Sip1 regulates sequential fate decisions through feedback signaling from postmitotic neurons to progenitor cells

Seuntjens E 1,2,*, Nityanandam A 3,*, Miquelajauregui A 3, Debruyn J 1,2, Stryjewska A 1,2, Goebels S 3, Nave KA 3, Huylebroeck D 1,2,#, Tarabykin V 3,4#

Supplementary Figure 1. Sip1 protein is largely ablated in the neocortex of Sip1|Emx1 and Sip1|NEX mutant mouse brains. (a-c) Immunostaining of control (a), Sip1|Emx (b) and Sip1|NEX (c) cortices, shown here at E15.5, confirms the absence of detectable levels of Sip1 in most cells. However, there is a small subpopulation of cells that continue to synthesize Sip1, including what appear to be interneurons that tangentially migrate from the ganglionic eminences into the neocortex as found also by additional Sip1 staining and in situ hybridization data in the forebrain of wild-type mice (data not shown). (d-g) In order to verify that the activity of Cre recombinase (expression driven by NEX promoter) is localized mostly to postmitotic neurons, NEX-Cre expressing E14.5 cortices were double immunostained for Cre (e) and the neuronal marker HuC/D (f). As can be seen, almost all Cre-positive cells also synthesize HuC/D (g).
Supplementary Figure 2. Cell cycle length is not significantly altered in the Sip1 mutant neocortex. (a) To estimate cell cycle length, we labeled cycling progenitors with IddU/BrdU (based on the method described by Martynoga et al, 2005). A pregnant mouse is injected with IddU at t = 0h to label S-phase cells. At t = 1.5h, the same mouse is injected with BrdU and at t = 2h, the mouse is sacrificed. This way, cells that are in the S-phase by t = 2h will be labelled with both BrdU and IddU (S\textsubscript{cells} - yellow), but cells that left the cell cycle in this 1.5h interval (T\textsubscript{i}) will be labelled only with IddU (L\textsubscript{cells}, red). (b) The cell cycle length is approx. 12 hrs at E12.5 in both control and Sip1\|Nestin cortices. Histograms show mean T\textsubscript{c} ± sem; t-test: P = 0.988. (c,d) IddU/BrdU immunohistochemistry of E12.5 control and Sip1\|Nestin mutant neocortex. Antibodies specific for both BrdU and IddU (red) and specifically for BrdU (green) were used. All cells are labeled with DAPI (blue). Cells were counted in 100µm-wide counting bins (white frames), and are shown here as different coloured dots (c). S\textsubscript{cells} or S-phase cells: yellow and marked with yellow dots in c; L\textsubscript{cells} or leaving fraction: red, marked with red dots in c; P\textsubscript{cells} or proliferating cells or total number of cells in the ventricular zone: yellow + red + blue. (e,f) A single administration of BrdU at E12.5 was followed by analysis of the neocortex after 20 hours for co-labeling of BrdU (red) and the cell cycle marker Ki67 (green). (g) We calculated the number of cells exiting the cell cycle during this 20-hour period between E12.5-13.5 (named Quitting Fraction Q = number of BrdU+ Ki67- cells/ total BrdU+ cells) between control (e) and mutant (f). If this Q-value is significantly higher in mutant mice (Q\textsubscript{ko}), this would imply that more cells leave the cell cycle within the 20-hour period, indicating premature differentiation. We analysed two regions R1 and R2, and found no statistically significant difference in Q (R1: P=0.08, R2: P=0.63, error bars indicate s.e.m.), although at this developmental stage more layer 2-5 neurons are generated in the mutant (see text).
Supplementary Figure 3. Increased non-ventricular proliferation of astrocytic precursors in the Sip1 mutant neocortex is preceded by a reduction in Tbr2 cells in the SVZ. (a-c) The extent of basal proliferation at E16.5 was assessed by immunostaining for Tbr2 (a,b): fewer (reduced by 37%) Tbr2-positive cells were detected in the SVZ in Sip1|Nex neocortex in comparison to the control (c), although the overall level of proliferation seemed to be unaffected. *P = 0.0043. Error bars indicate s.e.m. (d) The distribution of proliferating cells over the neocortex at E17.5 in Sip1|Emx1 is quantified. For this purpose, the entire radial unit from the ventricular to the pial surface in the region of the cingulate neocortex was divided into five equal-sized bins numbered 1 to 5 (shown in the y-axis). As can be seen, while in the control most dividing cells are located close to the ventricle, in Sip1|Emx1 they are dispersed over the SVZ and the IZ. bin2 **P = 0.0098, bin3 **P = 0.005, bin4 **P = 0.0019. (e,f) Co-localization of proliferation marker Ki67 and radial glial marker Blbp (differentiating astrocytic precursors) at E17.5 in control (e) and Sip1|NEX (f) cortices. Dividing cells (Ki67-positive) are not only dispersed over the IZ/CP in the mutant, but are also Blbp-positive, as can be seen clearly in the overlaid images (arrows).
Supplementary Figure 4. NT3 and Fgf9 mRNA expression in the neocortex.

(a-f) *in situ* hybridizations for NT3 at E13.5 (a,b) and E15.5 (c,d), and for Fgf9 at E17.5 (e,f) show that their mRNA expression remains low in the control neocortex (arrows). Overexpression of NT3 (mRNA) can already be detected in Sip1|Nestin neocortex at E13.5 (double arrows), and continues to be seen at E15.5 (d). Precocious upregulation of Fgf9 expression (mRNA) in Sip1|Nestin cortices at E17.5 can also be seen (f).
Supplementary Figure 5. Increased MAPK-p42/44 signaling in ventricular zone precursors in the absence of Sip1. (a-c) MAPK p42/44 activity was visualized by immunostaining of E14.5 brain sections of control and mutant (Sip1|Nestin or Sip1|NEX) animals with anti-phospho-p42/44 MAPK antibody. The hem (H) and antihem (AH) regions contain many cells with activated MAPK signaling. In the mutants, MAPK signaling increased massively in the cortical VZ, as indicated by the medial shift of the arrow compared to the control. (d-f) Anti-phospho-p38 MAPK antibody shows weak activity in the cortical hem and in the subplate/intermediate zone; this pattern was unchanged in the mutant neocortex at E14.5. (g-i) Similarly, no phosphorylated CREB could be found in the ventricular zone of either the control or the mutant neocortex at E14.5.
Supplementary Figure 6. Model for the role of Sip1 in neocortical cell fate decisions

(a) In the control neocortex, postmitotic neurons secrete NT3. The production of NT3 is controlled by Sip1, which keeps it at low levels during early development (E12.5). When enough postmitotic neurons are generated (E14.5), the NT3 levels cross a threshold, signal back to the progenitors, block production of deep layer neurons and stimulate production of upper layer neurons. Whether NT3 acts alone to induce this switch is not known (question mark). At E17.5, a similar mechanism induces the neurogenesis-gliogenesis switch. At this stage, Sip1 controls the level of Fgf9 production, which reaches its threshold only when neurogenesis is completed and Sip1 expression is downregulated. Fgf9 then induces proliferation of glial precursors. The dynamics of production of different layer-specific neurons followed by astrocytes during the course of neocorticogenesis is reflected by bell-shaped curves. 

(b) In the Sip1 mutant neocortex, NT3 and Fgf9 expression is no longer effectively repressed, such that these two secreted factors reach threshold levels earlier. This in turn induces precocious generation of upper layer neurons during early neocorticogenesis and glial precursors during later development. This temporal shift in the sequence of generation of neocortical cell types is illustrated once again through bell-shaped curves.