Nontelomeric splice variant of telomere repeat-binding factor 2 maintains neuronal traits by sequestering repressor element 1-silencing transcription factor

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Telomere repeat-binding factor 2 (TRF2) is critical for telomere integrity in dividing stem and somatic cells, but its role in postmitotic neurons is unknown. Apart from protecting telomeres, nuclear TRF2 interacts with the master neuronal gene-silencer repressor element 1-silencing transcription factor (REST), and disruption of this interaction induces neuronal differentiation. Here we report a developmental switch from the expression of TRF2 in proliferating neural progenitor cells to expression of a unique short nontelomeric isoform of TRF2 (TRF2-S) as neurons establish a fully differentiated state. Unlike nuclear TRF2, which enhances REST-mediated gene repression, TRF2-S is located in the cytoplasm where it sequesters REST, thereby maintaining the expression of neuronal genes, including those encoding glutamate receptors, cell adhesion, and neurofilament proteins. In neurons, TRF2-S–mediated antagonism of REST nuclear activity is greatly attenuated by either overexpression of TRF2 or administration of the excitatory amino acid kainic acid. Overexpression of TRF2-S rescues kainic acid-induced REST nuclear accumulation and its gene-silencing effects. Thus, TRF2-S acts as part of a unique developmentally regulated molecular switch that plays critical roles in the maintenance and plasticity of neurons.

brain development | RE1 element | Cajal–Retzius cells | alternative splicing | proteasomal degradation

During CNS development, the orderly acquisition and maintenance of neuronal traits is controlled by a complex network of transcriptional repressors and corepressors. A master repressor, repressor element 1-silencing transcription factor (REST), recruits various corepressors, suppressing neuronal programs in nonneuronal lineages (1–7). REST binds to a conserved 23-bp motif, repressor element 1 (RE1), of numerous neuronal genes (8, 9). During the early stages of neurogenesis, REST is greatly reduced by ubiquitin/proteasome degradation, resulting in derepression of genetic program for the neuronal phenotype (1, 2). Although it is unclear whether REST is present and functional in postmitotic neurons, emerging evidence suggests that REST dysfunction in adult neurons contributes to several neurological disorders, including cerebral ischemia (10) and epilepsy (11–13). In addition, Zuccato and colleagues demonstrated that a wild-type huntingtin protein inhibits the repressive effect of REST by sequestering it in the cytoplasm, whereas mutant huntingtin causes an aberrant nuclear accumulation of REST (14, 15). Because huntingtin is also expressed in nonneuronal cells, there is an ongoing search for factors that may regulate the functionality of REST, particularly in mature neurons in the adult brain (4, 16).

Alternative pre-mRNA splicing is a major contributor to proteomic diversity during development. From a single pre-mRNA species, multiple mRNA isoforms are generated by the shuffling of exons at intron–exon boundaries (17, 18). By containing or excluding certain pre-mRNA sequences, different splicing isoforms of an encoded protein may exhibit distinct subcellular localizations, protein–protein association, and/or enzymatic activities. Recent studies indicate that nearly 95% of human multiexon genes undergo alternative splicing (17). Neurons, in particular, exhibit an unusually large number of functionally relevant alternative splicing events in which specific protein isoforms are produced to regulate a range of neuronal properties, including excitability, neurite outgrowth, and synaptic plasticity (18).

Telomeres at the end of chromosomes are specialized DNA–protein complexes for maintaining chromosome stability. It has been suggested that telomere attrition is an important contributor to age-related diseases involving leukocytes (19), stem cells, and other proliferating cells (20, 21). However, little is known about the structure and function of telomeres in postmitotic neurons. Telomeres are protected by shelterin, a protein complex of six subunits, including telomere repeat-binding factors 1 (TRF1) and 2 (TRF2), protection of telomeres protein 1 (POT1), TRF1-interacting protein 2 (TIN2), tripeptidyl peptidase 1 (TPP1), and repressor/activator site-binding protein 1 (RAP1) (20–24). TRF2 serves as an anchoring molecule to facilitate the formation of telomeric cap-like structures, thereby preventing the ends of chromosomes from aberrant DNA recombination and degradation (20, 23, 24). Encoded by10 exons, TRF2 consists of a C-terminal telomere-binding Myb domain and an N-terminal dimerization domain (Fig. 1 *A* and *B*), which also facilitates nontelomeric interactions of TRF2 with other proteins (25, 26), including REST (27). For neural progenitor and tumor cells, experimentally induced loss of TRF2 can promote neuronal differentiation by a mechanism distinct from the typical telomere DNA damage responses of cell senescence or apoptosis (27, 28). Instead, TRF2 binds and stabilizes REST, thereby enforcing neuronal gene silencing. Disruption of the TRF2–REST interaction results in proteasomal degradation of REST and derepression of neuronal genes (27). Here, we report the discovery of a unique truncated TRF2 splice variant called TRF2-S (for TRF2 short) that is enriched in the cytoplasm of neurons, where it plays an important role in the acquisition and maintenance of the differentiated state of these postmitotic cells.

Results and Discussion

We screened for potential splicing isoforms of TRF2 in RNA samples from the cerebellums of rats, mice, and humans by RT-PCR with universal primers against exons 1 and 10 of the TRF2 mRNA (Fig. 1*A*). Two PCR fragments from rat (Fig. 1*A*) and human (Fig. S1 *A* and *B*) and three PCR fragments from mouse (Fig. 1*A*) were excised, subcloned, and sequenced. Clones having the longest PCR fragments (top bands in Fig. 1*A* and Fig. S1*B*) were identical to full-length TRF2 of National Center for Biotechnology Information (NCBI) reference sequences in those

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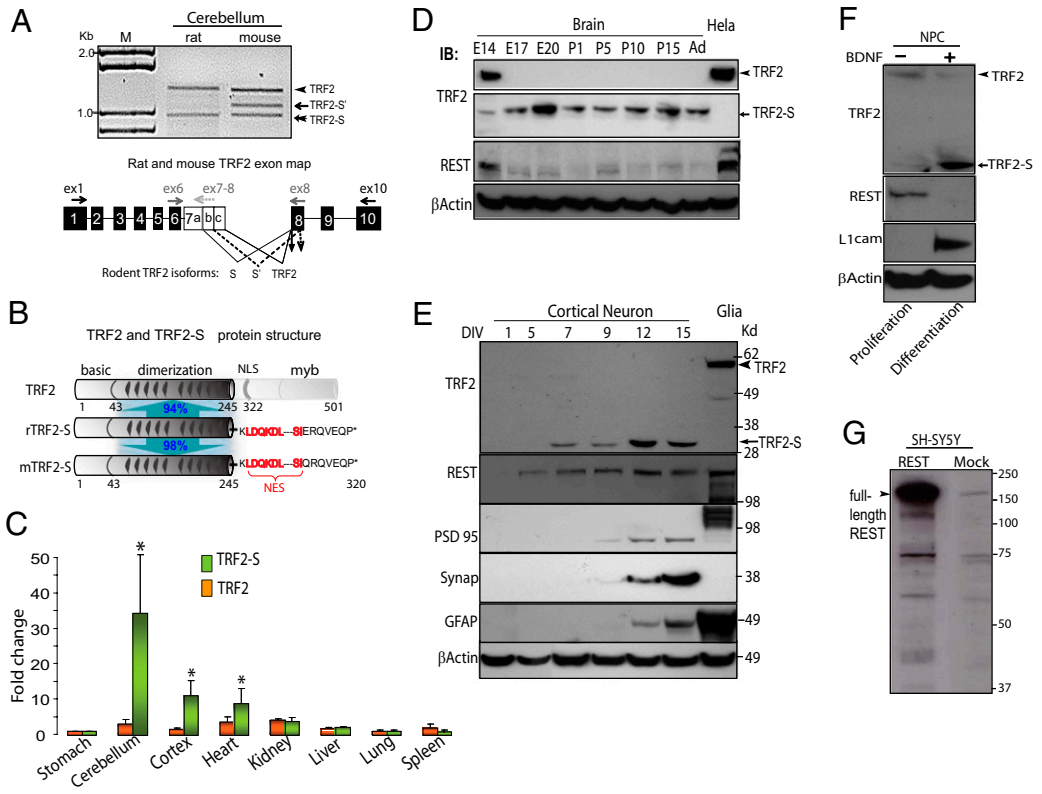

Fig. 1. Differential expression of TRF2 splice variants in rodent brains and cultured neuronal cells. (*A*) TRF2 isoforms were screened by RT-PCR using total RNAs from the cerebellums of rats and mice. (*Upper*) Top two bands were identical to full-length TRF2 (arrowhead). The bottom two bands represent unique short TRF2 isoforms (TRF2-S; heavy arrow). An additional mouse TRF2 isoform, TRF2-S′ (arrow), is described in Fig. S1 *A* and *C*. (*Lower*) Two universal primers against exons 1 and 10 are indicated by black horizontal arrows on the map. Downward arrows indicate the premature stop codons. (*B*) A conserved rodent TRF2-S isoform encodes a TRF2 protein lacking a Myb DNA-binding domain (myb) and a nuclear location signal (NLS) and containing a nuclear export signal (NES) at its C terminus. (*C*) qRT-PCR analysis of the expression of TRF2-S (green) and TRF2 (orange). Total RNAs were extracted from various rat tissues. The relative levels of TRF2 mRNAs from each tissue were normalized to those from stomach tissue. The rat-specific primers are indicated as gray arrows on the map in *A*. (*D*) Immunoblot analysis of TRF2 isoforms and REST during rat brain development. Brain extracts were collected from E14, E17, E20, P1, P5, P10, P15, and adult (Ad) rats. Blots were probed with antibodies against TRF2 (9143; Santa Cruz Biotechnology) and REST (07-579; Upstate Biotechnology). HeLa cell extract was used as a control. (*E*) Immunoblot analysis of TRF2 isoforms and REST in postmitotic neurons at different maturation stages and in proliferating glial cells. Membranes were reprobed with the neural maturation markers PSD95 and synaptophysin (Synap) as well as the glial cell marker glial fibrillary acidic protein (GFAP). (*F*) Primary cortical NPCs were either maintained as neurospheres in proliferation medium lacking BDNF (*SI Materials and Methods*) or differentiated by culture in medium containing 10 ng/mL BDNF. The expression of TRF2-S was induced by exposure of NPCs to BDNF for 7 d, accompanied by the down-regulation of REST and up-regulation of L1cam, a neuronal protein encoded by a REST target gene. (*G*) A REST antibody (07-579; Upstate Biotechnology) recognized full-length REST (~180 kDa) that was overexpressed in SH-SY5Y cells.

three species. In contrast, clones having the shortest PCR products (bottom bands in Fig. 1*A*) in both rodent samples represented a unique TRF2 isoform with deletions of 274 nt (rat) and 269 nt (mouse) in the exon 7bc region, resulting in a frameshift premature stop codon (Fig. 1*A* and Fig. S1 *A* and *C*). The latter isoform lacked the Myb domain and a nuclear location signal and included a unique nuclear export signal at its C terminus (Fig. 1*B*), suggesting a possible cytoplasmic localization of the protein. To confirm this result, we searched the NCBI database of nucleotide sequences and ESTs and found two ESTs (FM076256 and BG298303) and a full-length cDNA (AK160421) with an identical exon 7bc-skipping pattern. Here we designate this exon 7bc-deficient isoform as TRF2-S (GenBank accession no. HQ651915). The other TRF2 splice variants are shown in Fig. S1. The yeast TRF1 and TRF2 homolog Taz1 has also been reported to have splicing isoforms (29).

Differential Expression of TRF2 Splice Variants During Neuronal Development. To determine whether TRF2-S has a physiological function in the brain, we first compared the relative abundance of TRF2 and TRF2-S mRNAs in the brain with other tissues in the adult rat by quantitative RT-PCR (qRT-PCR). The rat-specific primers were designed to amplify the alternative splicing regions between exons 7 and 8 (Fig. 1*A*, gray arrows in map). qRT-PCR analysis demonstrated that expression levels of TRF2-S were significantly higher in cerebellum, cerebral cortex (forebrain), and heart compared with kidney, liver, lung, and spleen, whereas levels of TRF2 exhibited only modest variations among these tissues (Fig. 1*C*). TRF2-S mRNA was 10-fold, 7-fold, and 2.5-fold more abundant than TRF2 mRNA in cerebellum, cortex, and heart, respectively. These data suggest that TRF2-S is the predominant TRF2 isoform in tissues composed of postmitotic excitable cells.

To analyze dynamics of protein expression of TRF2 and TRF2-S during brain development, we used an antibody raised against the TRF2 dimerization domain. The specificity of this antibody was validated by using positive (overexpression of TRF2-S) and negative (shRNA-mediated knockdown) controls (Fig. S2*A*). TRF2 was expressed in high amounts in the developing rat brain before embryonic day 14 (E14); thereafter, TRF2 levels were greatly reduced, and TRF2-S expression was predominant (Fig. 1*D*). To further characterize the TRF2/TRF2-S switch, we compared postmitotic cortical neurons with proliferating glial cells in culture. As shown in Fig. 1*E*, TRF2 (~60 kDa) was undetectable in the cortical neurons but abundant in the glial cells. On the other hand, TRF2-S (~32 kDa) was absent in the glial cells, and, in the neuronal cultures, TRF2-S became abundant between days 7 and 15 as neuronal maturation occurred. We next induced the terminal differentiation in a homogenous population of cultured human neuroblastoma cells by sequential exposure to retinoic acid (RA) and brain-derived neurotrophic factor (BDNF) (Fig. S2*C*; ref. 30) and found that administration of RA and BDNF shifted TRF2 expression from TRF2 in the proliferative cells to TRF2-S in the neuronally differentiated cells (Fig. S2*B*). To further determine whether the TRF2/TRF2-S switch occurs upon the exposure of authentic proliferating neural progenitor cells (NPC) to a differentiation stimulus, we cultured primary cortical NPCs under conditions either for maintaining self-renewal (in the presence of FGF2 and EGF) or for promoting neural differentiation (in the presence of

BDNF and the absence of FGF2 and EGF) (Fig. S3 *A* and *B*). Whereas proliferating NPCs expressed only TRF2, the differentiating cells expressed TRF2-S as the predominant TRF2 isoform (Fig. 1*F*). Thus, in response to signals that induce the differentiation of neurons from proliferative precursor cells, a molecular switch from TRF2 to TRF2-S occurs, suggesting a role for TRF2-S in the acquisition and maintenance of a postmitotic neuronal phenotype.

TRF2-S Interacts with REST in the Cytoplasm of Mature Neurons. An abundance of REST in stem cells and nonneuronal somatic cell lineages is essential for suppressing the expression of neuronal genes (1–7). As NPCs differentiate into neurons, REST is largely degraded by the ubiquitin/proteasome system to derepress neuronal genes (Fig. 1*F*) (1, 2). When using an antibody to a high molecular-weight species of REST (~180 kDa; Fig. 1*G*), little or no REST was detected in young neurons before 5 d in vitro (DIV); TRF2 and TRF2-S were also undetectable in newly generated neurons. Unexpectedly, REST levels increased as neurons matured, coincident with up-regulation of TRF2-S and the markers of mature neurons synaptophysin and postsynaptic density protein 95 (PSD95) (Fig. 1*E*). Inhibition of proteasomal degradation with MG132 (1) markedly enhanced REST levels in older neurons (13 DIV) compared with younger neurons (6 DIV) (Fig. 2*A*). However, TRF2-S in mature neurons was not sensitive to proteasomal inhibition, likely because of a distinct regulatory pathway for its turnover. To evaluate REST subcellular distribution, we performed cell fractionation analysis of cortical neurons at different culture stages. As shown in Fig. 2*B*, levels of REST and TRF2-S in cytoplasmic fractions increased between 7 and 13 d in culture. In contrast, REST in the nuclear fraction remained at a low level during the process of neuronal maturation. We also observed that both TRF2-S and REST were enriched in the cytoplasm of class III β-tubulin (Tuj1)-positive neurons in differentiated NPC cultures (Fig. S3*C*) and in mature, but not in young, cortical neurons (Fig. S4*A*). In contrast, TRF2 immunoreactivity was concentrated in nuclear telomere-like foci of proliferating glial cells, whereas REST was distributed diffusely throughout these cells (Fig. S4*B*). These findings suggest a potential role for TRF2-S in sequestering REST in the cytoplasm.

To determine whether REST and TRF2-S interact in the cytoplasm of mature neurons, we used the bimolecular fluorescence complementation (BiFC) assay, a technique for visualizing protein interaction-induced fluorescent signals inside cells (31), to visualize REST–TRF2-S complexes in cortical neurons. Complementary fragments of Venus (VN173 and VC155) (32) were differentially tagged to REST, TRF2-S, and TRF2 (Fig. S5*A*) for cotransfection into cortical neurons (8 DIV). As shown in Fig. 2*C*, the fluorescent complexes of TRF2-S/VC155-REST/VN173 were localized in the cytoplasm and associated with clathrin-positive compartments. In contrast, TRF2/VC155-REST/VN173 complexes were concentrated in nuclear foci. We further mapped the domains of TRF2-S required for it to interact with REST by cotransfection of REST/VN173 with TRF2-S deletion mutants/VC155s into HeLa cells. The dimerization domain of TRF2-S was indispensable for forming TRF2-S–REST complexes, whereas the nuclear export signal region contributed to the cytoplasmic localization of REST (Fig. S5 *B* and *C*). As a control, we substituted REST with a well-known corepressor of REST, CoREST (33), in the BiFC assay. CoREST bound to TRF2 but not to TRF2-S (Fig. S6 *A* and *B*), providing further evidence for specificity of the TRF2-S interaction with REST. When overexpressed in HeLa cells, TRF2 and TRF2-S were both coimmunoprecipitated with endogenous REST (Fig. 2*D*). We also examined the endogenous association between TRF2-S and REST by using a reciprocal coimmunoprecipitation analysis with rat brain extract. We found that TRF2-S was readily detected in both TRF2 and REST immunoprecipitates. However, we failed to identify REST in TRF2 reciprocal immunoprecipitates (Fig. 2*E*). Altogether, our data indicate that TRF2-S interacts with REST but with less efficiency than the interaction of TRF2 with REST (27).

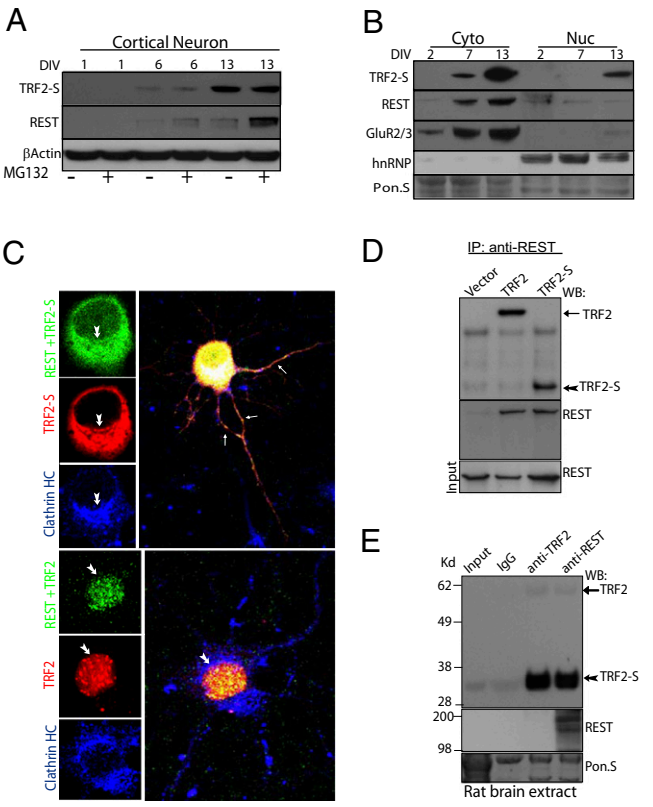

Fig. 2. Formation of TRF2-S and REST cytoplasmic complexes in mature neurons. (*A*) The treatment of proteasome inhibitor (10 μM MG132 for 5 h) markedly enhanced REST accumulation in mature neurons (13 DIV) but not in young neurons (1 DIV). (*B*) Concurrent elevation of TRF2-S and REST in the cytoplasm of mature neurons. Young (2 DIV), intermediate (7 DIV), and mature (13 DIV) cortical neurons were subjected to subcellular fractionation. The nuclear and cytoplasmic fractions were analyzed by immunoblotting. Membranes were reprobed with antibodies against the cytoplasmic marker GluR2/3 and the nuclear marker heterogeneous nuclear ribonucleoprotein (hnRNP). A total of 55 μg and 25 μg of protein from cytoplasmic and nuclear fractions was analyzed, respectively. Equal loading was assessed by Ponceau S (Pon.S) staining. (*C*) Analysis of cytoplasmic TRF2-S–REST complexes and nuclear TRF2–REST complexes. Cortical neurons (8 DIV) were subjected to Venus-mediated BiFC fluorescent (green) analysis and immunolabeling of TRF2-S/TRF2 (red) and the cytoplasmic vesicle marker clathrin heavy chain (blue). They were cotransfected with REST/VN175 in combination with either TRF2-S/VC155 (*Upper*) or TRF2/VC155 (*Lower*) for 24 h. (*Upper*) Interaction between TRF2-S and REST brought N- and C-terminal fragments of Venus (VN175 and VC155) into proximity to form a BiFC complex in cytosol (heavy arrow) and neurites (arrow). (*Lower*) Interaction between TRF2 and REST formed a BiFC complex in the nucleus (heavy arrow). (*D*) TRF2 (arrow) and TRF2-S (heavy arrow) were detected in REST immunoprecipitates in HeLa cells overexpressing either TRF2 or TRF2-S. (*E*) Rat brain extract was subjected to coimmunoprecipitation with TRF2 and REST antibodies. A distinct endogenous TRF2-S band at ~35 kDa (heavy arrow) and a faint TRF2 band at ~60 kDa (arrow) were detected in both TRF2 and REST immunoprecipitates, whereas REST immunoactivity was detected only in REST, but not in TRF2, immunoprecipitates.

Formation of TRF2-S and REST Cytoplasmic Complexes in Developing Brain. To explore the biological significance of the interaction of TRF2-S with REST, we determined whether the timing of their interaction is correlated with the maturation of early- and late-born neurons in brain development. During early development of the cerebral cortex, Reelin-expressing Cajal–Retzius (CR) cells are born and then migrate into the marginal zone as they mature at E12–E13, contributing to cortical lamination (34). At E14, newly generated neurons move to the cortical plate and are mature by E18. Of note, the genes encoding the neuron-specific proteins Tuj1, L1 cell-adhesion molecule (L1cam), reelin, and

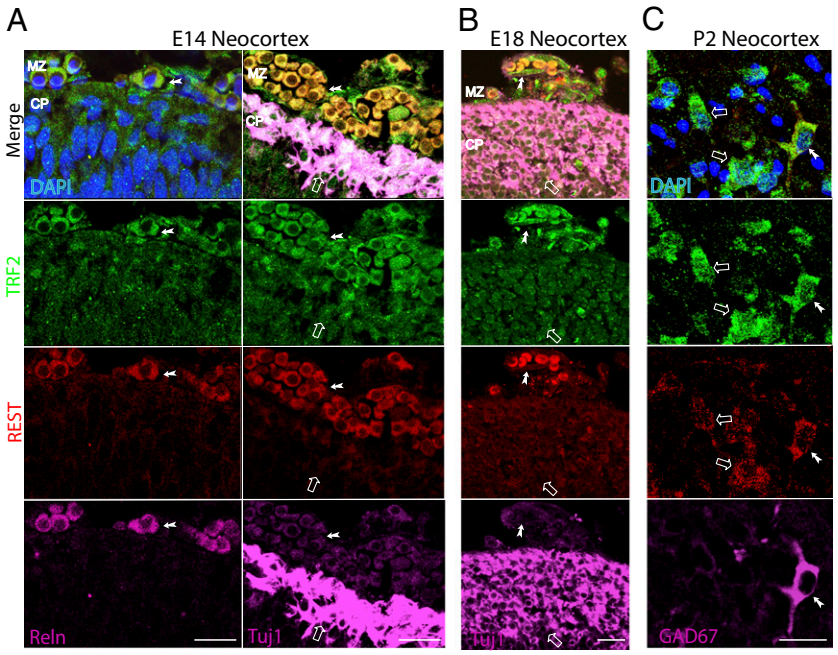

Fig. 3. Colocalization of TRF2-S and REST during neural maturation in developing neocortex. Coronal sections of E14, E18, and P2 neocortex were probed with TRF2 (9143; Santa Cruz Biotechnology) and REST (P18; Santa Cruz Biotechnology) antibodies in combination with the young neuron marker Tuj1 and the interneuron markers Reelin (Reln) and GAD67. (*A*) At E14, TRF2-S and REST were both concentrated in the cytoplasm of Reelin-positive CR cells that matured earlier and migrated in the marginal zone (MZ; heavy arrows), whereas TRF2-S and REST immunoreactivities were barely detectible in nascent Tuj1-positive pyramidal neurons that were migrated into cortical plate (CP; empty arrows). (*B*) In E18 neocortex, both CR cells (heavy arrows) and pyramidal neurons (empty arrows) appeared to have cytoplasmic coimmunoreactivity of TRF2-S and REST. (*C*) Cytoplasmic association of TRF2-S and REST in fully mature neurons (empty arrows), including GAD67-positive interneurons (heavy arrows) in P2 neocortex. DAPI (blue) was used for nuclear staining. (Bars: 20 μm.)

glutamate decarboxylase 67-kDa isoform (GAD67) are REST target genes (3, 35), and their expression therefore indicates lack of suppression by REST. At E14, Reelin-positive CR cells expressed the young neuronal markers Tuj1 and microtubule-associated protein 2 (MAP2) as well as the mature neuronal markers L1cam and NeuN (Fig. 3*A* and Fig. S7 *B* and *C*). The fully mature CR cells exhibited strong TRF2-S and REST coimmunoreactivity in the cytoplasm. Meanwhile, newly generated neurons expressed high levels of Tuj1 and MAP2, but little or no L1cam or NeuN, and only weak and diffuse TRF2-S and REST immunoreactivity (Fig. 3*A* and Fig. S7 *B* and *C*). Proliferating (Ki67-positive) neural progenitors also had little REST

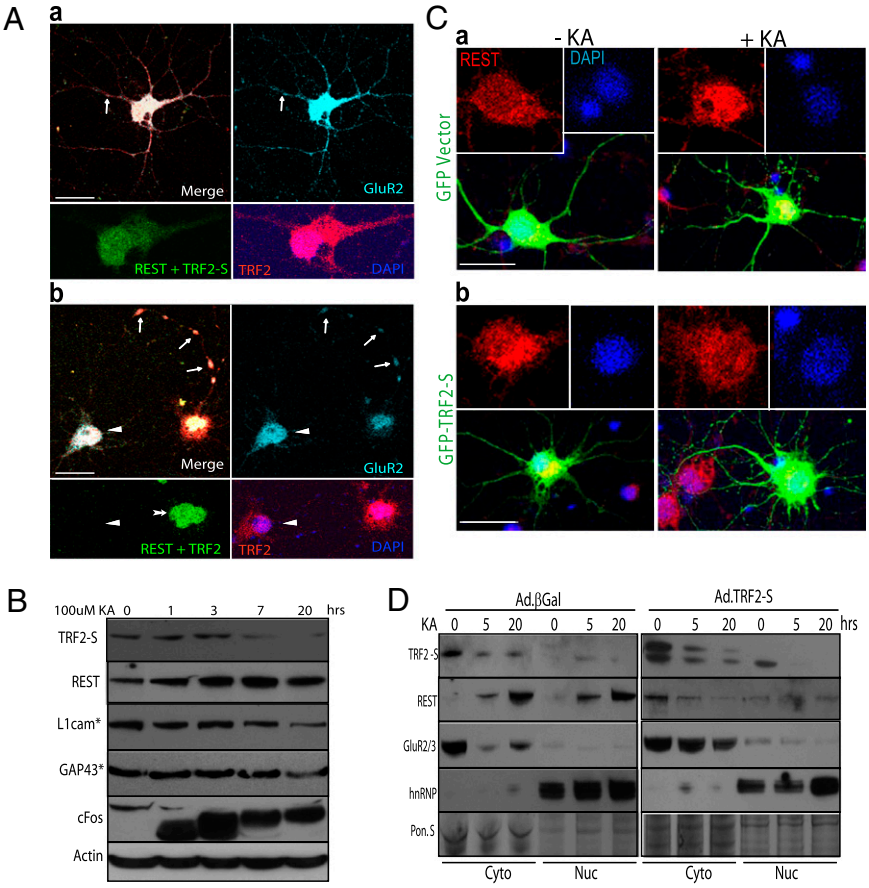

Fig. 4. Evidence of cytoplasmic sequestration of REST by TRF2-S. (*A*) Coexpression of REST with TRF2 (*Ab*) but not with TRF2-S (*Aa*) induced REST nuclear translocation (heavy arrow in *Ab*) and morphological abnormalities (deformed or beaded neurites and aberrant GluR2 accumulation in 8-DIV neurons; arrows in *Ab*). BiFC complexes (green) and immunostaining of TRF2 (red) and GluR2 (light blue) are shown. Arrowheads indicate untransfected neuron. (Bars: 20 μm.) (*B*) KA treatment of neurons up-regulated REST and depleted TRF2-S. Neurons (9 DIV) were treated with 100 μM KA for 0–20 h. Whole-cell lysates were subjected to immunoblotting, probed with TRF2 and REST antibodies, then reprobed with antibodies recognizing L1cam and GAP43. (Asterisks indicate REST target gene products.) cFos was positive control. (*C*) Blocking KA-induced REST nuclear accumulation by TRF2-S. Neurons (7 DIV) were transfected with either GFP vector alone (*Ca*) or GFP-tagged TRF2-S (*Cb*) for 24 h, then incubated with KA (+KA) or without KA (−KA) for an additional 20 h. Cells were fixed and immunostained with REST antibodies (red). DAPI (blue) was used for nuclear staining. (Bar: 20 μm.) (*D*) TRF2-S attenuated KA-induced REST nuclear translocation. Neurons (5 DIV) were infected with adenovirus bearing either βGal or GFP-tagged TRF2-S for 3 or 4 d then treated with KA for 5 or 20 h before cell fractionation and immunoblotting.

or TRF2 immunoreactivity (Fig. S7*A*). At E18, numerous young neurons were present in the cortical plate and began to exhibit TRF2-S and REST immunoreactivities in the cytoplasm (Fig. 3*B*). At postnatal day 2 (P2), neurons throughout the neocortex were fully mature and exhibited colocalization of TRF2-S and REST in their cytoplasm (Fig. 3*C*). Thus, in the developing neocortex, the temporal and spatial patterns of the expression of TRF2-S and REST are coincident, providing further evidence of the cytoplasmic association of TRF2-S and REST in mature postmitotic neurons.

TRF2-S Antagonizes Excitatory Amino Acid-Induced REST Nuclear Accumulation and Its Neural Gene-Silencing Effects. In excitotoxic neurodegenerative conditions, including cerebral ischemia (10) and epilepsy (12), the transcription of glutamate receptor subunit 2 (GluR2) is suppressed by nuclear accumulation of REST. Given that TRF2 expression was low in neurons and overexpressing TRF2 and REST facilitated the distinctive nuclear translocation of REST, we reasoned that coexpressing TRF2 and REST may recapitulate REST nuclear repressive effects in excitotoxic conditions. As a comparison, coexpressing TRF2-S and REST did not induce an aberrant expression of GluR2; instead, an enhanced immunoreactivity of GluR2 was found to spread throughout the soma and neurites of neurons (Fig. 4*Aa*). In contrast, overexpressing TRF2 and REST reduced the overall level of GluR2 and greatly disrupted the distribution of GluR2 such that the immunoreactivity of GluR2 was concentrated in large foci along the degenerative neurite (Fig. 4*Ab*). Thus, the key function of TRF2-S in blocking REST nuclear activity is not replaceable by TRF2. We next asked whether TRF2-S is dispensable for preventing REST nuclear accumulation upon exposure of neurons to the seizure-inducing excitotoxin kainic acid (KA) (11, 12). There was a rapid up-regulation of REST within 1 h after KA treatment and a reduction in TRF2-S levels evident at 7 h after KA treatment (Fig. 4*B*). Yet TRF2-S depletion was correlated with the down-regulation of REST target genes [e.g., L1cam and growth-associated protein 43 (Gap43) (Fig. 4*B*) as well as neuronal intermediate filament protein (Nfm), cyclin-dependent kinase 5 regulatory subunit 2 (Cdk5R2), and GluR2 (Fig. 5*D*)]. Importantly, KA-induced REST nuclear accumulation and its gene-silencing effect were remarkably rescued by elevating the expression of TRF2-S via adenoviral transduction (Fig. 4 *C* and *D*). These results suggest that TRF2-S is not necessary for regulating the overall level of REST, but it is essential for retaining REST in the cytoplasm, thereby maintaining the expression of neuronal genes. Of note, the increase in REST protein levels in response to KA occurred earlier and extended much longer than the increase in REST mRNA (Figs. 4*B* and 5*B*), indicating that the REST elevation is mainly the result of a posttranslational regulation event (1). As a control, KA-induced up-regulation of cFos had a typical transcriptional profile characterized by matched mRNA and protein dynamics.

We next determined whether TRF2-S counteracts the neuronal gene-silencing effects of KA and REST. As a consequence of KA-induced REST up-regulation and nuclear accumulation, REST occupancies in 11 of 26 potential RE1 sites were increased, as assayed by chromatin immunoprecipitation (ChIP) (Table S1). Strongly affected RE1s included L1cam −3.2k, GluR2 −0.1k, Nfm −0.9k, and Cdk5r2 +3.1k (Fig. 5*A*). The expression of RE1 element-regulated genes (e.g., *L1cam*, *GluR2*, and *Cdk5R2*) were evaluated by immunoblotting (Fig. 4 *B* and *D*) and RT-PCR (Fig. 5 *B* and *D*). The essential roles of these genes for neural functions and survival are listed in Table S1. Overexpression of TRF2-S markedly reduced REST binding to RE1s of the abovementioned genes as assayed at 5 and 20 h after KA treatment (Fig. 5*C*). RT-PCR measurements further demonstrated that TRF2-S rescued the expression of REST target genes that were otherwise suppressed by KA treatment (Fig. 5*D*). We also noticed that a non-RE1 promoter, brahma-related gene 1-associated factor 53B (BAF53b), and a non-REST target gene, GluR3, were affected by KA treatment. There are several plausible explanations for this result. Recent findings suggest that, in different subtypes of neurons and glial cells, REST-bound promoters contain a large number of previously uncharacterized non-RE1 motifs (16) that could potentially regulate neural-specific components (e.g., BAF53b) in switch/sucrose nonfermentable (SWI/SNF) chromatin

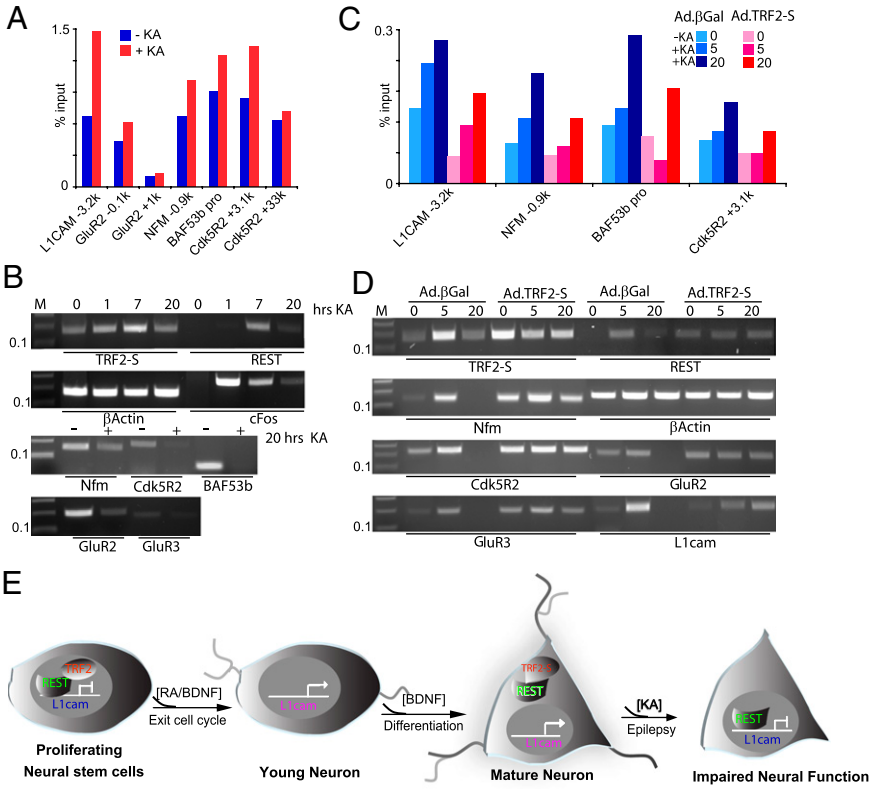

Fig. 5. TRF2-S attenuates KA-induced REST-mediated gene silencing. (*A*) ChIP was used to measure REST binding. Neurons (9 DIV) were treated with 100 μM of KA for 20 h. REST occupancies of its RE1 loci were normalized to the input DNA. Putative RE1 elements (RE1) in *L1cam*, *GluR2*, *Nfm*, and *Cdk5r2* genes as well as *BAF53b* promoters (pro) were tested. Binding to control IgG was 0.12. The results are representative of three independent experiments. (*B*) Semiquantitative RT-PCR analysis of RNAs from neurons treated with 100 μM KA. Expression levels of *TRF2-S*, *Rest*, and *cFos* (positive control) were analyzed along with housekeeping gene *β-actin* at 0, 1, 7, and 20 h. RE1 genes *GluR2*, *Nfm*, and *Cdk5r2*, non-RE1 gene *BAF53b*, and *GluR3* were analyzed at 0 and 20 h. (*C*) TRF2-S attenuates KA-induced REST binding to RE1 loci. Neurons (5 DIV) were infected with adenovirus bearing either βGal or GFP-tagged TRF2-S for 3 or 4 d, then treated with 100 μM KA for 5 or 20 h before ChIP assay. Binding to control IgG was 0.047. (*D*) Semiquantitative RT-PCR was performed on RNAs isolated from cells subjected to the same infections and KA treatment as indicated in *C*. (*E*) Model depicting interplay of TRF2, TRF2-S, and REST. Although TRF2 and TRF2-S both bind to REST, they exhibit opposite effects on REST nuclear activity at distinct stages of neural development because TRF2-S sequesters REST in the cytoplasm. Based on our current and previous results (27), L1cam, having the highest responses to REST-mediated repression, represents a group of REST target neural genes.

remodeling complexes. In addition, REST may indirectly regulate the expression of BAF53b and GluR3 via control of either positive or negative feedback loops. Furthermore, REST binding at unidentified distal or neighboring RE1 loci may contribute to repressing the expression of BAF53b and GluR3. Finally, REST might also have additional functions in the cytoplasm, including the regulation of translation initiation by binding RE1 elements in 5′ UTRs of mRNAs (36, 37).

Emerging findings suggest that REST has a biphasic activity in which REST-mediated neural-gene repressions occur not only in stem cells and nonneuronal lineages but also in varied subtypes of neurons (16, 38). Our findings reveal a biological process for the developmentally regulated switch from expression of TRF2, which stabilizes nuclear REST activity in NPCs, to the expression of TRF2-S, which antagonizes REST activity by retaining REST in the cytoplasm, in mature neurons. We propose a nonexclusive model that involves the interplay of TRF2 and TRF2-S with REST in a developmental stage-specific fashion (Fig. 5*E*). In self-renewing NPCs and proliferating glial cells, TRF2 is abundant and stabilizes REST in the nucleus, thereby enforcing neuronal gene silencing and inhibiting neurogenesis (27). As NPCs transit to postmitotic newly generated neurons, neither TRF2 nor TRF2-S is present for preventing REST from degradation. Thus, REST is largely depleted, resulting in the global expression of neuronal genes (1, 2). With further maturation and in response to differentiation signals such as BDNF, mature neurons express TRF2-S and REST. TRF2-S sequesters REST in the cytoplasm thereby preventing REST from repressing nuclear genes encoding proteins involved in synaptic transmission/plasticity and cell survival. Severe stress conditions, such as excessive activation of glutamate receptors, cause TRF2-S depletion, which allows REST to enter the nucleus where it silences neuronal genes that may cause the dedifferentiation/death of neurons (10, 14). Collectively, our findings provide a unique perspective for understanding mechanisms of brain development, and they also have implications for REST dysfunction-associated disorders, including glioblastoma (27), epilepsy (11–13), cerebral ischemia (10), and Huntington's disease (14, 15). A better understanding of the interactions of TRF2 and TRF2-S with REST may lead to novel therapeutic interventions for such disorders.

Materials and Methods

Methods for the culture of SH-SY5Y, HEK-293T, and HeLa cells as well as primary dissociated cultures of embryonic rat cerebral cortical neurons have been described previously (27, 28). BDNF was purchased from ProSpec Protein Specialists, MG132 was from Calbiochem, and KA and leptomycin B were from Sigma. Brains were immersion-fixed in 4% paraformaldehyde, and coronal sections were prepared for immunohistochemical staining. RT-PCR methods were similar to those described previously (28). The rat TRF2-S gene was cloned by using a two-round touchdown PCR protocol. Methods for transduction of neurons with adenoviral vectors for immunostaining and immunoblotting are detailed in *SI Materials and Methods*. For cell fractionation, cell pellets were subjected to nuclear and cytosol fractionation with NE-PER nuclear and cytoplasmic extraction reagents according to the manufacturer's protocol (Thermo Scientific). For immunoblot analysis, 55 μg of cytoplasmic protein or 25–35 μg of nuclear protein was used. BiFC assay was performed by cotransfection of REST/VN173 with VC155-conjugated full-length TRF2, TRF2-S, TRF2-S/ΔNES, and TRF2-S/B+NES in either HeLa cells or cortical neurons for 24 h (25–35 transfected neurons were analyzed for each condition). ChIP assay methods have been described previously (27).

Full details of all methods can be found in *SI Materials and Methods*.

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