Smad-interacting protein-1 (Zfhx1b) acts upstream of Wnt signaling in the mouse hippocampus and controls its formation

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Smad-interacting protein-1 (Sip1) [Zinc finger homeobox (Zfhx1b)] is a transcription factor implicated in the genesis of Mowat–Wilson syndrome in humans. *Sip1* expression in the dorsal telencephalon of mouse embryos was documented from E12.5. We inactivated the gene specifically in cortical precursors. This resulted in the lack of the entire hippocampal formation. *Sip1* mutant mice exhibited death of differentiating cells and decreased proliferation in the region of the prospective hippocampus and dentate gyrus. The expression of the Wnt antagonist *Sfrp1* was ectopically activated, whereas the activity of the noncanonical Wnt effector, JNK, was down-regulated in the embryonic hippocampus of mutant mice. In cortical cells, Sip1 protein was detected on the promoter of *Sfrp1* gene and both genes showed a mutually exclusive pattern of expression suggesting that *Sfrp1* expression is negatively regulated by Sip1. Sip1 is therefore essential to the development of the hippocampus and dentate gyrus, and is able to modulate Wnt signaling in these regions.

development | sfrp1 | knockout | telencephalon | cortex

Smad-interacting protein-1 (Sip1) is a transcription factor that interacts with Smads, implicating it as a regulator of TGFβ/BMP signaling (1), acting either as repressor or activator (2–4). SIP1 has been implicated in the genesis of Mowat–Wilson syndrome in humans (5–8). The syndrome exhibits microcephaly, agenesis of the corpus callosum, cerebral atrophy and poor hippocampal formation, as well as other non-brain-related congenital defects (6).

The molecular pathways leading to these brain related abnormalities have not yet been elucidated (9–11). Sip1-deficient mouse embryos show multiple defects at embryonic day (E)8.5 and die at E9.5 (12).

Here, we report on the generation of mice that lack *Sip1* throughout the entire dorsal telencephalon. Mutant mice survive to juvenile age but lack the entire hippocampus and corpus callosum by this stage. These mice have marked deficiencies in the development of the hippocampal formation similar to those reported in mice deficient in components of the Wnt signaling pathway. We found *Sfrp1* gene, which encodes the Secreted Frizzled-Related Protein 1, an extracellular inhibitor of Wnt factors (13), to be up-regulated in the hippocampus of Sip1 mutant mice. This was accompanied by a down-regulation of JNK activity in the hippocampus of Sip1 mutants. Sip1 protein was also detected on the promoter of Sfrp1 gene in cortical cells, and we demonstrate that expression of the two genes was mutually exclusive in the developing cerebral cortex. Our data provide evidence for a functional link between Sip1 and the control of Wnt/JNK signaling *in vivo*. In addition, the Sip1 mutant mouse provides a model system to clarify the brain-related abnormalities in Mowat–Wilson syndrome.

Results

***Sip1* mRNA Expression and Gene Ablation in the Dorsal Telencephalon.** In the developing mouse brain, Sip1 mRNA was predominantly detected in the telencephalon, basal ganglia (BG), and thalamus (Fig. 1). By the onset of corticogenesis (E12.5), the developing telencephalon showed strong *Sip1 in situ* hybridization (ISH) signals in the postmitotic area of the cortex, although less-intense signals were also found in the proliferative compartment, the ventricular zone (VZ) (Fig. 1 *a* and *b*). At later embryonic stages (E16.5 and E18.5), the strongest ISH signals in the developing neocortex and hippocampus were located in the intermediate zone (IZ) and cortical plate (CP) (Fig. 1 *c*, *e*, *g*, and *i*).

To inactivate Sip1 function specifically in the cerebral cortex, Sip1 mutants were generated by crossing the *Sip1*^{exo7flox} (14) and the *Emx1*^{IRESCre} (15) mouse lines. The specificity of the *Sip1*^{exo7} deletion in cortical tissue was verified by both PCR and radioactive ISH with a riboprobe specific for exon7 [Fig. 1 and supporting information (SI) Fig. 7].

In the nonmutant littermates (Fig. 1 *c* and *g*), this exo7 probe produced a signal identical to that of a full probe (data not shown) used previously in ISH studies with this gene. Conversely, in E16.5 mutant brains (Fig. 1*d*) the exo7 signal was not detected in the dorsal telencephalon but remained unchanged in the VZ of BG and in the thalamus. At later stages (E18.5), we detected some *Sip1* signal scattered throughout the dorsal telencephalon with relatively higher intensity in the hippocampus (Fig. 1*i*). Because *Sip1* was not targeted for deletion in the BG (where it is also expressed), the remaining Sip1 expression in the cortex could be attributed either to the migrating interneurons that invade the cortex tangentially from BG or to locally born cells that escaped Cre recombination.

***Sip1* Deletion Affects Hippocampal Development.** Sip1 mutants were born with the expected Mendelian frequency and usually reached the juvenile stage (3–4 weeks old) Overall brain size was smaller in the mutants, possibly because of a general growth retardation (Fig. 2 and SI Fig. 8). Analysis of Nissl-stained sections of adult Sip1 mutant brains showed a remarkable phenotype in which both the hippocampus and corpus callosum were consistently missing (Fig. 2). The first morphological onset of the phenotype was detected at E15.5 (SI Fig. 9 *Left*), when the developing mutant hippocampus appeared smaller than in control mice. Perinatally, the corpus

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Abbreviations: BG, basal ganglia; CP, cortical plate; DG, dentate gyrus; E*n*, embryonic day *n*; ISH, *in situ* hybridization; IZ, intermediate zone; VZ, ventricular zone.

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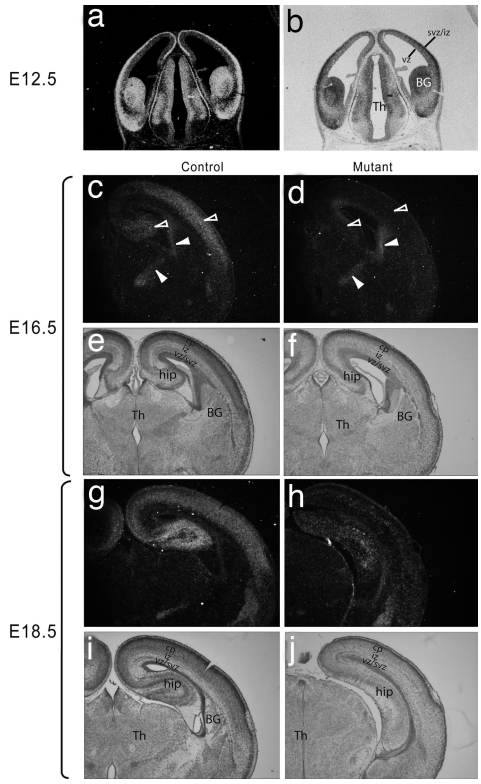

Fig. 1. *Sip1* is expressed in the developing mouse brain and is specifically deleted in the *Sip1*$^{flox/flox}$/ *Emx1*^{IRESCre} dorsal telencephalon. (*a* and *b*) Coronal sections of an E12.5 WT brain hybridized with a *Sip1*-specific riboprobe (*a*) show expression of *Sip1* in the cortex, BG and thalamus. In the cortex, *Sip1* expression is stronger in the CP and IZ than in the VZ and SVZ. At E16.5 (*c* and *e*) and E18.5 (*g* and *h*) *Sip1* transcripts continue to be expressed at a higher level in postmitotic areas than in mitotically active areas of the cortex. In *Sip1* mutants (*d*, *f*, *h*, and *j*) expression of WT *Sip1* mRNA is abolished in the developing Ncx and Hip (open arrowheads) but is maintained in other regions such as BG and thalamus (filled arrowheads). Hip, hippocampus; Ncx, neocortex; Th, thalamus; SVZ, subventricular zone. (*b*) Bright field of *a*. (*i* and *j*) Bright fields of *g* and *h*.

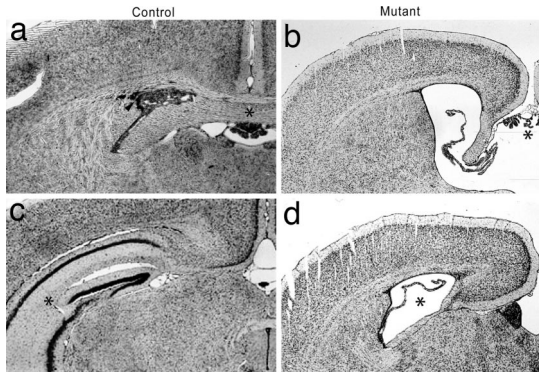

Fig. 2. Juvenile *Sip1* mutants lack the hippocampus and corpus callosum. Nissl-stained sections of 3-week-old control (*a* and *c*) and mutant (*b* and *d*) mice at two different rostro-caudal levels show, rostrally, the normal position of the corpus callosum between the two cerebral hemispheres and its absence in *Sip1* mutants (asterisks in *a* and *b*). Caudally, the entire hippocampal CA1–CA3 fields and DG are missing in mutant mice and replaced by an enlarged ventricle (asterisks in *c* and *d*).

callosum was absent, and all hippocampal fields were reduced in size (SI Fig. 9*c Center*). The presence of reduced CA1 and CA3 fields of the hippocampus was confirmed by ISH with the markers *SCIP* (16) and *KA1* (17), respectively. The dentate gyrus (DG) was almost absent, although a very few cells dorsal to CA fields expressed the molecular marker of the DG, *Prox1* (18) (SI Fig. 9*c Right*).

***Sip1* Ablation Increases Cell Death and Impairs Neural Progenitor Cell Proliferation in the Hippocampus and DG.** We first asked whether the absence of Sip1 would affect neuronal differentiation in the hippocampal formation, and thereby its normal size, by promoting premature differentiation. For this, we tested the presence of Hu and Tuj1 differentiation markers and nestin as a marker of neuronal progenitors at E13.5 and E15.5. Tuj1, Hu, and nestin were all normally present in the brain of Sip1 mutants (SI Fig. 10), which argues against the hypothesis of Sip1 function in suppressing premature differentiation of neuronal progenitors.

To assess whether Sip1 is required for the normal proliferation of hippocampal progenitors, we monitored the incorporation of BrdU into the nuclei of cycling cells at the morphological onset of the mutant phenotype (E14.5 and E15.5), using a 1.5-hour BrdU pulse. The number of BrdU$^+$ cells was quantified in two regions of the developing hippocampus: the prospective DG and the CA1-CA3 border of the hippocampus (Fig. 3 *a–g*). In the VZ of CA1-CA3 regions, the number of proliferating cells was ≈20% lower in Sip1 mutants than in control littermates (Fig. 3*g*). Moreover, a substantial decrease of ≈75% in proliferation rate was detected within the secondary proliferative population, the region responsible for the generation of DG cells. In the neocortical VZ, however, BrdU analysis revealed no significant difference in proliferation at E15.5 between mutant and control littermates (Fig. 3 *c*, *d*, and *g*).

We also performed a TUNEL assay to investigate whether the cells in the hippocampus had responded to Sip1 deficiency by activating apoptotic cell death. Before the morphological onset of the mutant phenotype at E15.5, we did not detect apoptotic cell death in either mutant or in control brains (data not shown). Starting from E16.5, however, we found an increase in the number of TUNEL-positive cells in the mutant hippocampi (Fig. 3 *h* and *i*). At this stage, cells undergoing apoptosis were located in the CP of the developing hippocampus, a region composed mainly of postmigratory neurons, but not in the hippocampal ventricular and IZs or in the neocortex. Later, at postnatal day (P)0, the majority of dying cells was detected in the IZ and ventral part of the CP of the hippocampus (Fig. 3 *j* and *k*). Increased apoptosis was also found at later postnatal stages, as revealed by the high number of TUNEL$^+$ cells in the remaining hippocampus of Sip1 mutants (Fig. 4 *l* and *m* and data not shown). Quantification of TUNEL$^+$ cells at E16.5, P0, and P8 (Fig. 4*n*) revealed a progressive increase in apoptotic cell death in the mutant hippocampus (3-, 4-, and 5-fold, respectively) (Fig. 3*n*). Thus, decreased proliferation and increased apoptotic cell death may account for the total loss of the hippocampus and DG in juvenile *Sip1* mutant mice.

Initial Patterning of Dorsal Telencephalon Is Not Affected in Sip1 Mutants. The cortical hem is a transient signaling center located between the presumptive hippocampus and choroid plexus (19). This center is a source of Wnt and BMP signaling and is essential for the normal development of the hippocampus (20). To determine whether the cortical hem is compromised in Sip1 mutant brains, we examined the expression of the hem-specific markers, *Wnt3a* and *Wnt5a* and found that the mRNA expression of both markers was not affected in Sip1 mutant embryos (SI Fig. 11 *c–f*). Because Sip1 is known to interact with BMP-SMADs (1), we asked whether *Sip1* deletion in the dorsal telencephalon would affect the expression of *Msx1*, a recognized downstream target of BMP, in the dorsal midline (21). We found that *Msx1* expression is not compromised in *Sip1* mutants (SI Fig. 11 *a* and *b*). Although we cannot completely rule out the possibility that some other aspects of BMP signaling are affected in *Sip1* mutants, we did not detect any signs

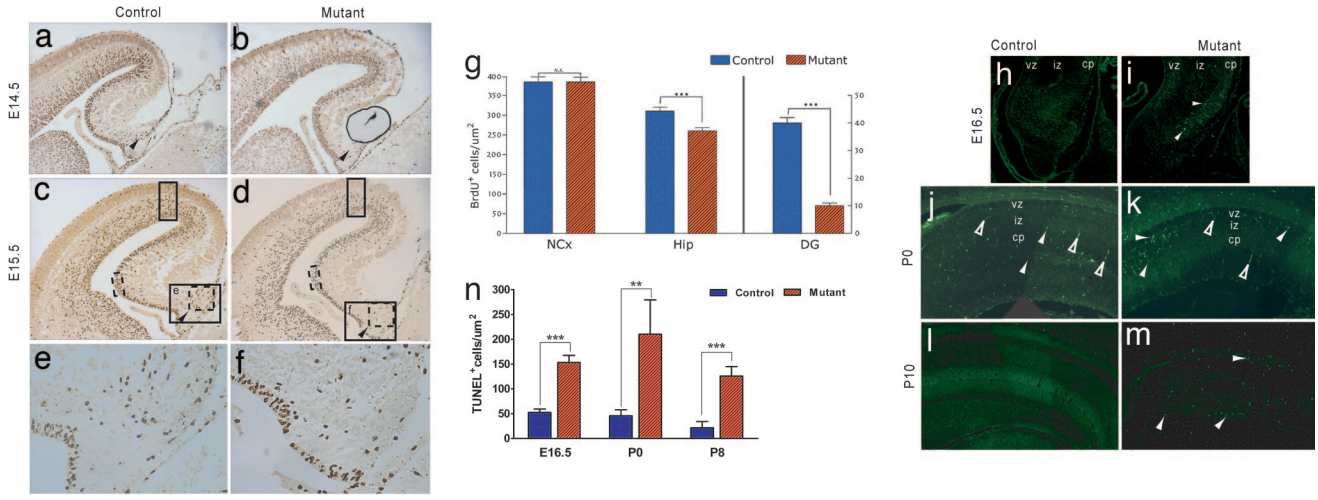

Fig. 3. Decreased proliferation and increased apoptotic cell death take place in the mutant hippocampus. Cells that incorporated BrdU after a 1.5-h pulse were detected on coronal sections of E14.5 (*a* and *b*) and E15.5 (*c* and *d*) brains. Note the reduced number of BrdU$^+$ cells of the secondary proliferative population (SPP) (arrowheads in *a–d*; *e* and *f* show high magnification of boxes in *c* and *d*) in the mutant brains (*b* and *d*) when compared with controls (*a* and *c*). The number of proliferating BrdU$^+$ cells at E15.5 (*c* and *d*) was quantified in the VZ of the developing hippocampus (small dashed box), neocortex (vertical box), and in the SPP (large dashed box) of three independent pairs of mutant and control brains in a several corresponding sections (mutant, $n = 29$; control, $n = 27$). Results were normalized to the area and expressed in micrometers squared. Statistical analysis was performed by using Student's *t* test. ***, $P < 0.0001$. Note the ≈20% and 75% decreases in cell proliferation in the SPP and VZ, respectively, of the mutant hippocampus (*g*). Apoptotic cell death was greater at E16.5 in the CP of the developing hippocampus in the mutant (*i*) than in the control (*h*) brains. At P0, substantial apoptosis was found in cells located in the ventral part of the hippocampal CP and IZ of the mutant brain (*k*) although a few scattered TUNEL$^+$ cells could also be found in control brains (*j*). At P10, many apoptotic cells were found in the gray matter (*m*) but none in the controls (*l*). The number of TUNEL$^+$ cells at E15.5, P0, and P8 (*n*) was quantified in the developing hippocampus of two independent pairs of mutant and control brains for each stage in corresponding sections (mutant, $n = 15$; control, $n = 17$).

of abnormal BMP signaling, such as a characteristic malformation of the choroid plexus, the most dorsal structure of the cortex (21)

We also addressed whether the cortico–hippocampal boundary was affected in Sip1 mutant brains. ISH with several molecular markers (Fzd8 and Satb2 are shown in SI Fig. 12; for Tcf3 and Id3, data not shown) did not reveal any anteriorisation of hippocampal fields or posteriorisation of the neocortex (SI Fig. 12 *Right*)

These data indicate that *Sip1* is neither required for the formation of the cortical midline and hem, nor for the initial patterning of the dorsal telencephalon, at least after the onset of Emx1 expression. This conclusion is consistent with *Sip1* being expressed in the dorsal VZ at a relatively low level (Figs. 1*a* and 5*a*).

The Negative Regulator of Wnt Signaling, *Sfrp1*, Is Up-Regulated in the Mutant Hippocampus. A series of genetic manipulations have shown that Wnt signaling plays a pivotal role in the development of the hippocampus. For example, mice deficient in *Wnt3a* show a loss of the entire hippocampal formation and corpus callosum (20). Similar defects appear in mice that have reduced function in downstream mediators of the canonical Wnt signaling pathway, ranging from Lef1/Tcf transcription factors (22) and β-catenin (23) to frizzled-9 (24). In addition, deletion of the transcription factor *Emx2*, a target gene of Wnt signaling in the cortex (25), leads to a reduced hippocampus and almost complete absence of the DG.

Sip1 is known to act as a transcriptional repressor (1), and *Sip1* deletion leads to gross morphological and cell proliferation defects that are comparable with those found in mice defective in components of the Wnt pathway. Hence, we reasoned that Sip1 could exert a negative regulation on certain inhibitors of Wnt signaling. Thus, we evaluated the expression of *Sfrp* genes known to be expressed in the dorsal telencepalon (26). The expression of *Sfrp2* was not altered (data not shown), but the expression of *Sfrp1* was dramatically up-regulated in the *Sip1* mutant. In the wild-type (WT) E14.5 dorsal telencephalon, *Sfrp1* expression has a very distinct rostrocaudal gradient (Fig. 4*A*); it is expressed at high levels in the neocortex, but it is not expressed in the hippocampus. Its expression at this stage is limited to the VZ and is not found in the CP. In the *Sip1* mutant mice, *Sfrp1* is ectopically expressed in the VZ of the hippocampus. At later stages, the *Sfrp1* up-regulation in the dorsal telencephalon of mutant mice becomes much more pronounced. Specifically, at E16.5–E18.5, it is strongly up-regulated in virtually all postmitotic cells (arrowheads Fig. 4*A*). The up-regulation of *Sfrp1* in the mutant is much stronger in postmitotic than in proliferating cells of the hippocampal VZ. This finding correlates with a higher level of *Sip1* transcripts in postmitotic than in VZ cells (Figs. 1*a* and 5 *a* and *b*).

Sip1 Protein Is Detected on the *Sfrp1* Promoter *in Vivo*, and the *Sip1* Expression Pattern Is Complementary to That of *Sfrp1*. The ectopic activation of *Sfrp1* in the Sip1 mutant hippocampus and neocortex could be either a primary or a secondary event in the Sip1-mediated pathway. We therefore investigated the possibility of *Sfrp1* being a transcriptional target of Sip1. To reveal a possible interaction between Sip1 and the *Sfrp1* promoter, we performed a chromatin immunoprecipitation assay (ChIP), using an antibody generated in our lab (see *SI Methods* for details). The specificity of Sip1 antibody was demonstrated by both Western blot and immunohistochemistry (IHC) analyses (SI Fig. 13). After chromatin precipitation, the presence of a Sip1 protein/Sfrp1 DNA complex was further analyzed by semiquantative PCR with several pairs of primers complementary to sites spanning 8 kb of *Sfrp1* upstream region. ChIP assay with two of these pairs demonstrated that Sip1 protein was detected within the region 2.5 kb upstream of *Sfrp1* transcription start. On the other hand, Sip1 protein was not detected within the distal most 5-kb region from exon1 (Fig. 5*h*).

In addition, we performed ISH with *Sip1* and *Sfrp1* probes on adjacent sections to correlate their expression patterns during cortical development. At E14.5, *Sfrp1* was highly expressed in the neocortical VZ with no expression in the IZ and CP. In contrast, *Sip1* expression was low in the VZ and high in the IZ/CP (Fig. 5). At P2, *Sfrp1* maintained its high expression in the VZ/SVZ with an additional domain of expression in the CP of the cingulate cortex.

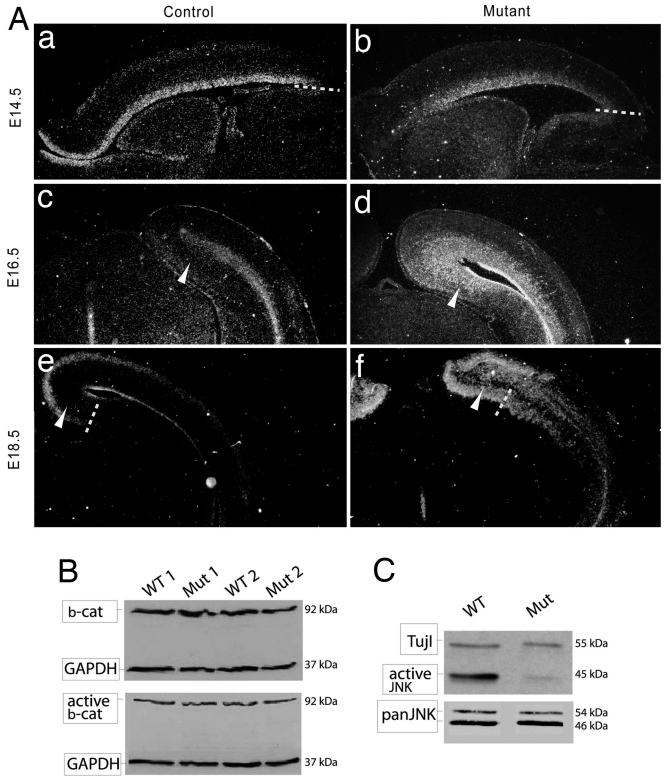

Fig. 4. Wnt pathway is affected in SIP1 mutant hippocampus. (*A*) Secreted frizzled-related protein 1 (*Sfrp1*) mRNA is up-regulated and ectopically expressed in *Sip1* mutants. Because E14.5 *Sfrp1* transcripts in the dorsal telencephalon are normally confined to the neocortical VZ but are excluded from the VZ of the hippocampal anlage at the onset of hippocampal development (*a*). In *Sip1* mutants, however, *Sfrp1* is not only found in the neocortical VZ but is also ectopically expressed in the hippocampal VZ (*b*). By E16.5 (*c* and *d*) and E18.5 (*e* and *f*), *Sfrp1* is strongly up-regulated in the mutant in both the proliferating and postmitotic areas of the cortex. In control brains at E16.5, *Sfrp1* is not expressed in postmitotic cells (arrowhead in *c*) but is up-regulated in postmitotic cells of the mutant (arrowhead in *d*). At E18.5 (*e*), *Sfrp1* starts to be expressed in some postmitotic cells of the neocortex but not in the hippocampus. In the mutant brains (*f*), however, *Sfrp1* is up-regulated in all cortical regions. (Dashed lines demarcate the normal medial border of *Sfrp1* expression.) (*B*) The activation of β-catenin is not impaired in the *Sip1* mutant hippocampus. Western blot analysis of E15.5 medial cortex total protein extract of two independent pairs of mutant (Mut) and control (WT) brains was performed by using the pan β-catenin antibody and an antibody recognizing only the active (nonphosporylated) form of β-catenin. In both cases, a ≈92-kDa band was detected with equal intensity in mutants and controls. (*C*) The active (phosphorylated) form of JNK1-3 (≈45 kDa) is down-regulated in the mutant E15.5 hippocampus, whereas the total level of JNK1-3 is not changed. The amount of protein loaded was controlled with anti-GAPDH and anti-TujI.

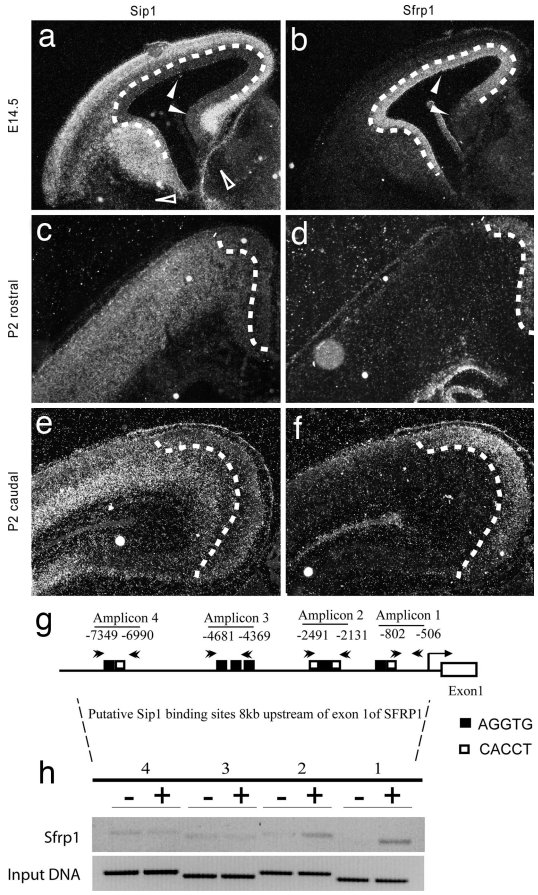

Fig. 5. SFRP1 is a target of SIP1 in the cerebral cortex. (*a–f*) *Sfrp1* and *Sip1* mRNA expression in the developing cortex. *Sip1* (*a*) and Sfrp1 (*b*) expression at E14.5. Filled arrowheads depict low but uniform expression of *Sip1* in the VZ of neocortex and hippocampus. Empty arrowheads depict regions with no *Sip1* expression. (*c–f*) *Sip1* (*a*) and *Sfrp1* (*b*) expression in the cerebral cortex at P2. Yellow dashed lines demarcate the borders of Sfrp1 expression. (*g*) Putative Sip1 binding sites and positions of amplicons for ChIP assay within the upstream genomic region of Sfrp1. (*h*) Semiquantitative PCR analysis of four amplicons containing putative Sip1 binding sites on SFRP1 promoter after ChIP (upper lane) and amplification of input DNA (lower row). Sip1 was detected within the 2.5-kb upstream region from the transcription initiation site (amplicons 1 and 2) but not within the more distal region (amplicons 3 and 4). Samples with (+) and without (−) added antibody against Sip1 are indicated.

Sip1 expression in the late CP was also complementary to that of *Sfrp1* expression, being excluded from *Sfrp1*-positive territory (Fig. 5). Collectively, these data suggest that *Sip1* is a direct negative regulator of *Sfrp1* expression.

Canonical Wnt Signaling Is Not Significantly Impaired in the *Sip1* Mutant Hippocampal CA1–CA3 Fields. We investigated whether the transcription of canonical Wnt downstream factors was affected in *Sip1* mutants. At the stages analyzed, neither the expression of Wnt mediator *Lef1*, nor of *Emx2* and *Axin2*, known downstream target genes of canonical Wnts (27), was affected (SI Fig. 12 *Right*). At the protein level, the hippocampus of *Sip1* mutants and control littermates showed a similar distribution of β-catenin immunoreactivity within the developing hippocampus (data not shown). The levels of total and the activated fraction of β-catenin appeared equivalent between mutant and control samples, as shown by Western blot analysis (Fig. 4*B*).

Activity of a Downstream Effecter of Noncanonical Wnt Signaling, JNK, Is Inhibited in the *Sip1* Mutant Medial Cortex During Embryonic Development. Next, we asked whether Sfrp1 up-regulation in *Sip1* mutants coincided with an impairment of noncanonical Wnt mediators. Noncanonical Wnt pathways are independent of β-catenin. At least two noncanonical Wnt pathways are known: one mediated by JNK, the other dependent on Ca^{2+} (28). The main effecter of the latter, the Ca^{2+}-dependent calmodulin kinase II, starts to be expressed postnatally in the murine brain. On the other hand, JNK is expressed in the dorsal telencephalon IZ already from embryonic stages (29), and JNK-mediated Wnt signaling has been shown to modulate dendritic development in cultured hippocampal cells.

We therefore looked for possible changes in JNK activity, using an antibody that specifically recognizes activated JNK1-3 (30). At E15.5, the developing medial cortex of *Sip1* mutants showed a dramatic decrease in the levels of the activated form of JNK1-3 when compared with WT littermates (Fig. 4*C*). Neither the total

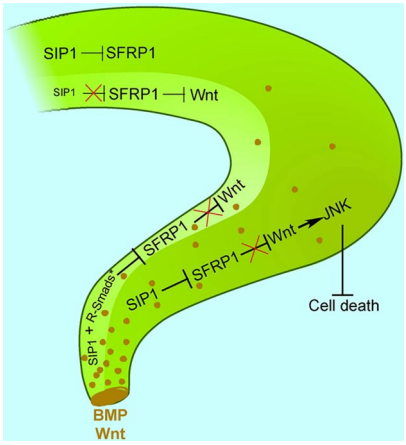

Fig. 6. Model of Sip1 function in the dorsal telencephalon. In the WT cortex, cells expressing *Sip1* at a very high level (postmitotic cells) do not coexpress *Sfrp1*. VZ cells of the neocortex, in contrast to VZ cells of the hippocampus, do coexpress *Sip1* and *Sfrp1*. It is likely that a high level of *Sip1* expression alone is enough to down-regulate *Sfrp1* expression in postmitotic cells. Conversely, the low level of expression of *Sip1* in the neocortical VZ cells is not sufficient to suppress the expression of *Sfrp1*. However, in VZ cells of the hippocampus, where *Sip1* expression is similar to that in neocortical VZ cells, *Sip1* and *Sfrp1* are not coexpressed. Thus, it is likely that *Sip1* (when expressed at a low level) requires other cofactors, such as BMP-Smads, to suppress *Sfrp1*. The hippocampus is situated closer to the localized source of BMP signals than is the neocortex. Therefore, it is conceivable that the level of activated BMP-Smads is higher in the hippocampus than in the neocortex. On the other hand, Sip1 can interact with Smads, making them good candidates to help Sip1 regulate *Sfrp1* in cells with low *Sip1* expression. In the hippocampus, Sip1 positively controls JNK activity possibly by negative regulation of Sfrp1 expression. In *Sip1* mutants, Sfrp1 may inhibit noncanonical Wnt signaling in the hippocampus, because it is no longer subject to Sip1-mediated repression. This may result in inactivation of JNK, which, in turn, induces apoptotic cell death.

levels of JNK1-3, nor those of the neuronal marker TujI differed between mutant and control hippocampi (Fig. 4*C*).

We also asked whether lower JNK1-3 activity is maintained in *Sip1* mutants at postnatal stages. To our surprise, we did not detect any significant differences in JNK1-3 activity at P0 or P1 between mutant and WT animals (data not shown). However, this might reflect the differences in tissue composition of the medial cortex between E15.5 and P0 mutant brains (see *Discussion* for details).

Discussion

Sip1 is expressed in the VZ of the developing dorsal telencephalon at a low level, and at a high level in the postmitotic cells. *Sip1* ablation in the dorsal telencephalon leads to a loss of the entire hippocampal formation and corpus callosum in the adult. We demonstrate that the loss of the hippocampus in *Sip1* mutant mice is a result of progressive degeneration by apoptosis. SIP1 has been implicated in the genesis of Mowat–Wilson syndrome in humans (5–8). The syndrome also exhibits, with variable penetrance, microcephaly, agenesis of the corpus callosum, cerebral atrophy, and poor hippocampal formation, as well as other non-brain-related congenital defects (6). Interestingly, although in humans Mowat–Wilson syndrome is caused by *SIP1* heterozygous mutations, we did not detect significant differences between heterozygous and WT mice. It might indicate that either humans are more sensitive to *Sip1* dosage than mice, or human mutations produce dominant-negative forms of Sip1 protein. Human patients exhibit microcephaly, as do our mutant mice. However, in mice, microcephaly does not seem to be an isolated condition but is possibly a result of general postnatal growth retardation and dwarfism. The physiological basis of the dwarfism in Sip1 mutants is not clear. It is unlikely to be caused by *Sip1* deletion in the cerebral cortex, because this brain region is not involved in the control of general growth. Probably, it is triggered by Emx1-Cre activity in another tissue where *Sip1* plays an important role. *Sip1* juvenile mutant mice also show some degree of lateral ventricle enlargement, but this is unlikely to cause hippocampal degeneration, because even in severe cases of hydrocephalus in rats, cell death in the hippocampus was not observed (31).

Because Sip1 is known to interact with BMP-Smads, the expected phenotype would reflect strong deficiencies in BMP signaling in the dorsal telencephalon. However, *Sip1* mutants show a normal choroid plexus, and the expression of *Msx1*, one of the few well characterized BMP targets, is not affected in the dorsal telencephalic midline, which suggest that BMP signaling is not severely impaired. Previous studies have shown that Wnt signaling is required for normal hippocampal development (20, 22–24). Here, we identify Sip1 as an agonist of the Wnt pathway in the hippocampus. It is noteworthy that morphological abnormalities in *Sip1* mutant brains are preceded by a strong up-regulation of Sfrp1, a known extracellular antagonist of the Wnt pathway. In the developing WT telencephalon, *Sfrp1* is expressed in the VZ of the neocortex but not in the VZ of the hippocampus. In the *Sip1* mutants, *Sfrp1* is strongly up-regulated at E14.5 in the VZ of hippocampus. On the other hand, in the WT cerebral cortex *Sfrp1* is expressed only in areas with low *Sip1* expression. We also detected Sip1 protein on the promoter of *Sfrp1* gene in cortical cells by ChIP assay. These findings suggest that up-regulation of *Sfrp1* in the hippocampus could be a molecular cause of the observed hippocampal abnormalities in *Sip1* mutant brains. It is not clear whether the Sfrp1 ectopic activation is the sole cause of hippocampal degeneration in *Sip1* mutants or whether there are other direct targets of Sip1 that also contribute to the phenotype. This needs to be addressed in detail in the future by studying, for example, *Sip1*/*Sfrp1* compound mutant mice.

Although the phenotype of the Sip1 mutant in the hippocampus is reminiscent of the Wnt3a or β-catenin mutant phenotypes, there are marked differences. In Wnt3a and β-catenin mutants the hippocampal formation is absent since early development, whereas in *Sip1* mutants, reduced hippocampal fields are still present during early development but disappear postnatally. The main cause of the underdeveloped hippocampus in the Wnt3a mutant was reported to be decreased proliferation, and no cell death was detected. In the case of the *Sip1* mutant, the proliferation rate is decreased, but apoptosis largely contributes to the reduction of hippocampal size. In contrast to *Sip1* mutants, no massive cell death was detected in Wnt3a or in β-catenin conditional mutants (20, 22, 23), which can be explained by stage-specific differences. The authors of refs. 20, 22, and 23 did not elaborate on their apoptosis analysis beyond E14.5, the stage where we also detected no cell death. Another possible explanation could be that noncanonical rather than canonical Wnt signaling is affected in *Sip1* mutants.

It has been shown that the compound mutation of *Jnk1* and *Jnk2* is associated with increased apoptosis in the forebrain (32). A recent report (24) suggests that noncanonical Wnt signaling can regulate cell death in the hippocampal formation. Increased apoptosis observed in the postmitotic regions of the developing hippocampus from in *Sip1* mutants is likely to be a consequence of the JNK inactivation. In this scenario, Wnt signaling controls not only cell proliferation of hippocampal cells but also cell survival by modulation of JNK activity. Surprisingly, we did not detect differences in JNK activity within the medial cortex in neonatal brains. It is possible that in the postnatal brain, molecules other than JNK can mediate apoptosis in the *Sip1*-deficient hippocampus. Alternatively, it might reflect differences in the composition of tissue samples extracted for the Western analysis at different stages. Indeed, when isolating tissue samples for Western analysis, we included hippocampus, DG, subiculum and part of the cingulate cortex. At E15.5, the relative proportions of these parts of the medial cortex did not differ drastically between WT and mutants. In contrast, in P2 mutant brains, a substantial part of hippocampus and DG had already degenerated (SI Fig. 9 *Middle*). In this case, most protein for

the Western was extracted from subiculum and cingulate cortex, two regions preserved in *Sip1* mutants. It is not clear whether Sfrp1 up-regulation is the main reason for JNK inactivation in the mutant hippocampus. However, given that Sfrp1 is a recognized inhibitor of both canonical and noncanonical Wnt pathways, its overexpression in the *Sip1* mutant hippocampus is likely to contribute to JNK inactivation (Fig. 6).

Collectively, our data demonstrate that *Sip1* is indispensable for the normal development and maintenance of the hippocampal formation. The absence of Sip1 induces up-regulation of *Sfrp1* expression in the developing hippocampus, inhibits JNK activity and eventually results in decreased proliferation of neuronal progenitors and in apoptosis of postmitotic cells. Our data suggest that in the hippocampus, Sip1 functions as a positive regulator of noncanonical Wnt signaling by regulating the expression of the Wnt inhibitor *Sfrp1*.

Methods

Mice. The animals were kept on a mixed CD1/C57B6 background. Genotyping was performed as described in ref. 12. All animal manipulations were carried out in accordance with German law and were approved by the Bezirksregierung Braunschweig. Pregnant females were killed by cervical dislocation. Brains were fixed either by immersion (embryonic and perinatal brains) into or perfused (adult brains) by freshly prepared 4% paraformaldehyde-PBS overnight at 4°C and then washed, dehydrated, and embedded in wax according to standard protocols.

ISH, IHC, and Cell Death Assay. Radioactive ISH and emulsion autoradiography were performed essentially as described in ref. 33 with the only modification that hybridization buffer contained 200 mg/ml of SPthio-ATP (Roche Diagnostics, Mannheim, Germany) to block nonspecific binding of labeled RNA. Nonradioactive ISH was performed as described in ref. 34. The following probes were used: SCIP and Prox1; Emx2 (Ep1.3), Wnt3a, Wnt5a, Wnt8b; Axin2; and Sfrp1 (RZPD, Berlin, Germany; entry no. 7305480).

IHC was performed according to standard protocols (See *SI Methods* for details). All experiments were repeated at least 3 times with tissue samples from independent litters.

The following antibodies were used: anti-BrdU (1:100; Roche Diagnostics; catalog no. 1170376), anti-Nestin (1:100; Chemicon, Temecula, CA; catalog no. MAB353), anti-TujI (1:300; Sigma–Aldrich, Seelze, Germany; catalog no. T8660), anti-β-Cat (IHC 1:100; BD Biosciences, San Jose, CA; catalog no. 610153), goat anti-mouse-HRP (1:5,000; Chemicon), and rabbit anti-mouse-Cy3 and goat anti-rabbit-Cy5 (Jackson ImmunoResearch, West Grove, PA). Cell death was assessed by TUNEL assay on paraffin sections, using the Apoptag fluorescein direct *in situ* apoptosis detection kit (Chemicon).

Western Blot Analysis. Tissue from the medial telencephalon of E15.5 embryos was extracted and suspended in Triton X-100 buffer. Western blot analysis was performed by using the ECL kit (Amersham Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The antibodies used were anti-b-Cat (1:500; BD Biosciences; catalog no. 610153), anti-Active b-Cat (1:250 Upstate Biotechnology, Lake Placid, NY; catalog no. 05-665), anti-GAPDH (1:500; Chemicon; catalog no. MAB374), and goat anti-mouse-HRP (1:5,000; Chemicon).

Generation of Antibodies Against Sip1 Protein. To produce antibody against Sip1 we generated a peptide based on predicted protein sequence: CDPPLRLTKSSHFTNI (754–769 aa). Antibody was produced in rabbit as described in ref. 35 and verified by Western blot analysis and IHC (see SI Fig. 13 and *SI Methods*).

ChIP Assay. Mouse embryonic cortex (E17.5 or E 18.5) was used as a tissue source of chromatin. ChIP and a semiquantitative PCR assay were performed as described in *SI Methods*.

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