Repressor element 1 silencing transcription factor (REST) controls radial migration and temporal neuronal specification during neocortical development

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Neurogenesis requires mechanisms that coordinate early cell-fate decisions, migration, and terminal differentiation. Here, we show that the transcriptional repressor, repressor element 1 silencing transcription factor (REST), regulates radial migration and the timing of neural progenitor differentiation during neocortical development, and that the regulation is contingent upon differential REST levels. Specifically, a sustained presence of REST blocks migration and greatly delays—but does not prevent—neuronal differentiation, resulting in a subcortical band heterotopia-like phenotype, reminiscent of loss of doublecortin. We further show that doublecortin is a direct gene target of REST, and that its overexpression rescues, at least in part, the aberrant phenotype caused by persistent presence of REST. Our studies support the view that the targeted down-regulation of REST to low levels in neural progenitors, and its subsequent disappearance during neurogenesis, is critical for timing the spatiotemporal transition of neural progenitor cells to neurons.

in utero electroporation | neuronal differentiation | neuronal cell fate

Nervous system development relies on extrinsic and intrinsic signaling to regulate the precise spatial and temporal acquisition of the different neural lineages. Neurons and glia arise from neural stem cells in a temporally defined order, where generation of neurons precedes glia (1–3). Furthermore, the generation and migration of neurons occur in a stereotyped pattern to construct the distinctive structure of the central nervous system. For example, the neocortex, which consists of six layers of neurons, is built through precisely orchestrated waves of newly born neurons that migrate past their precursors (2, 4). This orderly acquisition of the different neural lineages is mediated by specific networks of transcriptional activators and repressors in response to environmental and intrinsic cues (for reviews see refs. 3, 5, and 6). How the precise timing of this signaling cascade is accomplished and whether migration and differentiation are linked obligatorily during development is still obscure.

One key factor in this process could be the transcriptional repressor REST (also called NRSF), which regulates a large number of neuronal genes as well as brain-specific microRNA genes (7–11). In nonneuronal cells, REST binds to a conserved 23-bp DNA motif known as RE1 (repressor element 1), located in the regulatory regions of these genes, and blocks their transcription, via the corepressors CoREST and Sin3 (12–14). We showed previously that REST repression in pluripotent ES cells and multipotent neural stem/progenitor (NS/P) cells creates a chromatin status poised for subsequent activation (12, 14). Importantly, REST itself is regulated differentially throughout development, expressed to high levels in ES cells but present in minimal levels in NS/P cells. The down-regulation of REST in NS/P cells is mediated, at least in part, by targeted proteasomal degradation via the E3 ubiquitin ligase β-TRCP (15, 12). As NS/P cells differentiate into neurons, REST and its corepressors dissociate from the RE1 site, while the *REST* gene itself is transcriptionally repressed, allowing activation of neuronal genes (12, 14).

REST knockout mice are embryonic-lethal for unknown reasons (16), and die before most nervous system development. Overexpression of REST in the developing spinal cord of chicken embryos, however, causes neuronal pathfinding errors (17) and, in immature neuronal cell lines, blocks growth-factor induced acquisition of sodium channel excitability (18). Although the data taken together suggest an intimate link between terminal differentiation and REST function, whether REST has any role during the transition of NS/P cell to neuron and whether the down-regulation of REST in NS/P cells is critical for neurogenesis remains unknown.

To test this idea in a well-defined anatomical and temporal context in vivo, we used in utero electroporation to manipulate REST expression in the developing neocortex. At embryonic stages, cells overexpressing REST were arrested at the boundary of the ventricular/subventricular (VZ/SVZ) and the intermediate zone (IZ) and were in delayed transition between the NS/P and neuronal stages. Importantly, although the REST-expressing cells remained arrested at the white matter during postnatal stages, they eventually became neuronal. Furthermore, expression of doublecortin (DCX) rescued, at least in part, both migration and the neuronal differentiation defects caused by the presence of REST. Our data suggest that the targeted down-regulation of REST to minimal levels at the NS/P stage, and its subsequent disappearance as neurogenesis proceeds, is critical for regulating radial migration and the timing of neuronal differentiation, but not for neuronal cell-fate decision.

Results

Sustained Expression of REST During Neurogenesis Blocks Migration and Neuronal Differentiation. Our previous studies show that, in contrast to pluripotent ES cells where REST is expressed to high levels, in multipotent NS/P cells, although still functioning as a repressor, REST is maintained at minimal levels but absent in neurons (14). To understand the role and the significance of the down-regulation of REST in NS/P cells during neocortical development, we used a gain-of-function approach. To this end, we used in utero electroporation to introduce transgenes expressing GFP alone (pCA-IRES-GFP), GFP with full-length REST (pCA-REST-IRES-GFP), or GFP with a truncated form of REST that lacks the carboxy and amino terminal-repressor domains (pCA-RESTΔNΔC-IRES-GFP) (18) into the VZ of embryonic day (E) 14 rat embryos, and analyzed the fate of the GFP-expressing cells as development proceeded. In situ hybridization showed that at the time of electroporation (E14), endogenous *REST* expression is confined to the VZ of the developing neocortex, where the progenitor cells are actively dividing (Fig. S1 *A* and *B*, respectively). We analyzed the electroporated brains as early as 2 d

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postelectroporation (E16) and found that NS/P cells expressing GFP alone or with RESTΔNΔC, but not NS/P cells expressing REST, migrated deep into the IZ (Fig. 1*A*, *Upper*), suggesting that elevated levels of REST in NS/P cells interfere with the migration process during neurogenesis. Importantly, the transgenes were already expressed to high levels (Fig. 1*A*, *Lower*) when the cells were still at the NS/P stage, as evidenced by the presence of nestin and the cell division marker Ki67 (Fig. S2). This finding suggests that any effects of the transgene expression on neurogenesis initiate as early as the NS/P stage. To verify more quantitatively the interference of REST with migration, we measured the maximum distance of migration of the electroporated cells into the IZ and found that although cells expressing REST migrated only up to 20% of the IZ, cells expressing GFP alone or with RESTΔNΔC migrated up to 75% of the IZ interval (Fig. 1*B*). The lack of effect of RESTΔNΔC further suggests that the block in migration that occurs when REST is overexpressed (Fig. 1 *A* and *B*) is because of the presence and function of the two REST repressor domains.

We wished to further analyze the fate of the REST-expressing cells at a later time during neocortical development. Because REST is regulated posttranscriptionally in NS/P cells, potentially the REST-IRES-GFP transgene might also be down-regulated, precluding our ability to track fluorescence at these later time points. To eliminate this possibility, the GFP-expressing plasmids were coelectroporated with an mRFP-expressing plasmid (pCASSG-mRFP). When the control, GFP-expressing plasmid (pCA-IRES-GFP), was coelectroporated with pCASSG-mRFP, over 80% of NS/P cells expressing both GFP and mRFP migrated all of the way to the cortical plate (CP) 4 d after electroporation (E14–E18) [Fig. 2 *A* (*Upper*) and *B*]. In contrast, when the REST/GFP-expressing plasmid (pCA-REST-IRES-GFP) was coelectroporated with pCASSG-mRFP, over 90% of the cells expressing both mRFP and REST/GFP were arrested at the boundary of the VZ/SVZ and IZ [Fig. 2 *A* (*Bottom*), *B*, and *C*]. Interestingly, although REST was overexpressed and seemingly arrested the cells at the SVZ/IZ border at E16 (Fig. 1*A*), by E18 the REST-expressing cells had migrated from that position to a discrete boundary located about halfway into the IZ (Fig. 2*A*, yellow fragmented line). This finding indicates that the continuous presence of REST delays the initial migration (Fig. 1), but REST is unable to entirely block the process until the cells reach halfway through the IZ (Fig. 2). It should be noted that, at this stage, the majority of the cells expressing mRFP also expressed GFP and REST (Fig. 2 *A* and *C*), suggesting that 4 d after electroporation, REST and GFP are persistently expressed and were not significantly down-regulated posttranscriptionally or posttranslationally. As we showed above, RESTΔNΔC did not interfere with migration, and like NS/P expressing GFP alone, over 80% of the NS/P cells expressing RESTΔNΔC and GFP migrated to the CP (Fig. 2*B* and Fig. S3*A*) and differentiated into neurons, as indicated by the presence of the neuronal-specific marker microtubule associated protein 2 (MAP2) (Fig. S3*B*). Taken together, our data suggest that sustained presence of REST during neurogenesis clearly blocks the migration process and that this phenotype is dependent on the presence of the REST repressor domains.

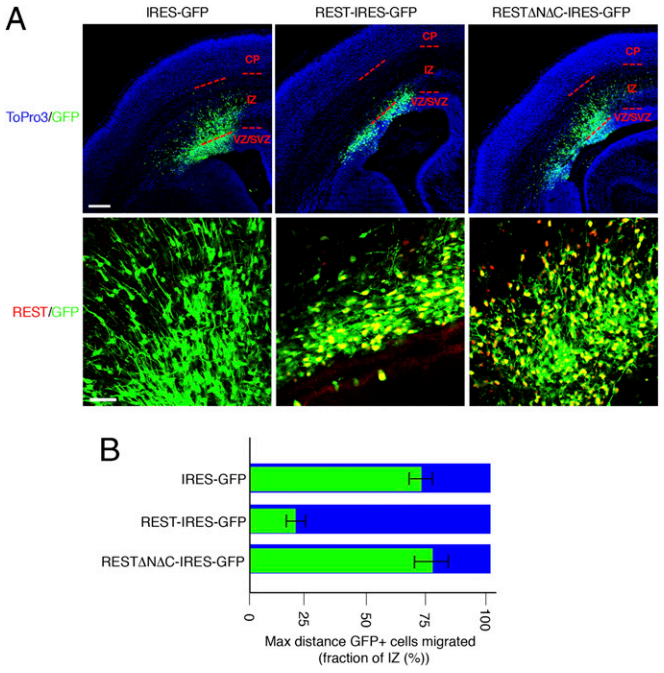

Fig. 1. Overexpression of REST during neurogenesis blocks migration. (*A*) Immunostaining of representative coronal brain sections, 2 d postelectroporation (E14–E16). (*Upper*) The migration of the electroporated GFP cells. ToPro3 (blue) represents nuclear staining and serves as a marker for the different cortical layers. (*Lower*) The coexpression of REST or RESTΔNΔC with GFP. The REST p73 antibody was used to detect both full-length REST and RESTΔNΔC (red). [Scale bars, 200 μm (*Upper*); 50 μm (*Lower*).] (*B*) Bar graphs represent the maximum distance in which GFP-expressing cells migrated into the IZ. The length of the IZ (blue bar graph) is taken as 100%; the maximum distance in which GFP-expressing cells (green) migrated (green bar graph) presented as a fraction of the total length of IZ (blue). Error bars represent SD based on an *n* value of at least 4 and three sections from each brain.

Next, we analyzed the nature of the arrested REST-expressing cells at E18, using cell-specific markers. Surprisingly, the REST-expressing cells were no longer at the NS/P stage, as evidenced by the lack of Ki67 as well as the lack of progenitor markers, such as nestin and the radial glia marker brain lipid-binding protein (BLBP) (Fig. 3*A*). These cells were also not apoptotic as indicated by the absence of Caspase3 (Fig. 3*A*). The arrested REST-expressing cells also lacked expression of the early neuronal markers DCX, involved in radial migration, and neuronal-specific β-tubulin (TUJ1), as well as the late neuronal marker MAP2 (Fig. 3). However, some of the arrested cells, which were still in the SVZ, expressed the T-domain transcription factor, TBR2, normally present in neurogenic intermediate progenitors localized mainly to the SVZ (Fig. 3*A*, *Middle*). Because most of the REST-expressing cells migrated passed the SVZ and the TBR2 progenitor layer, this finding may indicate that they are in transition toward neuronal differentiation. As the high density of cells and processes in tissue sections sometimes makes colocalization of cytoplasmic markers difficult to assess, we further analyzed the presence of several neural progenitor and neuronal markers in acutely dissociated cortical cells, 1 and 4 d postelectroporation. Consistent with the immunohistochemical analyses (Fig. 3 and Fig. S2), immunostaining of the acutely dissociated cells for the progenitor marker nestin clearly shows that, although it was present in most of the REST/GFP-expressing cells at 1 d postelectroporation (Fig. 3*B*a), it was absent in REST/GFP- and RESTΔNΔC/GFP-expressing cells 4 d after electroporation (Fig. 3*B*, b and c). Furthermore, consistent with the immunohistochemical analyses (Fig. 3*A*), none of the REST/GFP-expressing cells were positive for the late neuronal marker MAP2, whereas all of the RESTΔNΔC/GFP-expressing cells were positive at this stage (Fig. 3*B*, b and c, and Fig. S3*B*). However, unlike brain sections, in acutely dissociated cells it was noticeable that some of the REST/GFP-expressing cells weakly expressed the progenitor marker BLBP, and some weakly expressed the early neuronal marker DCX (Fig. 3*Bb*). This finding further suggests that 4 d postelectroporation, the REST-expressing cells may be in transition between the progenitor stage and immature neuronal stage.

Although the exit of the REST-expressing cells from the NS/P stage could be the result of progression of neuronal differentiation, it is also possible that expression of REST above normal levels itself causes the loss of NS/P identity, as shown for ES cells expressing a higher than normal level of REST (19). To distinguish between these two possibilities, we expressed REST/GFP or GFP alone, using the lentiviral vector pEF1α-IRES-GFP, in a primary culture of NS/P cells in the continuous presence of FGF-2, which maintains cells in a NS/P state. We analyzed the nature of the GFP-expressing cells 8 d postinfection, a time-frame past the 4-d interval postelectroporation. Immunostaining showed that the fraction of NS/P cells expressing either the progenitor marker

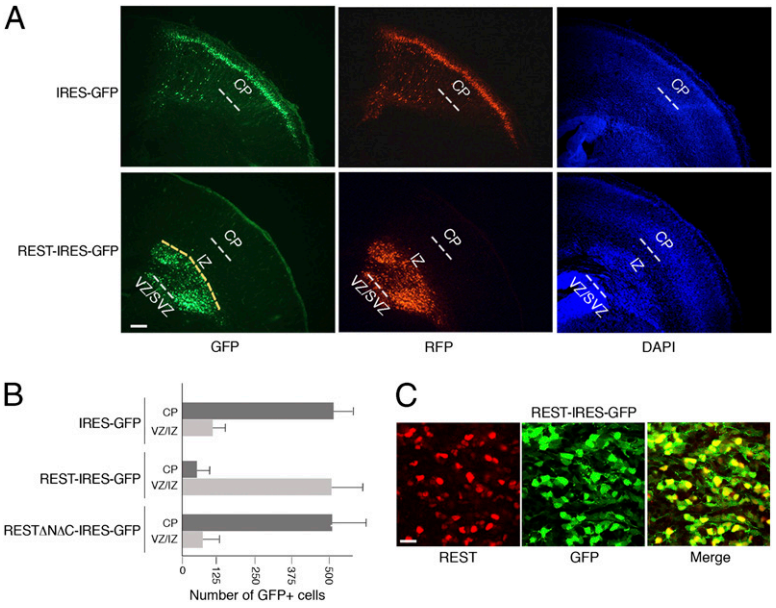

Fig. 2. The REST-expressing cells are arrested between the VZ/SVZ and IZ 4 d postelectroporation. (*A*) Expression of GFP (green) and mRFP (red) in representative coronal brain sections 4 d postelectroporation (E18). DAPI represents nuclear staining and serves as a marker for the different cortical layers. (Scale bar, 200 μm.) (*B*) Bar graphs show the number of GFP$^+$ cells in the VZ/IZ and in the CP. Error bars represent SD based on $n = 4$. (*C*) Immunostaining of the arrested REST-expressing cells for REST (red) and GFP (green) in representative coronal section of E18 mouse brain. (Scale bar, 20 μm.)

nestin or Ki67 was similar between the REST/GFP- and control GFP-expressing cells (Fig. 4). This finding suggests that the presence of higher than normal levels of REST in NS/P cells does not in itself compromise their identity, at least within an 8 d period, supporting the view that the exit of the REST-expressing cells from the NS/P stage during neocortical development is likely because of progression, albeit delayed, of neuronal differentiation.

Persistent Expression of REST Interferes with the Normal Spatiotemporal Transition of NS/P Cells to Neurons but Not with Acquisition of Neuronal Cell Fate. We further analyzed the REST-expressing cells at postnatal stages of the electroporated brains. For this analysis, the REST-IRES-GFP–expressing plasmid was coelectroporated with mRFP-expressing plasmid at E14 and the brains were analyzed at postnatal day 14 (P14) or 23 (P23). At these stages, the majority of the GFP-expressing cells were localized to the white matter where mainly glial cells normally reside (Fig. 5*A*, *Upper*), and consistent with their previous IZ arrested position during embryogenesis. Most of the mRFP-expressing cells also expressed GFP (Fig. S4*A*), indicating that at these postnatal stages, the bicistronic REST-IRES-GFP mRNA was still expressed and not significantly down-regulated posttranscriptionally. The REST-expressing cells were not apoptotic, as indicated by the absence of Caspase3 (Fig. S4*B*). Notably, 95% of the electroporated cells had differentiated into mature neurons, as indicated by the presence of the late neuronal marker NeuN (Fig. 5). At P14, most of the cells expressed NeuN weakly, except for the few cells localized close to or in cortical layer VI, which expressed NeuN to higher levels and extended long processes (Fig. 5*A*, arrows *Upper Left*). At P23, however, most of the cells expressed NeuN to high levels and extended processes as indicated by the intense GFP around the cell bodies (Fig. 5*A*, *Bottom*). Importantly, REST was still present in up to 85% of the GFP-positive cells, which expressed NeuN [Fig. 5 (see arrows in *A*, *Lower*) and Fig. S4*C*). Together, our data suggest that, although REST is a repressor of a large network of neuronal-trait genes, continuous presence of REST interferes with the spatiotemporal transition of NS/P cells into neurons, but not with neuronal cell-fate decision.

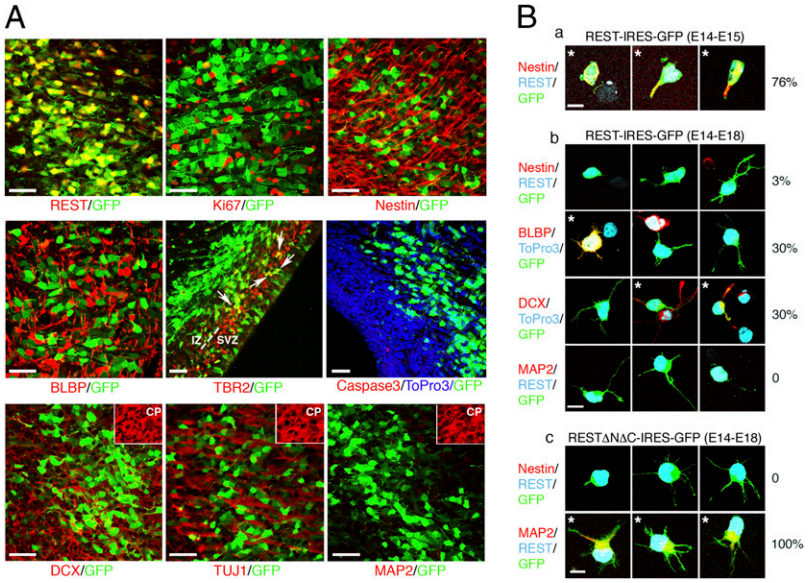

Fig. 3. The arrested REST-expressing cells are in transition between the NS/P and neuronal stage. (*A*) Immunostaining of representative coronal brain sections from E18 brains electroporated with pCA-REST-IRES-GFP at E14 for the progenitor markers: nestin, BLBP, TBR2, for the apoptotic marker Caspase3, or for the neuronal markers DCX, TUJ1, and MAP2 (all in red). Arrows show the few GFP$^+$ cells expressing TBR2. Fragmented line shows the boundary between the SVZ and IZ based on the location of the TBR2 progenitors. ToPro3 represents staining of nuclei. (*Insets*) Small sections of the CP area indicating the presence of neuronal markers at this stage. (Scale bars, 40 μm.) (*B*) Representative images of acutely dissociated cells immunostained for the indicated cell specific markers: (a) 1 d postelectroporation (E14–E15) with pCA-REST-IRES-GFP ($n = 5$); (b) 4 d postelectroporation (E14–E18) with pCA-REST-IRES-GFP ($n = 5$); (c) 4 d postelectroporation (E14–E18) with pCA-RESTΔNΔC-IRES-GFP ($n = 3$). Asterisks show cells that are positive for the indicated markers. Numbers on the right represent the percentage of positive cells labeled with the indicated markers out of total GFP$^+$ cells counted. (Scale bars, 10 μm.)

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DCX Overexpression Partially Rescues Radial Migration and the Timing of Neuronal Differentiation Defects Caused by REST. DCX, a microtubule-associated protein, is normally expressed in neural precursors and immature neurons that are migrating out of the VZ/SVZ to the cortical plate, and its absence interferes with the migration process (20). The REST migratory phenotype (Figs. 2, 3, and 5) was highly reminiscent of the subcortical band heterotopia phenotype caused by loss-of-function of DCX in the developing neocortex (20). In addition, similar to DCX loss-of-function, the sustained presence of REST during neurogenesis caused not only cell-autonomous but also noncell-autonomous disruption of radial migration, as evidenced by the aberrant location of not only $NeuN^+/GFP^+$ cells but also $NeuN^+$, GFP^- cells in the white matter of the electroporated hemisphere of P21 brains (Fig. S5). Based on these phenotypic similarities, we sought to examine whether expression of DCX could rescue, at least in part, the phenotype caused by sustained presence of REST during neurogenesis. To this end, we coelectroporated DCX and REST-IRES-GFP expression plasmids (pCAGGS-DCX and pCA-REST-IRES-GFP, respectively) at E14, and analyzed the brains at E19. Similar to what we described before, cells expressing only REST/GFP were arrested in the mid IZ (Fig. 6*A*, *Top Left*). In contrast, many cells coexpressing REST/GFP and DCX were now radially migrating toward the CP, and some of the cells reached the upper layer (Fig. 6*A*, *Top Center* and *Right*). Although cells expressing REST/GFP did not express DCX and lacked distinct morphology (Fig. 6*A*, *Middle Left*), cells in the IZ coexpressing REST/GFP and DCX extended processes and many appeared bipolar, and were migrating radially (Fig. 6*A*, *Middle Center* and *Right*, and Fig. S6). Indeed, it was shown previously that DCX is required for the multipolar-bipolar transition during the radial migration process (20). It should be noted that REST was retained in the migrating cells as well as in the cells that reached the upper layer (Fig. S6). Importantly, the migrating DCX-expressing cells expressed the late neuronal marker NeuN (Fig. S7) only as they migrated further in the IZ and became closer to the CP (Fig. S7), but cells that reached the upper layer of the CP expressed NeuN comparable to the neighboring nontransfected cells (Fig. 6*A*, *Bottom Center* and *Right*, respectively). These data suggest that expression of DCX rescued, at least in part, the migration and neuronal differentiation defects caused by the presence of REST and further indicate that the migration is coupled to the timing of neuronal differentiation.

To determine whether the *DCX* gene is a direct target of REST, we searched for the presence of an RE1 motif using the position weight matrix we generated previously for the serial analysis of chromatin occupancy of REST (8), and found a canonical RE1 motif in intron 3 of the *DCX* gene. Importantly, our ChIP analysis, using two different REST antibodies against the N and C termini of REST, clearly show that REST is bound to the RE1 motif in the *CDX* gene in NS/P cells (Fig. 6*B*). Taken together, our data suggest that REST regulates the radial migration coupled to neuronal differentiation, at least in part, by regulating the timing of *DCX* gene expression during neurogenesis.

Discussion

Using in utero electroporation to manipulate REST expression in NS/P cells in vivo, we analyzed the function and the significance of the differential presence of REST during nervous system development. Unexpectedly, our results show that although REST regulates a large network of neuronal trait genes, its continuous presence during neurogenesis does not interfere with the acquisition of neuronal fate per se but rather with the spatiotemporal acquisition of neuronal fate. Our results show that sustained presence of REST during neurogenesis interferes with migration and, therefore, with the timing of neuronal differentiation, likely because of the inability of the NS/P cells to clear out the higher than normal levels of REST via the proteasomal pathway. These results are further supported by previous studies showing that failure of the E3 ubiquitin ligase, β-TRCP, to degrade REST, attenuates ES cell differentiation into neurons in vitro (15). Thus, the down-regulation of REST from high

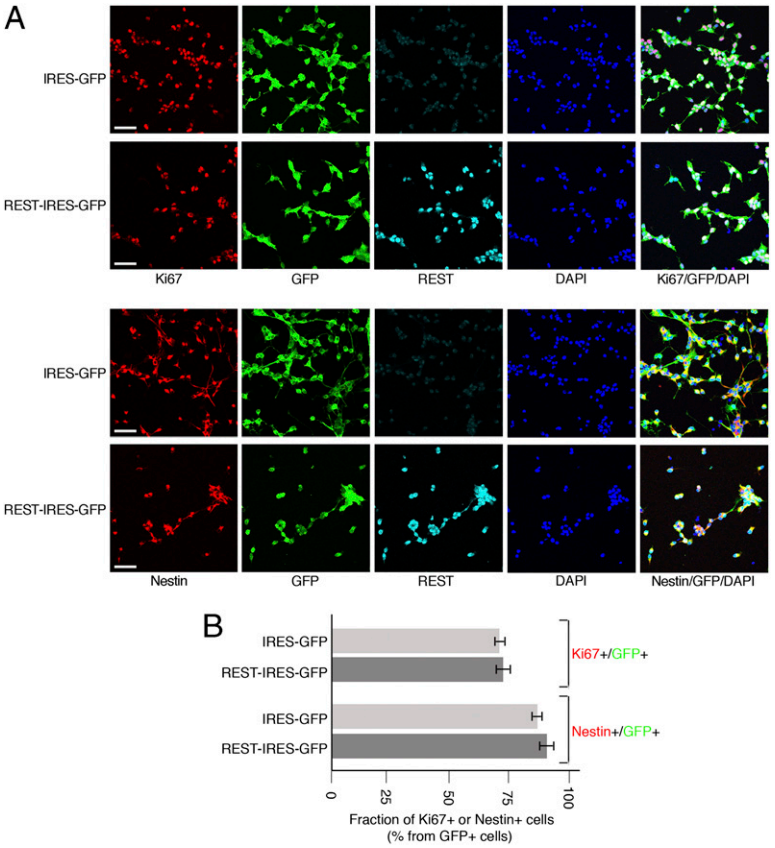

Fig. 4. Increased levels of REST in NS/P cells cultured in the presence of FGF-2 do not compromise the NS/P cell identity within an 8-d interval. (*A*) Immunostaining of GFP^+ E12.5 NS/P cells for nestin and Ki67, 8 d after transduction with lentivirus bearing pEF1α-IRES-GFP– or pEF1α-REST-IRES-GFP–expression vector. DAPI represents nuclear staining. Note the low level of endogenous REST. (Scale bars, 50 μm.) (*B*) Bar graphs represent the fraction of GFP^+ cells, which are Ki67- or nestin-positive. Error bars represent SD of three independent experiments based on counting 300 GFP^+ cells for each condition in each experiment.

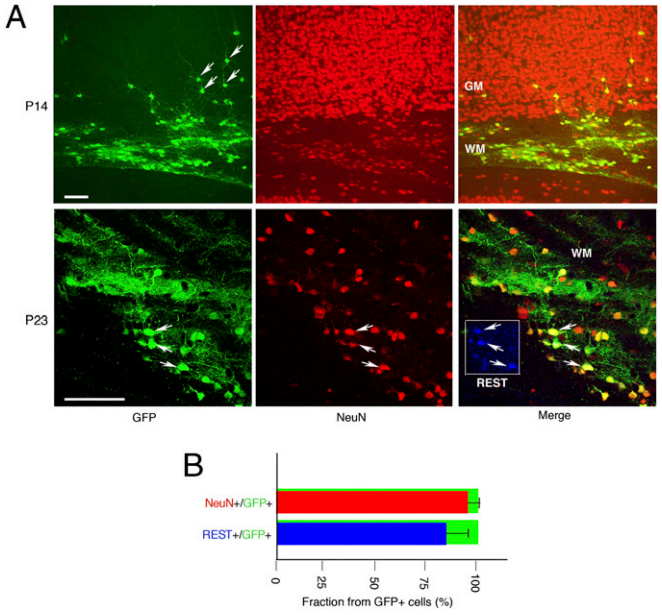

Fig. 5. The REST-expressing cells eventually differentiate into neurons during postnatal stages. Representative immunostaining of coronal brain sections. (*A*) pCA-REST-IRES-GFP was electroporated at E14 and brains were harvested at P14 (*Upper*) or P23 (*Lower*). (*Upper Left*) Arrows point to cells with long processes. (*Lower*) Arrows indicate cells that are GFP$^+$, NeuN$^+$, and REST$^+$. (Scale bars, 100 μm.) (*B*) Bar graphs represent the fraction of GFP$^+$ cells that are NeuN$^+$ or REST$^+$ at P23. Error bars represents SD on the basis of at least $n = 4$ and three sections from each brain. GM, gray matter; WM, white matter.

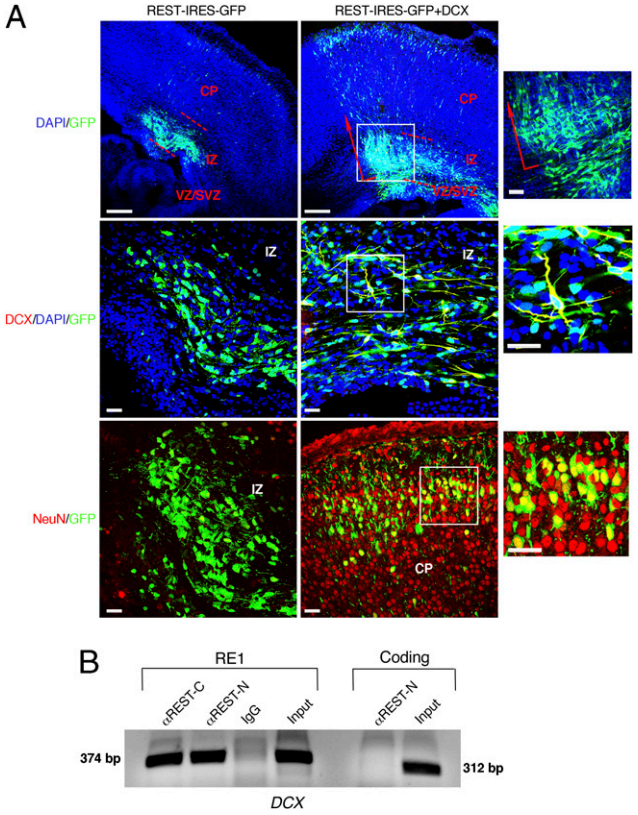

Fig. 6. Overexpression of DCX partially rescues the migration and neuronal differentiation defects caused by sustained REST expression. (*A*) Representative images of immunostained brain sections. Brains were electroporated at E14, either with REST-IRES-GFP alone or with the DCX-expression vector, and harvested at E19 ($n = 5$). Smaller panels on the *Right* are enlargements of the framed areas in the *Center*. (*Top*) Migration of the electroporated GFP cells. DAPI (blue) represents nuclear staining and serves as a marker for the different cortical layers (fragmented red lines). Red arrows show radial migration. (*Middle*) Coexpression of DCX with GFP. Note the bipolar radially migrating cell on the *Right*. (*Bottom*) Coexpression of NeuN with GFP. (Scale bars, all 50 μm, except *Top*, which are 200 μm.) (*B*) ChIP analysis on NS/P cells indicating binding of REST to the RE1 site in the *DCX* gene. Coding region of the *DCX* gene serves as a negative control.

levels in ES cells to low levels in NS/P cells and its subsequent loss during the transition to neurons are critical for proper progression of neurogenesis.

Potentially, the truncated form of REST that lacks the two repressor-domains (RESTΔNΔC) could act as a dominant-negative to inhibit endogenous REST function, as we showed previously in E12.5 NS/P cells (12). However, the 48-h time gap (Fig. 1*A*), between when RESTΔNΔC cDNA was electroporated into the VZ and when it was expressed to high levels, is sufficient to allow most of the electroporated cells to progress to an advanced stage of neurogenesis in which REST has been down-regulated. Therefore, the normal migration in the presence of RESTΔNΔC (Fig. 1*B*) cannot be interpreted as lack of loss-of-function effect of REST on this process. In fact, recent studies showed that loss-of-function of REST, at least during adult neurogenesis, resulted in a transient increase in neurogenesis, which eventually leads a diminished number of granule neurons (21).

Our sequential analysis of the fate of the REST-expressing NS/P cells as neocortical development proceeds, at embryonic and postnatal stages, indicates that the continuous presence of REST during the transition of NS/P cell to neuron delays—but does not prevent—the acquisition of neuronal fate in favor of progenitor or glial cell fates. This conclusion is lent support by our data showing that: (*i*) at earlier stages (E14–16 and E14–18), the numbers of GFP$^+$ cells expressing only GFP, or expressing GFP and RESTΔNΔC, or expressing GFP and full-length REST, are comparable to each other (Fig. 2*B*), indicating that the presence of functional REST did not expand the progenitor pool, although REST had been already overexpressed at the progenitor stage; and (*ii*) sustained expression of REST results in delayed migration of NS/P cells (E14–E16 and E14–E18) followed by exit from NS/P stage (E14–E18) and differentiation into neurons, but not glia (E14–P14 and E14–P23). These observations suggest that the presence of REST at low levels in NS/P cells and its subsequent disappearance during the transition to neurons is critical for the timing in which the NS/P cell is converted to neuron, but not for neuronal cell-fate specification.

Importantly, at postnatal stages, the REST-expressing cells were found in the white matter, a brain area that originates from the IZ. Despite the fact that the arrested cells were localized to the white matter where glial cells normally reside, and that normally glia and not neurons retain REST (22), these cells unexpectedly differentiated into neurons. This finding is surprising because REST is a repressor of neuronal-trait genes, suggesting that the REST-expressing cells circumvented the presence of REST and initiated neurogenesis, albeit with a delay. Earlier studies showed that extrinsic cues, to which NS/P cells are exposed in their environment, are a key determinant in their differentiation capacity. For example, embryonic cortical precursors generate neurons when cultured on embryonic cortical slices, but produce astrocytes when cultured on postnatal cortical slices (23). That the REST-expressing cells were arrested in the IZ and later in the white matter, yet did not differentiate into glia, suggests that the REST-expressing NS/P cells had been already committed to neurogenic fate and that the environmental cues were unable to switch their early neurogenic capacity to gliogenic. Importantly, although several bHLH proneural genes from the Neurogenin, Hes, and NeuroD gene families, as well as brain-specific microRNA genes, such as miR124, miR9, and others are REST targets (8, 10, 24; for review see refs. 25 and 26), neurogenic NS/P cells overexpressing REST remained neurogenic and finally acquired

neuronal fate. The inability to generate glia is also consistent with earlier studies showing that multipotent neural stem cells are not competent to make glia, even in the absence of bHLH proneural genes in the early phase of development (2, 27, 28), indicating that besides expression of these genes, other cell-intrinsic regulators are important for the gliogenic switch.

Our data show that between E16 and E18 the REST-expressing cells were able to proceed with slow migration, reaching half-way through the IZ before being arrested. Most of them progressed past the NS/P stage yet did not express DCX, which is required for radial migration (20). This finding suggests that migration has two phases: an initial phase that is DCX-independent and a second phase that requires DCX. Importantly, our data show that *DCX* is a direct REST target gene containing a canonical RE1 site and that REST binds to this site efficiently at the NS/P stage. Our data further show that overexpression of DCX rescues, at least in part, the migration and neuronal differentiation defects caused by sustained presence of REST, a phenotype reminiscent of the subcortical-band heterotopia caused by the loss of DCX. Thus, although REST is a repressor of many neuronal-trait genes, its sustained presence does not prevent terminal neuronal differentiation per se, but rather disrupts radial migration, and therefore the timing of terminal neuronal differentiation mediated at least in part, by continuous direct suppression of *DCX* gene expression during neurogenesis. This finding suggests that the low level of REST in NS/P cells and its subsequent disappearance as NS/P cells differentiate to neurons is critical for the proper timing of DCX expression, and thus for radial migration and the timing of neuronal differentiation. In support of our studies, recent studies show, that the loss of REST in adult NS/P cells elevates DCX expression (21). Interestingly, it was shown, that blocking miR124 also interferes with the timing of adult neurogenesis at the SVZ (29), suggesting that the activities of multiple key REST target genes are integrated to precisely time terminal differentiation.

Methods

The Institutional Animal Care and Use Committee at the University of Connecticut and Stony Brook University approved all of the animal studies.

Plasmids and in Utero Electroporation. The full-length human *REST* cDNA and the *REST*ΔNΔC cDNA (18) were subcloned into the pCA-IRES-GFP vector containing the CAG promoter (gift from C. Cepko, Harvard Medical School, Boston, MA). Expression of cDNA was verified by transfection into HEK293 cells and Western blot analysis. In utero electroporation was performed as previously described (20). One microliter of 1.5 μg/μL DNA, containing fast green and either one of plasmids pCA-REST-IRES-GFP, pCA- RESTΔNΔC-IRES-GFP, or pCA-IRES-GFP, was injected into the lateral ventricles of the embryos and electroporated (20). In experiments where the above plasmids were coelectroporated with pCAGGS-mRFP or pCAGGS-DCX, the plasmids (1.5 μg/μL) were mixed with 0.5 μg/μL pCAGGS-mRFP or 1.5 μg/mL pCAGGS-DCX.

Primary Cell Culture, Viral Infection, and Immunocytochemistry. For immunostaining of acutely dissociated cells after in utero electroporation, cortices were dissociated and plated on poly-D-lysine/laminin coverslips (30) and fixed after 4 h with 4% paraformaldehyde/PBS solution. Isolation and culture of E12.5 cortical NS/P cells was as previously described (12). For REST overexpression, the full-length human *REST* cDNA was subcloned into the lentiviral vector pEF1α-IRES-GFP (gift from I. Lemischka, Mount Sinai Medical Center, New York, NY) and NS/P cells were transduced and then cultured for 6 d. GFP^+ cells were sorted using FACS ARIA (Becton Dickinson) and cultured on coverslips for another 2 d before fixation. The primary antibodies used for immunocytochemistry are as described for immunohistochemistry (described in supplement) followed by the appropriate secondary antibody conjugated to Alexa Fluor 1:600 (Molecular Probes). Images were collected on a Zeiss confocal laser scanning LSM 510 microscope.

Chromatin Immunoprecipitation. ChIP assays were performed on E12.5 NS/P cultured in the presence of FGF, as described previously (18). Cross-linked chromatin was sonicated to generate fragments with an average length of 400 to 800 bp. For ChIP: anti–REST-N (REST p73) (9), anti–REST-C (12), or rabbit IgG (Santa Cruz) were used. DNA was subjected to 50 cycles of PCR. The primer sets used for the *DCX* gene: primer set flanking the RE1 site: forward 5′-GAT CCC TAG CTC TTA GGT AAA TAC ACA C, reverse 5′-AGC TCA TGG AGC TAA TGA CCA CCC. Primer set for coding region: forward 5′-ACA GAA CCA GAA CCT TGC AGG CAT, reverse 5′-TAA GCG AGG ATG TCC TTT CCC TCT.

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1. Qian X, et al. (2000) Timing of CNS cell generation: A programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. *Neuron* 28: 69–80.
2. Okano H, Temple S (2009) Cell types to order: Temporal specification of CNS stem cells. *Curr Opin Neurobiol* 19:112–119.
3. Edlund T, Jessell TM (1999) Progression from extrinsic to intrinsic signaling in cell fate specification: A view from the nervous system. *Cell* 96:211–224.
4. Gupta A, Tsai LH, Wynshaw-Boris A (2002) Life is a journey: A genetic look at neocortical development. *Nat Rev Genet* 3:342–355.
5. Hsieh J, Gage FH (2004) Epigenetic control of neural stem cell fate. *Curr Opin Genet Dev* 14:461–469.
6. Miller FD, Gauthier AS (2007) Timing is everything: Making neurons versus glia in the developing cortex. *Neuron* 54:357–369.
7. Schoenherr CJ, Anderson DJ (1995) The neuron-restrictive silencer factor (NRSF): A coordinate repressor of multiple neuron-specific genes. *Science* 267:1360–1363.
8. Otto SJ, et al. (2007) A new binding motif for the transcriptional repressor REST uncovers large gene networks devoted to neuronal functions. *J Neurosci* 27:6729–6739.
9. Chong JA, et al. (1995) REST: A mammalian silencer protein that restricts sodium channel gene expression to neurons. *Cell* 80:949–957.
10. Bruce AW, et al. (2004) Genome-wide analysis of repressor element 1 silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF) target genes. *Proc Natl Acad Sci USA* 101:10458–10463.
11. Johnson DS, Mortazavi A, Myers RM, Wold B (2007) Genome-wide mapping of in vivo protein-DNA interactions. *Science* 316:1497–1502.
12. Ballas N, Grunseich C, Lu DD, Speh JC, Mandel G (2005) REST and its corepressors mediate plasticity of neuronal gene chromatin throughout neurogenesis. *Cell* 121:645–657.
13. Lunyak VV, et al. (2002) Corepressor-dependent silencing of chromosomal regions encoding neuronal genes. *Science* 298:1747–1752.
14. Ballas N, Mandel G (2005) The many faces of REST oversee epigenetic programming of neuronal genes. *Curr Opin Neurobiol* 15:500–506.
15. Westbrook TF, et al. (2008) SCFbeta-TRCP controls oncogenic transformation and neural differentiation through REST degradation. *Nature* 452:370–374.
16. Chen ZF, Paquette AJ, Anderson DJ (1998) NRSF/REST is required in vivo for repression of multiple neuronal target genes during embryogenesis. *Nat Genet* 20:136–142.
17. Paquette AJ, Perez SE, Anderson DJ (2000) Constitutive expression of the neuron-restrictive silencer factor (NRSF)/REST in differentiating neurons disrupts neuronal gene expression and causes axon pathfinding errors in vivo. *Proc Natl Acad Sci USA* 97:12318–12323.
18. Ballas N, et al. (2001) Regulation of neuronal traits by a novel transcriptional complex. *Neuron* 31:353–365.
19. Yamada Y, Aoki H, Kunisada T, Hara A (2010) Rest promotes the early differentiation of mouse ESCs but is not required for their maintenance. *Cell Stem Cell* 6:10–15.
20. Bai J, et al. (2003) RNAi reveals doublecortin is required for radial migration in rat neocortex. *Nat Neurosci* 6:1277–1283.
21. Gao Z, et al. (2011) The master negative regulator REST/NRSF controls adult neurogenesis by restraining the neurogenic program in quiescent stem cells. *J Neurosci* 31: 9772–9786.
22. Kohyama J, et al. (2010) BMP-induced REST regulates the establishment and maintenance of astrocytic identity. *J Cell Biol* 189:159–170.
23. Morrow T, Song MR, Ghosh A (2001) Sequential specification of neurons and glia by developmentally regulated extracellular factors. *Development* 128:3585–3594.
24. Conaco C, Otto S, Han JJ, Mandel G (2006) Reciprocal actions of REST and a microRNA promote neuronal identity. *Proc Natl Acad Sci USA* 103:2422–2427.
25. Ross SE, Greenberg ME, Stiles CD (2003) Basic helix-loop-helix factors in cortical development. *Neuron* 39:13–25.
26. Li X, Jin P (2010) Roles of small regulatory RNAs in determining neuronal identity. *Nat Rev Neurosci* 11:329–338.
27. Tomita K, Moriyoshi K, Nakanishi S, Guillemot F, Kageyama R (2000) Mammalian achaete-scute and atonal homologs regulate neuronal versus glial fate determination in the central nervous system. *EMBO J* 19:5460–5472.
28. Nieto M, Schuurmans C, Britz O, Guillemot F (2001) Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. *Neuron* 29:401–413.
29. Cheng LC, Pastrana E, Tavazoie M, Doetsch F (2009) miR-124 regulates adult neurogenesis in the subventricular zone stem cell niche. *Nat Neurosci* 12:399–408.
30. Bittman KS, LoTurco JJ (1999) Differential regulation of connexin 26 and 43 in murine neocortical precursors. *Cereb Cortex* 9:188–195.