Transducin-like Enhancer of Split-1 (TLE1) Combines with Forkhead Box Protein G1 (FoxG1) to Promote Neuronal Survival*[S]

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Background: Although highly expressed in the adult brain, the role of TLE1 in mature neurons is poorly understood.
Results: TLE1 promotes the survival of postmitotic neuron in cooperation with FoxG1. Phosphorylation of TLE1 by casein kinase-II and of FoxG1 by Akt plays a key role.
Conclusion: TLE1 and FoxG1 cooperate to promote neuronal survival.
Significance: Our results provide new information on the molecular mechanisms regulating neuronal survival.

Transducin-like enhancer of split-1 (TLE1) plays a critical role in the regulation of neurogenesis by inhibiting the differentiation of neural progenitor cells into neurons. Although TLE1 is also expressed highly in the postnatal brain and through adulthood, its role in postmitotic neurons is not clear. Using cultures of cerebellar granule neurons, we show that expression of TLE1 is reduced in neurons primed to die. Reestablishment of elevated TLE1 levels by ectopic expression protects neurons from death, whereas suppression of TLE1 expression in otherwise healthy neurons induces cell death. These results show that TLE1 is necessary for the maintenance of neuronal survival. Experiments using pharmacological inhibitors as well as expression of point mutants indicate that phosphorylation of TLE1 by casein kinase-2 (CK2) at Ser-239 and Ser-253 is necessary for its survival-promoting activity. TLE1-mediated survival is also inhibited by pharmacological inhibition of PI3K-Akt signaling but not by inhibitors of Raf-MEK-ERK signaling or other molecules, including histone deacetylases, calcium calmodulin kinase, or CK1. The survival-promoting activity of TLE1 depends critically on interaction with FoxG1, another protein involved in the regulation of neurogenesis and shown previously to promote survival of postmitotic neurons. Likewise, the ability of FoxG1 to promote neuronal survival depends on TLE1. Taken together, our study demonstrates that TLE1 cooperates with FoxG1 to promote neuronal survival in a CK2- and PI3K-Akt-dependent manner.

The human transducin-like enhancer of split (TLE)[2] and mouse homologue, Groucho-related gene (Grg), proteins are mammalian counterparts of the *Drosophila* Groucho protein. Like Groucho, TLE proteins play fundamental roles in the regulation of nervous system development as well as a number of other developmental processes (1–7). Whereas *Drosophila* produces a single Groucho protein, several TLE/Grg proteins are expressed in mammals, which, based on their size, can be subdivided into two groups. The larger TLE proteins act as transcriptional corepressors. Although lacking in DNA binding activity of their own, these proteins are recruited to gene promoters through interaction with a number of repressors belonging to different protein families and including the HES class of basic helix-loop-helix proteins, the LEF-1/TCF transcription factors, Runt homology domain proteins, the homeodomain transcription factor Engrailed, and the winged helix domain protein FoxG1 (1, 2, 8, 9). *Drosophila* Groucho and mammalian TLE proteins possess a conserved amino-terminal glutamine-rich domain (Q domain), a glycine/proline-rich domain (GP domain), a region containing phosphorylation sites for Cdc2 and casein kinase 2 (CcN domain), a serine/proline-rich domain (SP domain), and a conserved carboxyl-terminal region containing several WD40-repeat domains in tandem (Fig. 1). Interaction with repressor proteins mostly involves the WD40-repeat region of TLE proteins.

A second subgroup of TLE/Grg proteins has been identified in mammals that are truncated versions of the TLE proteins described above. Two members of this subgroup are amino-terminal enhancer of split (AES)/Grg5 and TLE6/Grg6. AES contains only the amino-terminal region of larger TLE proteins and lacks the carboxyl-terminal half, including the WD40-repeat region (Fig. 1). On the other hand, TLE6/Grg6 displays a high degree of similarity with the carboxyl terminus of the TLE proteins but lacks much of the amino-terminal region that the larger TLE proteins possess (Fig. 1). The physiological significance of these two smaller proteins is not well understood. AES can heteromerize with the larger TLE/Grg proteins, antagoniz-

LK, low potassium; HK, high potassium; Q domain, glutamine-rich domain; GP domain, glycine/proline-rich; CcN domain, Cdc2 and casein kinase 2 domain; BME, basal medium Eagle; TBB, 4,5,6,7-tetrabromobenzotriazole; HCA, homocysteic acid.
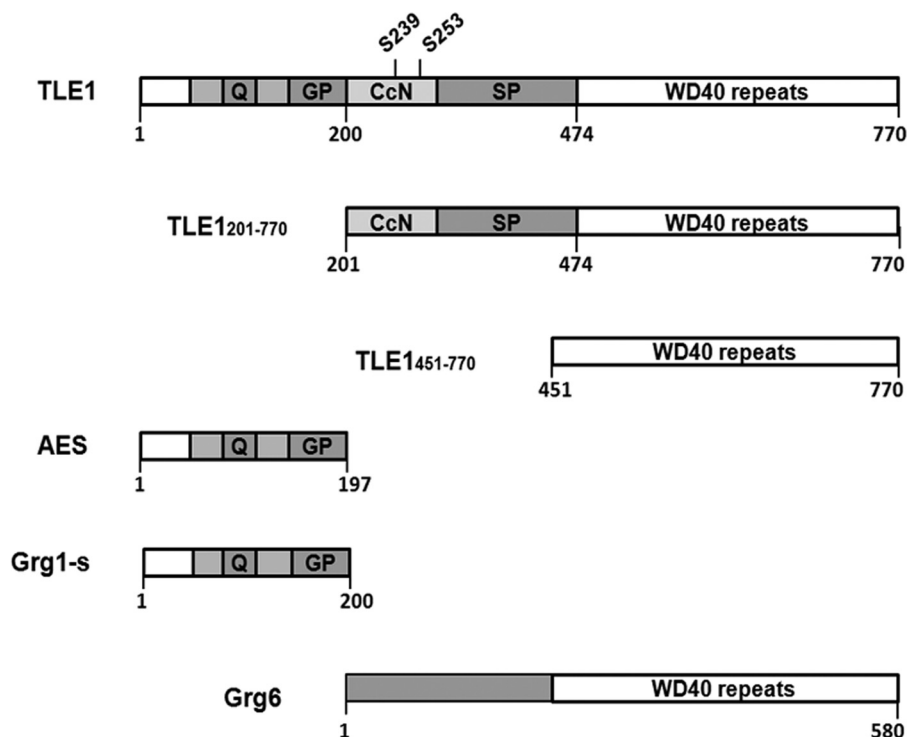
FIGURE 1. **Schematic representation of the different TLE1 constructs used in this study.** The amino terminus of TLE1 is characterized by the Q domain, which is required for dimerization; a GP domain; the CcN domain, which is involved in nuclear localization as well as phosphorylation by CK2 (at residues Ser-239 and Ser-253) and Cdc2; and an SP domain. The carboxyl terminus is characterized by WD40 repeats and is involved in binding to several proteins. TLE1(201–770)-FLAG is the deletion construct that lacks the dimerization domain, whereas TLE1(451–770) lacks the dimerization domain, the CcN domain, and the SP domain. AES, Grg1-s, and Grg6 proteins that were used in this study are also shown. AES lack the carboxy-terminal region of TLE1. Grg6 lacks the amino-terminal region of TLE1, including the Q domain.

ing their functions in a dominant negative manner; however, Grg6 cannot heteromerize with TLE1 (10, 11). It has also been proposed that AES/Grg5 have separate functions independent of TLE/Grg proteins regulating non-transcriptional events (12).

The best studied of the TLE proteins is TLE1. TLE1 inhibits the differentiation of neural progenitor cells into neurons during mammalian forebrain development (13–15). Forced TLE1 expression in cultures of undifferentiated cortical progenitor cells causes reduced neuronal differentiation and increased numbers of proliferating progenitors. Transgenic mice with elevated TLE1 expression exhibit a delay of forebrain neuronal differentiation during embryonic development (14). The inhibitory effect of TLE1 on neuronal differentiation is believed to be mediated through interaction with Hes1 (2). The transcriptional activity and functions of TLE1 can be regulated through phosphorylation by various kinases, including MAPK, Cdc2, and casein kinase-2 (CK2) (3, 8, 9). DHIPK2 phosphorylates *Drosophila* Groucho (16).

Consistent with its role in inhibiting neuronal differentiation, TLE1 expression is high in proliferating neural progenitor cells and is subsequently reduced concomitant to the transition of neural progenitors into postmitotic neurons (4, 13, 14). Interestingly, TLE1 expression is reactivated in mature neurons (4, 13, 14) and is high in the postmitotic brain through adulthood (supplemental Fig. 1). We recently described evidence indicating that TLE1 contributes to the survival of postmitotic neurons (11). Indeed, we described that a down-regulation of TLE1 expression precedes neuronal death and that reestablish-

ing an elevated level of TLE1 expression maintains neuronal survival even under apoptotic conditions (11). In this study, we investigated the mechanism by which TLE1 promotes neuronal survival. We report that the survival-promoting effect of TLE1 is mediated through interaction with FoxG1, another transcription factor that plays a critical role in the regulation of neurogenesis during early brain development. As described in the context of neurodevelopment, TLE1 and FoxG1 physically interact in mature neurons. Mutant forms of TLE1 that are unable to promote neuronal survival block FoxG1-mediated survival of neurons, whereas mutant forms of FoxG1 block the survival-promoting effect of TLE1, indicating that these two transcription factors function together. We report that activities of both CK2 and the PI3K-Akt pathway are necessary for TLE1- and FoxG1-mediated survival.

EXPERIMENTAL PROCEDURES

Materials—Unless indicated otherwise, all materials were purchased from Sigma-Aldrich. Tissue culture reagents, including culture medium and fetal calf serum, were purchased from Invitrogen. Antibodies used in this paper were as follows: FLAG (catalogue no. F1804, Sigma-Aldrich), HA (Y-11 (catalogue no. sc-805) and F-7 (catalogue no. sc-7392), Santa Cruz Biotechnology, Inc., Santa Cruz, CA), tubulin (TU-02 (catalogue no. sc-8035), Santa Cruz Biotechnology, Inc.), GFP (B-2 (catalogue no. sc-9996) and FL (catalogue no. sc-8334), Santa Cruz Biotechnology, Inc.), TLE1 (M-101 (catalog no. sc-9121), rabbit polyclonal, Santa Cruz Biotechnology, Inc.), IgG (3E8 (catalog no. sc-69786), Santa Cruz Biotechnology, Inc.), and

phospho-Akt substrate (catalog no. 110B7E, Cell Signaling (Danvers, MA)). In addition to these commercially available antibodies, a rabbit polyclonal FoxG1 antibody (17) and a rabbit polyclonal TLE1 antibody (2) were used. Primary antibodies were used at concentrations ranging from 1:250 to 1:1000 in 5% bovine serum albumin. Secondary antibodies (Pierce) were used at concentrations of 1:10,000. For immunocytochemistry, primary antibodies were used at a dilution of 1:200. Texas Red- or FITC-tagged secondary antibodies for immunocytochemistry were purchased from Jackson ImmunoResearch Laboratories and used at a dilution of 1:85.

Plasmids—The Grg1-S HA (18) plasmid was purchased from Addgene (Cambridge, MA). The TLE1-FLAG plasmid was described previously (19). From this construct, we generated TLE1(201–770)-FLAG and TLE1(451–770)-FLAG. The coding sequence of rat AES was amplified from RNA isolated from rats and subcloned into pTarget vector. All other constructs used were described in previous studies: FoxG1 plasmid constructs (17); TLE1$_{S239A}$-FLAG, TLE1$_{S239E}$-FLAG, and TLE1$_{S253A}$-FLAG (19); and Grg6 FLAG (20).

Neuronal Cultures—Cerebellar granule neurons (CGNs) were cultured from dissociated cerebella of 7–8-day-old Wistar rats as described previously (17, 21, 22) and plated on dishes coated with poly-L-lysine in 24-well dishes at a density of 1×10^6 cells/well or 1.2×10^7 cells/60-mm dish. Cytosine arabinofuranoside (10 μM) was added to the culture medium 18–22 h after plating to prevent replication of non-neuronal cells. Cultures were maintained for 6–7 days prior to experimental treatments. For treatment, the cells were rinsed once and then maintained in low potassium (LK) medium (serum-free BME medium, 5 mM KCl) or high potassium (HK) medium (serum-free BME medium, supplemented with 20 mM KCl). Treatment of cultures with pharmacological inhibitors was initiated at the time of switching the medium to HK or LK unless specified otherwise.

Embryonic cortical cultures were prepared from embryonic day 17 rat pups as described previously (17, 21). The cortical cultures were grown in DMEM + Glutamax-I medium (Invitrogen) supplemented with 10% FBS in the presence of penicillin/streptomycin (Invitrogen). About 26 h after plating, the cultures were treated with 15 μg/ml 5-fluoro-2-deoxyuridine and 35 μg/ml uridine to prevent mitotic cell proliferation.

Reverse Transcription-PCR—RNA was extracted from cultured neurons or HT22 cells using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA was normalized, and complementary DNA was made from 5 μg of RNA using the Thermoscript RT-PCR system (Invitrogen) according to the manufacturer's instructions. PCR was performed with PCR master mix (Promega, Madison, WI).

Transfection and Evaluation of Cell Viability—Transfection of CGNs was done as described previously (11, 17, 21). Briefly, 3 μg/well of DNA was precipitated by the calcium phosphate method for 5 min at room temperature. Cell culture medium of neurons was changed to DMEM without L-glutamine after one wash. Precipitated DNA was added to the cell culture medium for 30 min. Cells were then washed twice with DMEM without L-glutamine medium and placed in their original medium. 8 h after transfection, the cells were rinsed once and then main-

tained in serum-free BME medium containing either 5 mM KCl (LK) or 25 mM KCl (HK). Pharmacological inhibitors (dissolved in dimethyl sulfoxide) were added in the HK or LK condition at the time of treating. Transfected neurons were identified by immunostaining using FLAG antibody or GFP antibody as described previously (11, 17, 21). Neuronal viability was quantified by staining cell nuclei with 4′,6-diamidino-2-phenylindole hydrochloride (DAPI) as described previously (11, 17, 21). The proportion of successfully transfected cells (identified by immunocytochemistry) that displayed condensed or fragmented nuclei (visualized by DAPI) was quantified. In each individual experiment, more than 100 transfected cells were counted for each treatment or condition. Unless otherwise stated, cell viability data are presented as mean values $\pm$ S.E. from at least three separate experiments. Statistical significance was determined by using Student's t test. p values of <0.05 were deemed significant.

Transfections of cortical neurons were performed on 2–3-day-old cultures as described above for cerebellar granule neurons. When used, homocysteic acid (Sigma-Aldrich) was added at a 1 mM concentration 8 h after transfection for 15–18 h. Viability of successfully transfected cells was quantified following immunocytochemistry and DAPI staining.

shRNA-mediated Suppression—Two shRNA constructs against TLE1 and a control shRNA construct were purchased from Origene. These HuSH shRNA plasmids from Origene were designated as sh1 (catalog no. TG511107,34), sh2 (catalog no. TG511107,37), and control shRNA (catalog no. TR30007). The ability of these shRNAs to suppress TLE1 expression was evaluated in HEK293T and HT22 cells. Briefly, the cells were transiently transfected at around 85% confluence with the shRNA plasmid using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. A day after transfection, the cells were passaged using fresh medium supplemented with 1 μg/ml puromycin; this helps to enrich cells that are transfected with the shRNA plasmids. 72 h later, the cells were lysed using $1\times$ cell lysis buffer (Cell Signaling), and the protein lysate was harvested after centrifugation at $12,000 \times g$ for 12 min in 4 °C. Eastman Kodak Co. 1D software was used for densitometric analysis. For statistical analysis, one-way analysis of variance was performed using Bonferroni's multiple comparison tests. CGNs were transfected on day 4 with control shRNA or sh1 and sh2. Two days later, the neurons were treated with HK/LK for 24 h, after which the cells were fixed with 4% paraformaldehyde (diluted in phosphate-buffered saline). Viability of the GFP-expressing cells was quantified by DAPI staining.

Western Blotting and Immunoprecipitation—The culture medium was removed, and the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in $1\times$ cell lysis buffer (1% Triton, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na$_2$EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na$_3$VO$_4$, 1 μg/ml leupeptin, and 1 protease inhibitor mixture). Protein concentrations were measured and normalized using Bradford protein assay reagent (Bio-Rad). Following normalization, 40–80 μg of protein was subjected to Western blotting. Immunoreactivity was examined by enhanced chemiluminescence (Amersham Biosciences).
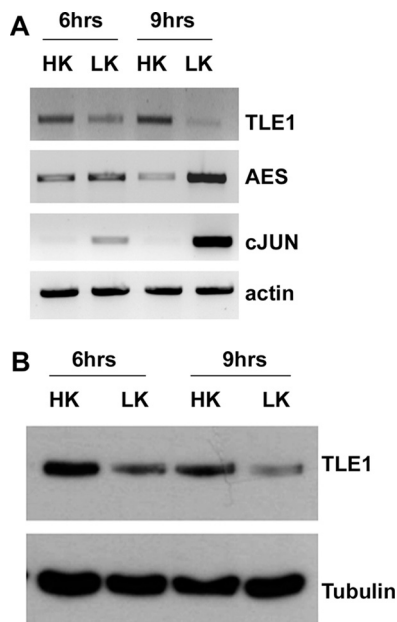
FIGURE 2. **Expression of TLE1 mRNA and protein during neuronal apoptosis.** *A*, RT-PCR analysis of RNA isolated from CGNs treated with HK or LK for 6 and 9 h. Expression of TLE1, AES, and c-Jun was evaluated. The level of TLE1 mRNA was down-regulated after 6 and 9 h of LK treatment. The level of AES was up-regulated after 9 h of LK treatment. c-Jun mRNA level was up-regulated after 6 and 9 h of LK treatment. Actin served as the loading control. *B*, Western blot analysis of TLE1 protein levels in CGN treated with HK or LK for 6 and 9 h, respectively. At the protein level also, TLE1 is down-regulated after 6 and 9 h of treatment with LK. Tubulin served as the loading control.

FIGURE 3. **Effects of TLE1 knockdown in CGNs.** *A*, Western blot analysis of endogenous TLE1 protein after knockdown using sh1 and sh2 shRNA in HEK 293T cells. sh1 was able to completely knock down endogenous levels of TLE1, whereas sh2 had a modest effect. Shown is densitometric analysis of endogenous TLE1 protein levels from three independent experiments after knockdown using sh1 and sh2 shRNA. *B*, viability of CGNs transfected with shRNAs, sh1 and sh2, against TLE1. Knockdown of endogenous TLE1 kills healthy neurons treated with HK. Viability was normalized with CGNs transfected with control shRNA treated with HK. *Error bars*, S.E. **, $p < 0.01$; ***, $p < 0.001$.

For immunoprecipitation experiments, cultured cells were treated accordingly and then washed twice with ice-cold PBS followed by lysis in lysis buffer. Lysates were centrifuged for 10 min at $12,000 \times g$ at 4 °C. The protein concentration of supernatant fractions was determined and normalized using Bradford protein assay reagent (Bio-Rad). Equal amounts of protein were incubated overnight with 1 μg of primary antibody at 4 °C. Samples were then incubated with 20 μl of Protein A/G PLUS-agarose (Santa Cruz Biotechnology, Inc.) for 2 h at 4 °C. Bead complexes were pelleted by centrifugation at $4000 \times g$ for 2 min at 4 °C and washed three times with $1\times$ cell lysis buffer. Pellets were then resuspended in 30 μl of $3\times$ SDS sample buffer, heated for 5 min at 95 °C, and subjected to Western blotting. Approximately 5–10% of the whole cell lysate was used as input to check for the expression of either overexpressed proteins or endogenous proteins.

RESULTS

TLE1 Is Required for Neuronal Survival—CGNs undergo apoptosis when switched from medium containing depolarizing levels of potassium (HK) to medium containing lower and non-depolarizing levels of potassium (LK) (22). LK treatment caused a reduction in the expression of TLE1 mRNA and protein, which was clearly discernible by 6 h (Fig. 2, *A* and *B*). Previous studies have established that commitment to death takes place within 4–6 h of LK treatment (23). These results further confirmed previously reported findings that TLE1 down-regulation is associated with neuronal death (11).

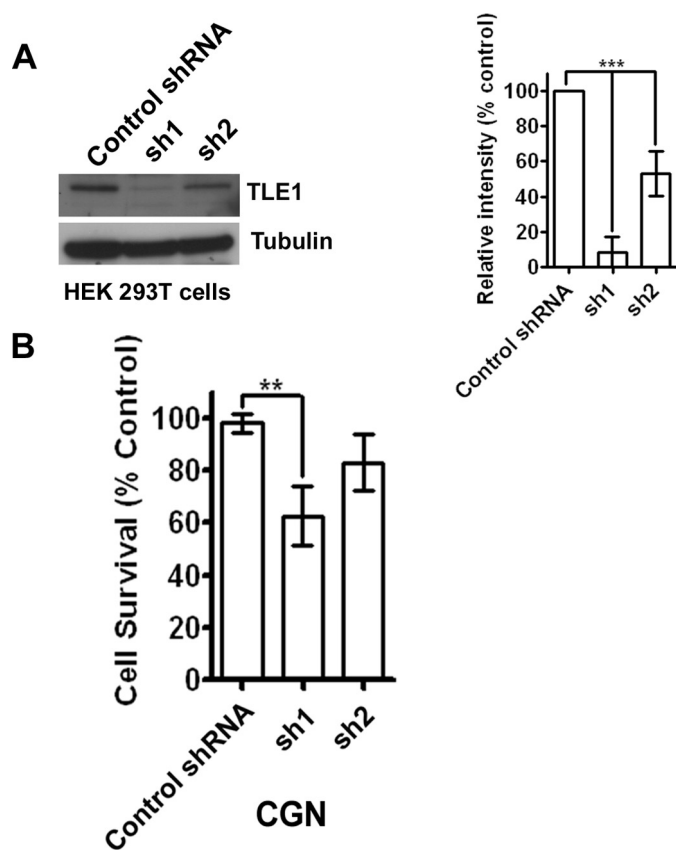The reduction in the levels of TLE1 mRNA and protein in apoptotic conditions suggests that elevated levels of TLE1 are necessary for neuronal survival. To examine this issue, we knocked down TLE1 expression in otherwise healthy CGNs using two separate shRNA constructs (denoted as sh1 and sh2). Control experiments performed in HEK293 cells revealed that sh1 reduced endogenous TLE1 levels substantially, whereas the effect of sh2 was modest (Fig. 3*A*). A similar difference in knockdown efficiency was observed in HT22 cells (data not shown). When expressed in CGNs, sh1 markedly reduced neuronal survival, whereas sh2 had a small but statistically insignificant effect (Fig. 3*B*). We also examined whether reestablishing elevated levels of TLE1 in LK-treated neurons could protect them from death. As shown in Fig. 4, *A* and *B*, overexpressing TLE1 completely prevented death in LK, confirming the need for elevated TLE1 expression in neuronal survival. In contrast to wild-type TLE1, TLE1(451–770), a mutant containing only the carboxyl-terminal region of TLE1 composed of the WD40 repeats, failed to promote neuronal survival (Fig. 4*B*). Another deletion mutant, TLE1(201–770), lacking the dimerization domain (11, 25, 26) also failed to protect against LK-induced death of CGNs (Fig. 4*B*). This result suggests that TLE1 dimerization is required for its survival-promoting effect.

To examine if TLE1 could promote survival of other neuronal types and in response to a different apoptotic stimulus, we
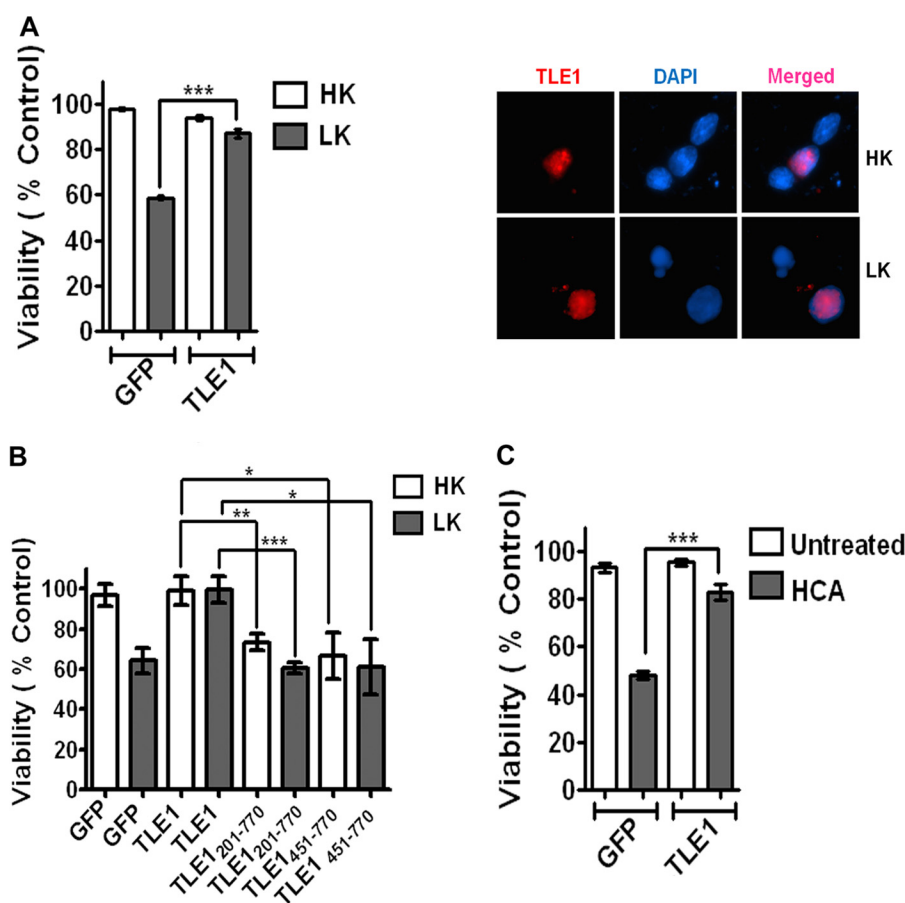
FIGURE 4. **Elevated expression of TLE1 promotes neuronal survival.** *A*, CGNs were transfected with GFP or TLE1-FLAG for 8 h and then treated with HK or LK for 24 h. The viability of transfected neurons was evaluated using DAPI staining. Neurons with condensed or fragmented nuclei were scored as dead. *B*, CGNs were transfected with GFP, TLE1, and two TLE1 deletion mutants, TLE1(451–770) and TLE1(201–770). Cell viability was normalized to GFP-transfected cells in HK. Elevated expression of TLE1, but not the two amino-terminal deletion mutants, protects neurons from LK-mediated cell death. *C*, cortical neurons were transfected with GFP or TLE1-FLAG for 8 h and then treated with or without HCA for 18 h. Viability was normalized to GFP-transfected cortical neurons without HCA (untreated). Elevated levels of TLE1-FLAG expression help to maintain neuronal survival in HCA-mediated proapoptotic conditions. *Error bars*, S.E. *, $p <$ 0.05; **, $p < 0.01$; ***, $p < 0.001$.

examined the effect of TLE1 overexpression in embryonic cortical neurons induced to die by HCA treatment, a stimulus that causes oxidative stress. In this model also, the forced expression of TLE1 inhibited cell death, suggesting a more general effect of TLE1 in the promotion of neuronal survival (Fig. 4*C*).

Promotion of Neuronal Survival by TLE1 Is Dependent on PI3K-Akt Signaling Pathway and Phosphorylation by CK2— Two of the best studied survival-promoting signaling pathways are the Raf-MEK-ERK and the PI3K-Akt signaling pathway (24). To examine if these pathways were involved in TLE1-mediated neuronal survival, we used pharmacological inhibitors that blocked each of these pathways. Inhibition of Raf-MEK-ERK signaling with PD98059 or U0126 had no effect on the neuroprotective effect of TLE1 (Fig. 5*A*). In contrast, treatment with two structurally distinct inhibitors of PI3K, wortmannin or LY294002, and an Akt inhibitor (Akt inhibitor X; *AktX*) blocked the protective effect of TLE-1 (Fig. 5, *A* and *B*). Inhibitors of other molecules known to promote neuronal survival, such as calcium calmodulin kinase (such as KN62), histone deacetylases (TSA-Trichostatin A), and CK1 (such as IC261) had no effect on TLE1-mediated protection (Fig. 4*A*).

Previous work by Nuthall *et al.* (19) demonstrated that CK2 phosphorylates TLE1 at Ser-239 and Ser-253 and that phos-

phorylation at these sites was necessary for the inhibitory effect of TLE1 on neuronal differentiation. We examined whether phosphorylation by CK2 was also necessary for the survival-promoting activity of TLE1. As shown in Fig. 5*A*, treatment with TBB, a potent and specific inhibitor of CK2, abolished the ability of TLE1 to promote survival. To further examine the role of CK2, we expressed two non-phosphorylatable forms of TLE1, in which Ser-239 or Ser-253 was substituted with an alanine residue, in which Ser-239 and Ser-253 were substituted with alanine residues (Fig. 1). Both TLE1$_{S239A}$ and TLE1$_{S253A}$ failed to protect CGN (Fig. 5*C*). On the contrary, expression of these mutants induced neuronal death even in HK (Fig. 5*C*), presumably by a dominant negative mechanism. Confirming that CK2 phosphorylation at Ser-239 is particularly important for TLE-1-mediated neuronal survival is the finding that TBB had no effect on the survival-promoting ability of TLE1$_{S239E}$, in which Ser-239 is replaced by a phosphomimetic arginine residue (Fig. 5*C*).

TLE1 and FoxG1 Cooperate to Promote Neuronal Survival— FoxG1 is a member of the winged helix family of Forkhead proteins acting as a DNA-binding transcriptional repressor (27–29). Like TLE1, FoxG1 has been studied primarily in the context of neurodevelopment, where it functions to regulate
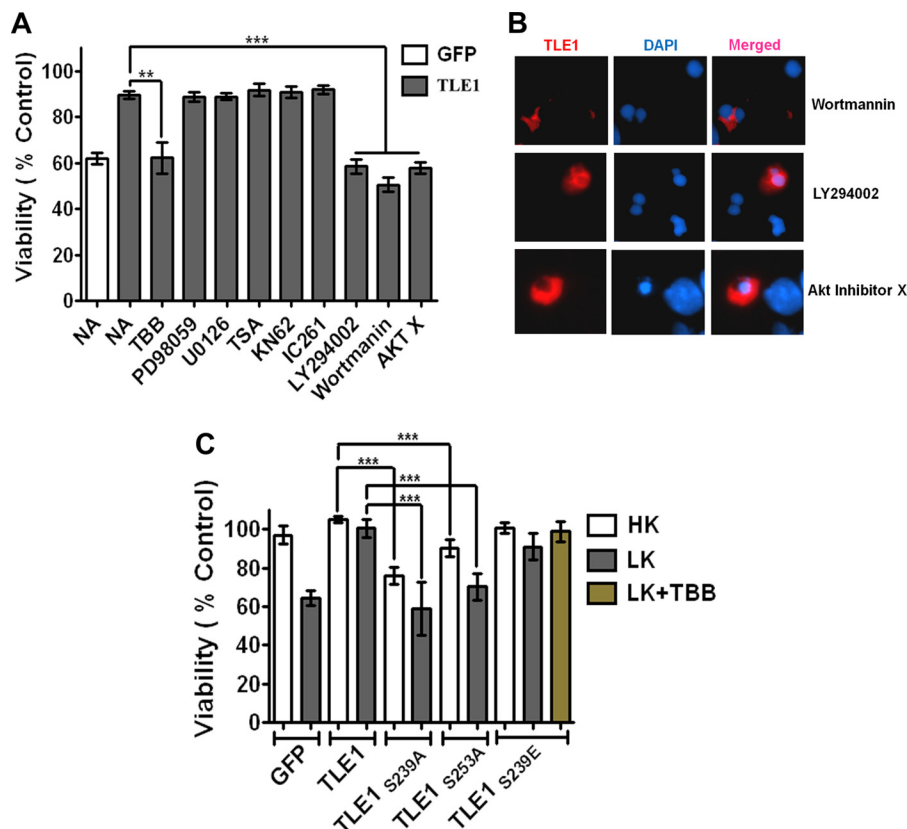
FIGURE 5. **TLE1 requires phosphorylation by CK2 and signaling via the PI3K-Akt pathway to promote neuronal survival.** *A*, CGNs were transfected with TLE1-FLAG and switched to LK medium with no additive (*NA*), LK medium with 10 μM TBB, 40 μM PD98059, 10 μM U0126, 1 μM TSA (Trichostatin A), 50 μM KN62, 10 μM IC261, 20 μM LY294002, 200 nM wortmannin, and 10 μM Akt inhibitor X. Viability of transfected neurons identified by immunocytochemistry was estimated after normalizing with respect to CGNs transfected with GFP with no additive. Inhibitors against CK2 (TBB) blocked survival by TLE1 as well as inhibitors against the PI3K-Akt pathway (LY294002, wortmannin, and Akt inhibitor X (*AktX*)). *B*, immunocytochemistry of CGNS transfected with TLE1-FLAG and treated with wortmannin, LY 294002, and Akt inhibitor X. DAPI staining was performed to identify the nuclei. *C*, viability of neurons transfected with GFP, TLE1-FLAG, TLE1$_{S239A}$-FLAG, TLE1$_{S253A}$-FLAG, and TLE1$_{S239E}$-FLAG for 8 h and treated with HK or LK or LK supplemented with 10 μM TBB (CK2 inhibitor) for 24 h. Viability was normalized to GFP-transfected neurons treated with HK. DAPI-stained nuclei were utilized to score for neurons that were transfected with the respective plasmid constructs. The non-phosphorylatable constructs of TLE1 were unable to maintain neuronal survival and even killed healthy neurons treated with HK. The phosphomimetic form of TLE1 was able to protect neurons in LK conditions as well as in LK conditions in the presence of CK2 inhibitor, TBB, used at a concentration of 10 μM. **, $p < 0.01$; ***, $p < 0.001$.

the rate of neurogenesis by keeping neural progenitor cells in a proliferative state and by inhibiting their differentiation into neurons (30–33). FoxG1 is also expressed in mature neurons of the postnatal and adult brain (17, 34). We recently showed that in mature neurons, FoxG1 promotes cell survival and does so in a PI3K-Akt-dependent manner (17). Indeed, FoxG1 is directly phosphorylated by Akt, a modification necessary for its neuronal survival-promoting activity (17). Because TLE1 and FoxG1 cooperate in the regulation of neurogenesis during early brain development, and because the survival-promoting activity of both TLE1 and FoxG1 in mature neurons requires PI3K-Akt signaling, we examined whether these two proteins cooperated to promote survival of mature neurons. As shown in Fig. 6*A*, TLE1 and FoxG1 interact robustly in neurons. Interaction was reduced in LK-treated neuronal cultures. Moreover, extensive co-localization is observed between TLE1 and FoxG1 when they are overexpressed in CGNs (supplemental Fig. 2). Interaction was also observed in HEK293 cells when the two proteins were ectopically expressed (Fig. 6*B*), as reported by other laboratories (2, 20). Interaction is preserved in TLE1(201–770) and TLE1(451–770), suggesting that FoxG1 binds to the carboxyl-terminal region of TLE1 (Fig. 6*B*), consistent with previous

reports mapping the interaction site to the carboxyl terminus of TLE1 (13).

We have previously described that deletion of the first 36 amino acids of FoxG1 abrogates the survival-promoting effect of FoxG1 (17). Interestingly, TLE1 failed to interact with FoxG1$_{36-481}$ while interacting normally with FoxG1$_{1-275}$, a deletion mutant lacking the carboxyl-terminal region of FoxG1 (Fig. 7*A*). This suggests that TLE1 binds to a sequence within the first 36 amino acids of FoxG1. To examine whether interaction with FoxG1 was necessary for the survival-promoting activity of TLE1, we co-expressed TLE1 with FoxG1$_{36-481}$. As shown in Fig. 7*B*, the survival-promoting effect of TLE1 was lost when co-transfected with FoxG1$_{36-481}$, suggesting that interaction with FoxG1 was necessary. We have previously shown that the survival-promoting effect of FoxG1 requires DNA binding and its phosphorylation by Akt at Thr-271. Thus, expression of FoxG1$_{N219A}$, which lacks DNA binding activity, or a non-phosphorylatable mutant, FoxG1$_{T271A}$, abolishes survival-promoting activity (17, 35). Moreover, like FoxG1$_{36-481}$, these mutants induce death of CGNs in HK probably by inhibiting signaling of endogenous FoxG1 through a dominant negative mechanism (Fig. 7*B*) (17). These results indicate that
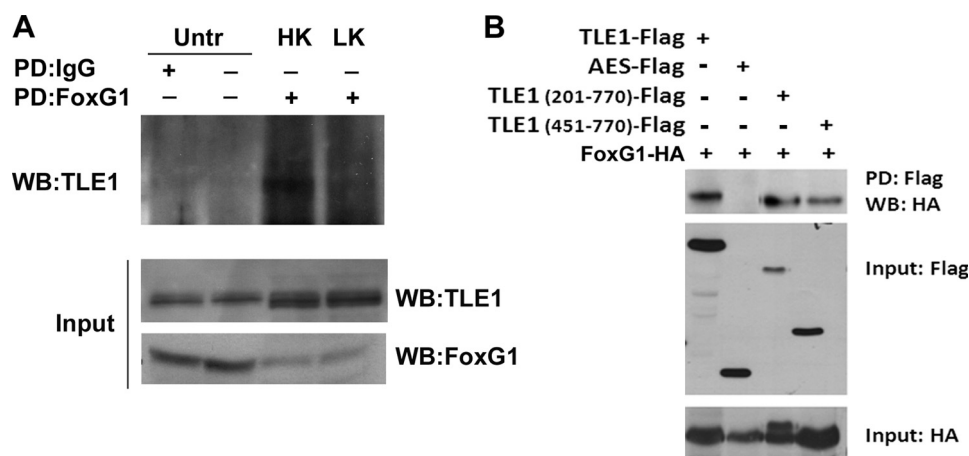
FIGURE 6. TLE1 and FoxG1 interact. *A,* lysates from CGNs treated with HK or LK for 6 h were immunoprecipitated with a FoxG1 antibody, and the immuno-precipitate was subjected to Western blot analysis with TLE1 antibody. 15% of the whole cell lysate was used as the input control and probed with TLE1 antibody and FoxG1 antibody. *B,* cell lysates were prepared from HEK293T cells co-transfected with FoxG1-HA and either TLE1-FLAG, AES-FLAG, TLE1(201–770)-FLAG, or TLE1(451–770)-FLAG. The lysates were subjected to a co-immunoprecipitation assay using FLAG antibody for pull-down, and the immunoprecipitate was subjected to Western blotting using a HA antibody. An aliquot of the preimmunoprecipitated lysate (10%) was used as the input control. FoxG1 was able to interact with full-length TLE1 as well as the two amino-terminal deletion constructs of TLE1, but it did not interact with AES. These results demonstrate that TLE1 requires its carboxyl-terminal WD40R domain to interact with FoxG1. *PD,* pull-down; *WB,* Western blot.

TLE1 needs FoxG1 to be functionally active for its own survival-promoting activity.

To examine whether functional TLE1 is also required for the survival-promoting activity of FoxG1, we co-expressed wild-type FoxG1 with TLE1$_{S239A}$. The ability of FoxG1 to promote survival was abrogated by TLE1$_{S239A}$ expression (Fig. 7*C*) indicating the need for functional TLE1. Interestingly, the survival-promoting effect of FoxG1 was also abolished by co-expression of TLE1(201–770) (Fig. 7*C*). Because TLE1(201–770) is capable of interacting with FoxG1, this result suggests that in addition to interaction with TLE1, dimerization of TLE1 is also required for FoxG1-mediated neuronal survival.

Consistent with the requirement of Akt phosphorylation at Thr-271 for the survival-promoting effect of FoxG1 (17, 35), treatment with inhibitors of PI3K-Akt signaling abolished the survival-promoting effect of FoxG1 (17). As shown in Fig. 5*A*, PI3K-Akt inhibitors also blocked the survival-promoting effect of TLE1. Surprisingly, an examination of the TLE1 sequence revealed the absence of a consensus sequence for Akt phosphorylation. This suggested that although necessary for the survival promotion by TLE1, Akt acted by phosphorylating FoxG1 rather than TLE1 itself. To confirm that TLE1 was not phosphorylated by Akt, we used a phospho-Akt substrate-specific antibody. Although FoxG1 phosphorylation was obvious, phosphorylation of TLE1 was not discernible (Fig. 8*A*). As observed with Akt and TLE1, the examination of the FoxG1 sequence revealed the absence of a consensus CK2 phosphorylation site, yet treatment with TBB inhibited the ability of FoxG1 to prevent LK-induced death (Fig. 8*B*), suggesting that CK2 influenced the activity of FoxG1 through its phosphorylation of TLE1. This is consistent with the abrogation of FoxG1-mediated survival by the co-expression of the CK2 phosphorylation-deficient TLE mutant, TLE1$_{S239A}$ (Fig. 7*C*).

Role of Other Grg Proteins in Regulation of Neuronal Survival by FoxG1 and TLE1—AES is a truncated member of the TLE family of proteins, which is capable of heterodimerizing with TLE1 through its Q domain (Fig. 1). In CGNs treated with LK,

AES expression remains unchanged for about 6 h (6) but is then increased substantially within the next few h (Fig. 2*A*), suggesting that the increased levels of AES promote neuronal death. Indeed, as reported previously (11), the overexpression of AES induces the death of otherwise healthy CGNs (Fig. 9, *A* and *B*). AES also promotes the death of cortical neurons when overexpressed (Fig. 9*C*).

Although it is generally believed that AES acts as a TLE1 antagonist through heteromerizing with it, recent studies have suggested that AES may have its own activity independent of TLE1 inhibition (12). To investigate this issue, we studied the effect of Grg1-s on TLE1-mediated neuronal survival. Grg1-s is a naturally occurring truncated form of Grg1/TLE1 that is generated through alternative splicing (Fig. 1). Although resembling AES in overall structure (it lacks the carboxyl-terminal domain of TLE1 but possess a dimerization domain for association with TLE1), Grg1-s shares only about 60% overall sequence homology with AES (18). Like AES/Grg5, Grg1-s has no transcriptional repressor activity (18). As expected and as described previously (18), we found that Grg1-s interacts with TLE1 (Fig. 9*D*). Expression of Grg1-s promoted apoptosis of otherwise healthy CGNs as well as cortical neurons (Fig. 9, *A* and *C*). As observed for AES, Grg1-s overexpression did not increase the extent of death induced by LK in CGNs or HCA-induced death in cortical neurons (Fig. 9, *A* and *C*). The almost identical effects of these two truncated proteins, which share overall structure but differ significantly in their sequence, suggest that AES acts through TLE1 antagonism in this context.

Because AES can heteromerize with TLE1, it was possible that an elevation in its expression following LK treatment or by overexpression induced neuronal death by the sequestration of limiting amounts of TLE1, thus disrupting the interaction between TLE1 and FoxG1. Consistent with this hypothesis, elevating TLE1 levels by co-expressing it inhibited the ability of AES to promote death (Fig. 10*A*). Also in support of the hypothesis is the finding that co-expression of AES with FoxG1 inhibits the ability of FoxG1 to maintain survival in HK or prevent
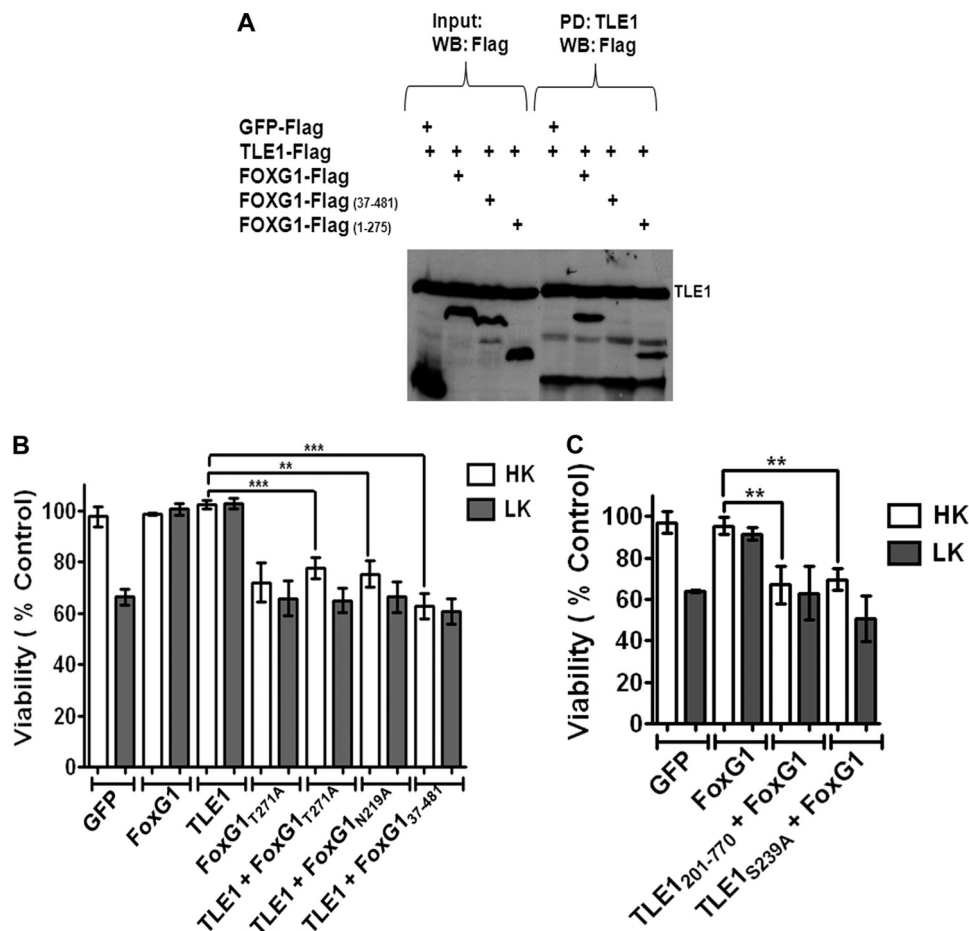
FIGURE 7. **FoxG1 and TLE1 cooperate to promote neuronal survival.** *A*, cell lysates were prepared from HEK 293T cells co-transfected with TLE1 and either GFP or FoxG1 or co-transfected with TLE1 and either FoxG1$_{37-481}$-FLAG or FoxG1$_{1-275}$-FLAG. The lysates were immunoprecipitated using TLE1 antibody, and the immunoprecipitate was subjected to Western blotting using a FLAG antibody. Full-length FoxG1 interacted with TLE1, but the 36-amino acid-deleted FoxG1 construct at the amino terminus failed to do so. 10% of the input lysate was checked for proper expression of the constructs. *PD*, pull-down; *WB*, Western blot. *B*, viability of CGNs transfected with GFP, FoxG1-FLAG, TLE1-HA, FoxG1$_{T271A}$-FLAG, FoxG1$_{N219A}$-FLAG, and FoxG1$_{37-481}$-FLAG as indicated in the graph and then treated with HK or LK for 24 h. Mutants of FoxG1 that do not promote neuronal survival (17) block the ability of TLE1 to promote neuronal survival. Viability was normalized to GFP-transfected neurons treated with HK. *C*, viability of CGNs transfected with GFP, FoxG1-FLAG, TLE1(201–770)-FLAG, and TLE1$_{S239A}$-FLAG as indicated in the graph and then treated with HK or LK for 24 h. Viability of CGNs was normalized to GFP-transfected cells treated with HK. The results show that fully functional TLE1 is required by FoxG1 to mediate neuronal survival. **, $p < 0.01$; ***, $p < 0.001$.

LK-induced death. To verify that AES disrupted the interaction between TLE1 and FoxG1, we performed a co-immunoprecipitation experiment in which TLE1 and FoxG1 were expressed by themselves or along with AES. As shown in Fig. 10*B*, the level of interaction between TLE1 and FoxG1 is reduced in the presence of AES.

Grg6 is another member of the subfamily of truncated TLE proteins. In contrast to AES and Grg1-s, which lack the carboxyl-terminal part of the larger TLE proteins, Grg6 does not possess the amino-terminal half of the larger TLE1 proteins (Fig. 1). Because Grg6 lacks the Q domain, it cannot heteromerize with TLE1 (20), but because it possesses the region equivalent to the carboxyl-terminal region of TLE1, Grg6 interacts with FoxG1 (20). Overexpression of Grg6 could thus be expected to disrupt TLE1-FoxG1 interaction through the sequestration of FoxG1. As observed with AES, the overexpression of Grg6 induced death of CGNs in HK and increased the extent of neuronal death in LK (Fig. 10*C*). This neurotoxic effect of Grg6 was inhibited when FoxG1 was co-expressed but not when co-expressed with TLE1 (Fig. 10*C*). Taken together, these results

suggest that disruption of the interaction between TLE1 and FoxG1 results in neuronal death (see model in Fig. 11).

DISCUSSION

TLE1 has been extensively studied for its role in the development of the telencephalon, where it functions to prevent premature differentiation of neural progenitor cells. In this context, it acts as a transcriptional corepressor in association with DNA-binding repressors, including FoxG1 and Hes1. Although TLE1 expression is reduced in progenitor cells at the time they differentiate into neurons, it is once again expressed robustly in the postnatal brain and through adulthood (supplemental Fig. 1). We extend our previous work to show that TLE1 promotes the survival of postmitotic neurons. Indeed, TLE1 expression is reduced in neurons concomitant to the time at which the neurons become committed to undergo apoptosis. Suppression of TLE1 expression in otherwise healthy neurons leads to their death, whereas reestablishment of elevated TLE1 protects neurons from death. The ability of TLE1 to promote neuronal survival depends on its ability to heteromerize, as suggested by the
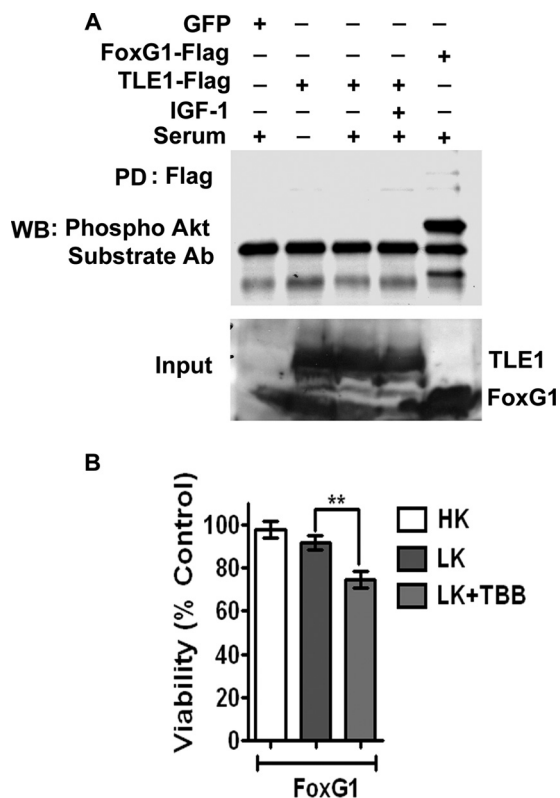
FIGURE 8. **Phosphorylation by Akt and CK2 regulate survival promotion by TLE1-FoxG1.** *A*, cell lysates were prepared form HEK293T cells transfected with GFP, TLE1-FLAG, and FoxG1-FLAG and subjected to a co-immunoprecipitation assay using FLAG antibody (*Ab*). The immunoprecipitate was subjected to Western blot analysis using a phospho-Akt substrate-specific antibody, which will only identify proteins phosphorylated by Akt. 10% of the input lysate was checked for proper expression of the constructs. The results show that FoxG1 is being phosphorylated by Akt, whereas TLE1 is not. *PD*, pull-down; *WB*, Western blotting. *B*, viability of CGNs transfected with FoxG1-FLAG and treated with HK, LK, or LK supplemented with TBB at 10 μM (a CK2 inhibitor) for 24 h. Viability was normalized to CGNs transfected with FoxG1 and treated with HK. Treatment with TBB abrogates the role of FoxG1 in mediating neuronal survival. **, *p* <0.01.

FIGURE 9. **Elevated expression of AES and Grg1-s promotes neuronal death.** *A*, viability of CGNs transfected with GFP, AES-FLAG, and Grg1-s-HA and then treated with HK or LK for 24 h. Viability was normalized to CGNs transfected with GFP and treated with HK. Overexpression of AES and Grg1-s killed neurons even in HK. *B*, immunocytochemical analysis of AES-transfected neurons treated with HK or LK. *C*, viability of cortical neurons transfected with GFP, AES-FLAG, and Grg1-s-HA for 8 h and then either left untreated or treated with 1 mM HCA. Viability was normalized to GFP-transfected cortical neurons without any treatment (*Untreated*). AES and Grg1-s killed healthy cortical neurons. *D*, cell lysate of HEK293 cells co-transfected with GFP-FLAG, TLE1-FLAG, and Grg1-s-HA, as indicated in the figure, was obtained. The lysate was subjected to co-immunoprecipitation analysis using HA antibody, and the immunoprecipitate was subjected to immunoblotting using FLAG antibody. 10% of the input lysate was checked for proper expression of the constructs. *PD*, pull-down; *WB*, Western blot. ***, *p* < 0.001.

abolishment of survival-promoting activity in TLE1 mutants lacking the heterodimerization domain. In addition, phosphorylation by CK2 is necessary. Indeed, pharmacological inhibition of CK2 blocks neuronal survival by TLE1. Moreover, mutation of either Ser-239 or Ser-253, previously identified as CK2 phosphorylation sites, completely abolishes the survival-promoting activity of TLE1. Interestingly, activity is fully restored by a phosphomimetic form of Ser-239, suggesting that phosphorylation at either of these sites might be sufficient for neuronal survival-promoting activity.

In neuronal progenitor cells, the inhibitory effect of TLE1 on differentiation involves interaction with proteins, including FoxG1 (1, 2). Like TLE1, FoxG1 controls neurogenesis in progenitor cells but promotes survival in postmitotic neurons. As observed in neural progenitor cells, we find that TLE1 also interacts with FoxG1 in postmitotic neurons. Furthermore, the survival-promoting effect of both TLE1 and FoxG1 is dependent on the proper functioning of the other protein such that TLE1 is unable to maintain neuronal survival if co-expressed with mutant forms of FoxG1 that are deficient in survival-promoting activity and *vice versa*. We have previously demonstrated that the survival-promoting activity of FoxG1 requires
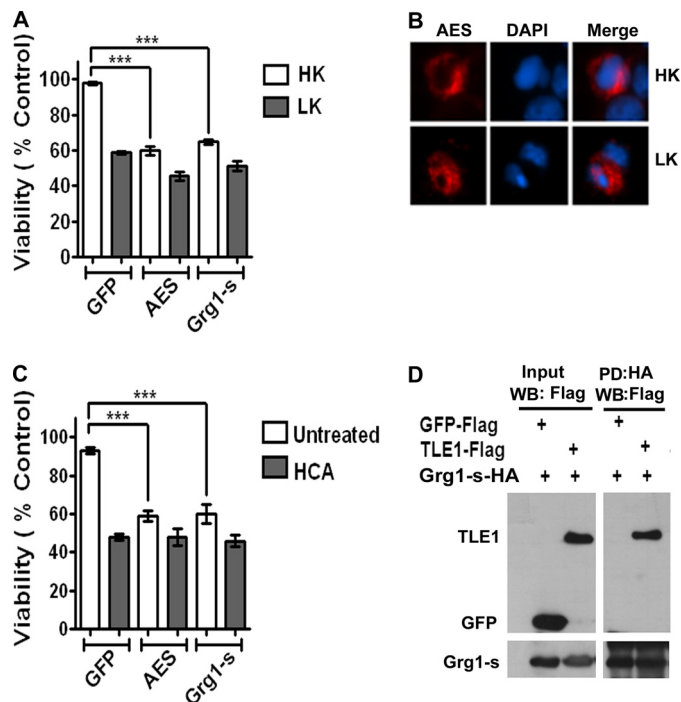
its phosphorylation by Akt at Thr-271. Mutation of this residue to a non-phosphorylatable one abolishes the survival-promoting activity of FoxG1 as does treatment with inhibitors of PI3K-Akt signaling. Inhibitors of PI3K-Akt signaling also inhibit TLE1-mediated survival. Analysis of the TLE1 sequence reveals the absence of a consensus site for Akt phosphorylation, suggesting that the effect of PI3K-Akt inhibitors on TLE1-mediated neuronal survival is through inhibition of FoxG1 phosphorylation. TLE1 can also be phosphorylated at by MAPK at two sites within the Q domain (36). However, pharmacological inhibition of Raf-MEK-ERK signaling has no effect on the survival-promoting effect of TLE1. Similarly, inhibition of histone deacetylases and calcium calmodulin kinase has no effect on TLE1-mediated survival.

We have also studied the role of two of the truncated TLE proteins in the regulation of neuronal survival. AES can heteromerize with TLE1 but lacks the region corresponding to the carboxyl-terminal half of TLE1. It therefore does not interact with FoxG1. Its inhibition of TLE1-mediated neuronal survival both in HK- and LK-treated neurons when it is overexpressed by itself can be explained by sequestration of endogenous TLE1 away from FoxG1. In comparison with AES, Grg6 can interact with FoxG1 but, because it lacks the heterodimerization

FIGURE 11. **Model to explain promotion of neuronal survival by TLE1.** *A*, TLE1 is phosphorylated by CK2 and functions as a dimer. Although the exact stoichiometry of FoxG1 to TLE1 is not clear, interaction with FoxG1 is necessary for the survival-promoting activity of TLE1. *B*, AES promotes death by heteromerizing with TLE1, thus disrupting the interaction between TLE1 and FoxG1. This occurs during LK treatment when the levels of endogenous AES are increased and the expression of TLE1 is reduced or when AES is overexpressed. *C*, Grg6 promotes death by disrupting the interaction of TLE1 with FoxG1 through direct interaction with FoxG1.

domain, cannot associate with TLE1. Grg6 inhibits TLE1-mediated survival but not survival by expression of FoxG1. Expression of elevated amounts of Grg6 could be expected to reduce TLE1-FoxG1 interaction by sequestering limiting amounts of endogenous FoxG1 through direct interaction. Supporting this model is the finding that co-expression of FoxG1 blocks the neurotoxic effect of Grg6 in both HK and LK. Similarly, co-expression of TLE1 blocks AES-induced neuronal death.

In summary, we have identified five regulatory mechanisms that are necessary for the survival-promoting effect of TLE1 in postmitotic neurons: (*a*) elevated expression of TLE1, (*b*) homodimerization (although heterodimerization with another of the longer TLE proteins cannot be excluded), (*c*) phosphorylation by CK2, (*d*) interaction with FoxG1, and (*e*) PI3K-Akt signaling. In addition, the expression of members of the truncated TLE protein subfamily, such as AES and Grg6, can negatively regulate the ability of TLE1 to promote neuronal survival by disrupting the interaction between TLE1 and FoxG1.

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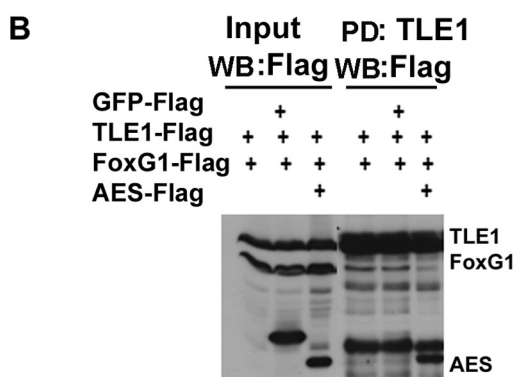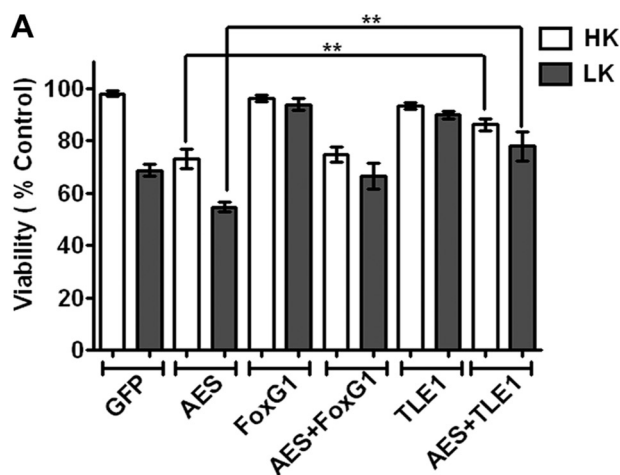
FIGURE 10. **AES blocks FoxG1-TLE1-mediated neuronal survival.** *A*, CGNs were transfected with GFP, AES-FLAG, FoxG1-FLAG, and TLE1-FLAG as indicated in the graph for 8 h and then treated with HK or LK for 24 h. Viability of neurons was normalized to CGNs transfected with GFP and treated with HK. Overexpression of AES and TLE1 was able to rescue AES-mediated cell death. *B*, cell lysate of HEK 293T cells co-transfected with GFP-FLAG, TLE1-FLAG, FoxG1-FLAG, and AES-FLAG, as indicated in the figure, were obtained. The lysates were subjected to co-immunoprecipitation analysis using TLE1 antibody, and the immunoprecipitate was subjected to Western blot analysis using FLAG antibody. 10% of the input lysate was checked for proper expression of the constructs. The results show that AES interferes with interaction between FoxG1 and TLE1. *PD*, pull-down; *WB*, Western blot. *C*, viability of CGNs transfected with GFP, FoxG1-HA, Grg6-FLAG, and TLE1-HA, as indicated in the graph, and then treated with HK or LK for 24 h. Viability was normalized to GFP-transfected neurons treated with HK. **, $p < 0.01$; ***, $p < 0.001$.
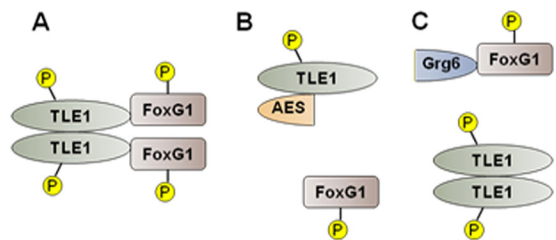
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