Supplemental Material

Interaction and Antagonistic Roles of NF-kappaB and Hes6 in the Regulation of Cortical Neurogenesis

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SUPPLEMENTAL FIGURES AND LEGENDS

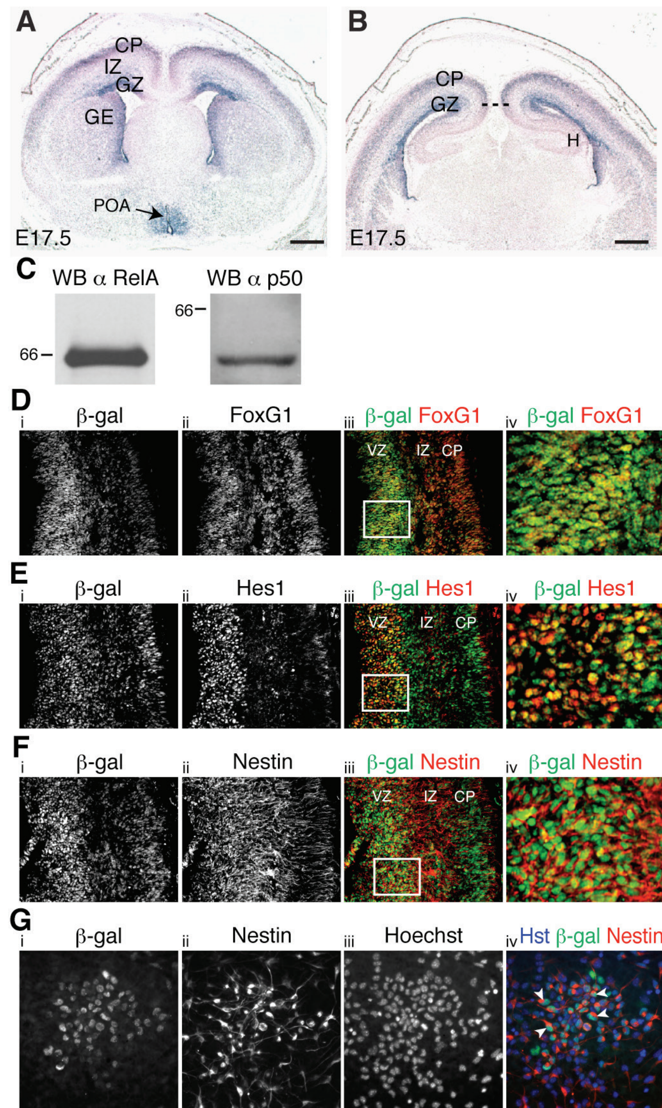

FIG S1 Activation of NF-κB signaling in neocortical neural progenitor cells. (A and B) Coronal views of the distribution of β-gal activity (blue staining) in the telencephalon of E17.5 NF-κB^{LacZ} embryos at two successive rostrocaudal levels. β-gal expression is robust in the pallial germinal zone (GZ), decreased in the intermediate zone (IZ), and again robust in the cortical plate (CP). Dotted line in (B) roughly demarcates the dorsal

boundary of the β-gal^{+} territory in the pallium. Dorsal is up and ventral down. Other abbreviations: GE, ganglionic eminence; H, hippocampus; POA, preoptic area. Scale bars: 500 μm. (C) Expression of endogenous RelA and p50 in dissected telencephalon from E13.5 mouse embryos, as detected by Western blotting. (D-F) Double-label immunofluorescence analysis of β-gal and either FoxG1 (D), Hes1 (E), or Nestin (F) expression in the pallium of E14.5 NF-κB^{LacZ} embryos. β-gal is expressed in neural progenitor cells located in the neocortical VZ. Panels iv represent high-magnification views of the boxed areas in panels iii. Dorsal is up and lateral is right in all panels. Abbreviations: VZ, ventricular zone. (G) Double-label immunofluorescence analysis of primary cultures of cortical neural progenitor cells obtained from mixed E13.5 wildtype and NF-κB^{LacZ} embryos, showing that most β-gal^{+} cells coexpress Nestin *in vitro*. Arrowheads point to examples of double-labeled cells.

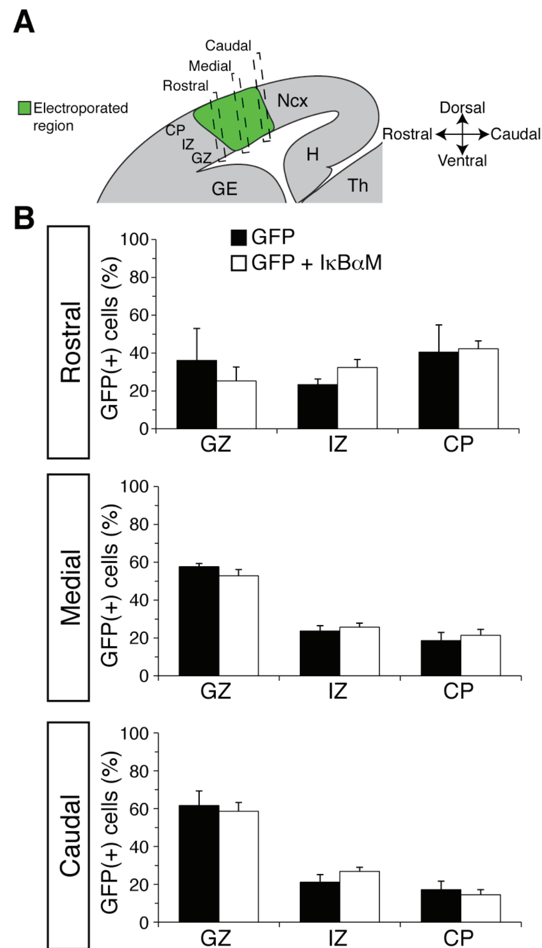

FIG S2 Distribution of cells electroporated with GFP alone or together with IκBαM in the developing neocortex. (A) Cartoon depicting a sagittal view of an electroporated forebrain. The region depicted in green represents the electroporated area; this area was operationally divided into rostral, medial, and caudal levels, as indicated. Abbreviations: CP, cortical plate; GE, ganglionic eminence; GZ, germinative zone; H, hypothalamus; IZ, intermediate zone; Ncx, neocortex; Th, thalamus. (B) Quantification of the numbers of of GFP^+ cells in the germinal zone (GZ), intermediate zone (IZ), or cortical plate (CP) at three different rostro-caudal levels of brains electroporated with plasmids expressing GFP

alone or together with IκBαM. Data are shown as the mean ± SEM (n=5 electroporated embryos per condition, no statistically significant difference, *t*-Test).

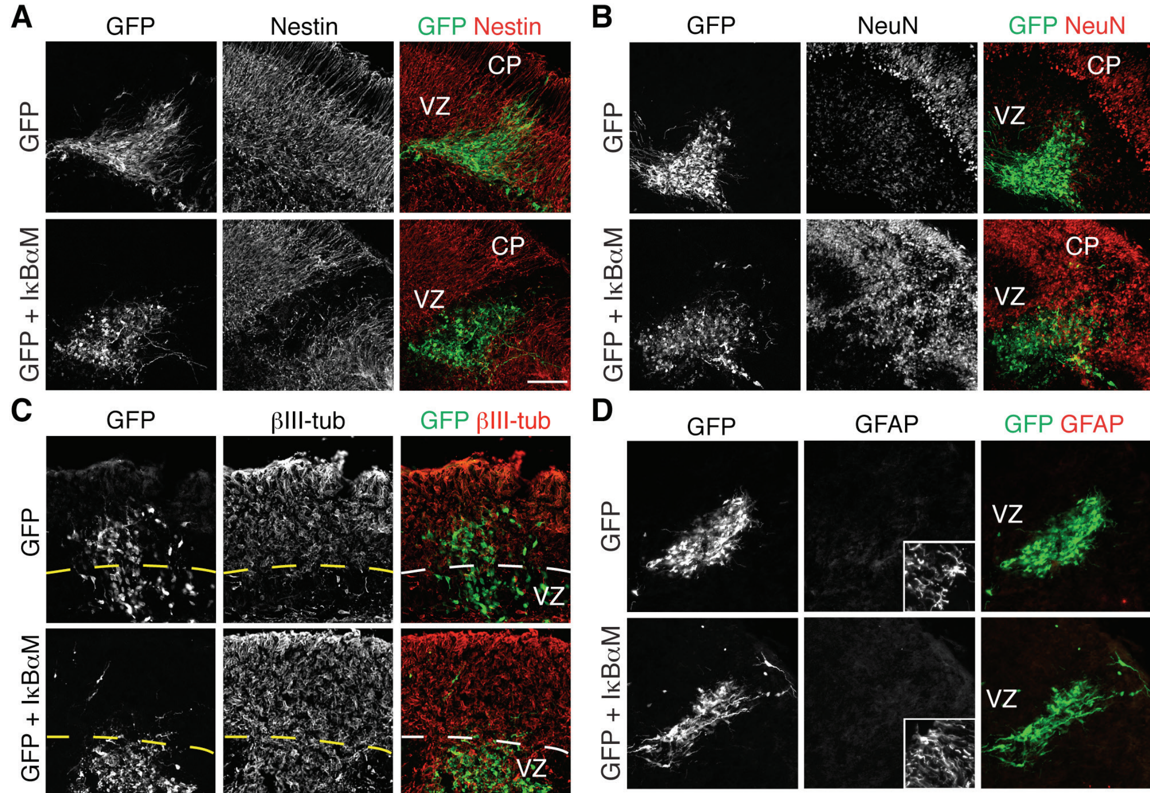

FIG S3 NF-κB signaling is important to prevent premature neuronal differentiation in the embryonic neocortex *ex vivo*. (A-D) Double-label immunofluorescence analysis of GFP and either Nestin (A), NeuN (B), βIII-tubulin (βIII-tub) (C), or GFAP (D) expression in organotypic slice cultures from E13.5 mouse forebrain, 48 hr after electroporation of plasmids encoding the indicated proteins. (D) Insets show presence of GFAP-expressing cells in other regions of the brain, demonstrating the application and efficacy of the anti-GFAP antibody. Abbreviations: CP: cortical plate; VZ: ventricular zone. Scale bar: 100 μm.

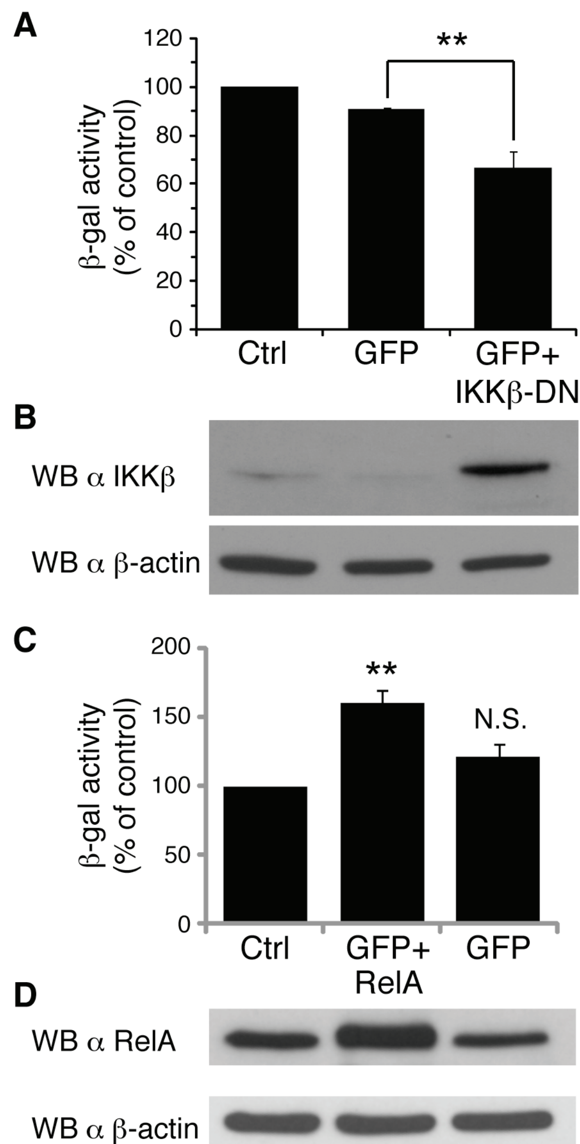

FIG S4 (A and B) IKKβ-DN-mediated inhibition of NF-κB signaling in cortical progenitor cells. (A) Quantification of β-gal activity in primary cultures of cortical progenitor cells from E13.5 NF-κB^{LacZ} embryos. Cells were either not infected ("Ctrl"; considered as 100%) or infected with adenovirus expressing GFP alone or GFP+IKKβ-DN, as indicated. Determination of β-gal activity was performed 48 h after trasnduction. Data are shown as the mean ± SEM (**, $p < 0.01$, n=3 separate experiments performed in

duplicates, *t*-Test). (B) Expression of IKKβ-DN in the transduced cells was detected by Western blotting with an anti-IKKβ antibody. (C and D) RelA-mediated activation of NF-κB signaling in cortical progenitor cells. (C) Quantification of β-gal activity in primary cultures of cortical progenitor cells from E13.5 NF-κB^{LacZ} embryos. Cells were either not infected ("Ctrl"; considered as 100%) or infected with adenovirus expressing GFP+RelA or GFP alone, as indicated. Determination of β-gal activity was performed 24 h after transduction. Data are shown as the mean ± SEM (**, p <0.01, n=4 separate experiments performed in duplicates, *t*-Test). Increased expression of RelA in the transduced cells was detected by Western blotting with an anti-RelA antibody.

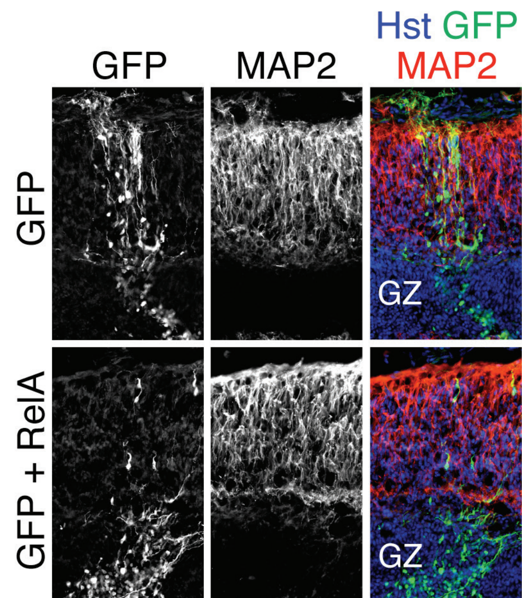

FIG S5 Double-label immunofluorescence analysis of GFP and MAP2 expression in organotypic slice cultures from E13.5 mouse forebrain, 96 h after electroporation of plasmids encoding GFP or GFP+RelA. Abbreviations: GZ, germinative zone; Hst, Hoechst.

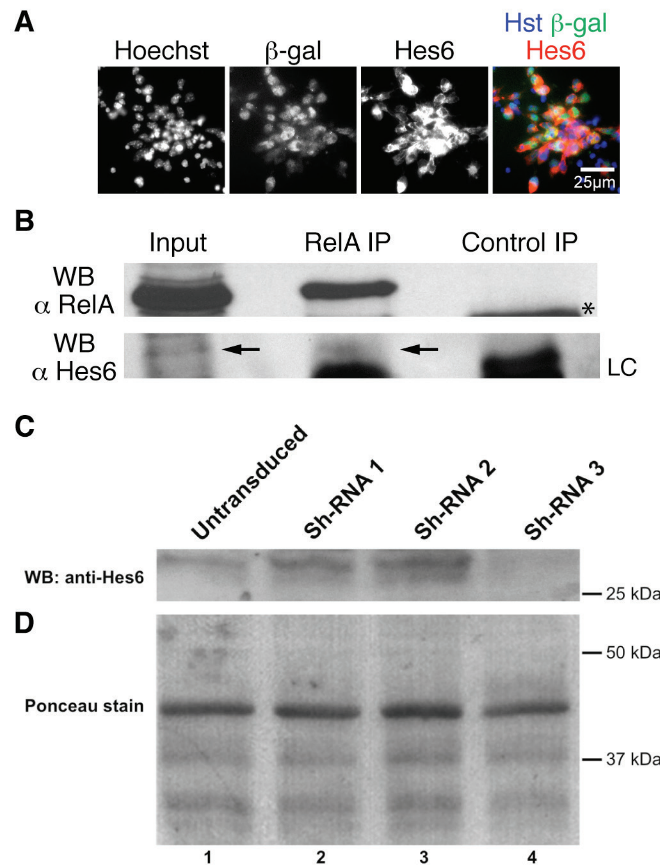

FIG S6 Expression of Hes6 in cortical progenitor cells in which NF-κB is activated. (A) Double-label immunofluorescence analysis of β-gal and Hes6 expression in primary cortical neural progenitor cells from E13.5 NF-κB^{LacZ} embryos. Hst, Hoechst. Scale bar: 25 µm. (B) Nuclear extracts from dissected cortices from E14.5 CD1 mouse embryos were subjected to immunoprecipitation (IP) with anti-RelA or control antibodies. Immunoprecipitates were analyzed with each input lysate by Western blotting (WB) with anti-RelA (top panel) or anti-Hes6 (bottom panel) antibodies. Arrow points to position of Hes6 immunoreactive band. LC, immunoglobulin light chain. Asterisk in lane 3 in top panel indicates a non-specific band. (C and D) Primary cultures of cortical neural

progenitor cells were established from E13.5 CD1 mouse embryos. After 3 days *in vitro*, cells were transduced with lentiviral particles expressing three different shRNA sequences under the control of the human H1 promoter (GenTarget Inc.), followed by culture for an additional period of 48 hours and preparation of cell lysates. (C) Lysates were subjected to Western blotting using rabbit anti-Hes6 antibody (1/1,000, Novus Biologicals). shRNA directed against mouse Hes6 ("shRNA 3"; GenTarget Inc.; sequence, TGCAGGTCCCTAGCACTATTT) caused a significant decrease in the ~28-kDa band recognized by the anti-Hes6 antibody (lane 4). No changes were observed when shRNA reagents 1 and 2 were used (lanes 2 and 3). (D) Ponceau S staining prior to Western blotting was used to compare protein loading in the different lanes. Molecular size markers are indicated.