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Neurog2 Simultaneously Activates and Represses Alternative Gene Expression Programs in the Developing Neocortex

Christopher Kovach[1,], Rajiv Dixit[1], Saiqun Li[1], Pierre Mattar[1,3], Grey Wilkinson[1], Gina E. Elsen[2], Deborah M. Kurrasch[1], Robert F. Hevner[2] and Carol Schuurmans[1]

[1]University of Calgary, Hotchkiss Brain Institute, Alberta Children's Hospital Research Institute, Health Sciences Centre, Calgary, Alberta, Canada [2]Center for Integrative Brain Research, Seattle Children's Research Institute, Seattle, WA 98101, USA and [3]Present address: Cellular Neurobiology Research Unit, Institut de recherches cliniques de Montréal, Montréal, Québec, Canada

C.K., R.D., and S.L. contributed equally to this work.

Address correspondence to Carol Schuurmans, Health Sciences Centre 2277, 3330 Hospital Dr NW, Calgary, Alberta, Canada T2N 4N1. Email: cschuurm@ucalgary.ca

Progenitor cells undergo a series of stable identity transitions on their way to becoming fully differentiated cells with unique identities. Each cellular transition requires that new sets of genes are expressed, while alternative genetic programs are concurrently repressed. Here, we investigated how the proneural gene *Neurog2* simultaneously activates and represses alternative gene expression programs in the developing neocortex. By comparing the activities of transcriptional activator (*Neurog2-VP16*) and repressor (*Neurog2-EnR*) fusions to wild-type *Neurog2*, we first demonstrate that *Neurog2* functions as an activator to both extinguish Pax6 expression in radial glial cells and initiate Tbr2 expression in intermediate neuronal progenitors. Similarly, we show that *Neurog2* functions as an activator to promote the differentiation of neurons with a dorsal telencephalic (i.e., neocortical) identity and to block a ventral fate, identifying 2 *Neurog2*-regulated transcriptional programs involved in the latter. First, we show that the Neurog2-transcriptional target *Tbr2* is a direct transcriptional repressor of the ventral gene *Ebf1*. Secondly, we demonstrate that *Neurog2* indirectly turns off *Etv1* expression, which in turn indirectly regulates the expression of the ventral proneural gene *Ascl1*. *Neurog2* thus activates several genetic off-switches, each with distinct transcriptional targets, revealing an unappreciated level of specificity for how *Neurog2* prevents inappropriate gene expression during neocortical development.

Keywords: binary fate choice, genetic off-switch, neocortical development, Neurog2 proneural gene, transcriptional activator or repressor

Introduction

The neocortex is comprised of 6 layers of glutamatergic projection neurons that are sequentially generated from dorsal telencephalic progenitors (also designated cortical progenitor cells) between embryonic day (E) 10 to E17 in mouse (Takahashi et al. 1999). Cortical progenitors undergo a series of stable identity transitions over this period. They begin as neuroepithelial cells that span the apicobasal axis of the ventricular zone (VZ), where they undergo symmetric proliferative divisions to expand the progenitor pool (Kriegstein and Noctor 2004). Then, at ~E10.5, cortical neuroepithelial cells differentiate into radial glial cell (RGC) progenitors that retain their apical and basal contacts while initiating asymmetric neurogenic mitoses at the apical surface of the VZ (Malatesta et al. 2003; Gotz and Huttner 2005). Next, at ~E11.5, a subset of RGCs give rise to intermediate neuronal progenitor cells (INPs) that initially reside in the VZ, but later lose their apical contacts and migrate outward to form the subventricular zone (SVZ), a more basally-located germinal zone (Haubensak et al. 2004; Miyata et al. 2004; Noctor et al. 2004). SVZ INPs have a limited proliferative capacity and undergo symmetric, neurogenic divisions after 1–2 cell cycles (Noctor et al. 2004; Farkas et al. 2008).

Cortical progenitor cell transitions depend on the coordinated activation and repression of distinct genetic programs. RGCs initially express the homeodomain transcription factor *Pax6* and later, as they differentiate into VZ INPs, they turn on the T-box transcription factor *Tbr2*, before finally extinguishing *Pax6* expression as they transform into SVZ INPs (Englund et al. 2005; Kowalczyk et al. 2009). *Pax6* is required to maintain a RGC identity and to initiate the RGC to INP transition (e.g., Estivill-Torrus et al. 2002; Quinn et al. 2007). Likewise, *Tbr2* is required to specify an INP identity (Arnold et al. 2008; Sessa et al. 2008). To promote the RGC to INP transition, *Pax6* functions as an activator, initiating transcription of *Tbr2* (Holm et al. 2007; Sansom et al. 2009) as well as *AP2γ*, which is required for INP differentiation in the caudal cortex (Pinto et al. 2009). In contrast, *Tbr2* may have transcriptional repressor activity, as it cell autonomously blocks *Pax6* expression (Sessa et al. 2008), although it is not yet known if this is due to direct transcriptional repression.

Progression through the different phases of cortical progenitor cell maturation is coordinated with the initiation of neuronal differentiation, which occurs in a restricted subset of progenitors at any given time (Shimojo et al. 2008). In the embryonic neocortex, neurogenesis is induced by the proneural basic helix-loop-helix (bHLH) transcription factors *Neurog1* and *Neurog2* (Fode et al. 2000; Schuurmans et al. 2004; Mattar et al. 2008). *Neurog1/2* regulates several binary cell fate decisions during neocortical development, all of which require the simultaneous activation and repression of alternative genetic programs. For instance, *Neurog2* promotes the RGC to INP transition, likely by directly initiating *Tbr2* transcription in INPs and repressing *Pax6* in RGCs, the latter through unknown mechanisms (Miyata et al. 2004; Britz et al. 2006; Ochiai et al. 2009). *Neurog2* also dictates the type of neuron that differentiates, specifying a dorsal, glutamatergic projection neuron identity while simultaneously repressing an alternative ventral, GABAergic interneuronal identity (Fode et al. 2000; Schuurmans et al. 2004; Mattar et al. 2008). Thus, *Neurog2* must activate dorsal genetic pathways and repress ventral genes, such as *Ascl1*, a proneural bHLH protein that specifies a ventral telencephalic identity (Fode et al. 2000; Schuurmans et al. 2004; Mattar et al. 2008).

To date, the *Neurog2*-regulated cell fate decisions for which a mechanism of transcriptional repression has been identified include the neuronal versus glial fate choice (Sun et al. 2001),

and the control of neuronal migration (Ge et al. 2006; Heng et al. 2008; Pacary et al. 2011). Mechanistically, *Neurog2* represses astrocytic genes such as *GFAP*, as well as a negative regulator of migration, *RhoA*, in an indirect, non-DNA–binding–dependent fashion, sequestering CREB-binding protein (CBP) and other activator proteins from the regulatory regions of these genes (Sun et al. 2001; Ge et al. 2006; Heng et al. 2008; Pacary et al. 2011). In contrast, it is not yet known how *Neurog2* represses *Pax6*, *Ascl1*, or other ventral genes. We set out to determine if *Neurog2* acts as a direct transcriptional repressor to turn off alternative genetic programs during neocortical development, or if instead, *Neurog2* functions as a transcriptional activator, switching off inappropriate gene expression by sequestering co-activators, or more directly, by initiating the expression of downstream transcriptional repressors. For this purpose, we generated *Neurog2* fusion proteins that function as obligate transcriptional repressor (*Neurog2-EnR*) or activator (*Neurog2-VP16*) proteins. We predicted that if *Neurog2* directly represses the expression of genes in alternate pathways, *Neurog2-EnR* would phenocopy wild-type *Neurog2* (*Neurog2-WT*), recruiting co-repressor proteins to shut off the expression of inappropriate genes. Conversely, we predicted that if *Neurog2* functions indirectly, *Neurog2-VP16* would mimic *Neurog2-WT*, perhaps by initiating the expression of downstream transcriptional repressors, or by sequestering transcriptional activators away from target promoters in alternate pathways. Here, we demonstrate that *Neurog2-VP16* phenocopies *Neurog2-WT* in gain-of-function assays in vitro and in vivo, suggesting that *Neurog2* functions as a transcriptional activator to switch off alternative gene expression. Furthermore, we identify a *Neurog2-Tbr2-Pax6* transcriptional cascade that turns off *Pax6* expression during cortical progenitor maturation, and *Neurog2-Tbr2-Ebf1* and *Neurog2-Etv1-Hes5-Ascl1* transcriptional cascades that turn off ventral gene expression in cortical cells. Combined, our data demonstrate that *Neurog2* functions as a transcriptional activator to initiate the expression of several genetic off-switches, each of which repress distinct gene expression programs during neocortical development.

Materials and Methods

Animal Breeding and Maintenance

All animal procedures were compliant with the Guidelines of the Canadian Council of Animal Care and were approved by the University of Calgary Animal Care Committee under animal protocol M08003. CD1 (outbred) mice were intercrossed to generate timed pregnancies for in utero electroporation experiments. Embryos were staged by taking the date of the vaginal plug as embryonic day (E) 0.5. The *Neurog2*^{KIGFP} null allele was maintained on a CD1 background and PCR genotyping was performed as previously described (Britz et al. 2006).

Generation of Expression and Reporter Constructs

For electroporation, cDNA was cloned into pCIG2, a bicistronic expression vector that includes an internal ribosome entry site (IRES) green fluorescent protein (GFP) cassette as described (Mattar et al. 2008). A p*NeuroD*^{1kb} *luciferase* reporter was previously generated (Huang et al. 2000). To generate a pCMV-*NeuroD*$^{1\ kb}$ *luciferase* reporter, the 1 kb 5′ *NeuroD* promoter/enhancer elements were amplified by PCR, and cloned into pMIR-Report, which contains a CMV-*luciferase* cassette. To generate a *Pax6*-5 kb–P1 telencephalic-specific reporter, PCR was used to amplify 5130 bp between the P1 and P0 promoters of *Pax6*, a region encompassing regulatory elements that drive reporter expression in the dorsal telencephalon (Kammandel et al. 1999). The 5130 bp *Pax6* fragment was cloned into pGL3 Basic (Promega).

Protein Half-Life Assay

Proteins were transcribed and translated in vitro using a TNT rabbit reticulocyte lysate kit (Promega) and radiolabeled using ^{35}S-methionine according to the manufacturer's instructions. Protein degradation assays were performed as described (Nguyen et al. 2006). Additional details are described in the Supplementary Material.

Cell Culture and Luciferase/β-Galactosidase Assays

The P19 embryonic carcinoma cell line (ATCC# CRL-1825) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, United States of America). Cells were maintained in complete medium, containing Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (FBS), 5% horse serum (HS), 10 units/mL penicillin, 10 μg/mL streptomycin, and 29.2 mg/mL L-glutamine. DNA for transfection was prepared using Qiagen plasmid plus maxi kit as described by the manufacturer. Details of the luciferase and β-galactosidase assays are provided in the Supplementary Material.

In Utero Electroporation

Endotoxin-free plasmid DNA for electroporation was generated using a column-based purification system (Qiagen, Mississauga, ON, Canada). In utero electroporation was performed as previously described (Dixit et al. 2011). Briefly, DNA (1–3 μg/μL) mixed with Fast Green FCF dye (1:200) was injected into the telencephalic (i.e., lateral) ventricles at defined embryonic stages using pulled borosilicate needles and a Femtojet microinjector. Next, 7 pulses of 45–55 mV were applied within a 7-s interval to the uterus surrounding the head of the embryo using a BTX electroporator. The uterus was replaced in the body cavity, the peritoneum was sutured and skin stapled and embryos were allowed to develop until the designated stage of analysis.

Tissue Processing and RNA In Situ Hybridization

Whole brains were dissected in cold phosphate buffered saline (PBS) and then fixed for 24 h at 4 °C in 4% paraformaldehyde/1× PBS. Tissues were washed in 1× PBS and then cryoprotected overnight (O/N) at 4 °C in 20% sucrose/1× PBS. For electroporated brains, fixation and cryoprotection were performed in the dark to avoid GFP degradation. Tissues were then embedded in optimal cutting temperature (Tissue-TEK) and stored at −80 °C. Coronal sections (10 μm) were collected on Superfrost/Plus slides (Fisher Scientific, Ottawa, ON, Canada) using a Leica cryostat (Richmond Hill, ON, Canada). RNA in situ hybridization using digoxygenin-labeled probes was performed as described previously (Alam et al. 2005). Probes are described in the Supplementary Material.

Immunohistochemistry

Sections were blocked for 1 h in 10% HS/1× PBS with 0.1% Triton X-100 (PBST) at room temperature (RT). Briefly, primary antibodies were diluted in blocking solution and applied to sections O/N at 4 °C. Sections were then washed 3× in PBST, and then secondary antibodies were appropriately diluted in blocking solution and applied to the sections for 2 h at RT in the dark. Sections were washed 3× in PBST, incubated 5 min with 4′,6-diamidino-2-phenylindole (DAPI; Sigma), diluted 1/10 000 in PBS, washed 3× in PBS, and mounted using Aqua Polymount (Polysciences Inc., Woodbridge, ON, Canada). Primary and secondary antibodies are described in the Supplementary Material.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments were performed as previously described with some modifications (Demers et al. 2007). Cells from E14.5 mouse telencephalons were dissociated by

incubating the tissue in 0.05% trypsin–ethylenediaminetetraacetic acid (Invitrogen# 25300-054) for 10 min at 37 °C. The cells were cross-linked at RT for 10 min with 1% (w/w, final) formaldehyde. Sonication was performed with a Bioruptor (Diagenode) at high power for 30 cycles (30 s on/30 s off). Immunoprecipitations with rabbit anti-Tbr2 (Abcam) or control antibodies (FBS) were performed with 500 μg of chromatin per assay. qPCR was performed with Bio-Rad DNA Engine Opticon 2 real-time PCR detection systems and a Quanti-Fast SYBR Green Kit for quantification (Qiagen). PCR products were also loaded on a 2% agarose gel to verify the size of the amplification product. Primer design is described in the Supplementary Material.

Quantitative and Statistical Analysis

For the analysis of *luciferase/β-galactosidase* assays, *luciferase* data were normalized by dividing raw light readings by the corresponding A_{420} *β-gal* or Renilla values. Reported *n*-values correspond to the number of individual experiments performed, each comprised of 3 replicates per sample. For in vivo experiments, brains from at least 3 independent experiments, and at least 3 section for each brain, were processed (*n*-values refer to # of brains analyzed). Comparisons between control and experimental conditions were performed using a 2-tailed Student's *t*-test (to compare 2 values), while comparisons between multiple samples were performed by applying a 1-way analysis of variance and Tukey's multiple comparison test using the Graphpad Prizm software (Graphpad Software Inc., La Jolla, CA, United States of America). Statistical variation was determined using the standard error of the mean (SEM).

Results

Generation of Obligate Activator and Repressor Forms of Neurog2

Neurog2 both positively and negatively regulates the expression of downstream genes to carry out its myriad of neocortical functions (Fig. 1*A*). Despite considerable understanding of how *Neurog2* can function as a transcriptional activator, its ability to repress alternative gene expression programs is less well understood. To date, *Neurog2* has been shown to repress *RhoA* (Ge et al. 2006) and *GFAP* (Sun et al. 2001) by sequestering transcriptional activators or co-activators away from target promoters (Fig. 1*A*). Here, we asked if *Neurog2* employs a similar indirect strategy to suppress *Pax6* and *Ascl1* transcription, or if instead, *Neurog2* functions as a transcriptional repressor. For this purpose, we generated *Neurog2* fusion proteins that bind to DNA, but which have obligate activator (*Neurog2-VP16*) or repressor (*Neurog2-EnR*) activity. We reasoned that if *Neurog2* represses transcription of target genes directly, by binding DNA and recruiting transcriptional co-repressors, then the *Neurog2*-repressor fusion would have the same effects as *Neurog2-WT* (Fig. 1*B*). If, on the other hand, *Neurog2* represses transcription of target genes indirectly, either by sequestering co-activators or by inducing the expression of downstream transcriptional repressors, we expected that the *Neurog2*-activator fusion would function like *Neurog2-WT* (Fig. 1*C*).

To generate an activator fusion, we linked the *Neurog2* C-terminus to the VP16 transactivator domain (Fig. 1*C*), which interacts with basal transcriptional machinery and histone acetylases to transactivate target genes (Hall and Struhl 2002). To generate an obligate repressor, the *Neurog2* C-terminus was fused to the engrailed repressor domain (EnR; Fig. 1*B*), which interacts with Grg/transducin-like enhancer of split (TLE) co-repressors that in turn recruit histone deacetylases to target promoters (Buscarlet and Stifani 2007). To ensure that the functions of the *Neurog2* fusion proteins could be directly compared, we measured protein half-lives and demonstrated that *Neurog2-WT* protein had a half-life of ~0.5 h, consistent with our previous results (Nguyen et al. 2006), while *Neurog2-VP16* and *Neurog2-EnR* proteins had half-lives of ~0.7 h and ~1.2 h, respectively (Fig. 1*D*). Thus, while the EnR and VP16 domains had modest stabilizing effects, *Neurog2-VP16* and *Neurog2-EnR* retained relatively short intracellular half-lives, allowing us to compare their transcriptional and biological activities to *Neurog2-WT*.

To assess the transcriptional activities of the *Neurog2* fusion proteins, reporter assays were performed in P19 embryonic carcinoma cells, which can differentiate into neural lineages, notably in response to *Neurog2* (Farah et al. 2000). Importantly, P19 cells express the co-repressors *TLE1*, *TLE2*, and *TLE4* (Yao et al. 1998), indicating that necessary co-repressors for *Neurog2-EnR* were available to be recruited, and that these cells are an appropriate system to test *Neurog2-EnR* function. To monitor *Neurog2* transcriptional activity, we used a transcriptional reporter for *NeuroD1* (p*NeuroD*^{1kb}; Fig. 1*E*), a direct target of *Neurog2* (Huang et al. 2000). Twenty-four hours post-transfection, *Neurog2-WT* promoted a 17-fold increase ($n = 10$; $P < 0.0001$) in *NeuroD1* reporter activity ($n = 9$; normalized to 1), while *Neurog2-VP16* stimulated a 32-fold increase ($n = 6$; $P < 0.0001$), significantly higher than *Neurog2-WT* ($P < 0.0001$; Fig. 1*E*). In contrast, *Neurog2-EnR* did not transactivate the *NeuroD* reporter ($n = 9$; $P = 0.16$), nor did the *VP16* ($n = 6$; $P = 0.72$) or *EnR* ($n = 4$; $P = 0.44$) domains expressed alone (Fig. 1*E*). The inactivity of *Neurog2-EnR* was not due to a failure of the encoded fusion protein to translocate to the nucleus, as immunostaining of cortical cells transfected with IRES-enhanced green fluorescent protein (EGFP) expression vectors for *Neurog2-EnR* and *Neurog2-VP16* revealed that both fusion proteins localized to the nucleus (Fig. 1*F–G″*), as previously shown for *Neurog2-WT* (Mattar et al. 2008). Finally, transactivation of the *NeuroD* promoter was dependent on DNA binding as mutation of 2 critical amino acids in the DNA-binding domain of *Neurog2-WT* (*N2*$^{NR>AQ}$; Sun et al. 2001; $n = 4$; $P = 0.08$; Fig. 1*D*) and *Neurog2-VP16* (*Neurog2-VP16*$^{NR>AQ}$; $n = 5$; $P = 0.16$; data not shown) ablated transactivation. *Neurog2-WT* and *Neurog2-VP16* thus both function as transcriptional activators, with *Neurog2-VP16* displaying enhanced transcriptional activity, either because of the modest increase in protein stability or because of the enhanced transactivation strength of the VP16 domain.

The low basal levels of *pNeuroD*^{1kb} luciferase activity made it difficult to assess *Neurog2-EnR* repressor function, so we implemented 2 additional assays. First, in co-transfection assays, we showed that *Neurog2-EnR* was able to act in a dominant negative fashion to reduce the transcriptional activity of *Neurog2-WT* on the *NeuroD1* promoter (1.2-fold decrease; $n = 5$; $P < 0.01$; Fig. 1*H*). Secondly, we generated a luciferase reporter with high basal transcriptional activity by cloning a *Neurog2*-responsive enhancer (i.e., E-box cluster from *NeuroD1* promoter) (Huang et al. 2000) downstream of a CMV-luciferase cassette (pCMV-*NeuroD1*$^{1\,kb}$; Fig. 1*I*). *Neurog2-EnR* suppressed the basal transcriptional activity of pCMV-*NeuroD1*$^{1\,kb}$ (2.3-fold decrease; $n = 5$; $P < 0.05$), while both *Neurog2-WT* (1.6-fold increase; $n = 8$, $P < 0.05$) and *Neurog2-VP16* (1.8-fold increase; $n = 8$; $P < 0.05$) modestly increased the constitutive levels of transactivation from the CMV

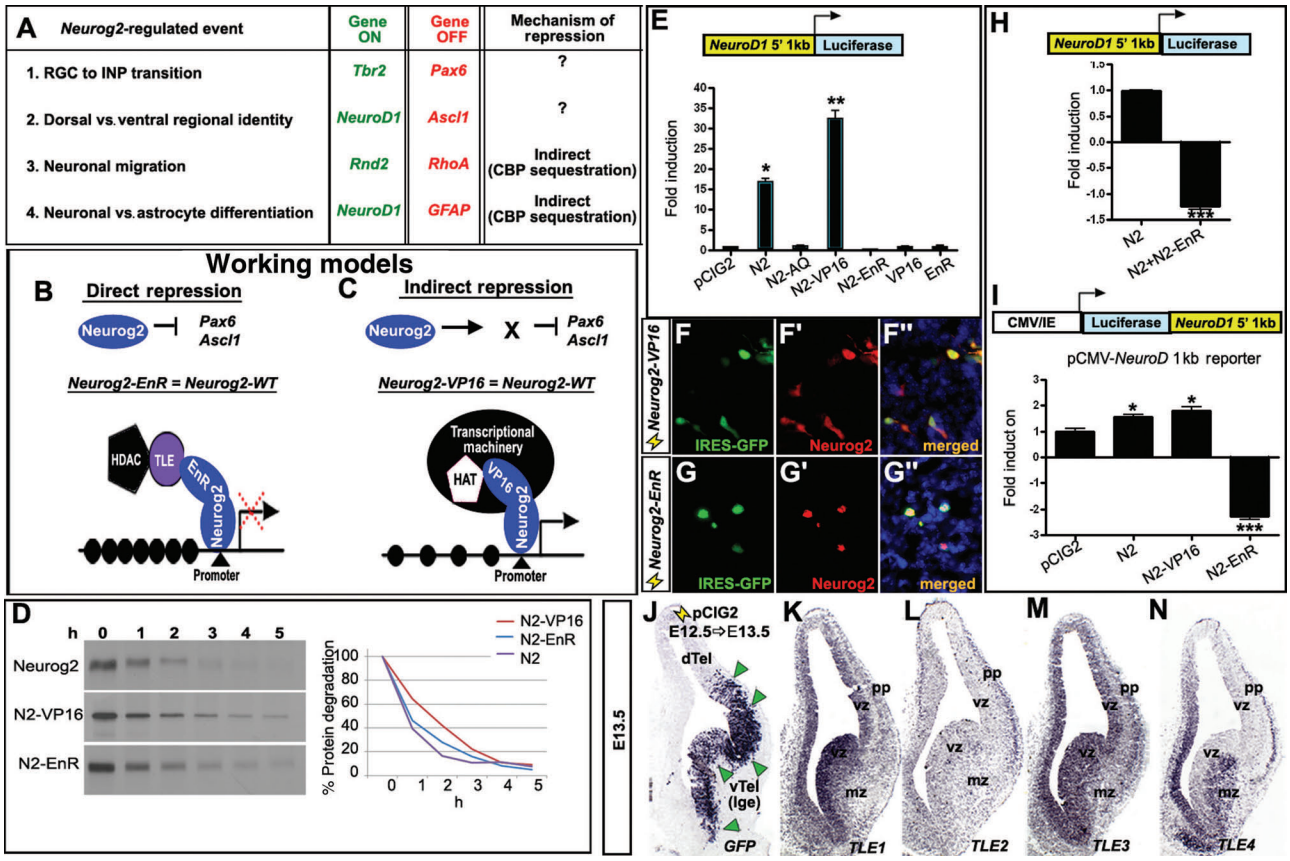

Figure 1. Generation and validation of *Neurog2* activator and repressor fusion proteins. (*A*) *Neurog2* both activates and represses gene expression to carry out its functions in the developing neocortex. Neurog2 sequesters CBP and other co-activators to negatively regulate *RhoA* (Ge et al. 2006) and *GFAP* (Sun et al. 2001) transcription. (*B* and *C*) Generation of obligate repressor (*Neurog2-EnR*; *B*) and activator (*Neurog2-VP16*; *C*) forms of *Neurog2*. Our working model predicts that if *Neurog2* functions as a direct transcriptional repressor, *Neurog2-EnR* will phenocopy *Neurog2-WT* (*B*). If instead *Neurog2* represses gene expression indirectly, *Neurog2-VP16* will phenocopy *Neurog2-WT*. (*D*) *Neurog2* protein half-life assay. (*E*) Transcriptional reporter assay in P19 cells using a p*NeuroD*$^{1\ kb}$ reporter. (*F* and *G*) Neurog2 (red; *F*′, *F*″, *G*′, G″) immunostaining of E12.5 → E15.5 electroporated cortical cells (GFP^{+}, green; *F*, *F*″, *G*, *G*″) showing nuclear localization (arrowheads). Blue is DAPI nuclear stain (*F*″ and *G*″). (*H* and *I*) Transcriptional reporter assays in P19 cells using a p*NeuroD*$^{1\ kb}$ reporter (*H*) and pCMV-*NeuroD*$^{1\ kb}$ (*I*) showing that *Neurog2-EnR* is a dominant negative repressor of *Neurog2*. (*J*) Example of E12.5 → E13.5 telencephalon electroporated with pCIG2 and analyzed with a *GFP* riboprobe (arrowheads mark transfected cells in *J*). (*K*–*N*) Distribution of *TLE1* (*K*), *TLE2* (*L*), *TLE3* (*M*), and *TLE4* (*N*) transcripts in the E13.5 telencephalon. *$P < 0.05$, **<0.01, ***<0.005. Error bars indicate SEM. dTel, dorsal telencephalon; e, embryonic day; HAT, histone acetylase; HDAC, histone deacetylase; lge, lateral ganglionic eminence; mz, mantle zone; N2, Neurog2; pp, preplate; vTel, ventral telencephalon; vz, ventricular zone.

promoter (Fig. 1*I*). *Neurog2-EnR* therefore acts as a transcriptional repressor, and furthermore, functions as a dominant negative inhibitor of *Neurog2-WT*, likely through its ability to dimerize with either endogenous *Neurog2* protein itself, or with E-proteins, which are essential *Neurog2* cofactors.

Neurog2 Acts as a Transcriptional Activator to Suppress a Radial Glial Cell Identity and Promotes the Transition to an Intermediate Neuronal Progenitor

Previous loss- and gain-of-function studies have suggested that *Neurog2* is required and sufficient to promote the RGC to INP transition (Miyata et al. 2004; Britz et al. 2006; Ochiai et al. 2009). Accordingly, expression of the INP marker *Tbr2* is reduced in *Neurog2* mutant cortices (Schuurmans et al. 2004), while the RGC marker *Pax6* is ectopically expressed (Britz et al. 2006). Moreover, *Tbr2* has been identified as a direct transcriptional target of *Neurog2* (Ochiai et al. 2009), suggesting that *Neurog2* likely functions as a transcriptional activator to initiate an INP fate. In contrast, the mechanism by which *Neurog2* represses *Pax6* expression and an RGC identity is unknown, both in the neocortex, and in the spinal cord, where a similar negative regulatory interaction between *Neurog2* and *Pax6* is observed (Bel-Vialar et al. 2007).

To determine how *Neurog2* promotes the Pax6^{+} RGC to Tbr2^{+} INP fate transition, we introduced expression constructs for the *Neurog2* activator and repressor fusion proteins into E12.5 neocortical progenitors via in utero electroporation (Dixit et al. 2011). The pCIG2 expression construct had an IRES-GFP cassette, allowing transfected cells to be visualized via GFP epifluorescence or RNA in situ hybridization using a GFP riboprobe (Fig. 1*J*). We first confirmed that *Neurog2-EnR* would be able to recruit co-repressors in vivo by examining the expression of *TLE1–4* in the E13.5 telencephalon. *TLE1* and *TLE3* were expressed in dorsal (i.e., neocortical) and ventral (lateral ganglionic eminence) telencephalic germinal zones, where progenitor cells are located (Fig. 1*K–N*). *TLE2*, *TLE3*, and *TLE4* were expressed in the neocortical preplate and *TLE4* was expressed in the ventral mantle zone, where differentiated neurons reside (Fig. 1*K–N*). Therefore, *Neurog2-EnR* has the potential to associate with TLE proteins and functions as a transcriptional repressor in dorsal and ventral telencephalic progenitors and post-mitotic neurons.

RGCs undergo interkinetic nuclear migration during the cell cycle such that G2/M-phase nuclei are positioned at the ventricular surface, while INPs lose their apical contacts and divide in non-surface or basal positions (Englund et al. 2005; Kowalczyk et al. 2009). We first asked how the different *Neurog2* fusion proteins influenced cortical progenitor cell maturation by examining the ratio of apical to basal cell divisions following the misexpression of these proteins. For this purpose, we quantitated the number of GFP$^+$-transfected cells that expressed phospho-histone H3 (pHH3), a late-G2/M-phase marker. In E12.5 → E13.5 control electroporations of pCIG2, most GFP$^+$/pHH3$^+$ cells were apically located (Fig. 2*A*, *M*,*N*; Supplementary Table S1), consistent with the inherent bias of this technique to transfect cortical cells with apical contacts (Britz et al. 2006; Kowalczyk et al. 2009). Unexpectedly, however, all 3 forms of *Neurog2* (*-WT*, *-VP16*, and *-EnR*) promoted non-surface cell divisions as evidenced by the increased basal-to-apical ratio of mitotic figures (Fig. 2*B–D*,*M*,*N*; Supplementary Table S1). Notably, the reduction in surface-dividing apical progenitors was not due to an increase in apoptosis since overall numbers of electroporated EGFP$^+$ cells expressing activated caspase 3, a marker of cells committed to the cell death pathway, was similar following electroporation with all constructs (Supplementary Fig. S1*A–E*). We

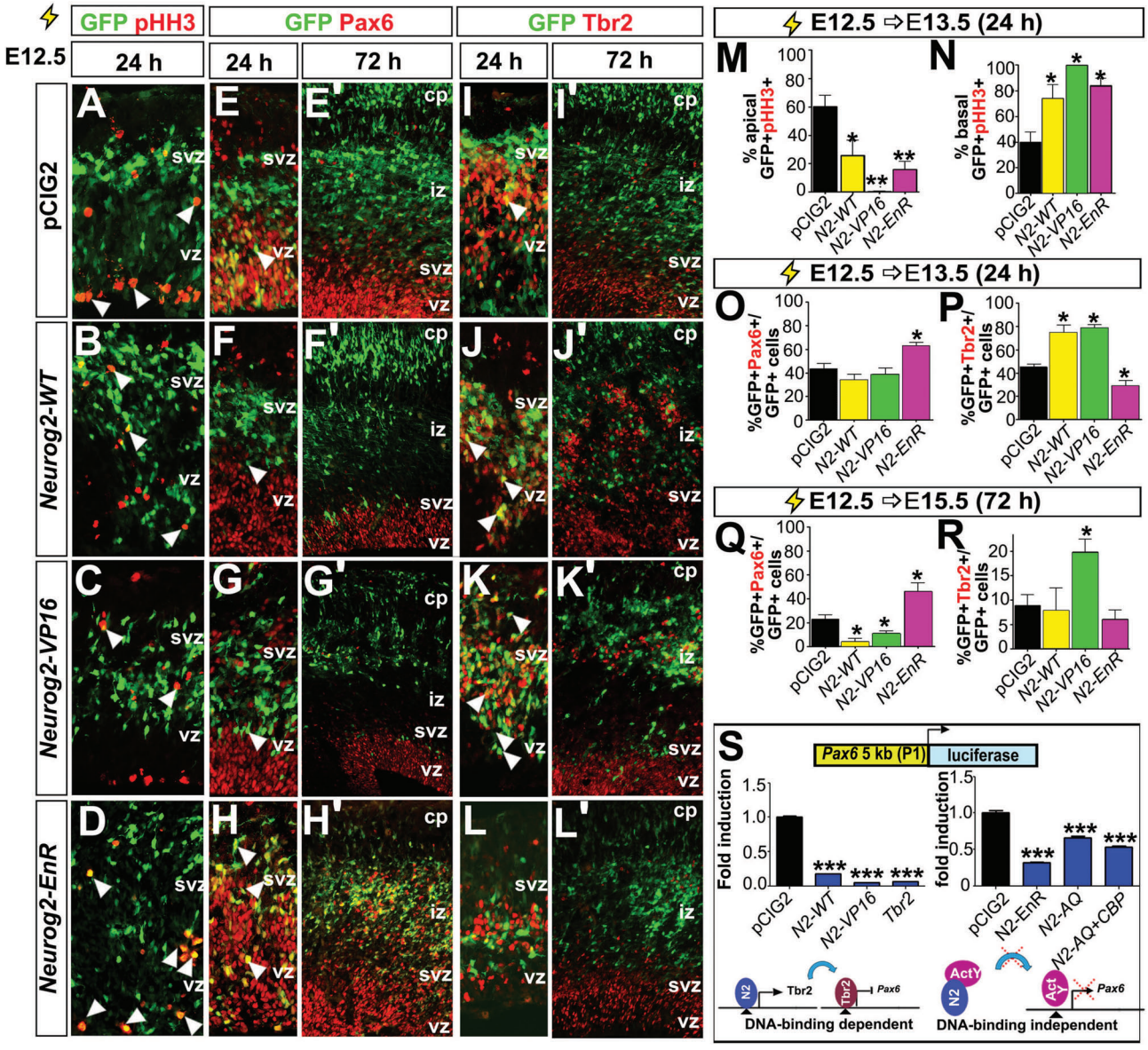

Figure 2. *Neurog2* functions as a transcriptional activator to suppress an RGC identity and promote an INP fate. (*A–L*) E12.5 → E13.5 and E12.5 → E15.5 (*E′–L′*) electroporations of the dorsal telencephalon with pCIG2 (*A*,*E*,*E′*,*I*,*I′*), *Neurog2-WT* (*B*,*F*,*F′*,*J*,*J′*), *Neurog2-VP16* (*C*,*G*,*G′*,*K*,*K′*), and *Neurog2-EnR* (*D*,*H*,*H′*,*L*,*L′*) analyzed for co-expression of GFP (green) with pHH3 (red; *A–D*), Pax6 (red; *E–H*, *E′–H′*), and Tbr2 (red, *I–L*, *I′–L′*). White arrowheads indicate double-labeled cells. (*M–R*) Ratios of apical (*M*) and basal (*N*) GFP$^+$pHH3$^+$ cells 24 h post-electroporation at E12.5. Ratios of GFP$^+$Pax6$^+$/total GFP$^+$ cells 24 h (*O*) and 72 h (*Q*) post-electroporation at E12.5; and GFP$^+$Tbr2$^+$/total GFP$^+$ cells 24 h (*P*) and 72 h (*R*) post-electroporation at E12.5. (*S*) Transcriptional reporter assays in P19 cells using a *Pax6*-5 kb/P1-luciferase reporter, with dorsal telencephalic-specific regulatory elements (Kammandel et al. 1999). *Neurog2*-mediated repression of *Pax6* was only partially dependent on DNA binding as mutation of a critical amino acid in the DNA-binding domain (*Neurog2*$^{NR > AQ}$) still retained some repressive ability (Sun et al. 2001). *$P < 0.05$, **<0.01, ***<0.005. Bars indicate SEM. CP, cortical plate; dTel, dorsal telencephalon; IZ, intermediate zone; lge, lateral ganglionic eminence; SVZ, subventricular zone; VZ, ventricular zone.

interpreted the basal bias of *Neurog2-VP16*– and *Neurog2-WT*–transfected cells as a direct role for *Neurog2* in promoting an INP fate by functioning as a transcriptional activator (Miyata et al. 2004). In contrast, we suggest that *Neurog2-EnR* may increase basal cell divisions because of its ability to induce *Ascl1* expression (see below). Indeed, we have previously demonstrated that the increase in *Ascl1* expression in *Neurog2* mutant cortices is a likely cause of the increase in basal cell divisions (Britz et al. 2006).

To further explore how *Neurog2* regulates the RGC to INP transition, we used molecular markers to phenotype the cortical cells misexpressing the different forms of *Neurog2* within 24–72 h post-electroporation. Both *Neurog2-WT* and *Neurog2-VP16* rapidly induced the expression of Tbr2, a marker of INPs committed to a neuronal fate (Englund et al. 2005; Arnold et al. 2008; Kowalczyk et al. 2009), while significantly fewer *Neurog2-EnR*–transfected cells expressed Tbr2 compared with controls (Fig. 2*I–L,I′–L′,P,R*; Supplementary Table S2). This is consistent with previous reports demonstrating that *Neurog2* directly induces *Tbr2* transcription and the acquisition of an INP fate (Ochiai et al. 2009). Conversely, the number of $GFP^+/Pax6^+$ cells observed in *Neurog2-WT* and *Neurog2-VP16* transfected cortices was reduced after 72 h compared with controls, while significantly more $GFP^+/Pax6^+$ cells were evident in *Neurog2-EnR*–transfected cortices within 24 h (Fig. 2*E–G,E′–G′,O,Q*; Supplementary Table S2). Notably, *Neurog2-WT* had a similar capacity to induce Tbr2 and block Pax6 expression whether it was electroporated at a concentration of 3 μg/μL or <2 μg/μL (Supplementary Table S4), indicating that the observed effects were not due to non-specific toxicity of higher DNA concentrations.

Taken together, our data support the model that *Neurog2* functions as a transcriptional activator to promote the RGC to INP transition. Furthermore, our data suggest that *Neurog2* represses *Pax6* transcription and an RGC identity through indirect means, a model we tested further using transcriptional assays.

Neurog2 Acts as a Transcriptional Activator to Repress Pax6 Transcription

There are at least 2 indirect ways by which *Neurog2* may repress *Pax6*: 1) By inducing the expression of a downstream transcriptional repressor or 2) by sequestering co-activators away from *Pax6* regulatory elements. To understand how *Neurog2* functions, we implemented a transcriptional reporter assay in P19 cells, using previously validated promoter and enhancer sequences for *Pax6* that drive reporter expression in the dorsal telencephalon (Kammandel et al. 1999). As expected, both *Neurog2-WT* (5.7-fold decrease; $n = 3$; $P < 0.0001$) and *Neurog2-VP16* (20.8-fold decrease; $n = 3$; $P < 0.0001$) repressed *Pax6* transactivation below control levels ($n = 3$; Fig. 2*S*). These data confirm that *Neurog2* functions as a transcriptional activator to repress *Pax6*, indicating that the repression must be at least in part indirect (i.e., *Neurog2* does not directly bind to *Pax6* regulatory elements). Given that *Tbr2* is a direct transcriptional target of *Neurog2*, and that *Tbr2* is able to repress *Pax6* cell autonomously (Sessa et al. 2008), the most parsimonious model is that *Neurog2* indirectly suppresses *Pax6* transcription by initiating *Tbr2* transcription, which then feeds forward to repress *Pax6*. Consistent with this model, *Tbr2* also repressed the *Pax6* reporter (16.7-fold; $n = 3$; $P < 0.0001$; Fig. 2*S*).

Notably, the repressor form of *Neurog2* (*-EnR*; 3.1-fold decrease; $n = 3$; $P < 0.0001$), which can bind DNA and should repress transcription, and a mutant form of *Neurog2* that does not bind DNA (*Neurog2-AQ*; 1.5-fold decrease; $n = 3$; $P < 0.0001$), was also able to inhibit *Pax6* transactivation, albeit to a much lesser extent (Fig. 2*S*). This data suggested that *Neurog2* may also suppress *Pax6* expression at least in part through indirect methods, such as the CBP sequestration model shown for *RhoA* (Ge et al. 2006) and *GFAP* (Sun et al. 2001) reporters. However, CBP was not able to rescue the inhibition of *Pax6* transactivation by *Neurog2-AQ*, suggesting that other co-activator or activator proteins must be involved in this indirect mode of repression, at least in vitro (Fig. 2*S*).

Taken together, our in vivo and in vitro data support the idea that *Neurog2* regulates the RGC to INP transition of cortical progenitors through a simple repressor loop, directly initiating *Tbr2* transcription, which in turn acts as a direct transcriptional repressor of *Pax6*. However, other indirect mechanisms may also contribute to the repressive effects of *Neurog2* on *Pax6* expression and an RGC identity.

Neurog2 Functions as a Transcriptional Activator to Initiate a Cortical-Specific Neuronal Differentiation Program

Neurog2 and the related gene *Neurog1* promote precocious neuronal differentiation when misexpressed in cortical progenitors, directly initiating the transcription of several neuronal differentiation genes both in vitro and in vivo (Schuurmans et al. 2004; Mattar et al. 2008). To confirm that *Neurog2* functions as a transcriptional activator to induce cortical neurogenesis, we electroporated E12.5 neocortices with expression constructs for each *Neurog2* fusion protein, and then quantitated the number of GFP^+ cells that co-expressed the panneuronal marker NeuN or the cortical-specific neuronal markers Bhlhb5 (Beta3/Bhlhe22) and Tbr1 (Hevner et al. 2001; Joshi et al. 2008) at 24 h and 72 h post-electroporation. As expected, both *Neurog2-WT* and *Neurog2-VP16* induced the differentiation of more $GFP^+/NeuN^+$ neurons compared with pCIG2 controls (Fig. 3*A–H,U,U′*; Supplementary Table S2). These ectopic neurons expressed cortical-specific markers, as evidenced by the similar increases in $GFP^+/Bhlhb5^+$ and $GFP^+/Tbr1^+$ double-positive cells in *Neurog2-WT* and *Neurog2-VP16* transfections compared with empty vector controls (Fig. 3*I–P,V,V′,W,W′*, Supplementary Table S2). Conversely, *Neurog2-EnR* suppressed neurogenesis, reducing the numbers of $GFP^+/NeuN^+$, $GFP^+/Bhlhb5^+$, and $GFP^+/Tbr1^+$ neurons that differentiated (Fig. 3*D,H,L,P,T,U–W,U′–W′*, Supplementary Table S2). Furthermore, *Neurog2-WT* and *Neurog2-VP16* reduced the number of GFP^+ cells expressing the pan-proliferative marker $Ki67^+$, while *Neurog2-EnR* did not influence the overall number of cycling cells (Fig. 3*Q–T,X*, Supplementary Table S2). *Neurog2-WT* and *Neurog2-VP16* thus have similar capacities to promote cell cycle exit and neuronal differentiation in the dorsal telencephalon, acting in an opposing fashion to *Neurog2-EnR*, which inhibits neurogenesis.

We next asked if *Neurog2* similarly functions as a transcriptional activator to induce a cortical identity when misexpressed in an ectopic site. In E12.5 → E15.5 electroporations of the ventral telencephalon, both *Neurog2-VP16* and

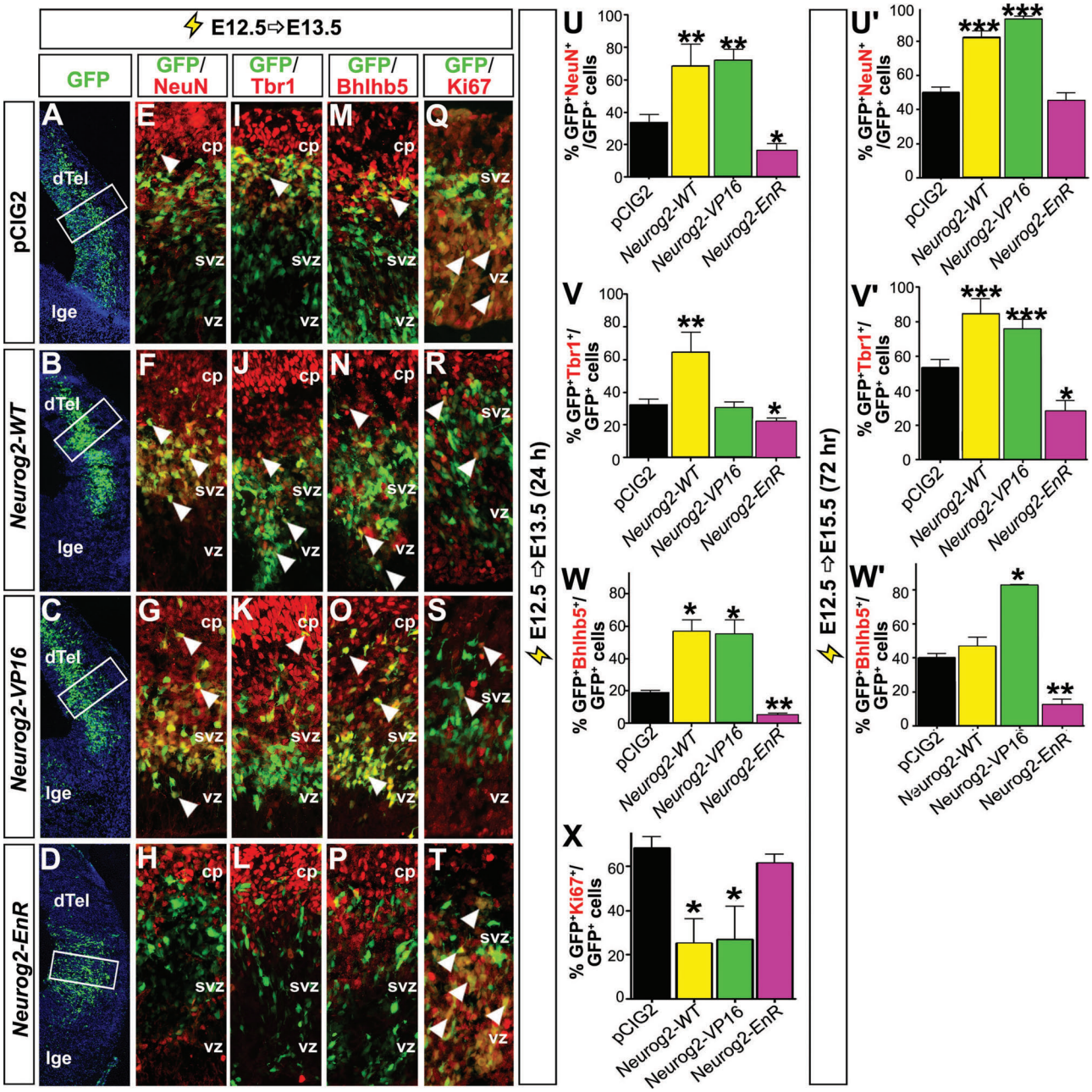

Figure 3. *Neurog2* functions as a transcriptional activator to promote cortical neuronal differentiation in the dorsal telencephalon. (*A–T*) E12.5 → E13.5 electroporations of the dorsal telencephalon with pCIG2 (*A,E,I,M,Q*), *Neurog2-WT* (*B,F,J,N,R*), *Neurog2-VP16* (*C,G,K,O,S*), and *Neurog2-EnR* (*D,H,L,P,T*) analyzed for the co-expression of GFP (green) with NeuN (red; *E–H*), Tbr1 (red, *I–L*), Bhlhb5 (red, *M–P*), and Ki67 (red, *Q–T*). White arrowheads indicate double-labeled cells and blue is DAPI nuclear stain. (*U–X*) Ratios of GFP^+NeuN^+/total GFP^+ cells 24 h (*U*) and 72 h (*U′*) post-electroporation at E12.5; GFP^+Tbr1^+/total GFP^+ cells 24 h (*V*) and 72 h (*V′*) post-electroporation at E12.5; $GFP^+Bhlhb5^+$/total GFP^+ cells 24 h (*W*) and 72 h (*W′*) post-electroporation at E12.5; and GFP^+Ki67^+/total GFP^+ cells 24 h post-electroporation. (*X*) $^*P < 0.05$, $^{**}<0.01$, $^{***}<0.005$. Bars indicate SEM. CP, cortical plate; dTel, dorsal telencephalon; lge, lateral ganglionic eminence; SVZ, subventricular zone; VZ, ventricular zone.

Neurog2-WT (as shown in Mattar et al. 2008) had a similar capacity to promote the differentiation of $NeuN^+$ neurons (Fig. 4*A′–D′,E*) that expressed cortical-specific markers, including Tbr1 (Fig. 4*A″–D″,F*), Bhlhb5 (Fig. 4*A‴–D‴,G*), *NeuroD6* (Supplementary Fig. S2*M–P*), *Sox5* (Supplementary Fig. S2*Q–T*), and *Nhlh2* (Supplementary Fig. S2*U–X*; Supplementary Table S3). Notably, following *Neurog2-WT* and *Neurog2-VP16* overexpression, GFP^+ cells formed distinct cellular aggregates or heterotopias in the ventral telencephalon, consistent with an alteration in the neuronal identity and hence, adhesive properties (Fig. 4*B–B‴,C–C‴*; Supplementary Fig. S2*F,G,J,K*). In contrast, pCIG2 and *Neurog2-EnR*-transfected GFP^+ cells were sparsely distributed in the ventral telencephalon, suggesting that neither construct altered the regional identity or adhesive properties of the transfected cells (Fig. 4*A–A‴,D–D‴*; Supplementary Fig. S2*E,H,L*). Furthermore, *Neurog2-EnR* did not alter the number of GFP^+/$NeuN^+$, GFP^+/$Bhlhb5^+$, or GFP^+/$Tbr1^+$ neurons that differentiated in the ventral telencephalon compared with pCIG2 controls (Fig. 4*D–D‴*, Supplementary Table S3).

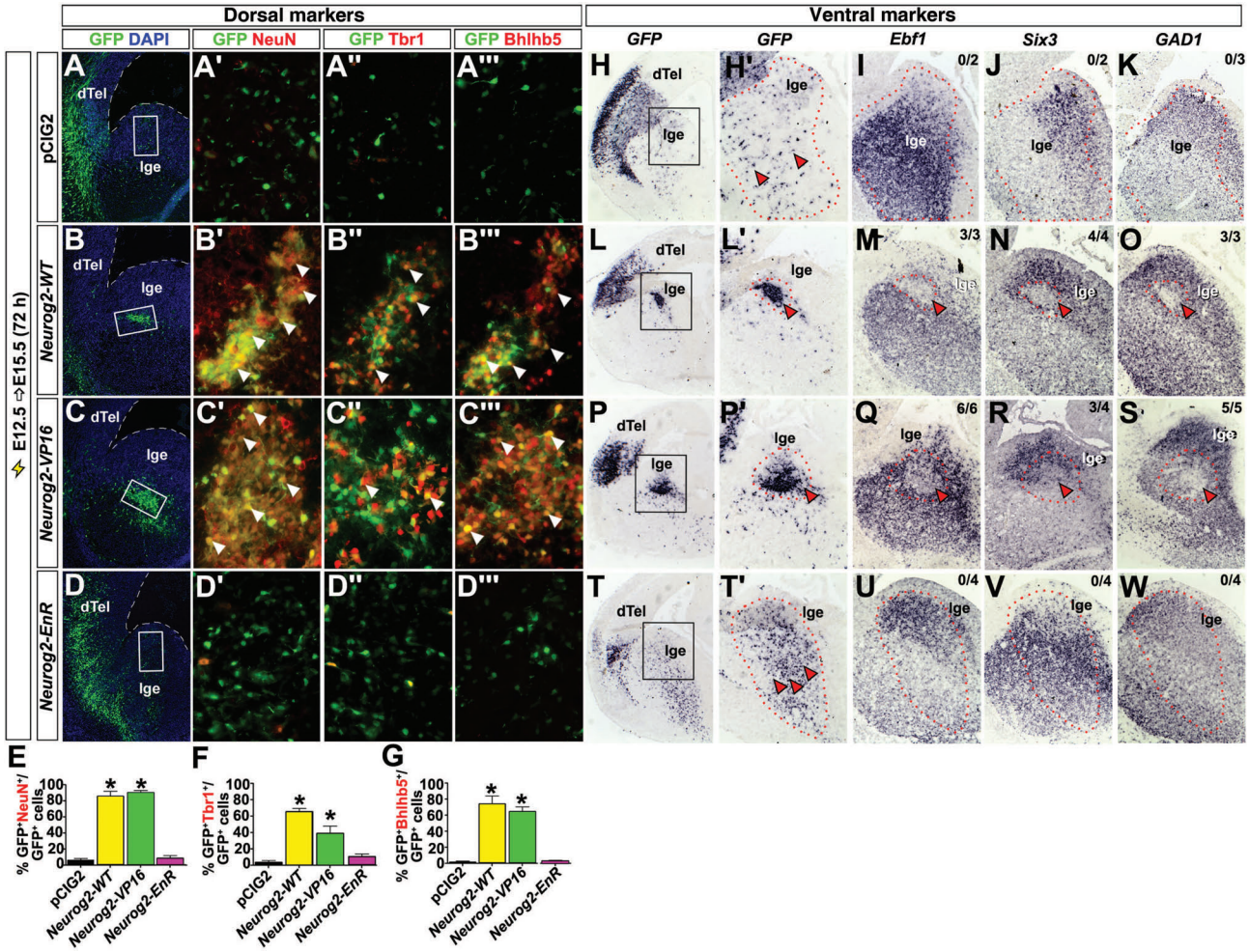

Figure 4. *Neurog2* functions as a transcriptional activator to initiate dorsal-specific and to repress ventral-specific neuronal differentiation programs in the ventral telencephalon. (*A–G*) E12.5 → E15.5 electroporations of the ventral telencephalon with pCIG2 (*A–A‴*), *Neurog2-WT* (*B–B‴*), *Neurog2-VP16* (*C–C‴*), and *Neurog2-EnR* (*D–D‴*) analyzed for the co-expression of GFP (green) with NeuN (red; *A′–D′*), Tbr1 (red; A″–*D″*), and Bhlhb5 (red; *A‴–D‴*). White arrowheads indicate double-labeled cells and blue is DAPI nuclear stain. (*E–G*) Quantitation of GFP^+NeuN^+/total GFP^+ cells (*E*), GFP^+Tbr1^+/total GFP^+ cells (F), and $GFP^+Bhlhb5^+$/total GFP^+ cells (*G*) 72 h post-electroporation of the E12.5 ventral telencephalon. $^*P < 0.05$, $^{**}<0.01$, $^{***}<0.005$. Bars indicate SEM. (*H–R*) E12.5 → E15.5 electroporations of the ventral telencephalon with pCIG2 (*H,H′,I–K*), *Neurog2-WT* (*L,L′,M–O*), *Neurog2-VP16* (*P,P′,Q–S*), and *Neurog2-EnR* (*T,T′,U–W*) analyzed for the expression of *GFP* (*H,H′,L,L′,P,P′,T,T′*), *Ebf1* (*I,M,Q,U*), *Six3* (*J,N,R,V*), and *GAD1* (*K,O,S,W*). Boxed areas in (*H,L,P,T*) indicate positions of higher magnification images in (*H′,I–K,L′,M–O,P′,Q–S,T′*, and *U–W*), respectively. Dotted red lines outline transfected region and red arrowheads indicate suppression of gene expression. The number of brains in which gene suppression was observed is indicated in the top-right corner. dTel, dorsal telencephalon; lge, lateral ganglionic eminence.

Taken together, these data indicate that *Neurog2* does indeed function as a transactivator to initiate cortical-specific neuronal differentiation, both in its normal territory and in ectopic sites in the telencephalon.

Neurog2 Functions as a Transcriptional Activator to Suppress Ventral Differentiation Programs, in Part Via a Neurog2-Tbr2-Ebf1 Transcriptional Cascade

Neurog2 is not only required to specify a dorsal, glutamatergic neuronal identity, but also to repress an alternative ventral, GABAergic fate in neocortical progenitors (Fode et al. 2000; Schuurmans et al. 2004). We thus asked if *Neurog2* functions as a transcriptional activator or repressor to suppress ventral identities in telencephalic progenitors by performing E12.5 → E15.5 ventral electroporations. Both *Neurog2-WT* and *Neurog2-VP16* efficiently suppressed the expression of several ventral neuronal genes, including *Ebf1*, *Six3*, and *GAD1*, while pCIG2 and *Neurog2-EnR* had no effect on the expression of these ventral markers (Fig. 4*H–S*). Conversely, in E12.5 → E15.5 electroporations of the dorsal telencephalon, neither *Neurog2-WT*, *Neurog2-VP16*, nor *Neurog2-EnR* influenced the expression of "ventral" markers, including those expressed by GABAergic interneurons (*Dlx1*, *Ebf1*, *Six3*, and *GAD1*) and oligodendrocytes (*Olig2*; Supplementary Fig. S3), the exception being *Ascl1*, which was induced by *Neurog2-EnR* (Fig. 6, Supplementary Fig. S3*L*, see below).

Taken together, these data indicate that *Neurog2* functions as a transcriptional activator to suppress a ventral telencephalic identity, indicative of an indirect mode of repression. To explore the indirect repression mechanism further, we first examined *Tbr2*, given our demonstration above that this gene functions as a downstream effector of *Neurog2*-mediated repression. We first asked if *Tbr2* had any capacity to suppress ventral differentiation programs in the neocortex by electroporating *Tbr2* into E12.5 cortical progenitors. As previously reported, misexpression of *Tbr2* does not initiate cortical

neuronal differentiation (Sessa et al. 2008), as exemplified by the lack of induction of Tbr1 expression (Fig. 5*A*,*A*′,*B*–*B*″). However, in E12.5 → E15.5 electroporations of the ventral telencephalon, *Tbr2*-overexpressing cells formed tight cellular aggregates (Fig. 5*C*), similar to the heterotopias evident following the misexpression of *Neurog2* in ventral domains (Fig. 4). The clustering of *Tbr2*-overexpressing cells was suggestive of a change in the regional identity and adhesive properties of these cells. Strikingly, we observed a clear repression of the ventral marker *Ebf1* in the *Tbr2*-transfected patch (Fig. 5*D* vs. control side in *D*′), but surprisingly, other components of the ventral differentiation program, such as *Six3* and *GAD1* (Fig. 5*E*,*F*), were expressed normally in *Tbr2*-overexpressing cells.

To further investigate regulatory interactions between *Tbr2* and *Ebf1*, we examined *Ebf1* expression in *Tbr2* mutant cortices. In E14.5 wild-type embryos, *Ebf1* expression was restricted to the mantle zone of the ventral telencephalon, with no expression detected in dorsal telencephalic domains, not even in migrating cortical interneurons (Fig. 5*H*,*H*′). Strikingly, in E14.5 *Tbr2* mutants, *Ebf1* was ectopically expressed in the dorsal telencephalon, specifically within the SVZ (Fig. 5*I*,*I*′), where *Tbr2* is normally expressed (Fig. 5*G*,*G*′). We reasoned that the ectopic expression of *Ebf1* in *Tbr2* mutants was most likely due to a cell autonomous requirement for *Tbr2* to repress Ebf1, rather than the ectopic migration of *Ebf1*-expressing cells from ventral to dorsal domains, as interneuron migration is strongly reduced in *Tbr2* mutants (Sessa et al. 2010). To test if *Tbr2* was indeed a direct repressor of *Ebf1* transcription, we used 2 approaches. First, we performed luciferase assays using previously generated reporters containing regulatory sequences from the proximal and distal promoters of *Ebf1* (Roessler et al. 2007). We found that *Tbr2* was able to suppress transactivation of the *Ebf1* proximal promoter (1.8-fold decrease; $n = 3$; $P < 0.0001$; Fig. 5*K*) and not the distal promoter ($n = 3$; $P > 0.05$; Fig. 5*J*). Accordingly, multiple conserved *Tbr2*-binding sites were identified in the *Ebf1* proximal promoter region (Fig. 5*L*). To test if Tbr2 could directly bind the *Ebf1* proximal promoter, we performed anti-Tbr2 ChIP experiments. Tbr2 was specifically enriched on *Ebf1* proximal promoter sequences in chromatin isolated from E14.5 cortices (Fig. 5*M*,*N*).

Taken together, we have identified a novel *Neurog2*-*Tbr2*-*Ebf1* repressor loop in the embryonic neocortex that involves direct transcriptional interactions, with *Neurog2* turning on the expression of *Tbr2* (Ochiai et al. 2009), and *Tbr2* in turn binding to and repressing the transcription of *Ebf1*.

Neurog2 Indirectly Suppresses Ascl1 Expression Through DNA-Binding–Dependent and –Independent Mechanisms

We were struck by the inability of *Tbr2* to repress all ventral genes, such as *Six3* and *GAD1*, which are also negatively regulated by *Neurog2* (Fode et al. 2000; Schuurmans et al. 2004). This suggested that *Tbr2* was not the only downstream effector in *Neurog2*-mediated repressor cascades. To further investigate how *Neurog2* represses ventral fates, we focused on *Ascl1*, a proneural bHLH gene that is negatively regulated by *Neurog2* in cortical progenitors (Fode et al. 2000; Schuurmans et al. 2004; Britz et al. 2006; Mattar et al. 2008). *Ascl1* is necessary and sufficient to promote the differentiation of oligodendrocytes (Parras et al. 2007) and neurons with a ventral regional identity and GABAergic neurotransmitter phenotype (Casarosa et al. 1999; Fode et al. 2000; Schuurmans et al. 2004; Britz et al. 2006; Mattar et al. 2008). To first test if *Neurog2* regulates *Ascl1* cell autonomously, we examined Ascl1 expression in heterozygous and homozygous E15.5 *Neurog2*^{GFP} knock-in (KI) embryos. While very few GFP$^+$/Ascl1$^+$ double-positive progenitors were observed in *Neurog2*$^{GFPKI/+}$ cortices (Fig. 6*A*,*B*,*B*′), many double-positive cells were observed in *Neurog2*$^{GFPKI/GFPKI}$ cortices (Fig. 6*C*,*D*,*D*′), indicating that *Ascl1* is upregulated within *Neurog2* mutant cortical cells. We next asked if *Neurog2* functions as a transcriptional activator or repressor to regulate *Ascl1*. In E12.5 → E13.5 electroporations of the dorsal telencephalon, both *Neurog2-WT* and *Neurog2-VP16* reduced the number of GFP$^+$/Ascl1$^+$ progenitors compared with control transfections (Fig. 6*E*–*G*,*E*′–*G*′,*M*; Supplementary Table S2). Conversely, *Neurog2-EnR* induced the ectopic expression of *Ascl1* at both the transcript (Fig. 6*K*,*L* vs. 6*I*,*J* control transfection; Supplementary Fig. S3*L*) and the protein level (Fig. 7*H*,*H*′,*M*; Supplementary Table S2), promoting a 3.9-fold increase in the percentage of GFP$^+$/Ascl1$^+$ cells. *Neurog2* thus acts as a transcriptional activator to cell autonomously suppress *Ascl1* expression.

To understand the mechanism(s) by which *Neurog2* indirectly suppresses *Ascl1* expression, we subcloned 5 kb of the *Ascl1* upstream regulatory and promoter sequences into a luciferase reporter. Consistent with an indirect mode of transcriptional repression, both *Neurog2-WT* (3.9-fold decrease; $n = 3$; $P < 0.0001$) and *Neurog2-VP16* (7.5-fold decrease; $n = 3$; $P < 0.0001$) repressed *Ascl1* transactivation below control levels within 24 h post-transfection of P19 cells, whereas *Neurog2-EnR* ($n = 3$; $P > 0.05$) had no effect on the *Ascl1* reporter (Fig. 6*N*). To further probe how *Neurog2* represses *Ascl1*, we also examined the activities of *Neurog2* fusion proteins carrying a mutation in the DNA-binding domain (AQ mutants; Sun et al. 2001). While *Neurog2-WT-AQ* (1.5-fold decrease; $n = 3$; $P < 0.01$) and *Neurog2-VP16-AQ* (2.9-fold decrease; $n = 3$; $P < 0.0001$) had some ability to repress the *Ascl1* reporter, their repressive activities were less than the proteins that retained their DNA-binding capacity (Fig. 6*N*). Furthermore, CBP had no capacity to rescue *Neurog2*-mediated repression of the *Ascl1* reporter, suggesting that this sequestration model does not apply to *Ascl1* regulation (Fig. 6*O*). Thus, *Neurog2* represses *Ascl1* transcription through novel DNA-binding–dependent and –independent mechanisms. We focused on identifying the novel DNA-binding–dependent mechanisms of *Neurog2*-mediated repression.

Etv1 is a Neurog2-Regulated Gene That Regulates Expression of the Ventral Identity Determinant Ascl1

Our data suggested that *Neurog2* functions as a transcriptional activator to repress *Ascl1* expression, but the intermediaries of this indirect regulation are unknown. To identify such molecules, we examined the expression of several candidate transcriptional regulators in E13.5 *Neurog2* mutant neocortices, at a stage when *Ascl1* expression is upregulated (Fode et al. 2000; Schuurmans et al. 2004). Of the genes tested, Tbr2 (Fig. 7*A*,*A*′,*B*,*B*′) and *Sox5* (Fig. 7*C*,*C*′,*D*,*D*′) were expressed at reduced levels in *Neurog2* mutant neocortices, while Pax6 (Fig. 7*E*,*E*′,*F*,*F*′), *Etv1* (Fig. 7*G*,*G*′,*H*,*H*′), and *Sox6* (not shown)

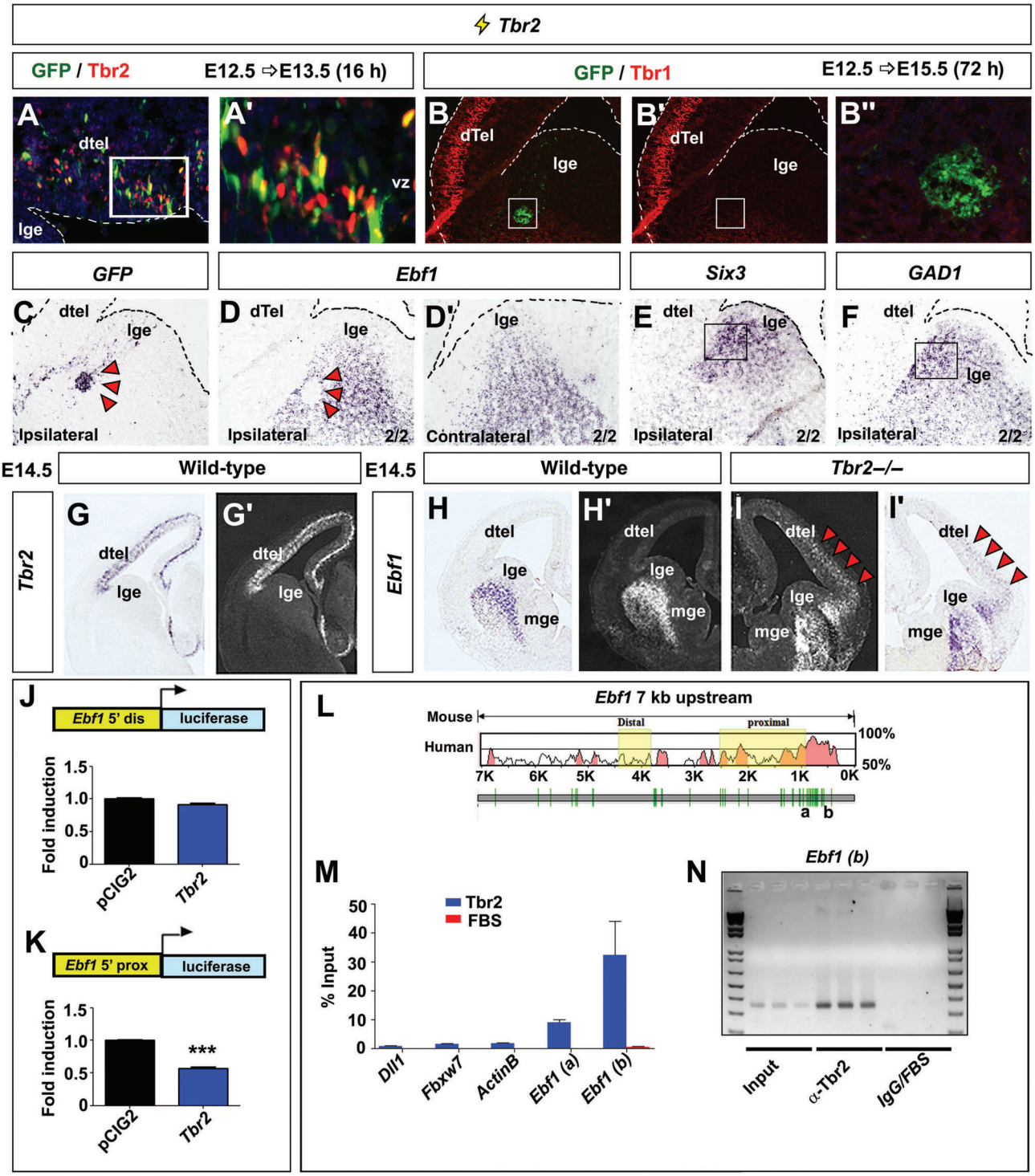

Figure 5. *Neurog2* suppresses *Ebf1* transcription via *Tbr2*. (*A*–*F*) Electroporation of *Tbr2* into the dorsal (*A* and *A*′) and ventral (*B*,*B*′,*C*–*F*) telencephalon analyzed 16 h (*A* and *A*′) and 72 h (*B*–*F*) post-electroporation for the expression of GFP (green) with Tbr2 (*A* and *A*′) and Tbr1 (*B*–*B*″) protein, or the distribution of transcripts for *GFP* (*C*), *Ebf1* (*D* and *D*′), *Six3* (*E*), and *GAD1* (*F*). Contralateral hemisphere in (*D*′) serves as a negative control (i.e., un-electroporated side) for comparison to the electroporated ipsilateral side in (*D*). Arrowheads in (*C* and *D*) and boxes in (*B*,*B*′,*E*,*F*) mark electroporated patch. (*G*–*I*′) Distribution of *Tbr2* (*G* and *G*′) and *Ebf1* (*H*,*H*′,*I*,*I*′) transcripts in wild-type (*G*,*G*′,*H*,*H*′) and *Tbr2* mutant (*I* and I′) telencephalons at E14.5. Arrowheads in (*I* and *I*′) mark ectopic *Ebf1* expression. (*J* and *K*) Transcriptional reporter assays in P19 cells using *Ebf1* proximal and distal promoter elements showing that *Tbr2* represses transactivation of the *Ebf1* proximal promoter. ***$P < 0.001$. Bars indicate SEM. (*L*–*N*) Identification of conserved *Tbr2*-binding sites in the *Ebf1* proximal and distal promoter of mouse and human (*L*). Anti-Tbr2 ChIP experiments show that *Tbr2* protein binds to proximal promoter elements of *Ebf1* in E14.5 dorsal telencephalic cells, but does not bind to negative control elements in *Dll1*, *Fbxw7*, and *actinB*. The *Ebf1* amplified product from input chromatin, and ChIP with IgG/FBS (negative control) and anti-Tbr2 are shown on an agarose gel in (*N*). The data are plotted as the means of 2 independent ChIP assays and 3 independent qPCR amplifications.

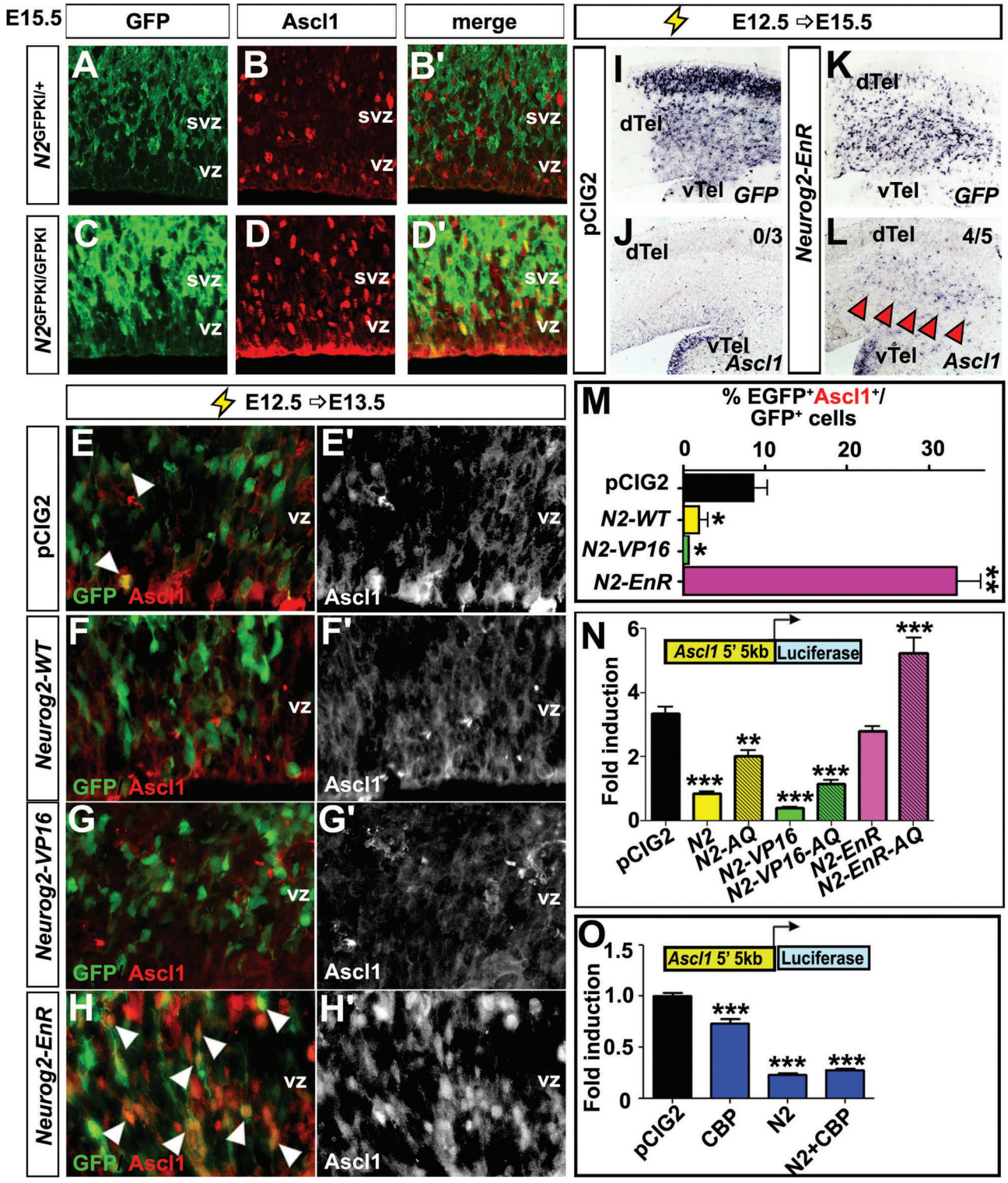

Figure 6. *Neurog2* functions as a transcriptional activator to repress *Asc1l* expression. (*A–D*) Expression of GFP (green) and Ascl1 (red) in E15.5 *Neurog2*$^{GFPKI/+}$ (*A*,*B*,*B′*) and *Neurog2*$^{GFPKI/GFPKI}$ (*C*,*D*,*D′*) cortices. (*E–H*) E12.5 → E13.5 electroporations with pCIG2 (*E* and *E′*), *Neurog2-WT* (*F* and *F′*), *Neurog2-VP16* (*G* and *G′*), and *Neurog2-EnR* (*H* and *H′*) showing co-labeling of GFP (green, *E–H*) and Ascl1 (red, *E–H*; white in grayscale images, *E′–H′*). White arrowheads indicate ectopic double-positive cells in (*H*). (*I–L*) E12.5 → E13.5 electroporations with pCIG2 (*I* and *J*) and *Neurog2-EnR* (*K* and *L*) showing GFP (*I* and *K*) and Ascl1 (*J* and *L*) expression. Arrowheads mark ectopic *Ascl1* expression in (*L*), with the number of brains with ectopic *Ascl1* expression indicated in the top-right corner. (*M*) Quantitation of GFP$^+$Ascl1$^+$/total GFP$^+$ cells following E12.5 → E13.5 electroporations with the indicated constructs. (*N*) Transcriptional reporter assays in P19 cells using a p*Ascl1* 5 kb reporter showing that *Neurog2* represses *Ascl1* transcription through mechanisms that are DNA-binding–dependent and –independent (i.e., repression also seen with mutations in the DNA-binding domain in AQ constructs). (*O*) Transcriptional reporter assays in P19 cells using a p*Ascl1* 5 kb reporter showing that CBP cannot rescue *Neurog2*-mediated repression of *Ascl1* transcription. *$P < 0.05$, **<0.01, ***<0.005. Bars indicate SEM. VZ, ventricular zone; dTel, telencephalon; lge, lateral ganglionic eminence.

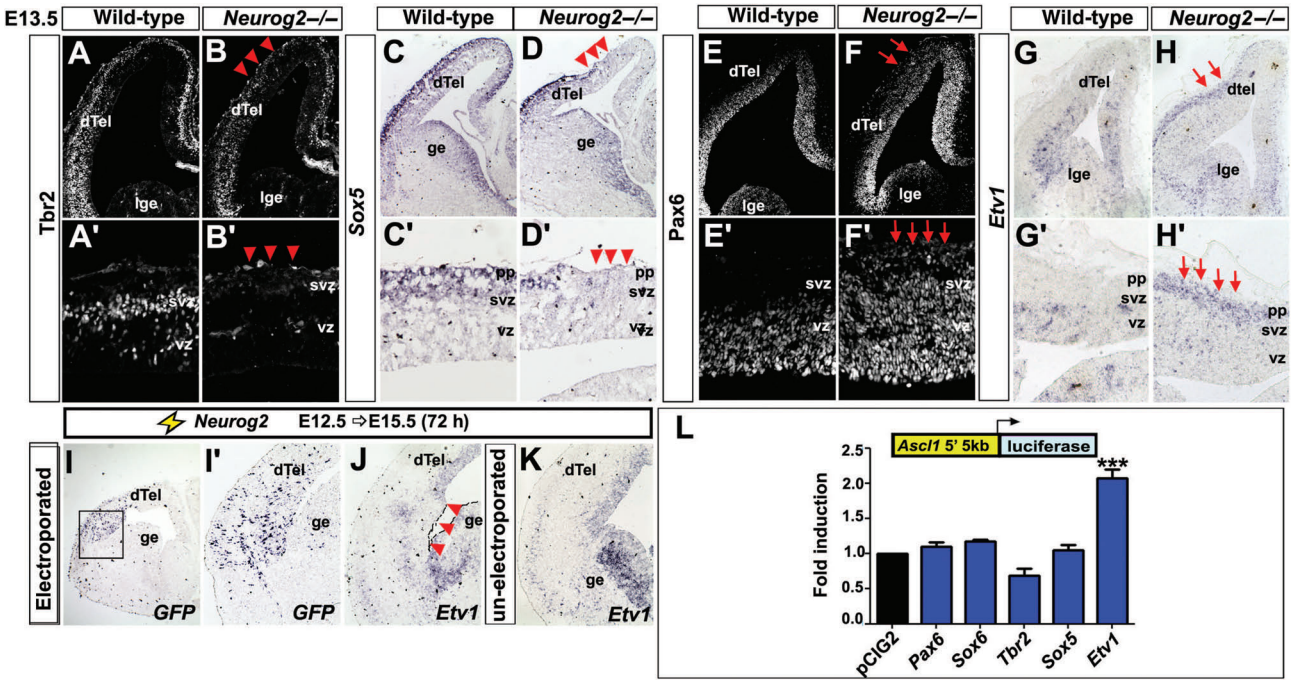

Figure 7. *Neurog2* suppresses *Ascl1* expression by negatively regulating the expression of *Etv1*. (*A–H*) Expression of Tbr2 (*A*,*A*′,*B*,*B*′), *Sox5* (*C*,*C*′,*D*,*D*′), Pax6 (*E*,*E*′,*F*,*F*′), and *Etv1* (*G*,*G*′,*H*,*H*′) in E13.5 wild-type and *Neurog2* mutant neocortices. Arrowheads in (*B*,*B*′,*D*,*D*′) mark reduced marker expression in *Neurog2* mutant neocortices, while arrows in (*F*,*F*′, *H*,*H*′) mark ectopic marker expression. (*I–K*) E12.5 → E15.5 *Neurog2* electroporation showing the distribution of *GFP* (*I* and *I*′) and *Etv1* (*J* and *K*) on the electroporated (*J*) and un-electroporated (*K*) side. *Etv1* expression is repressed by *Neurog2* (arrowheads in *J*). (*L*) Transcriptional reporter assay in P19 cells using a p*Ascl1* 5 kb reporter. ***$P < 0.001$. Bars indicate SEM.

were upregulated (Schuurmans et al. 2004; Britz et al. 2006; Azim et al. 2009). Conversely, in E12.5 electroporations, *Neurog2* and *Neurog2-VP16* increased Tbr2 (Fig. 2*J*,*J*′,*K*,*K*′,*P*, *R*) and *Sox5* (Supplementary Fig. S2*R*,*S*) expression in the dorsal and ventral telencephalon, respectively, while Pax6 (Fig. 2*F*,*F*′,*G*,*G*′,*Q*) and *Etv1* (Fig. 7*I*,*I*′,*J*,*K*) expression were repressed by *Neurog2* in cortical progenitors.

We predicted that those transcription factors positively regulated by *Neurog2* (i.e., *Tbr2*, *Sox5*) might repress *Ascl1* expression, while those negatively regulated by *Neurog2* (i.e., *Etv1*, *Pax6*, *Sox6*) may induce *Ascl1* transcription. To directly test this, we used the 5 kb *Ascl1* transcriptional reporter assay in P19 cells. Introduction of *Tbr2*, *Sox5*, *Sox6*, and *Pax6* did not have significant effects on *Ascl1* reporter activity, suggesting that these transcriptional regulators do not directly repress *Ascl1* transcription—at least not through the 5 kb of *Ascl1* regulatory sequence tested (Fig. 7*L*). Because of the inherent limitations of this in vitro assay, we did further tests to determine if *Tbr2* repressed *Ascl1* expression, even though it did not repress the *Ascl1* reporter in vitro ($n = 3$; $P > 0.05$; Fig. 7*L*). In E12.5 electroporations of the ventral telencephalon, *Tbr2* did not reduce the number of progenitors that expressed Ascl1 (pCIG2 control: $57.45 \pm 2.11\%$ GFP^+Ascl1^+/GFP^+, $n = 5$ vs. Tbr2: $50.64 \pm 5.52\%$ GFP^+Ascl1^+/GFP^+, $n = 3$; $P = 0.21$; 16 h post-electroporation). Moreover, *Ascl1* expression was not altered in *Tbr2* mutant cortices (Robert F. Hevner, unpublished observation). We thus conclude that *Tbr2* is not a transcriptional repressor for *Ascl1*, validating the results of our in vitro reporter assay.

Of the transcription factors repressed by *Neurog2*, *Etv1* was the only factor to increase *Ascl1* reporter activity (2.0-fold increase; $n = 3$; $P < 0.0001$; Fig. 7*L*). Accordingly, in E12.5 → E15.5 *Etv1* cortical electroporations, ectopic *Ascl1* expression was detected in the transfected patch (Fig. 8*A–E*). We thus conclude that *Etv1* is able to initiate ectopic *Ascl1* expression, and suggest that *Neurog2* may be required to repress *Etv1* so that *Ascl1* is not ectopically expressed in cortical cells.

Etv1 Represses Hes5 Expression to Suppress Ascl1 Transcription

Etv1 expression is initiated downstream of fibroblast growth factor signaling, which has recently been shown to regulate Notch signaling (Akazawa et al. 1992). We thus speculated that *Etv1* may regulate *Ascl1* expression via regulatory interactions with the Notch pathway. Indeed, the Notch effector proteins *Hes1* and *Hes5* are well known suppressors of proneural gene expression in neural progenitors (e.g., Arber et al. 2000; Nieto et al. 2001; Hansen et al. 2010), with Hes1 directly recruiting TLE co-repressors to *Ascl1* regulatory elements (Ju et al. 2004). We first examined if like *Etv1*, *Hes5* expression levels were regulated by *Neurog2*. *Hes5* transcript levels were reduced in the dorsal telencephalon of E13.5 *Neurog2* mutants compared with wild-type controls (Fig. 8*F*, *G*). Moreover, *Neurog2* induced ectopic *Hes5* expression in E12.5 → E15.5 cortical electroporations (Fig. 8*H–K*). Finally, *Neurog2* induced a 10-fold increase ($n = 3$; $P < 0.0001$) in the activity of a 3 kb *Hes5*-luciferase reporter (Nieto et al. 2001). *Neurog2* is thus required and sufficient to promote *Hes5* expression in cortical progenitors.

To determine if *Etv1* also regulates *Hes5* transcription, we generated obligate activator (*Etv1-VP16*) and repressor (*Etv1-EnR*) fusions and tested their activities in vivo and in vitro. In E12.5 → E13.5 cortical electroporations, *Etv1-VP16*

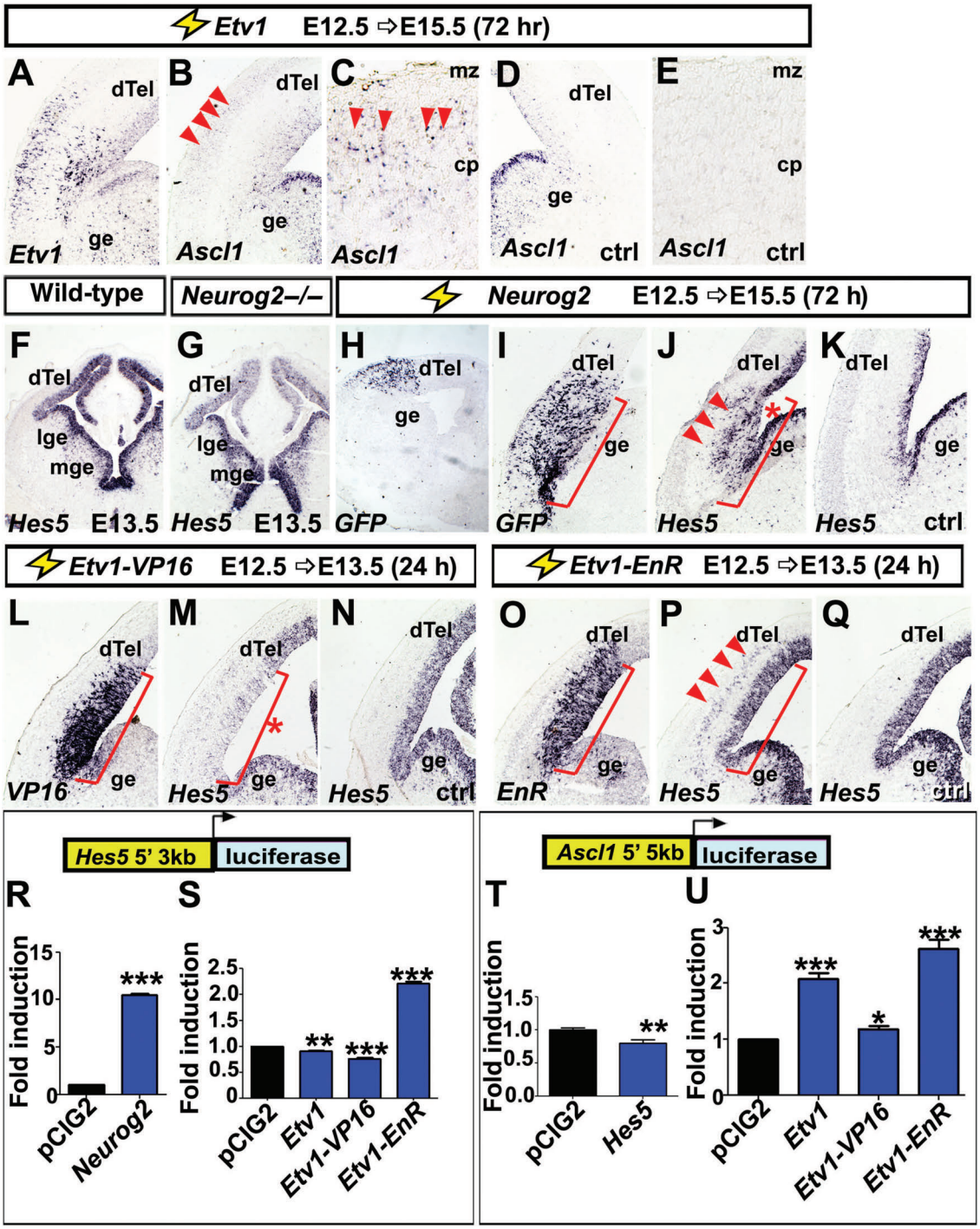

Figure 8. *Etv1* regulates *Hes5* and *Ascl1* expression in cortical progenitors. (*A–E*) E12.5 → E15.5 electroporation of *Etv1*. The electroporated patch is marked by the ectopic expression of *Etv1* (*A*). Expression of *Ascl1* on the electroporated (*B* and *C*) and un-electroporated (*D* and *E*) side, with ectopic expression of *Ascl1* marked with arrowheads in (*B* and *C*). (*F* and *G*) Expression of *Hes5* in E13.5 wild-type (*F*) and *Neurog2* mutant (*G*) neocortices. (*H–K*) E12.5 → E15.5 electroporations with *Neurog2* showing the distribution of *GFP* (*H* and *I*) in the electroporated patch (marked with red bracket), and *Hes5* expression in the electroporated (*J*) and un-electroporated (*K*) side. *Hes5* expression is upregulated by *Neurog2* (arrowheads in *J*). (*L–Q*) E12.5 → E13.5 electroporations of *Etv1-VP16* (*L–N*) and *Etv1-EnR* (*O–Q*). The electroporated patch is marked by the ectopic expression of *VP16* (*L*) or *EnR* (*O*) and outlined with a red bracket. Expression of *Hes5* on the electroporated (*M* and *P*) and un-electroporated (*N* and *Q*) side. *Etv1-VP16* represses *Hes5* (asterisk, *M*), while *Etv1-EnR* induces the ectopic expression of *Hes5* (arrowheads in *P*). (*R–U*) Transcriptional reporter assays in P19 cells using a *Hes5* 3 kb reporter (*R* and *S*) and *Ascl1* 5 kb reporter (*T* and *U*). ***$P < 0.001$. Bars indicate SEM.

repressed *Hes5* expression (Fig. 8*L–N*), while conversely, *Etv1-EnR* led to the ectopic expression of *Hes5* (Fig. 8*O–Q*). A similar trend was observed in reporter assays in vitro, with *Etv1* ($n = 3$; $P = 0.02$) and *Etv1-VP16* ($n = 3$; $P < 0.0001$) repressing while *Etv1-EnR* ($n = 3$; $P < 0.0001$) activated a *Hes5* 3 kb transcriptional reporter (Fig. 8*S*). Furthermore, we confirmed that *Hes5* was able to repress transactivation of the *Ascl1* reporter in vitro (Fig. 8*T*), while *Etv1* ($n = 3$; $P < 0.0001$)

and *Etv1-VP16* ($n = 3$; $P = 0.03$) activated this reporter (Fig. 8*U*). Unexpectedly, *Etv1-EnR* was also a strong activator of the *Ascl1* reporter activity (2.1-fold increase; $n = 3$; $P < 0.0001$; Fig. 8*U*), despite the strong induction of *Hes5* expression by this construct. Moreover, both *Etv1-VP16* and *Etv1-EnR* were able to induce ectopic *Ascl1* expression in E12.5 → E15.5 electroporations (Supplementary Fig. S4). While *Etv1-EnR's* ability to induce ectopic *Ascl1* expression was unexpected, a previous report has demonstrated that *Hes5* is converted to an activator of *Ascl1* transcription with the onset of neuronal differentiation (Ju et al. 2004).

In summary, we have uncovered a novel repressor cascade in neocortical progenitors, demonstrating that *Neurog2* indirectly suppresses *Etv1* expression, which in turn indirectly represses *Ascl1* transcription, possibly through interactions with Notch signaling and the *Hes* genes (Fig. 9*A*).

Discussion

Neurog2 encodes a proneural bHLH transcription factor that is required in the embryonic neocortex to promote progenitor cell maturation, neuronal fate specification, neuronal differentiation, and migration (Fode et al. 2000; Schuurmans et al. 2004; Hand et al. 2005; Britz et al. 2006; Ge et al. 2006; Heng et al. 2008; Pacary et al. 2011). To control these events, *Neurog2* initiates the expression of several downstream genes and genetic programs, such as *NeuroD1* and a dorsal glutamatergic fate, *Tbr2* and an INP fate, and *Rnd2* to promote radial migration (Huang et al. 2000; Seo et al. 2007; Heng et al. 2008; Ochiai et al. 2009; Pacary et al. 2011). Here, we investigated the regulatory logic underlying *Neurog2*'s ability to simultaneously repress alternative gene expression programs in neocortical lineages, focusing on the repression of *Pax6* and a RGC fate, and *Ascl1* and a ventral GABAergic neuronal identity (Fode et al. 2000; Schuurmans et al. 2004; Britz et al. 2006; Ge et al. 2006). Our studies confirm that *Neurog2* functions as a transcriptional activator to carry out its neocortical functions, including its ability to repress alternative cell fates. Furthermore, we identify *Tbr2*, which is a direct *Neurog2* transcriptional target (Ochiai et al. 2009), as a key effector of *Neurog2*-mediated transcriptional repression. Specifically, we show that *Tbr2* represses both the transcription of *Pax6* and an RGC fate, and *Ebf1* and a ventral neuronal identity. Finally, we identify *Etv1* as a *Neurog2*-repressed gene that can indirectly promote *Ascl1* expression, both in vitro and in vivo. Mechanistically, we suggest that *Etv1* may indirectly promote *Ascl1* expression through its ability to influence *Hes5* transcription, which in turn regulates *Ascl1* (Ju et al. 2004). Our studies highlight the importance of transcriptional repression in controlling cell fate decisions in the neocortex, and provide evidence for a derepression mode of cell fate specification, whereby cortical cells acquire their identities at least in part by repressing alternative cell fates.

Tbr2 is a Downstream Effector in the Neurog2-Mediated Repression of a RGC Identity

The progression from RGC to INP involves the activation and repression of distinct genetic pathways. Here, we found that *Neurog2* functions as a transcriptional activator to promote *Tbr2* transcription in INPs and to repress *Pax6* expression in RGCs, thereby promoting the RGC to INP transition. The ability of *Neurog2-WT* and the activator fusion (*Neurog2-VP16*) to induce *Tbr2* expression was expected as *Tbr2* is a direct transcriptional target of *Neurog2* (Ochiai et al. 2009). In

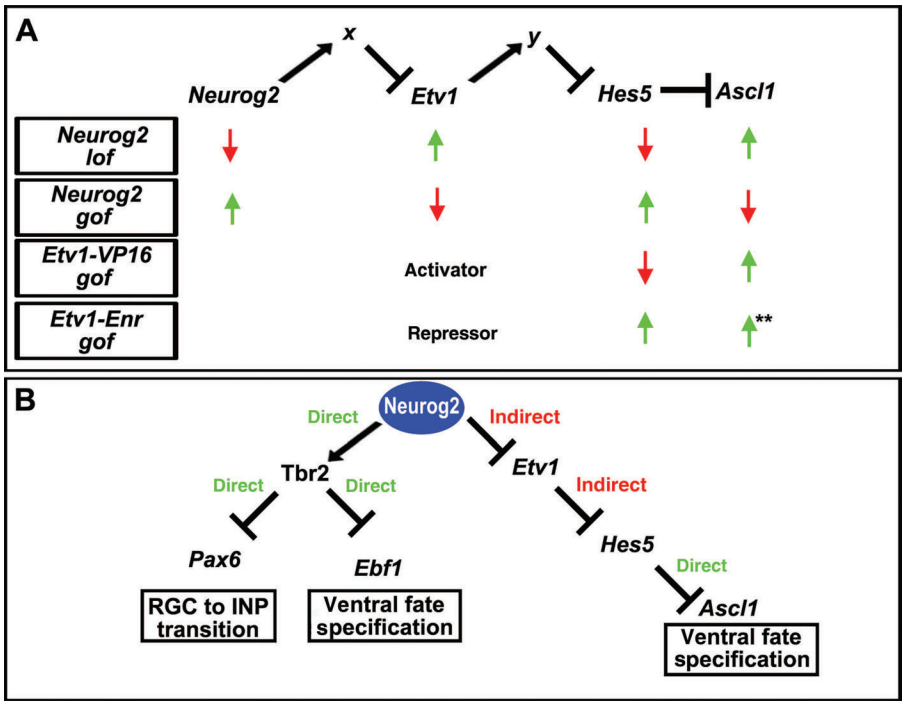

Figure 9. *Neurog2* functions as a transcriptional activator to repress alternative gene expression programs in the neocortex. (*A*) Summary of the *Neurog2* transcriptional cascade that represses *Ascl1*. *Neurog2* represses *Etv1*, which in turn represses *Hes5*; these interactions are indirect, invoking the existence of unknown transcriptional repressors *x* and *y*. The increase in *Ascl1* expression in *Etv1-EnR* electroporations may be due to the sequestration of TLE co-repressor proteins by *Etv1-EnR*, thereby preventing *Hes5* from binding TLE proteins, switching *Hes5* to an activator of *Ascl1* transcription (asterisks). (*B*) *Neurog2* activates several repressor cascades to suppress inappropriate gene expression in the neocortex.

contrast, the mechanism by which *Neurog2* represses *Pax6* expression was previously unknown. We found that *Neurog2* functions through indirect means to repress *Pax6* transcription. The most parsimonious model that fits our data is that *Neurog2* induces the expression of *Tbr2*, which is known to be a direct *Neurog2* transcriptional target (Ochiai et al. 2009), and that *Tbr2* in turn functions as a transcriptional repressor of *Pax6*. Indeed, in our transcriptional reporter assay, *Tbr2* could repress *Pax6* transcription. Furthermore, misexpression of *Tbr2* in cortical progenitors suppresses *Pax6* expression in a cell autonomous fashion (Sessa et al. 2008).

In previous gain-of-function studies, *Tbr2* misexpression was shown to sustain INP proliferation, delaying cell cycle exit and partially blocking the expression of neuronal markers (Sessa et al. 2008). This is consistent with our data, where we found that *Tbr2* did not induce the expression of the cortical neuronal marker *Tbr1* in the ventral telencephalon. Furthermore, we found that misexpression of *Neurog2-VP16*, a strong transcriptional activator, led to a sustained upregulation of *Tbr2* expression after 72 h post-electroporation, which was accompanied by a delay in the onset of *Tbr1* expression. The zinc finger transcriptional repressor *Insm1* is another *Neurog2*-regulated gene that is sufficient to promote an INP fate, but does not induce neuronal differentiation when misexpressed in cortical progenitors (Farkas et al. 2008). *Tbr2* and *Insm1* thus behave very differently than *Neurog2* in gain-of-function assays, as *Neurog2* induces neuronal differentiation and cell cycle exit. If *Neurog2's* sole function is to induce neuronal differentiation, the rationale for inducing the transcription of downstream genes (e.g., *Tbr2*, *Insm1*) that block this process is at first glance contradictory. However, our study supports the idea that *Tbr2* (and possibly *Insm1* by extension) plays an essential role in stabilizing the INP progenitor state, and that they achieve this in part by repressing alternative gene expression programs (e.g., *Pax6* and an RGC identity). This allows for the orderly progression through different progenitor states and stages of differentiation.

Given that *Tbr2* and *Insm1* do not promote neuronal differentiation, additional *Neurog2*-regulated genes must promote the conversion of INPs to differentiated neurons. Possibilities include the *Neurog2*-regulated genes *NeuroD1* and *NeuroD4* (Mattar et al. 2008), which may be required to bias $Tbr2^+$ INPs to undergo differentiative, neurogenic divisions. In such a model, *Neurog2* might rapidly induce the expression of *Tbr2* to suppress an RGC fate and promote an INP identity, followed by the activation of *NeuroD1/D4* (and other genes) to promote neuronal differentiation once the INP fate is stabilized. Consistent with such a sequential model, we previously showed that it takes 72 h for *Neurog2* to induce *Neurod1* expression in the ventral telencephalon (Mattar et al. 2008), whereas in this study, we found that *Neurog2* can induce *Tbr2* transcription in ventral domains within 24 h post-electroporation.

Tbr2 is a Downstream Effector in the Neurog2-Mediated Repression of an $Ebf1^+$ Ventral Neuronal Identity

To promote the differentiation of glutamatergic projection neurons, *Neurog2* must simultaneously activate cortical-specific neuronal genes while suppressing the acquisition of a ventral, GABAergic neuronal fate. A failure to suppress competing ventral developmental programs leads to the misspecification of cortical lineages in several mutants, including *Neurog2*, *Emx2*, and *Pax6* loss-of-function mice (Fode et al. 2000; Muzio et al. 2002; von Frowein et al. 2006; Mangale et al. 2008). Here, we demonstrated that *Neurog2* functions as a transcriptional activator to induce the expression of several cortical markers (e.g., *NeuroD6*, *Sox5*, *Nhlh2*, *Tbr1*, *Bhlhb5*). Furthermore, we showed that *Neurog2* represses a ventral identity through indirect means given that both *Neurog2* and *Neurog2-VP16* can block the expression of *Ascl1* and markers expressed in ventral interneuron lineages (*Ebf1*, *Six3*, *GAD1*). How does *Neurog2* repress ventral marker expression by functioning as an activator? Our study demonstrates that *Neurog2* regulates the expression of several transcriptional regulators that may contribute to the repression of ventral marker expression. Included is *Tbr2*, which we found plays a critical yet specific role in repressing a ventral neuronal identity, suppressing the expression of *Ebf1* and not other genes expressed in ventrally-derived neurons. The specific requirement for *Tbr2* was verified by examining *Tbr2* mutant cortices, demonstrating that *Ebf1* was ectopically expressed. This ectopic expression is unlikely due to the aberrant migration of ventrally-derived, $Ebf1^+$ interneurons into the *Tbr2* mutant neocortex as interneuron migration is reduced rather than elevated in *Tbr2* mutants (Sessa et al. 2010).

Ascl1 is upregulated in *Neurog2* mutant cortices, where it is an essential component of the pathways underlying the ventral misspecification of cortical lineages (Fode et al. 2000; Schuurmans et al. 2004). Interestingly, misexpression of *Neurog2-EnR* in the cortex, which blocks *Neurog2* function, similarly promotes the ectopic expression of *Ascl1* and not other ventral markers, such as *Dlx* and *GAD1*. This is reminiscent of *Sox6* mutant cortices, in which *Ascl1* is upregulated, while downstream genes such as *Dlx1* and *GAD1* are not (Azim et al. 2009). One possibility is that the ectopic expression of *Ascl1*, which is required and sufficient to promote the expression of ventral differentiation programs (Casarosa et al. 1999; Britz et al. 2006), does not reach sufficiently high levels in *Neurog2-EnR* transfections (or in *Sox6* mutants). Alternatively, in our E12.5 cortical transfections, *Ascl1* may not be induced by *Neurog2-EnR* sufficiently early, as the competence window for cortical progenitors to be respecified in response to *Ascl1* is short, with only early-born neocortical neurons (i.e., <E14.5) responding to ectopic *Ascl1* in transient (i.e., in utero electroporation) and chronic ($Neurog2^{KIAscl1}$ allele) gain-of-function experiments (Parras et al. 2002; Britz et al. 2006).

Using a candidate approach, we identified *Tbr2* and *Sox5* as 2 potential transcriptional repressors that were downregulated in *Neurog2* mutant cortices, and could thus be involved in suppressing a ventral telencephalic identity in cortical neurons. Surprisingly, however, *Tbr2* was not able to suppress the expression of *Ascl1*, either in our transcriptional assay in vitro, or in cortical progenitors in vivo, suggesting that other *Neurog2*-regulated genes carry out this function. Furthermore, *Sox5* was not able to repress our *Ascl1* transcriptional reporter, and while this is an artificial system, both *Sox5* and *Ascl1* are upregulated in *Sox6* mutant cortices (Azim et al. 2009), making it unlikely that *Sox5* represses *Ascl1* directly, at least in the absence of *Sox6*. Interestingly, *Sox6* was upregulated in *Neurog2* mutant cortices, along with *Pax6* and *Etv1*, but of these factors, only *Etv1* was able to transactivate

the *Ascl1* reporter. In an effort to better understand how *Etv1* regulates *Ascl1* transcription, we examined potential indirect interactions with *Hes5*. Our data suggest that *Etv1* may contribute to the repression of *Ascl1* within cortical progenitors by indirectly downregulating *Hes5*, which is itself a direct transcriptional repressor of *Ascl1*, at least in dividing neural progenitors (Ju et al. 2004). Thus, in a *Neurog2* mutant cortex in which *Etv1* is ectopically expressed, *Hes5* expression is reduced, thereby reducing the transcriptional repression of *Ascl1*. While our data support a model, whereby *Etv1* regulates *Hes5* transcript levels, we cannot rule out the possibility that *Neurog2* regulates *Hes5* expression more directly, through its ability to initiate transcription of the *Dll1* Notch ligand. However, there are precedents for other transcription factors directly regulating *Hes5* gene expression independent of Notch signaling, most notably the zinc finger transcription factors *Fezf1* and *Fezf2* (Nieto et al. 2001).

In other developmental systems such as the spinal cord, transcriptional repressors specify cell fates by repressing the expression of other transcriptional repressors. This implies that cells acquire their identities by repressing alternative cell fates—a paradigm that has been termed the derepression mode of cell fate specification (Muhr et al. 2001; Bylund et al. 2003). In this model, inappropriate cell fates are inhibited by transcriptional repressors. Here, we found some parallels in the neocortex, demonstrating that *Neurog2* functions as a transcriptional activator to ensure that alternative differentiation pathways (i.e., ventral programs) are not derepressed, and a cortical identity is specified.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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Notes

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