Directed Migration of Cortical Interneurons Depends on the Cell-Autonomous Action of Sip1

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SUMMARY

GABAergic interneurons mainly originate in the medial ganglionic eminence (MGE) of the embryonic ventral telencephalon (VT) and migrate tangentially to the cortex, guided by membrane-bound and secreted factors. We found that Sip1 (Zfhx1b, Zeb2), a transcription factor enriched in migrating cortical interneurons, is required for their proper differentiation and correct guidance. The majority of Sip1 knockout interneurons fail to migrate to the neocortex and stall in the VT. RNA sequencing reveals that Sip1 knockout interneurons do not acquire a fully mature cortical interneuron identity and contain increased levels of the repulsive receptor Unc5b. Focal electroporation of Unc5b-encoding vectors in the MGE of wild-type brain slices disturbs migration to the neocortex, whereas reducing Unc5b levels in Sip1 knockout slices and brains rescues the migration defect. Our results reveal that Sip1, through tuning of Unc5b levels, is essential for cortical interneuron guidance.

INTRODUCTION

The mammalian telencephalon is critical to higher brain functions such as processing of sensory and motor input, learning, and memory. This higher-order information processing relies on both excitatory projection neurons and inhibitory γ-aminobutyric acid (GABAergic) interneurons, which are essential to modulate the electrical activity of the projection neurons onto which they synapse. In the cortex, interneurons comprise a minority (20%-30%) of neurons compared to excitatory neurons, but they display a remarkable diversity and can be classified based on morphological, physiological, molecular, and synaptic features (Markram et al., 2004; Ascoli et al., 2008). In mice, cortical interneurons originate in the medial and caudal ganglionic eminences (MGE and CGE) and preoptic area (POA) (Fogarty et al., 2007; Miyoshi et al., 2007, 2010; Gelman et al., 2009; Rubin et al., 2010). Besides cortical interneurons, the MGE also generates interneurons destined for the striatum and hippocampus, and oligodendrocytes and projection neurons for the globus pallidus, amygdala, and septum (Kessaris et al., 2006; Xu et al., 2008). The specification, migration, and integration of cortical interneurons are complex but precisely orchestrated processes, and disturbances in interneuron development and function have been linked to various neurodevelopmental disorders (Levitt et al., 2004).

Once specified in the ganglionic eminences, interneurons migrate to different telencephalic structures, including the neocortex. For this, they need to interpret guidance information supplied by a range of cues in the surrounding ventral telencephalon (VT). Cortical interneurons express the receptor EphA4 and are repulsed by ephrinA5 in the ventricular zone (VZ) of the MGE and by ephrinA3 in the stratum (Zimmer et al., 2007; Rudolph et al., 2010). Neuropilin receptors (Nrp1, Nrp2) prevent cortical interneurons from entering the stratum, which produces the
repulsive Sema3a and Sema3f ligands (Marín et al., 2001). Deletion of Robo1 results in an increased influx of interneurons in the striatum and cortex (Andrews et al., 2006; Andrews et al., 2008). Robo1 interacts with Nrp1 and modulates semaphorin-neuropilin/plexin signaling to direct cortical interneurons around the striatum (Hernández-Miranda et al., 2011). Different isoforms of neuregulin-1 act as short- and long-range attractants for migrating cortical interneurons, which express the receptor gene ErbB4 (Flames et al., 2004). Stromal-derived factor-1 (SDF1, Cxcl12) and its receptors Cxcr4 and Cxcr7 are implicated in chemotaxis and positioning of interneurons in the cortex (Stumm et al., 2003; Li et al., 2008; López-Bendito et al., 2008; Sánchez-Alcaino et al., 2011; Wang et al., 2011).

Transcription factors are ideal candidate proteins to specify but also sort the different types of interneuron through controlling the synthesis of such guidance cues and receptors (for recent reviews, see Chédotal and Rijli, 2009; Corbin and Butt, 2011). Persistent expression of Nkx2-1 allows a subset of MGE-derived interneurons to downregulate Nrp2 and migrate into the striatum, whereas interneurons destined to the cortex downregulate Nkx2-1, maintain high Nrp2 levels, and avoid the striatum (Nóbrega-Pereira et al., 2008). However, evidence for a functional link between other transcription factors and guidance cues for migrating interneurons remains limited.

Sip1 (also named Zeb2, Zfhx1b) is a transcription factor implicated in embryonic development and in epithelial-to-mesenchymal transition (EMT) (for a recent review, see Conidi et al., 2011). Sip1 contains two clusters of zinc fingers that mediate binding to two spaced E-box sequences in regulatory regions of its target genes. Furthermore, it has domains that bind activated Smads, CtBP-1/2, and the chromatin-remodelling corepressor complex NuRD, respectively (Verschueren et al., 1999; van Grunsven et al., 2007; Verstappen et al., 2008). In humans, mutations in one ZFHXB1 allele cause Mowat-Wilson syndrome (MWS), characterized by severe intellectual disability and typical facial features, and many patients present with seizures, corpus callosum agenesis, Hirschsprung disease, and congenital heart disease (Zweier et al., 2002; Garavelli and Mainardi, 2007).

Using various conditional knockout (KO) mice, we showed that Sip1 regulates, in a non-cell-autonomous manner, hippocampal development and the timing of cortical neurogenesis and gliogenesis (Miquelajauregui et al., 2007; Seuntjens et al., 2009). Here, using both loss- and gain-of-function approaches in vivo and ex vivo, we show that Sip1 is essential for cortical interneuron migration. Deletion of Sip1 leads to a severe reduction in the number of interneurons in the embryonic and postnatal cortex. Sip1 KO MGE-derived interneurons do not acquire a fully mature cortical interneuron identity, and contain increased levels of the guidance receptor Unc5b. Overexpression of Unc5b in wild-type (WT) MGE largely abrogates interneuron migration to the cortex, while reduction of Unc5b levels in Sip1 mutant interneurons in vitro or in vivo rescues their migration defect. Thus, we discovered a role for Sip1 as a critical transcription factor regulating Unc5b mRNA levels during cortical interneuron migration.

RESULTS

Sip1 Is Abundantly Present in Migrating Cortical Interneurons

We documented the presence of Sip1 in the mouse VT by immunohistochemistry at embryonic day (E) 14.5 (Figure 1; Figure S1 available online). Sip1 is present at low levels in the VZ of the MGE and at higher levels in the mantle zone (Figures S1A, S1A', S1B, and S1B'). In the LGE, we detected Sip1 in a sickle-shape pattern in the subventricular zone (SVZ), and in scattered Sip1-positive (+) cells in the mantle zone (Figures S1A, S1A', S1B, and S1B'). Sip1 was also present in the CGE (Figures S1C and S1C'). Furthermore, Sip1+ cells were found across the pallial-subpallial boundary (PSB) and in the SVZ/intermediate zone (IZ) and cortical plate (asterisk in Figure S1A).
To assess whether these cells were MGE-derived interneurons destined to the cortex, we traced them by crossing the Nkx2-1-Cre mouse line (Kessaris et al., 2006) with RCEfl/fl (i.e., R26R\textsuperscript{CAG-rtTA-STOP-rtTA-EGFP}) reporter mice (Sousa et al., 2009). As shown previously (Kessaris et al., 2006; Fogarty et al., 2007), cells derived from the POA and MGE, except those from its most dorsal part, were labeled. We monitored green fluorescent protein (GFP) and Sip1 in the E14.5 telencephalon (Figure 1) and confirmed that Sip1 was present at low levels in the VZ of the MGE and at increasingly higher levels in the mantle zone (Figure 1A). Most GFP+ cells migrating through the LGE contained Sip1 (Figures 1A and 1B). The majority of MGE-derived cells crossing the PSB were Sip1+ (Figure 1C) and they maintained high Sip1 levels while migrating through the neocortex (Figure 1D), demonstrating that Sip1 is present in MGE-derived migrating interneurons. Sip1+ but GFP− cells were also observed in the cortical SVZ/IZ (Figure 1C), presumably representing CGE-derived interneurons.

We investigated the role of Sip1 in interneurons by removing Sip1 using the Nestin-Cre mouse line (Tronche et al., 1999), which inactivates Sip1 in the entire embryonic CNS and the Nkx2-1-Cre line (for details on the crossing schemes, see Supplemental Experimental Procedures). We refer to Nestin-Cre;Sip1fl/KO as “Sip1[Nestin] (KO) mice and to the Nestin-Cre;Sip1fl/WT control mice as “WT[Nestin]” mice; a similar convention is used for crosses with other Cre lines. We confirmed Sip1 removal from the entire telencephalon in Sip1[Nestin] mice (Figures S1E and S1E'), and in the POA/MGE (except its most dorsal portion) in Sip1[Nkx2-1 mutants (Figures S1F and S1F') when compared to control mice at E12.5 (Figures S1D and S1D'). At E12.5, when the first MGE-derived interneurons reach the dorsal telencephalon in control embryos (Figures 2A and 2A'), none or only few GABAergic interneurons (marked by Gad67) migrated in the cortical anlage in control embryos (arrowhead) (magnification in A'). None or only few Gad67+ interneurons are detected in the cortex of Sip1[Nestin] (B and B') and Sip1[Nkx2-1 (C and C') mutants. (D–L) In E16.5 control embryos, GABAergic interneurons are spread throughout the cortical plate (D and magnification in H; G is a schematic representation of sections shown in D–F and indicates the area of the magnifications in H–L). In the Sip1[Nestin] mutant, almost no interneurons are detected in the cortex at E16.5 (E, I). Sip1 deletion in the MGE (Nkx2-1–Cre, F and K) or in the CGE, LGE, and a portion of the MGE (Gsh2-Cre, L) mainly reduces the number of interneurons in the cortical MZ and IZ. In the Sip1[Dlx5/6 mutant, only few interneurons are found in the cortex (J). (M, M', N, and N') The RCE\textsuperscript{ff} reporter mouse line shows that the majority of the Sip1 mutant Nkx2-1-Cre-derived cells (M and M') do not migrate to the cortex when compared to a WT control (N and N'). Scale bar in (C') corresponds to 50 μm (A′–C′), and scale bar in (L) and (N') to 100 μm (H–L, M′, N′).

The Absence of Sip1 Hampers Migration of GABAergic Interneurons to the Cortex without Affecting Early Regionalization of the Ventral Telencephalon

We investigated the role of Sip1 in interneurons by removing Sip1 using the Nestin-Cre mouse line (Tronche et al., 1999), which inactivates Sip1 in the entire embryonic CNS and the Nkx2-1-Cre line (for details on the crossing schemes, see Supplemental Experimental Procedures). We refer to Nestin-Cre;Sip1fl/KO as “Sip1[Nestin] (KO) mice and to the Nestin-Cre;Sip1fl/WT control mice as “WT[Nestin]” mice; a similar convention is used for crosses with other Cre lines. We confirmed Sip1 removal from the entire telencephalon in Sip1[Nestin] mice (Figures S1E and S1E'), and in the POA/MGE (except its most dorsal portion) in Sip1[Nkx2-1 mutants (Figures S1F and S1F') when compared to control mice at E12.5 (Figures S1D and S1D'). At E12.5, when the first MGE-derived interneurons reach the dorsal telencephalon in control embryos (Figures 2A and 2A'), none or only few GABAergic interneurons (marked by Gad67) migrated in the cortical anlage in control embryos (arrowhead) (magnification in A'). None or only few Gad67+ interneurons are detected in the cortex of Sip1[Nestin] and Sip1[Nkx2-1] mice (Figures 2B, 2B', 2C, and 2C'). At E16.5, hardly any Gad67+ cells were found in the Sip1[Nestin] cortex (Figures 2E and 2I; control in Figures 2D and 2H), while several were still present in the Sip1[Nkx2-1] cortex (Figures 2F and 2K), although lower in number than in controls. Those Gad67+ cells in the...
Sip1 in Cortical Interneuron Migration

Sip1/Nkx2-1 mutant cortex might have originated from the untargeted CGE. Lineage tracing with the RCE\textsuperscript{fl/fl} reporter in the Nkx2-1 model confirmed that the migration of MGE-derived cells to the cortex was severely compromised in the absence of Sip1 (Figures 2M, 2M', 2N, and 2N').

We substantiated these observations using two additional Cre mouse lines: Dlx5/6-Cre, which targets the entire VT, except its VZ (Stenman et al., 2003), and Gsh2-Cre, which produces Cre in the LGE, CGE, and a portion of the MGE (Kessaris et al., 2006). Both Sip1\textsuperscript{Dlx5/6} and Sip1\textsuperscript{Gsh2} brains had reduced numbers of Gad67\textsuperscript{+} cells in the cortex, comparable to those found in Sip1\textsuperscript{Nestin} and Sip1\textsuperscript{Nkx2-1} mutants, respectively (Figures 2J and 2K). In contrast to Sip1\textsuperscript{Dlx5/6} and Sip1\textsuperscript{Nestin} mice, which die at birth, Sip1\textsuperscript{Nkx2-1} and Sip1\textsuperscript{Gsh2} mice are viable. Interestingly, in the latter animals, we occasionally observed myoclonic seizures during the third postnatal week.

Next, we assessed whether the lack of Sip1 modified the expression of acknowledged MGE/VT markers. We performed in situ hybridization for Mash1, Dlx2, Nkx2-1, and Lhx6, and immunohistochemistry for Gsh2 in E12.5 control and KO sections. At this stage, all markers were still present in their appropriate domains in Sip1\textsuperscript{Nestin} and Sip1\textsuperscript{Nkx2-1} embryos (Figures 3A–3E), suggesting that deletion of Sip1 did not affect early regionalization of the VT.

Defective Migration of Sip1-Deleted Cortical Interneurons Is Due to Removal of Cell-Autonomous Effects of Sip1

In the cortex, Sip1 controls a non-cell-autonomous feedback mechanism that emanates from Sip1+ postmitotic neurons to progenitor cells, thereby timing neurogenesis and gliogenesis (Seuntjens et al., 2009). We therefore investigated whether the action of Sip1 in the MGE would be cell autonomous or not. We performed focal electroporations (Figure 4A) with Cre plasmid pCIG-Cre in the MGE of E13.5 Sip1\textsuperscript{fl/fl} organotypic brain slices (Figures 4D and 4D'). After 3 days in vitro (DIV), we counted the total number of GFP\textsuperscript{+} cells in the slice and calculated the percentage of GFP\textsuperscript{+} neurons that reached the cortex. As controls, we electroporated pCIG-Cre in WT slices or a GFP-encoding plasmid (pCIG) in Sip1\textsuperscript{fl/fl} slices (Figures 4B, 4B', 4C, and 4C'). In both controls, targeted GFP\textsuperscript{+} cells migrated to the cortex (58.3% for pCIG in Sip1\textsuperscript{fl/fl} slices, n = 11; 56.8% for pCIG-Cre in WT slices, n = 13 slices; quantification in E). By contrast, only 14% of the Sip1-deleted cells (pCIG-Cre in Sip1\textsuperscript{fl/fl} slices, n = 19 slices, p < 0.001; two independent experiments) migrated to the cortex (Figures 4D and 4E).

The failure of the neighboring Sip1\textsuperscript{+} cells to rescue the defective tangential migration of Sip1 mutant interneurons

Figure 3. Sip1 Deletion Does Not Influence Early Regionalization of the Ventral Telencephalon

(A and A') The expression domain of Mash1, an important regulator of neurogenesis, is unchanged upon Sip1 deletion.
Sip1-Deficient Interneurons Are Intrinsically Able to Migrate

We next investigated whether Sip1-deficient cells had the intrinsic capacity to migrate. We cultured MGE explants from WT;+ and Sip1;RCE in Matrigel (Figure S2A) according to (Wichterle et al., 1999) and measured the maximum migration distance of the cells from the explants after 1 and 2 DIV (Figures S2B and S2B). Sip1 KO interneurons migrated about 15% less far from the explants than WT counterparts after 1 DIV (WT: 252.95 ± 13.70 μm, n = 29 versus KO: 215.77 ± 15.41 μm, n = 27; p = 0.0530; n = number of explants) or 2 DIV (WT: 685.06 ± 33.82 μm, n = 17 versus KO: 579.84 ± 22.68 μm, n = 21; * p = 0.0076) (Figure S2C), suggesting reduced migration speed. If the latter would be the sole cause of the observed reduction in interneuron numbers in the cortex of Sip1 KO embryos, then the defect could be restored at later stages, when all targeted cells had sufficient time to populate the cortex. However, in Sip1;RCE/Nkx2-1 mutants, only a few GFP+ cells were found in the cortex at later stages (E18.5; Figures S2D and S2E).

A large group of misrouted Sip1 KO cells was found in the caudal VT of Sip1;RCE/Nkx2-1 brains at E16.5 (Figures 5A, 5A', S3A, and S3A'). This ectopic group of cells was positive for Nkx2-1 (Figures 5B and 5B') and Lhx6 (Figures 5C and 5C'). Similar ectopia were found in the other Sip1 mutants (Sip1;Nestin [see Figures 5 and S3]; Sip1;Dlx5/6 and Sip1;Gli2 [data not shown]). These GFP+ ectopic cells did not locate to the globus pallidus (GP) area; also, they were Er81+ indicating this is not an ectopic GP (Figures S3A and S3A'), where GP is indicated by an asterisk and ectopic cells by an arrowhead; results not shown). The ectopia was also positive for neuropeptide-Y (NPY), somatostatin (Sst), and Sox6 (Figures 5D and 5E, and data not shown), as well as for receptors typically present in migrating cortical interneurons, such as Nrp2 and ErbB4, indicating cortical interneuron features (Figure S3, all panels B and C).

We investigated whether Sip1;RCE/Nkx2-1 cells present in the cortex 3 weeks after birth contributed to the cortical interneuron lineages. Quantification of the number of GFP+ cells in the cortex showed there were much less MGE-derived interneurons in the Sip1 mutants (48% of the control number) (Figure 6E). Furthermore, Sip1 KO cells largely failed to populate the more medial parts of the cortex and remained clustered in the lateral cortex (KO versus control in lateral, 60.4% compared to 35.8% of the total population of GFP+ cells for each genotype [i.e., 61 compared to 75 cells]; intermediate, 25.5% compared to 78 cells); and medial, 14.1% compared to 75% [i.e., 14 compared to 56 cells]; Figures 6A–6C, 6A, 6B, 6C, 6D, 6E, S4A, S4B, and S4E). Interestingly, the deep layers (marked by Ctip2) seemed to contain fewer GFP+ neurons when compared to control mice (Figures 6A–6C, 6A'–6C', 6D, 6E, S4A, S4B, and S4E). We also analyzed the expression of parvalbumin (PV), Sst, calretinin (CR), and NPY in Sip1-deleted cortical interneurons. Most of the WT;RCE/Nkx2-1 cells in the cortex were PV+ or SST+ interneurons, less were NPY+, and almost none contained CR, as expected for MGE-derived cells (Figures 6F–6I, quantification in 6J and 6K). We found significantly fewer PV+, SST+, and NPY-containing interneurons in the cortex of Sip1;RCE/Nkx2-1 mutants (absolute numbers, p < 0.0001 for PV and SST, p = 0.0128 for NPY) (Figures 6F–6I, and 6J). Interestingly, the relative contribution of each of these interneuron subtypes to the total amount of targeted (GFP+) cells was not changed compared to control mice (Figure 6K).
Taken together, Sip1 KO MGE-derived interneurons have the intrinsic capacity to migrate, yet largely fail to reach the cortex during embryogenesis. Instead, a large portion appears to be misrouted in the VT, indicating a guidance problem. Three weeks after birth, the small number of Sip1 KO interneurons in the cortex do not distribute properly, yet still contribute to the different interneuron subtypes.

RNA Sequencing Reveals that Transcript Levels of Differentiation Factors and Guidance Cues Are Affected in Sip1 Mutant Interneurons

To further characterize the nature of the Sip1 KO cells and to understand the cause of the misrouting, we compared control and Sip1 mutant transcriptomes via RNA sequencing (RNA-seq) of fluorescence-activated cell sorting (FACS) sorted MGE-derived cells, obtained from WT- and Sip1;RCE/Nkx2-1 telencephali (three biological repeats, Illumina HiSeq-2000 platform), respectively. This approach identified differentially expressed genes irrespective of the cells’ position in the telencephalon.

Gene expression levels were derived from the read counts via HT-Seq and normalized across the six samples using DE-Seq (false discovery rate [FDR] < 0.05 cutoff) (Anders and Huber, 2010). Hence, the obtained values are normalized mean read counts allowing direct comparison of control and mutant samples. We assessed the correlation among the three biological repeats (Figure S5 A). The control samples and the KO samples clustered in two highly different groups, indicating that the expression differences in our samples reflected changes caused by Sip1 deletion. A principal component analysis showed grouping of the samples according to the most important first principal component (Figure S5 B). The drastic decrease of mapped reads on the floxed exon7 confirmed the efficiency of Sip1 deletion (Figure S5 C). Differential expression analysis using HT-Seq and DE-Seq identified 505 significantly upregulated and 366 significantly downregulated genes in Sip1;RCE/Nkx2-1 compared to control cells (Table S7).

To study the impact of Sip1 deletion on differentiation of MGE-derived cell populations, we listed the expression differences of transcription factors (TFs) related to these populations in control versus Sip1 KO cells (Table S1). We found that transcript levels of Dlx1, Dlx2, and Lhx6 were not affected. Some TFs present in cortical interneurons (Cux2, Maf, and Mafb) were clearly reduced (gray overstrike when > 2-fold reduction), whereas others (Satb1 and Sox6) were not changed or even increased. Also, the transcript levels for oligodendrocyte TFs Olig1, Olig2, and Sox10 were downregulated, whereas Id4, an inhibitor of oligodendrocyte differentiation, was upregulated in the absence of Sip1.

Some TFs related to striatal (Nkx2-1), cholinergic (Lhx8 and Isl1), or pallidal (Gbx1) development were increased in Sip1 KO, but none exceeded 2-fold upregulation. Furthermore, we compared the mRNA levels of 11 additional genes (Cxcr4, Gria1, Ets1, Cxcrl7, Grik1, Cntnap4, Gria1, Ch1, Cacng2, Csd2, and Scn1a) previously reported as enriched in embryonic cortical interneurons (Batista-Brito et al., 2008; Marsh et al., 2008; Faux et al., 2010) and included Nrp2, a gene related to migration of cortical interneurons (Nóbrega-Pereira et al., 2008). In the Sip1 KO samples, 10 of these 11 genes were downregulated, whereas Nrp2 was upregulated. Most of the
Figure 6. *Sip1* Mutant Cells Distribute Abnormally in the Postnatal Cortex but Still Contribute to the Different Interneuron Populations

(A–C and A′–C′) Three weeks after birth, few MGE-derived interneurons are found in the cortex of *Sip1;RCE*Nkx2-1 mice. Compared to the control (A–C), the bulk of *Sip1* KO cells remains in the lateral cortex (A′), while only a minority are found in more medial regions (B′ and C′).

(D) Boxed areas represent the three cortical regions in the pictures in A, A′ to C, C′.

(E) Quantification of the absolute number of GFP+ cells in each of these regions shows a significant reduction in the intermediate and medial areas (KO versus control in lateral, 61 compared to 75 cells; intermediate, 26 compared to 78 cells; and medial areas, 14 compared to 56 cells).

(F–I and F′–I′) Coexpression of GFP with the interneuron markers PV, SST, CR, and NPY was compared between WT;RCE*Nkx2-1 (F–I) and *Sip1;RCE*Nkx2-1 (F′–I′). White arrowheads indicate double-positive cells, empty arrowheads point to GFP+/marker− cells and an asterisk marks GFP−/interneurons. Green and red channels are shown for the indicated cells.

(J) The absolute number of GFP/marker double-positive cells was significantly decreased in the *Sip1* mutant (KO versus control for PV, 8 compared to 30 cells; SST, 7 compared to 20 cells; NPY, 1 compared to 3 cells) except for CR (KO versus control for CR, 1 compared to 1 cell).

(K) The relative contribution of each of the different MGE-derived cell types was not changed (KO versus control for PV, 32% compared to 40%; SST, 30% compared to 28%; CR, 25% compared to 2%; NPY, 4% compared to 5%).

Scale bar in C′ is 250 μm (A, A′ to C, C′) and 50 μm in I′ (F′, F′ to I′, I′). Error bars represent SD. Statistical significance was determined via the Student’s t-test (*p < 0.0001, *p < 0.05).

See also Figure S4.
downregulated genes are related to interneuron function or migration in the cortex itself, suggesting that Sip1 deletion disturbed the maturation of MGE-derived cells to functional cortical interneurons.

Next, we performed an unbiased gene ontology (GO) enrichment analysis using the GOrilla tool (http://cbl-gorilla.cs.technion.ac.il) (Tables S2 and S3). Genes related to the GO terms “cell cycle” and “mitosis” were enriched among the downregulated genes (Table S2). However, although proliferation at E12.5 in the MGE of Sip1/Nestin animals was decreased by 25% (n = 5 for each condition, p = 0.0023), cell cycle exit was not affected and we could not detect a similar reduction in proliferation in Sip1/Nkx2-1 animals (data not shown). On the other hand, genes related to “axon guidance” were enriched (to a factor 6.5; Table S3) among the most upregulated genes in the Sip1 mutants. As predicted from our observed cell-autonomous action mode of Sip1, their gene products localized preferentially to the membrane and were implicated in “signaling by transmembrane receptors” (Table S3). Intriguingly, all four genes relating to the GO term “Netrin receptor activity” (i.e., Deleted-in-colorectal-carcinoma [Dcc], Unc5a, Unc5b, and Unc5c) were ranked within the first 294 of all 8,485 genes listed (Enrichment = 28.86; p = 3.76E-6). In fact, many of the ligands or receptors involved in the Netrin/Unc5 pathway had upregulated expression in Sip1 KO cells (Figure S6A). Transcripts encoding the Netrin1 receptor Unc5b (5.3-fold up) and the ligand Netrin1 (Ntn1) itself (2.4-fold up) were particularly increased (Figure S6A), which was confirmed by quantitative PCR (qPCR) (Figure S6B).

Taken together, E14.5 Sip1 KO cells downregulate markers of maturing cortical interneurons, and possess disturbed levels of guidance cues, especially those related to Netrin/Unc5 signaling.

Increased Unc5b Levels Lead to Aberrant Interneuron Migration

We examined the expression of several receptors and ligands of the Netrin/Unc5 system. Unc5b mRNA was barely detectable in the control MGE, whereas Unc5b+ cells accumulated in the Sip1;Rce/Nkx2-1 MGE (Figures 7A, 7A’, 7B, and 7B’). Ntn1 levels were increased around the striatal area as well as in the caudal ectopia (Figures S6C and S6C’). Unc5c levels increased in the VT of Sip1 mutants, whereas Dcc expression was unchanged (Figures S6D, S6E–D’, and S6E’). The fibronectin and leucine-rich membrane proteins Firt2 and Firt3 are also ligands for Unc5b (Karaulanov et al., 2009; Yamagishi et al., 2011). Firt2 was abundant in the LGE and striatal anlage in control and Sip1/Nkx2-1 telencephalon (Figures S6F and S6F’), whereas Firt3, present at the border between the LGE and the MGE and at low level in the MGE in the control, seemed to be expanded in the mutant VT (Figures S6G–S6G’). Taken together, both qPCR and in situ hybridization analysis confirmed the increased transcript levels of Unc5b and Ntn1 found by RNA-seq in Sip1;Rce/Nkx2-1 cells.

To test which candidate cue(s) is (are) likely to (mis)guide cortical interneurons, we focally electroporated WT MGEs with Ntn1 or Unc5b expression vectors. Overexpression of Ntn1 had no obvious effect on interneuron migration when compared to the GFP control (Figures S7A–S7C). In contrast, using either a mouse or rat Unc5b construct, we found that Unc5b overexpression disrupted the migration of interneurons toward the cortex (mUnc5b: 16.2%, n = 11 versus GFP: 47.6%, n = 16 slices; p < 0.0001) (Figures 7E–7G, quantification in H; Figures S7A, S7B, and S7D). Interestingly, cells overproducing Unc5b tended to migrate in a ventral direction (Figure 7G).

As dependence receptor, Unc5b may also trigger cell death in absence of its ligand. We therefore investigated presence of cleaved caspase-3 as indicator of apoptosis in electroporated slices (Figures S7E and S7F). We did not observe a difference in cell death in GFP or Unc5b electroporated cells, suggesting that the migration defect induced by Unc5b overproduction is not a consequence of apoptosis. We also never observed increased cell death in the MGE of Sip1 mutants (E14.5; data not shown).

If too high levels of Unc5b hamper the migration of Sip1 KO interneurons, then we should be able to rescue their migration by downregulating Unc5b. We therefore electroporated Unc5b small interfering RNA (siRNA) or a nontargeting (NT) mouse siRNA pool in the MGE of Sip1/Nkx2-1 mutant slices. To visualize targeted Sip1 KO cells, a conditional dsRed-expressing construct (pCALNL) was coelectroporated (Figure 7I). Electroporation of Unc5b siRNA almost doubled the number of Sip1 mutant cells reaching the cortex (NT siRNA: 9.04%, n = 16 versus Unc5b siRNA: 13.82%, n = 11 slices; p < 0.0001) (Figures 7J and 7K, quantification in L). This indicates that decreasing the levels of endogenous Unc5b partially rescues the migration defect of Sip1 KO interneurons.

In addition, we could also rescue the interneuron migration in vivo by using a Sip1 complementary DNA (cDNA)-encoded transgene conditionally expressed from the ROSA locus (R26-Sip1Y’/y’). This mouse delivers a relatively small but constant dose of Sip1 to cells that express Cre (M. Tatari and G. Berx, personal communication). Because the RCE reporter is also ROSA based, only hemizygous R26-Sip1 (R26-Sip1y’/y’) samples could be obtained from FACS-sorted cells. Introduction of R26-Sip1y’/y’ increased Sip1 levels, while it reduced Unc5b levels in E14.5 Sip1 KO cells, as measured by qPCR (representative samples shown in Figure 7D). Introduction of two R26-Sip1-based alleles (R26-Sip1y’/y’) in a Sip1/Nkx2-1 KO mouse abolished the increase in Unc5b levels in the MGE (Figure 7C’–C”) and rescued the migration of interneurons to the cortex, as seen at E16.5 (Figures 7M–7O and 7M’–7O’).

 Altogether, our data show that Sip1 is needed for the proper regulation of the expression level of Unc5b in MGE-derived interneurons. Furthermore, our results also indicate that the local tuning of Unc5b expression in the MGE mantle zone is essential to direct the migratory path of these interneurons to the cortex.

DISCUSSION

In this study, we identify Sip1 as a critical, cell-autonomously acting transcription factor required in GABAergic interneurons, for their efficient migration to the cortex. Deletion of Sip1 in the MGE results in misrouting of MGE-derived interneurons, indicating a guidance defect. Transcriptome analysis using RNA-seq followed by gain-of-function studies show that increased levels of Unc5b inhibit interneuron migration to the cortex.
Moreover, knockdown of Unc5b ex vivo in Sip1 mutant brain slices or conditional expression of a Sip1 transgene in vivo in a Sip1 KO background rescue this phenotype. Hence, we identify Unc5b as a key Sip1-modulated guidance receptor in directed interneuron migration.

The Sip1 mutant mice studied here recapitulate some features of MWS, in particular seizures, which are common in MWS patients (Garavelli and Mainardi, 2007). Defects in cortical interneuron migration typically cause seizures in mice (Powell et al., 2003; Levitt et al., 2004). In line with this, we observed...
spontaneous seizures in Sip1/Gsh2 mutant mice during the third postnatal week, but not in young Sip1/Nkx2-1 mice, suggesting that Sip1 deletion in CGE as well as MGE-derived interneurons (in Sip1/Gsh2 mice) is more detrimental than in the MGE alone (as in Sip1/Nkx2-1 mice).

Sip1 is produced in larger amounts by migrating interneurons than in progenitors, consistent with previous observations (Batista-Brito et al., 2008; Faux et al., 2010). Removal of Sip1 from the entire CNS (Nestin-Cre) leads to a phenotype similar to that observed in Sip1/Dlx5/6 mice, in which VZ progenitors of the VT are not targeted, suggesting that Sip1 functions at the level of the SVZ and/or in postmitotic cells to drive differentiation. Similarly, during neural induction, as well as during mouse embryonic stem cell differentiation, Sip1 controls the formation of definitive neural stem cells from progenitor cells (van Grunsven et al., 2007; Dang et al., 2012).

Our transcriptome analysis shows that E14.5 Sip1 KO cells have reduced levels of several cortical interneuron markers such as Cux2, Mat, Cxcr4, and Cxcr7, which may indicate a differentiation deficit. Persistent levels of Nkx2-1 in particular, instead of its downregulation in WT mice, may even suggest that these interneurons acquire a striatal or cholinergic fate. Unfortunately, at present, no other factors are known to be uniquely expressed in embryonic striatal interneurons, making a firm distinction between cortical or striatal interneuron fates based on transcriptome analysis rather difficult. It cannot be excluded that Sip1-deficient interneurons remain cortical in character but fail to differentiate fully, either because of the absence of Sip1 function(s) or rather because of a failure of these cells to be exposed to cortical maturation factors that allow them to articulate a fully mature cortical phenotype. Indeed, once in the neocortex, Sip1 KO interneurons were able to contribute to the PV+ or SST+ cell populations.

Clearly, a large portion of Sip1 KO cells never reach the cortex. Our unbiased GO analysis shows that axon guidance and cell adhesion factors were enriched among upregulated genes. In addition to other TFs, Sip1 is known to affect cell-cell adhesion in various contexts such as EMT and cancer, due to direct regulation of E-cadherin expression. Because Sip1 directly represses E-cadherin (Cornijn et al., 2001; van Grunsven et al., 2003) gain-of-function or high levels of Sip1 correlate with death/desheath, increased invasion, and/or worse prognosis in some cancers (Peinado et al., 2007), whereas loss-of-function of Sip1 promotes adhesion and leads to delayed/reduced delamination of cranial neural crest cells (Van de Putte et al., 2003). Rather than possibly affecting cell-cell adhesion, our results show that Sip1 deletion in interneurons deregulates their directed migration. During embryogenesis, an ectopic group of MGE-derived Sip1 KO cells was found in the caudal VT. A comparable misrouting was described in the Dlx1/2 double-mutant brain, accompanied with ectopia of cortical interneuron-like cells (Marin et al., 2001; Long et al., 2009).

Our data indicate that upregulation of Unc5b mRNA levels in interneurons results in their aberrant migration. Overexpression of Unc5b by focal electroporation in cells of the MGE indeed changes their direction of migration without influencing their differentiation into cortical interneurons, leading to a dramatic reduction in migration of interneurons to the cortex. Moreover, using a conditional overexpression approach, we show that Sip1 levels are inversely correlated with Unc5b levels. On the other hand, Sip1 chromatin immunoprecipitation on conserved regions of the Unc5b upstream regulatory region and the first intron (100 kb around the transcription start site) did not detect any direct binding of Sip1, suggesting that Sip1 represses Unc5b expression indirectly (data not shown). Further work is needed to identify the Sip1 transcriptional target that directly represses Unc5b.

Which ligands could cause the misrouting of these Unc5b-overexpressing MGE cells? Ntn1 mediates repellent responses via Unc5b, alone or in combination with the receptor Dcc (Rajasekharan and Kennedy, 2009). Although Netrins have been implicated in cell and axon migration and Ntn1 is present along the migratory routes of GABAergic interneurons, the cortices of Ntn1-/- and Slit1/2-/-;Ntn1-/- mutants as well as Dcc-/- mutants display normal interneuron numbers at birth, suggesting that these proteins are dispensable for tangential migration (Anderson et al., 1999; Marin et al., 2003). However, Ntn1 interacts with a3beta1 integrin, which is present on interneurons, promotes their migration. Deletion of both Ntn1 and a3 integrin (Itga3) results in a large ectopic aggregation of interneurons in the VT, suggesting that Ntn1 signaling provides directional information to migrating interneurons (Stanco et al., 2009). In addition to Ntn1, Unc5 receptors also bind Flt2 and Flt3, which results in a repellant interaction, based on observations with Unc5d and Flt2 during radial migration of cortical projection neurons (Karaulanov et al., 2009; Yamagishi et al., 2011). We detect both Flt2 and Flt3 in the LGE, a region through which MGE-derived interneurons migrate en route to the cortex. Moreover, Ntn1 is present in the VZ of the LGE and MGE, as well as in the striatal anlage. High levels of Unc5b in interneurons could repel them from these Ntn- and Flt-rich areas. Cells overexpressing Unc5b via focal electroporation preferentially migrate in a ventral direction, suggesting that they indeed avoid these Ntn- and Flt-rich areas. Further studies are necessary to define which ligand(s) is (are) primarily causing Sip1-deficient cells to deviate from their normal path in the VT.

In conclusion, our results identify Sip1 as an essential transcription factor for cortical interneuron migration and maturation. Furthermore, we demonstrate that the regulation of precise Unc5b levels by Sip1 represents a way of sorting the different MGE cell types generated during embryogenesis. In general, defining a global guidance code for each of the migrating cell types in the VT will be a challenge for the future.

**EXPERIMENTAL PROCEDURES**

**Animals**

Mice were maintained in a CD1/Swiss background and were kept at KU Leuven in accordance to Belgian and EU regulations. Mice carrying a floxed (exon 7) Sip1 allele (Sip1<sup>fl/fl</sup>) (Higashi et al., 2002) were crossed with the following Cre mouse lines: Nkx2-1-Cre and Gh22-Cre (Kessaris et al., 2006), Nestin-Cre (Tronche et al., 1999), Dlx5/6-Cre (Stenman et al., 2003), with...
Expressions were determined via the Student’s t test. The number of marker+, GFP+, and double GFP+/marker+ cells was quantified. Three animals (age P20–P24) were used for each genotype. Cells were counted via ImageJ software and results are represented as mean ± SD. Statistical significance was determined using the Student’s t test.

qPCR
RNA was obtained from FACS-sorted E14.5 WT;RCE−, Sip1;RCE−, and Sip1;R26-Sip1;RCE/Nkx2-1 telencephalic cells and cDNA was made via the SuperScript III First-Strand Synthesis System (Invitrogen). qPCR was carried out in duplicate on a LightCycler 480 Instrument (Roche) using SYBR Green PCR Master Mix (Roche). Relative quantitation was determined using qBasePLUS software.

Plasmids
Expression constructs used for focal electroporation were based on pCIG, a pCAGGS-IRES-eGFP plasmid obtained via P. Vanderhaeghen (Meganess and McMahon, 2002). In the multiple cloning site of this vector, we cloned (1) the mouse Unc5b coding sequence which was isolated from a pdNA3-mUnc5b construct (Yamagishi et al., 2011), (2) the rat Unc5b-coding sequence isolated from the eGFP-N1/rUnc5b construct (Larrivée et al., 2007), and (3) the mouse Netrin1 coding sequence isolated from a Ntr−1 expression plasmid (RCKp6014G0516Q, ImaGenes). To trace electroporated cells in RCE/Nkx2-1 brain slices, the pcALNL plasmid (Addgene) was used. To delete Sip1 in Sip1fl/fl brain slices, a pcIG-Cre plasmid was used (P. Vanderhaeghen).

Focal Electroporation
Focal electroporation of MGEs from E13.5 WT, Sip1fl/fl, or RCE/Nkx2-1 embryos was done as described previously (Passante et al., 2008) and carried out with the aforementioned plasmids at 1 μg/μl and 4% fast green (Sigma). For overexpression, pcIG-mUnc5b was mixed in a 1:1 ratio with pcIG (0.5 μg/μl) and 1 μg/μl was used for the pcIG-rUnc5b construct. To rescue the migration of Sip1 KO interneurons, mouse Unc5b siRNAs (Smartpool, ON-TARGET plus, Thermo Scientific) or a NT pool of mouse siRNAs were used (200 nM) and mixed with pcALNL plasmid (1 μg/μl) to trace the electroporated cells. Electroporated slices were cultured for 3 DIV using an air-interface protocol (Polleux and Ghosh, 2002). Slices were fixed with 4% paraformaldehyde and analyzed via confocal microscopy (Nikon A1R Eclipse Ti). For each condition, we quantified (via ImageJ software) the total amount of GFP+ or RFP+ cells in the slice and calculated the percentage of GFP+ or RFP+ neurons that reached the cortex. Statistical significance was determined using the χ2 test.

Immunohistochemistry in Electroporated Slices
Slices were immunoprecipitated for 1 hr with PBS containing 0.3% triton (PBST) and 10% normal donkey serum. Primary antibodies (rabbit anti-cleaved caspase-3, 1:500, Cell Signaling Technologies; goat anti-GFP, 1:200, Abcam) were added overnight at 4°C. After washes in PBST, secondary antibodies (donkey anti-rabbit CY3 and donkey anti-goat Dylight 488, both at 1:1,000, Jackson ImmunoResearch) were applied overnight at 4°C. Slices were washed in PBST and mounted in Mowiol, and pictures were taken with a Nikon A1R Eclipse Ti confocal microscope.

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REFERENCES


