulation while BRN2 overexpression diminished nPTB induction in shPTB-treated HAFs (Supplementary Fig. 5a,b). To further test these miRNAs in post-transcriptional regulation of nPTB, we expressed specific sponges for these miRNAs in hNPCs, finding significantly increased nPTB expression (Fig. 5d,e). The sponges similarly prevented nPTB downregulation and diminished NeuN expression in HAF-derived neurons (Fig. 5f), consistent with early works that established that both miRNAs are essential in maintaining neuron viability of differentiated NPCs43,44.

Next we verified the deduced targeting sites in the 3’ UTR of nPTB. We constructed a luciferase reporter containing the nPTB 3’ UTR and tested the effects of transfected miRNA mimics, with a universal siRNA as a negative control. We found that both mir-124 and mir-9 mimics repressed the reporter activity, and specific mutations in the predicted target sites diminished the miRNA-induced downregulation of the reporter (Fig. 5g). Of the two potential miR-124 target sites in the nPTB 3’ UTR, the first seemed to be the primary functional site because mutation of that site largely prevented mir-124-mediated downregulation (Fig. 5g).

We emphasize that, although forced miR-124 overexpression or mir-124 depletion by sponge were able to affect nPTB expression, it is the BRN2–miR-9–nPTB axis that seems to be critical for neuronal maturation under physiological conditions, as immature neurons induced by shnPTB alone expressed high levels of mir-124. To further demonstrate that diminished nPTB expression is key to neuronal maturation, we expressed a shRNA-resistant form of nPTB in neurons induced from HAFs by shPTB and shnPTB. This reduced MAP2 to basal levels and, more importantly, completely prevented the induced expression of NeuN (Fig. 5h).

Considered together, the data presented in Figures 3–5 suggest a previously unrecognized loop for driving neuronal maturation in PTB-depleted HAFs (Supplementary Fig. 6). In this loop, nPTB downregulation directly or indirectly triggers the activation of BRN2; activated BRN2 binds and transcriptionally induces MIR9; and, finally, elevated mir-9 post-transcriptionally diminishes nPTB. Therefore, like the PTB–REST–miR-124 loop for neuronal conversion, this nPTB–BRN2–miR-9 loop, once triggered, becomes self-sustaining for neuronal maturation.

DISCUSSION

Physiological relevance of the sequential regulatory loops

Neuronal differentiation is a tightly controlled process, as indicated by the sequential expression of transcriptional and post-transcriptional regulators. The two separate yet highly intertwined loops we have elucidated are consistent with the neurogenesis program in vivo. PTB is well known to maintain neural stem cell pools, as its ablation causes precocious neural differentiation and massive depletion of neural stem cells in the mouse45. The effect of PTB knockout is largely phenocopied by REST knockout, suggesting that they function in a common pathway during neuronal induction46. As both PTB and REST are constitutively expressed in non-neuronal cells, it is likely that their inactivation in developing neurons is triggered by induced MIR124 expression42. Our previous work28 revealed a double-negative feedback loop consisting of PTB, mir-124, and REST (Supplementary Fig. 6). Once triggered, the loop becomes self-sustaining and dismantled REST is responsible for de-repressing a large number of neuron-specific genes to drive neurogenesis28. It is important to note here that REST dismantling induced by mir-124 is fundamentally distinct from REST knockout, as residual levels of REST appear to be required for ensuring the healthy state of differentiated neurons46.

A key functional consequence of PTB inactivation is de-repression of nPTB due to the inclusion of a PTB-suppressed exon34,42,47, a process that occurs in the early phase of neural development35. Our new data show that nPTB is also negatively regulated by mir-124, which may counteract PTB knockdown–induced nPTB expression, thereby maintaining relatively stable nPTB expression during the transition from immature to mature neurons. The transient nPTB expression is coincident with the induction of both BRN2 and MIR-9 around E13.5; however, it has been unclear how nPTB is switched off during neuronal maturation39,40. We now establish that these three components form a feedforward loop (Supplementary Fig. 6) and the full activation of this loop underlies gradual nPTB switch-off in more mature neurons36,48. It remains to be defined which developmental cue(s) triggers this loop in human brain.

Key difference between mouse and human neurogenic programs

Mouse has been a prominent model for studying neurogenesis and neurodegeneration in humans. However, mouse and human are
evolutionarily different in many ways. One of the fundamental differences is neocortex expansion, which is mainly due to increased amplification of neural progenitor cells during embryonic corticogenesis\(^1\). The tightly controlled neuronal conversion and maturation loops might serve as gatekeepers to ensure programmatic switches in a sequential manner during brain development, as simultaneous knockout of PTB and nPTB produced a lethal phenotype, even in cell lines. Notably, while the optimal neuronal conversion protocol developed for mouse cells is insufficient to produce functional neurons from human cells, the optimized protocol developed in human cells caused a lethal phenotype in mouse cells, suggesting that cells from different mammals may have their own built-in timing mechanisms.

Such separate timing mechanisms between mouse and human cells are underscored by the observation that the PTB- and nPTB-mediated loops seem to be automatically connected in murine cells but need to be separately activated in human cells. While we are still early in understanding such species-specific mechanisms, one potential mechanism might be due to distinct regulatory programs for inducing miR-124 and miR-9 between mice and humans. miR-124 is known to be gradually induced during neurogenesis, while the induction of miR-9 expression is largely coincident with neuronal maturation\(^1\). It is therefore conceivable that the initial induction of miR-124 might be responsible for downregulating both PTB and nPTB in mouse cells, but predominantly PTB in human cells. It is likely that miR-124 is more efficient in targeting PTB than nPTB even in mouse cells, as simultaneous inactivation of both PTB and nPTB would severely compromise the viability of differentiating neurons.

Regulation of BRN2 expression is an obvious contributor to neuronal maturation, but other neuron-specific transcription factors, such as NeuroD1, may be also involved\(^14\). One of those mature-neuron-specific transcription factors may be responsible for further elevation of miR-124, joining force with BRN2-induced miR-9 in later phases of neurogenesis. Therefore, various combinations of empirically tested transcription factors might all be able to partially activate both neuronal conversion and maturation programs but unable to fully activate these programs in all transfected cells. As neuronal reprogramming of human cells appears to be stringently controlled by some critical gatekeepers, including the two elucidated in the present study, such opportunistic activation of the neural program may thus account for the overall low conversion efficiency in human cells.

Mechanisms underlying PTB- and nPTB-regulated gene expression

PTB and nPTB are well-known splicing regulators\(^34,36,42,47,48,50\). Despite their similar biochemical properties in RNA binding and biological functions in regulated splicing, they clearly have nonredundant functions in development, as based on knockout study results in mice\(^36,45,48\). Our recent study reveals that, besides its traditional role as a splicing regulator, PTB also binds numerous sites in the 3′UTR of many genes to either compete for miRNA targeting or alter RNA secondary structure to promote miRNA targeting, thus modulating miRNA functions in both directions\(^28\). Our earlier work\(^28\) demonstrated that this new function of PTB is largely responsible for miR-124-mediated dismantling of the REST complex during neuronal conversion.

Here we have uncovered a critical role of regulated nPTB expression in neuronal maturation. Previous studies have elucidated the function of nPTB in the regulation of neuron-specific alternative splicing events, which undoubtedly contribute to various phenotypes associated with mature neurons\(^34,36,48\). We now demonstrate a key role of nPTB downregulation in driving neuronal maturation via the activation of the nPTB–BRN2–miR-9 loop. In this loop, BRN2 likely activates transcription from MIR9 and induced miR-9 in turn inactivates nPTB via its targeting site in the 3′ UTR, but it is unclear how downregulation of nPTB leads to BRN2 activation. Future studies will thus address how nPTB downregulation may activate BRN2, either directly via a transcriptional de-repression mechanism or indirectly through induced splicing events of other genes in mature neurons. The established cellular model will empower this and other mechanistic studies in a biologically relevant context.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Gene Expression Omnibus: GSE77524.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Y.X. and X.-D.F. conceived the project. Y.X. designed and performed most biochemical experiments. H.Q. was responsible for all electrophysiology analysis and inNPC differentiation with input from A.K. and Z.P.B.Z. performed bioinformatics analysis and Y.Z. and X.H. contributed to RNA-seq analysis. J.H. performed EdU labeling, ChiP-qPCR, and FACS analysis. Y.X. and X.-D.F. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Hobert, O. Regulation of terminal differentiation programs in the nervous system. Annu. Rev. Cell Dev. Biol. 27, 681–696 (2011).
2. Molyneaux, B.J., Arlotta, P., Menezes, J.R. & Macklis, J.D. Neuronal subtype specification in the cerebral cortex. Nat. Rev. Neurosci. 8, 427–437 (2007).
3. Guillemot, F. Spatial and temporal specification of neural fates by transcription factor codes. Development 134, 3771–3780 (2007).
4. Bang, A.G. & Goulding, M.D. Regulation of vertebrate neural cell fate by transcription factors. Curr. Opin. Neurobiol. 6, 25–32 (1996).
5. Florio, M. et al. Human-specific gene ARHGAP11B promotes basal progenitor amplification and neocortex expansion. Science 347, 1465–1470 (2015).
6. Herculano-Houzel, S. The human brain in numbers: a linearly scaled-up primate brain. Front. Hum. Neurosci. 3, 31 (2009).
7. Lepski, G. et al. Delayed functional maturation of human neuronal progenitor cells in vitro. Mol. Cell. Neurosci. 47, 36–44 (2011).
8. Takahashi, K. et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861–872 (2007).
9. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663–676 (2006).
10. Yu, J. et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 318, 1917–1920 (2007).
11. Dimos, J.T. et al. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. Science 321, 1218–1221 (2008).
12. Wernig, M. et al. Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson’s disease. Proc. Natl. Acad. Sci. USA 105, 5856–5861 (2008).
13. Ciaiazzo, M. et al. Direct generation of functional dopaminergic neurons from mouse and human fibroblasts. Nature 476, 224–227 (2011).
14. Pang, Z.P. et al. Induction of human neuronal cells by defined transcription factors. Nature 476, 220–223 (2011).
15. Pfister, U. et al. Direct conversion of human fibroblasts to dopaminergic neurons. Proc. Natl. Acad. Sci. USA 108, 10343–10348 (2011).

16. Son, E.Y. et al. Conversion of mouse and human fibroblasts into functional spinal motor neurons. Cell Stem Cell 9, 205–218 (2011).

17. Vierbuchen, T. et al. Direct conversion of fibroblasts to functional neurons by defined factors. Nature 463, 1035–1041 (2010).

18. Broccoli, V., Calazzo, M., & Dell’Anno, M.T. Setting a highway for converting skin into neurons. J. Mol. Cell Biol. 3, 322–323 (2011).

19. Amamoto, R. & Arlotta, P. Development-inspired reprogramming of the mammalian central nervous system. Science 343, 1239882 (2014).

20. Cahan, P. et al. CellNet: network biology applied to stem cell engineering. Cell 158, 903–915 (2014).

21. Morris, S.A. et al. Dissecting engineered cell types and enhancing cell fate conversion via CellNet. Cell 158, 889–902 (2014).

22. Hou, P. et al. Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds. Science 341, 651–654 (2013).

23. Li, Y. et al. Generation of iPSCs from mouse fibroblasts with a single gene, Oct4, and small molecules. Cell Res. 21, 196–204 (2011).

24. Alkayed, N. et al. Biochemical modulation of gene expression improves neural conversion of human ES and iPS cells. Nat. Cell Biol. 13, 724–735 (2011).

25. Ambasudhan, R. et al. Direct reprogramming of adult human fibroblasts to functional neurons under defined conditions. Cell Stem Cell 9, 113–118 (2011).

26. Xue, Y. et al. Direct conversion of fibroblasts to neurons by reprogramming PTB-regulated microRNA circuits. Cell 152, 82–96 (2013).

27. Chambers, S.M. et al. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD-signaling. Nat. Biotechnol. 27, 275–280 (2009).

28. Li, W. et al. Rapid induction and long-term self-renewal of primitive neural precursors from human embryonic stem cells by small molecule inhibitors. Proc. Natl. Acad. Sci. USA 108, 8299–8304 (2011).

29. Liu, M.L. et al. Small molecules enable neurogenin 2 to efficiently convert human fibroblasts into cholinergic neurons. Nat. Commun. 4, 2183 (2013).

30. Ladewig, J. et al. Small molecular agonists can act as master regulators of neuronal development. Nature 499, 275–280 (2013).

31. Prasad, K.N. & Hsie, A.W. Morphologic differentiation of mouse neuroblastoma cells induced in vitro by dibutyryl adenosine 3′,5′-cyclic monophosphate. Nat. New Biol. 233, 141–142 (1971).

32. Boulton, P.L. et al. A post-transcriptional regulatory switch in polypyrimidine tract-binding proteins reprograms alternative splicing in developing neurons. Genes Dev. 21, 1636–1652 (2007).

33. Zheng, S. et al. PSD-95 is post-transcriptionally repressed during early neural development by PTBP1 and PTBP2. Nat. Neurosci. 15, 381–388 (2012).

34. Li, Q. et al. The splicing regulator PTBP2 controls a program of embryonic splicing required for neuronal maturation. Elife 3, e01201 (2014).

35. Hagiwara-Yamagishi, K. et al. Predominant expression of Brn-2 in the postmitotic neurons of the developing mouse neocortex. Brain Res. 752, 261–268 (1997).

36. Marchetto, M.C. et al. A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells. Cell 143, 527–539 (2010).

37. Dominguez, M.H., Ayoub, A.E. & Rakic, P. POU-III transcription factors (Bm1, Bm2, and Oct6) influence neurogenesis, molecular identity, and migratory destination of upper-layer cells of the cerebral cortex. Cereb. Cortex 23, 2632–2643 (2013).

38. Shibata, M., Nakao, H., Kiyonari, H., Abe, T. & Aizawa, S. MicroRNA-9 regulates neurogenesis in mouse telencephalon by targeting multiple transcription factors. J. Neurosci. 31, 3407–3422 (2011).

39. Krivtsov, A.M., King, K.S., Donahue, C.P., Khrapko, K. & Kosik, K.S. A microRNA array reveals extensive regulation of microRNAs during brain development. RNA 9, 1274–1281 (2003).

40. Makeyev, E.V., Zhang, J., Carrasco, M.A. & Maniatis, T. The MicroRNA mir-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. Mol. Cell 27, 435–448 (2007).

41. Akesson, M. et al. MicroRNA-124 is a subventricular zone neuronal fate determinant. J. Neurosci. 32, 8879–8889 (2012).

42. Giusti, S.A. et al. MicroRNA-9 controls dendritic development by targeting REST. Elife 3 (2014).

43. Shibusaki, T. et al. PTB deficiency causes the loss of adherens junctions in the dorsal telencephalon and leads to lethal hydrocephalus. Cereb. Cortex 23, 1824–1835 (2013).

44. Gao, Z. et al. The master negative regulator REST/NRSF controls adult neurogenesis by restraining the neurogenic program in quiescent stem cells. J. Neurosci. 31, 9772–9786 (2011).

45. Spellman, R., Liorian, M. & Smith, C.W. Crossregulation and functional redundancy between the splicing regulator PTB and its paralogs nPTB and ROD1. Mol. Cell 27, 420–434 (2007).

46. Ichihashi, K. et al. Ptbp2 represses adult-specific splicing to regulate the generation of neuronal precursors in the embryonic brain. Genes Dev. 26, 1626–1642 (2012).

47. Ravetch, J.V. et al. The extracellular domain of CD277 inhibits CD27 signals. Nature 447, 699–704 (2007).

48. Ichihashi, K. et al. Ptbp2 represses adult-specific splicing to regulate the generation of neuronal precursors in the embryonic brain. Genes Dev. 26, 1626–1642 (2012).

49. Rakic, P. Evolution of the neocortex: a perspective from developmental biology. Nat. Rev. Neurosci. 10, 724–735 (2009).

50. Xue, Y. et al. Genome-wide analysis of PTB-RNA interactions reveals a strategy used by the general splicing repressor to modulate exon inclusion or skipping. Mol. Cell 36, 996–1006 (2009).
**Online Methods**

Cell culture, virus production and infection. Primary human dermal fibroblasts from a 42-year-old male were purchased from ATCC and expanded in Dulbecco’s Modified Eagle Medium (DMEM)/F12 medium (20% FBS, 1× penicillin/ streptomycin) or fibroblast medium (DMEM/F12, 5 ng/ml recombinant bFGF, 1 μg/ml hydrocortisone, 50 μg/ml ascorbic acid, 5 μg/ml of recombinant human insulin, 2% FBS). Primary human adult fibroblasts from an 86-year-old female were a gift from Dr. John Ravits. HEK293T cells were cultured in DMEM containing 10% FBS and 100 U of penicillin/streptomycin. Human neuronal progenitor cells (hNPC) were expanded in neural growth (NG) medium consisting of DMEM/F12, Glutamax, 0.5× N2, 0.5× B27, and 1× penicillin/streptomycin. Stempro Accutase (Life Technologies) was used to detach hNPCs. Plates for passageing NPCs were sequentially treated with 10 μg/ml of poly-t-ornithine hydrobromide overnight and 2.5 μg/ml of laminin for 3 h.

Lentiviral shRNAs previously used to target human PTB were shuttled to the pLKO.1-Hygromycin vector (Addgene, #24150). Lentiviral plasmids containing transcription factors ASCL1, BRN2, MYT1L and NEUROD1 were from Addgene (#27150, 27151, 27152, and 30129). The transcription factor the AgeI and ClaI sites. The target sequence (TCGACATAATCTCTGTATTATA) was inserted into the EcoRI site. To build inducible shPTB constructs, the target sequences (GAAGAGAGGATCTGACGAACTA, TAAGAAAGACAGCGCTCTAATA, CGAGGAGACGGCTTCAATAG) were inserted into the pTRIPZ-RFP vector between the EcoRI and XhoI sites. To simultaneously express shPTB and BRN2 in a doxycycline-dependent manner, the target sequence (TGCACTAATCCTGCTTATA) was inserted into the pTRIPZ-RFP vector first, and RFP was replaced with the BRN2 cDNA between the AgeI and ClaI sites.

For neuronal reprogramming, human adult fibroblasts were first infected with shPTB lentivirus for 16 h. After selection with hygromycin B for 3 d, the cells were switched to N3 medium (DMEM/F12 plus 25 μg/ml insulin, 50 μg/ml transferrin, 30 nM sodium selenite, 20 nM progesterone, 100 nM putrescine) for 2 weeks to induce neuronal morphology. To test the enhancement effects of small molecules, selected cells were primed in N3 basal medium (1:1 mix of DMEM/F12 and Neurobasal, 25 μg/ml insulin, 50 μg/ml transferrin, 30 nM sodium selenite, 20 μM progesterone, 100 μM putrescine, 0.4% B27) for 2 d before the addition of small molecules Chir99021 (1 μM), SB341542 (10 μM) and Dbc-cAMP (1 mM) to N3 basal medium plus 10 ng/ml of FGF2, followed by culturing for another 2 d. Cells were then switched to N3 basal medium containing FGF2, BDNF, GDNF, NT3 and CNTF and 2% FBS for 8–10 weeks to induce functional neurons. Media were half-changed every other day. To measure synaptic currents, induced neurons were seeded onto a monolayer of glia cells from P1 GFP transgenic rats. For Western blotting, cells were lysed in SDS loading buffer without bromophenol blue and, after quantification, bromophenol blue was added back to a final concentration of 0.1%. 25 μg of total protein was resolved on 10% Nupage Bis-Tris gel and probed with following antibodies: mouse anti-PTBP1 (monoclonal BB7, ATCC, CRL-2501), rabbit anti-PTBP2 IS2 (gift from Dr. Douglas Black of UCLAl), PTBP2 monoclonal antibody (M1, clone 2D10-B2, Abnova), mouse monoclonal to PTBP2 (ab57619, Abcam), rabbit anti-BRN2 (Cell Signaling Technology, 12137) and anti-GAPDH (14C10, Cell Signaling Technologies), anti-Flag (Sigma, F3165) and mouse anti-ACTB (A2228, Sigma).

Total RNA was extracted with Trizol (Life Technology), except that 10 μg/ml glycogen was used at the precipitation step to enrich for small RNAs. Total RNA was first treated with DNase I (Promega) and reverse-transcribed with the Superscript III kit (Life Technology). RT-qPCR was performed with gene-specific primers and Fast Start Universal SYBR Green Master Mix (Roche) on a Step-One Plus PCR machine (Applied Biosystems). Statistical significance was determined by Student’s t-test based on triplicated experiments. All primers used for plasmid construction and real-time PCR are listed in Supplementary Table 3.

**Electrophysiological recording.** Whole-cell patch clamp recordings were performed as previously described. The only exception was that cultured cells were incubated with oxygenated (95% O2, 5% CO2) artificial cerebrospinal fluid (150 mM NaCl, 5 mM KCl, 1 mM CaCl2, 2 mM MgCl2, 10 mM glucose, 10 mM HEPES, pH 7.4) at 37 °C for 30 min. Recordings under whole-cell voltage clamp mode were performed at a holding potential of ~75 mV. The pipette resistance was 5–8 MΩ. Action potentials were elicited by injecting step depolarizing currents under current clamp recording mode. The pipette solution contained 150 mM KCl, 5 mM NaCl, 1 mM MgCl2, 2 mM EGTA, 1 mM Mg-ATP, and 10 mM HEPES (pH 7.2, adjusted with KOH). 1 mM GABA was used to evoke GABA currents. The following concentrations of channel inhibitors were used: TTX: 1 μM; PTX: 50 μM; NBOX: 20 μM; APV: 50 μM APV. All recordings were performed at room temperature (20–22 °C).

**Immunocytochemistry.** Cells were seeded on a coverslip precoated with Matrigel (BD, 356234) for 1 h at 37 °C and then fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 15 min at room temperature, followed by permeabilization with 0.1% Triton X-100 in PBS for 15 min on ice. After washing twice with PBS, cells were blocked in PBS containing 3% BSA for 1 h at room temperature. The following primary antibodies were incubated on the cells for 1 h: rabbit anti-Tuj1 (Covance, MRB-435P:1,000), mouse anti-Tuj1 (Covance, MMS-435P,1:1,000), mouse anti-AMAP2 (Millipore, MAB3418:1,000), mouse anti-NeuN (Millipore, MAB377:1,200), rabbit anti-synapsin1 (Synaptic Systems, 106001,1:5,000), rabbit anti-VGLUT1 (Synaptic Systems, 135-303-1,200), rabbit anti-GABA (Sigma, A2052,1:1,000), rabbit anti-BRN2 (Cell Signaling Technology, 12137,1:500), mouse anti-βIII-tubulin (DSHB, 13G8B7.500), mouse anti-vimentin (Dyelight 594 (Abcam, EPR73767.1:1,000), mouse anti-P63 (Thermo Fisher, MA1-21871,1:300), goat anti-MIF (Santa Cruz, sc-10999,1:1,000), rabbit anti-K5 (Thermo Fisher, RM2160S,1:300), rabbit anti-NGF receptor P75 (Millipore, AB 1554,1:100), goat anti-SOX2 (Santa Cruz, sc-17319,1:200), mouse anti-Pax6 (DSHB, PAX3,1:250), mouse anti-Pax7 (DSHB, PAX7-c,1:250), mouse anti-NKX2.2 (DSHB, 74.SAS,c,1:100), mouse anti-NCAM (DSHB, 5AS-c,1:100), mouse anti-Olig1 (Neuram, 75-180,1:100), rabbit anti-GFAP (Neuram, 75-240,1:100), rabbit anti-Tbr1 (Millipore, AB10554,1:300), mouse anti-GFP (Life Technology, A-11120,1:400), rabbit anti-SOX10 (Abgent, AP5854c,1:50), mouse anti-BRN3A (Bios USA, bs-3669R,1:50). Fluorescence-labeled secondary antibodies (Life Technologies, A-21207, A-21202, A-21203, and A-21206) and
were applied to the cells for another 1 h and 20 min respectively. After six washes with PBS, Fluoromount-G mounting medium was applied to the glass slides, which were examined under an Olympus Fluoview FV1000.

Identification of BRN2 targets by ChIP-seq. Human NPCs were cross-linked with 1% formaldehyde and quenched by adding 1/20th volume of 2.5 M glycine to a final concentration of 0.125 M. After three washes with ice-cold PBS, cells were scraped from the plate and collected by centrifugation at 2,500 rpm in a table-top centrifuge (Micro 17R) from Thermo Scientific. Cell pellets were suspended in 1 ml of Cyto lysis buffer, mixed briefly, and incubated on ice for 10–15 min with occasional inversion every 2 min. Nuclei were pelleted for 5 min at 3,500 rpm in the Micro 17R centrifuge, 4 °C. Supernatant was collected in a 15-ml tube. The supernatant was diluted 1:10 with dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-Cl, pH 8.1, protease inhibitor cocktail) and incubated with 50 μl of nuclear lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-Cl, pH 8.1, protease inhibitor cocktail) for another 4 h. Magnetic beads were blocked with PBS + 5 mg/ml BSA-fraction V and incubated at 4 °C for another 4 h. Magnetic beads were harvested on a magnetic stand and beads washed sequentially at 4 °C, twice with 0.7 ml of TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), twice in TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), twice in buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0), and finally, twice with 1 ml of TE buffer at 4 °C. Eluted beads were twice washed with 150 μl of elution buffer (TE buffer, 1% SDS) by vortexing at 70 °C, 1,000 rpm in an Eppendorf ThermoMixer C centrifuge for 10 min. The eluents were heated at 65 °C overnight. DNA fragments were purified with QIAquick Spin PCR purification Kit (Qagen, CA).

ChIP-seq libraries were constructed by using the TruSeq ChiP Sample Prep Kit (Illumina). To include a spike-in control, 700 ng of soluble chromatin from Drosophila melanogaster was added at the immunoprecipitation step to 200 μg of the soluble chromatin from hNPCs before and after differentiation, followed by the addition of 5 μg of anti-BRN2 and 1 μg of Drosophila-specific H2AV antibodies (Active Motif, 39716). After deep sequencing, total sequencing tags from Drosophila were used for normalization. ChIP-seq reads were aligned to the human genome with Bowtie 2 (ref. 52) using default parameters with local alignment model. Duplicated reads and low-mapping-quality reads were removed with SAMtools53. Binding peaks were identified by MACS54 with default parameters. The identified BRN2 targets are listed in Supplementary Table 2.

Statistical analysis. Statistical differences were calculated by two-tailed Student t-test with SigmaPlot 12 software and the degree of freedom (υ) were usually defined by the number of experiments in group 1 plus the number of experiments in group 2 minus two (n1 + n2 − 2). The variance was similar between groups being statistically compared. Data distribution was assumed to be normal, but this was not formally tested. All data collection and analyses were performed by experimenters blind to the treatment conditions. The image fields were randomly selected under 20× magnification. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those generally employed in the field.

A Supplementary Methods Checklist is available.

51. Sánchez, S. et al. A cAMP-activated pathway, including PKA and PI3K, regulates neuronal differentiation. Neurochem. Int. 44, 231–242 (2004).
52. Langmead, B. & Salzberg, S.L. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357–359 (2012).
53. Li, H. et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25, 2078–2079 (2009).
54. Zhang, Y. et al. Model-based analysis of ChIP-Seq (MACS). Genome Biol. 9, R137 (2008).