Protooncogene Ski cooperates with the chromatin-remodeling factor Satb2 in specifying callosal neurons

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First insights into the molecular programs orchestrating the progression from neural stem cells to cortical projection neurons are emerging. Loss of the transcriptional regulator Ski has been linked to the human 1p36 deletion syndrome, which includes central nervous system defects. Here, we report critical roles for Ski in the maintenance of the neural stem cell pool and the specification of callosal neurons. *Ski*-deficient callosal neurons lose their identity and ectopically express the transcription factor Ctip2. The misspecified callosal neurons largely fail to form the corpus callosum and instead redirect their axons toward subcortical targets. We identify the chromatin-remodeling factor Satb2 as a partner of Ski, and show that both proteins are required for transcriptional repression of *Ctip2* in callosal neurons. We propose a model in which Satb2 recruits Ski to the *Ctip2* locus, and Ski attracts histone deacetylases, thereby enabling the formation of a functional nucleosome remodeling and deacetylase repressor complex. Our findings establish a central role for Ski–Satb2 interactions in regulating transcriptional mechanisms of callosal neuron specification.

cortical development | cell fate | transcriptional regulation

During embryonic development, proliferating neural stem and progenitor cells located in the germinal zones of the dorsal telencephalon give rise to molecularly and functionally distinct projection neurons of the six-layered neocortex (1). Subtypes of postmitotic projection neurons located in different cortical layers express distinct sets of genes, whose functions are only starting to be elucidated (2). For example, Tbr1, Sox5, Fezf2, and Ctip2 are involved in the specification of deep layer (DL) neurons that project to subcortical targets, whereas Satb2 is a determinant of callosally projecting neurons.

Ski is a transcriptional regulator that exerts its functions in concert with various intracellular partners, and has been implicated in diverse cellular processes. Numerous reports established interactions of Ski with transcription factors, corepressors, or nuclear hormone receptors in cell lines under overexpression conditions (3). In mouse embryos, *Ski* transcripts are expressed in many tissues, including the developing cortex (4). Accordingly, $Ski^{-/-}$ pups die at birth and display complex phenotypes, including defects in the development of the nervous system (5, 6).

Here, we investigated the cell type-specific expression and function of Ski protein during the development of the neocortex. We demonstrate that Ski forms protein complexes with Satb2 in callosal neurons and is part of the chromatin-remodeling complex that binds to regulatory regions of *Ctip2* in vivo. Our data show that Ski recruits the transcriptional repressor complex onto the *Ctip2* locus and suppresses the genetic program of subcortically projecting neurons.

Results

Ski Protein Is Expressed in Subsets of Neural Progenitor Cells and in Projection Neurons. We first analyzed Ski protein expression by immunohistochemistry, and found that it was expressed throughout the neuroepithelium at E10.5 (Fig. S1*A*), where it colocalized with neural stem cell markers (Fig. S1 *B* and *C*). The specificity of the anti-Ski antibody was demonstrated by the lack of signal on E10.5 $Ski^{-/-}$ sections (Fig. S1*D*). Ski expression was maintained in the ventricular zone (VZ) of the telencephalon at E12.5 (Fig. S1*E*). Notably, Ski was down-regulated in Tbr2-positive intermediate progenitor cells in the subventricular zone (SVZ) and absent from the earliest-born HuC/D-positive neurons, which form the preplate (PP; Fig. S1 *F* and *G*). Ski continued to display strong expression in the VZ at E14.5 (Fig. 1*A*, *Left*). In addition, it appeared in postmitotic cells of the developing cortical plate (CP) at E14.5, and was strongly expressed in the dorsomedial part of the rostral telencephalon at E17.5 (Fig. 1*A*, *Right*). Higher-magnification images demonstrate that most Ski-positive cells reside in superficial layers of the CP, with a distribution similar to that of the Satb2-expressing population (Fig. 1*B*). A smaller number of Ski-positive cells was present in layer V, and even fewer in layer VI—layers that are defined by immunoreactivity for Ctip2 and Tbr1, respectively (Fig. 1*B*). To characterize the subpopulation of Ski-expressing neurons, we performed double immunostainings for Ski and a panel of layer-specific markers, including Satb2, Ctip2, and Tbr1 (Fig. 1 *C*–*F*). At E17.5, Satb2 is expressed in callosal projection neurons in upper layers, as well as in subsets of DL neurons, whereas Ctip2 and Tbr1 are expressed in different subsets of DL neurons that project to subcortical targets. We find that subpopulations of Ski-positive neurons express Satb2 in superficial layers of the CP (~85%) and in deep layers V and VI (~30%; Fig. 1*C*). Conversely, the majority of Satb2-positive cells coexpressed Ski in upper layers, although many Satb2-positive cells in deep layers were Ski negative (Fig. 1*C*). In addition, virtually all layer V neurons expressing high levels of Ctip2 were positive for Ski (Fig. 1*D*). Notably, very few cells were triple positive for Ski, Satb2, and Ctip2 (Fig. 1*E*, arrow), which is in agreement with previous findings demonstrating that <5% of Satb2-positive cells express Ctip2 in deep layers (7). Little coexpression was observed for Ski and Tbr1 at E17.5 (Fig. 1*F*). The dynamic expression pattern of Ski points to a temporally restricted and cell type-specific function of Ski in cortical cells.

Ski Ablation Affects Differentiation of Neural Progenitor Cells. We used $Ski^{-/-}$ mice (5) to investigate the requirement for Ski during cortical development. Analysis of the forebrain at E10.5 revealed a reduction in radial neuroepithelial thickness in $Ski^{-/-}$ compared with WT (WT: 101 ± 10 μm; $Ski^{-/-}$: 74 ± 2 μm, $n = 6$, $P \leq 0.001$; Fig. S2*A*). Moreover, immunostainings for the M-phase

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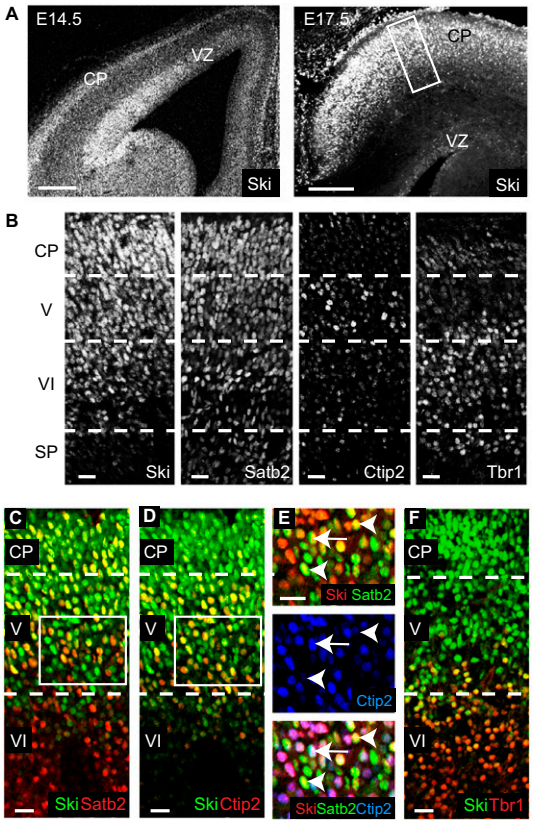

Fig. 1. Ski is expressed in postmitotic cells of the developing neocortex. (*A*) Ski immunostaining on coronal brain sections is predominantly detected in the VZ of the dorsal and ventral telencephalon, and in postmitotic cells of the neocortex at E14.5 and E17.5. (*B*) Higher-magnification images of the rostrodorsal neocortex at E17.5 (boxed region in *A Right*) show that neurons expressing high levels of Ski are mainly located in the superficial layers of the CP and in layer V as visualized by layer-specific markers Satb2, Ctip2, and Tbr1. (*C* and *D*) Ski shows high coexpression with Satb2 in upper layers of the CP and to a minor extent in layer V (yellow in the corresponding overlays) (*C*), and with Ctip2 in layer V neurons (*D*). (*E*) Triple immunostainings for Ski, Satb2, and Ctip2 in higher-magnification images (boxed region in *C* and *D*) show that most Ski and Satb2 double-positive cells (yellow in *Top*, arrow and arrowheads) do not express Ctip2 (blue in *Middle*, arrowheads). A rare triple-positive cell is depicted (white in *Bottom*, arrow). (*F*) Ski and Tbr1 are coexpressed to a minor extent in layer VI neurons. (Scale bars: *A*, 200 μm; *B–F*, 20 μm.)

marker phosphohistone H3 (pHH3; Fig. S2*A*) showed fewer proliferating apical progenitor cells in mutant forebrains (Fig. S2*B*). Similarly, the pool of intermediate progenitors was also affected in $Ski^{-/-}$ embryos (Fig. S2 *E* and *F*). Concomitantly, we observed increased numbers of cells positive for doublecortin (Dcx; marker of migrating postmitotic neurons) in $Ski^{-/-}$ vs. WT forebrains (Fig. S2 *C* and *D*). Thus, our findings suggest that as a result of Ski deletion, cells differentiate precociously into neurons, leading to a reduced progenitor pool at initial stages of corticogenesis. Despite this defect, $Ski^{-/-}$ brains revealed only a slight reduction of forebrain size at E17.5, and DAPI-stained coronal sections showed only a moderate reduction of cortical thickness compared with WT (Fig. S3 *A* and *B*). To further investigate the timing of neuronal generation, pregnant females were injected with BrdU at E10.5, E12.5, or E14.5 to label proliferating cells, and the total number of pulse-labeled progeny was assessed at E17.5 (Fig. S3 *C* and *D*). The percentage of total BrdU-positive neurons generated at E10.5 and E12.5 was lower in $Ski^{-/-}$ (Fig. S3*D*), presumably because of the decreased pool of progenitor cells at these time points (Fig. S2 *B* and *F*). However, we found an excess number of neurons born at E14.5 in the mutant (Fig. S3*D*). At the same time, the number of proliferating intermediate progenitors was increased in $Ski^{-/-}$ vs. WT (Fig. S4 *A*, *B*, and *D*), and the pool of Tbr2-positive progenitors was comparable between genotypes by E14.5 (Fig. S4*C*). Thus, during midcorticogenesis, intermediate progenitors are likely to substitute for the lack of sufficient numbers of progenitors during early corticogenesis, ultimately leading to comparable thickness and cell numbers in corresponding WT and mutant cortical layers at E17.5 (Fig. S3*B*).

Ski Is Required for Maintaining the Identity of Callosal Neurons. We next examined neuronal identities in mutant cortices. For this, we assessed and compared the distribution of cell type-specific markers by immunohistochemistry at E17.5 in the neocortex of WT and mutant (Fig. 2). Satb2 was expressed normally in all layers (CP, V, VI, SP) in the absence of Ski (Fig. 2*A*). In contrast, in $Ski^{-/-}$ mutants, expression of Ctip2, a marker for corticosubcortical projection neurons, was markedly expanded to the superficial layers of the CP (Fig. 2*B*). Further, the number of cells expressing Tbr1 in the UL neurons was reduced in Ski-deficient embryos, whereas Tbr1 expression in layer VI and in the subplate remained unchanged (Fig. 2C). To further characterize cells expressing Ctip2 ectopically, coronal sections were double stained for Satb2 and Ctip2 (Fig. 2 *D* and *E*). In the superficial layers of the CP, Satb2-positive cells were negative for the deep-layer marker Ctip2 in the WT (Fig. 2 *D* and *E*) (8), whereas in the mutant, most Satb2-positive cells ectopically expressed Ctip2 (Fig. 2 *D* and *E*). In deep layers, ∼2% (42/2,286) of total cells coexpressed Satb2 and Ctip2 in the WT (Fig. 2*D*), whereas in the mutant, the percentage of double-stained cells increased to ∼7% (166/2,444; Fig. 2 *D* and *E*). Collectively, these results demonstrate that subpopulations of Satb2-positive neurons in both upper and deep layers ectopically express Ctip2 upon loss of Ski, but that this effect is more prominent in UL neurons at the examined time point. To investigate the origin of the Ctip2 and Satb2 double-labeled cells in the upper layers, we performed BrdU pulse labeling at E12.5 and E14.5, and determined the distribution of BrdU-positive neurons among those expressing Ctip2, Satb2, or the specific UL marker Cux1 (Fig. 2*F* and Fig. S5 *A* and *B*). We find that in contrast to the WT, Ctip2-positive cells born at E14.5 reach the upper layers in $Ski^{-/-}$ (Fig. 2*F*). Mutant Ctip2-positive cells born at E12.5, however, migrate to deep layers as in the WT, and do not aberrantly reach the upper layers by E17.5 (Fig. 2*F*). Further, our results show that the distribution of Cux1-posititve neurons that were born at E14.5 is similar between genotypes at E17.5, suggesting that UL neurons are generated at the expected time point and migrate to the expected layers in the mutant (Fig. S5*A*). In support of this notion, very low numbers of Cux1-positive UL neurons were born at earlier stages, both in WT and $Ski^{-/-}$ (Fig. S5*B*). The analysis of Satb2-positive neurons born at E14.5 showed a similar distribution in WT and $Ski^{-/-}$ (Fig. S5*A*). In summary, UL neurons in the $Ski^{-/-}$ mutants are born at the expected time point, but ectopically express Ctip2. In support of this conclusion, we find increased expression of *Ctip2* mRNA in mutant cortices (Fig. 2*G*). These experiments show definitively that subpopulations of Ski-deficient Satb2-positive neurons change their genetic program. To further characterize the phenotype of the mutant callosal neurons, we performed in situ hybridization assays, including fate- and layer-specific markers (Fig. S5 *C–E*) (8). In addition to *Ctip2*, other DL-specific genes, such as *Clim1*/*Ldb2* and *Cdh13*, displayed an expansion of expression into upper layers in E18.5 *Ski* mutants. However, another DL-specific gene, the transcription factor *Fezf2*, was expressed normally, suggesting that Ski-deficient callosal neurons acquire some but not all characteristics of WT subcortical projection neurons (Fig. S5*C*). In addition, the expression of callosal projection neuron (CPN)-specific genes, including the expression of *Cdh10*, *Lmo4*, and *Ptn*, was up-regulated upon loss of Ski, revealing further changes in the regulation of the genetic program in callosal neurons (Fig. S5*D*). We also tested layer-specific genes, and found that *Cux2* was elevated in the intermediate zone, whereas

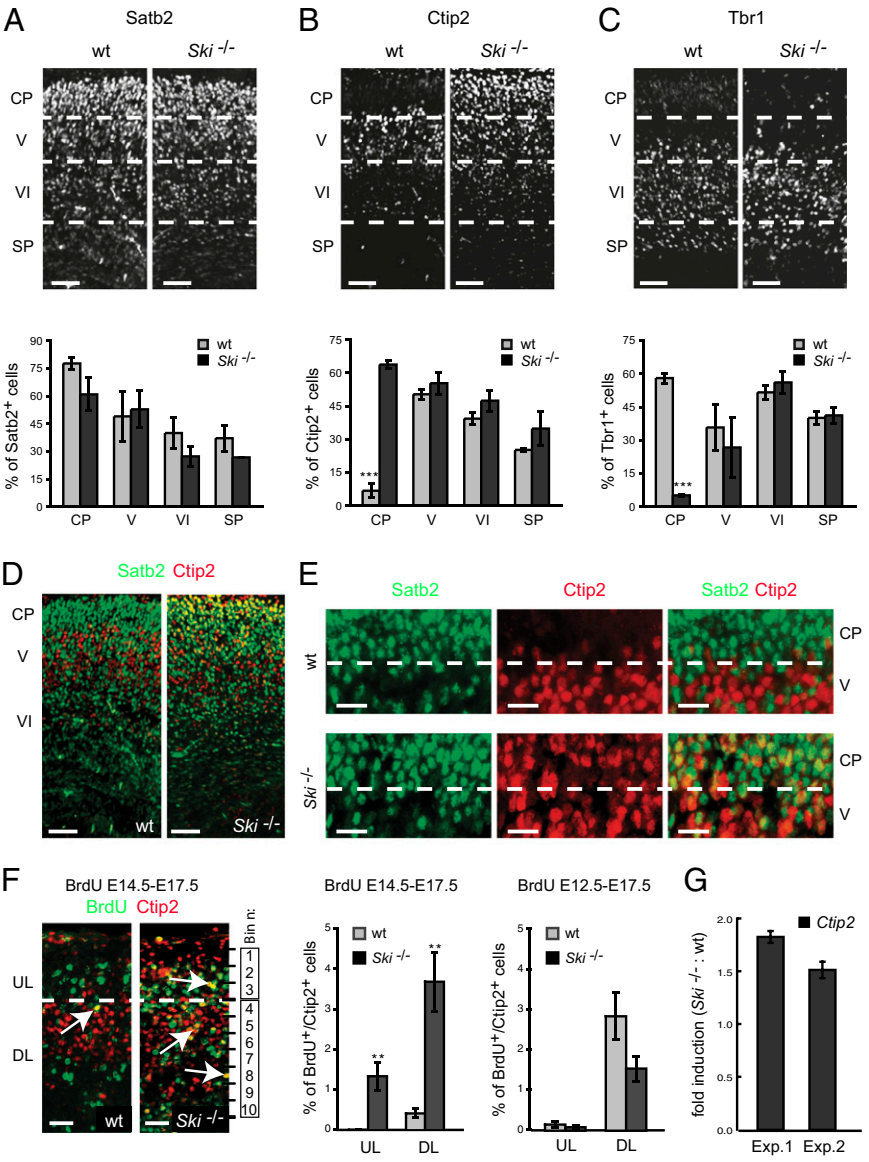

Fig. 2. *Ski* deletion affects Ctip2 and Tbr1 expression patterns in the dorsal telencephalon. (*A–C*) Satb2 immunostaining of E17.5 coronal brain sections is similar in WT and $Ski^{-/-}$ cortex (*A*). Ctip2 immunoreactivity has expanded to the superficial layers of the CP in the absence of *Ski* (*B*), and fewer cells in the upper layers of the CP express Tbr1 in $Ski^{-/-}$ mice (*C*). Quantification of Satb2-, Ctip2-, and Tbr1-positive neurons is shown for the superficial layers of the CP, the deep layers V and VI, and the subplate (SP) as a percentage of total DAPI-stained nuclei per field within the respective layer in WT (gray bars) and $Ski^{-/-}$ (black bars). Statistically significant differences were found in the numbers of Ctip2-positive cells (*B*) and Tbr1-positive cells (*C*) in the upper layers of the CP. (*D* and *E*) Double immunostainings for Satb2 and Ctip2 on E17.5 coronal brain sections in WT and $Ski^{-/-}$ (*D*). Higher-magnification images reveal ectopic expression of Ctip2 (red) in Satb2-positive cells (green) in *Ski* mutants (*E Lower*), whereas Ctip2 expression is absent in Satb2-positive cells of the WT (*E Upper*). (*F*) Photomicrographs of neocortical sections show the representative distribution of E14.5 BrdU birth date-labeled, Ctip2-positive cells in WT and $Ski^{-/-}$ (arrows). For the quantification of labeled cells the cortical thickness was divided into 10 equal bins. Bins 1–3 correspond to the upper layers (UL), and bins 7–10 to the deep layers (DL) of the cortical plate. The percentage of BrdU-labeled cells, double positive for Ctip2 in each region (UL, DL) relative to the total number of DAPI-stained nuclei per field, was determined in WT (gray bars) and $Ski^{-/-}$ (black bars; *Center* and *Right*). The analysis shows that the numbers of E14.5-born Ctip2-positive cells that populate the UL and DL are significantly increased in the mutant. However, the increase in Ctip2-positive cells in the mutant UL is not due to a precocious generation of these cells, because Ctip2-positive cells born at E12.5 are predominantly found in the DL in both genotypes. (*G*) Quantitative RT-PCR was performed to determine *Ctip2* mRNA levels in WT and $Ski^{-/-}$ cortices at E18.5. Ctip2 values were normalized to HPRT1 mRNA. cDNA from brains of two WT /$Ski^{-/-}$ littermates (experiments 1 and 2) were generated. Results are presented as ratios of *Ctip2* levels in $Ski^{-/-}$ and WT, demonstrating ~1.5- and 1.8-fold induction of *Ctip2* in the $Ski^{-/-}$ mutant. (Scale bars: *A–D*, 50 μm; *E* and *F*, 20 μm.) Data are the mean of at least three embryos per genotype. Error bars indicate SD in *A–C* and SEM in *F* and *G*. Student *t* test, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

expression of the transcription factor *bHLHb5* in layers II–V was reduced in the *Ski* mutant (Fig. S5*E*). However, not all expression patterns of genes were altered. For example, the expression of the signaling molecule *Dkk3*, and the layer-specific markers *Cux1*, *RORβ*, and *Bcl6*, remained normal in the absence of Ski (Fig. S5 *D* and *E*). Overall, Ski-deficient callosal neurons display a phenotype reminiscent of and partially overlapping with that of Satb2-deficient mice, where *Satb2* mutant neurons acquired ectopic expression of *Ctip2* and other DL-specific genes, and lost their identity as callosal projection neurons (7, 8).

We next evaluated whether the formation of the corpus callosum was also impaired in the *Ski* mutants (Fig. 3). Immuno-

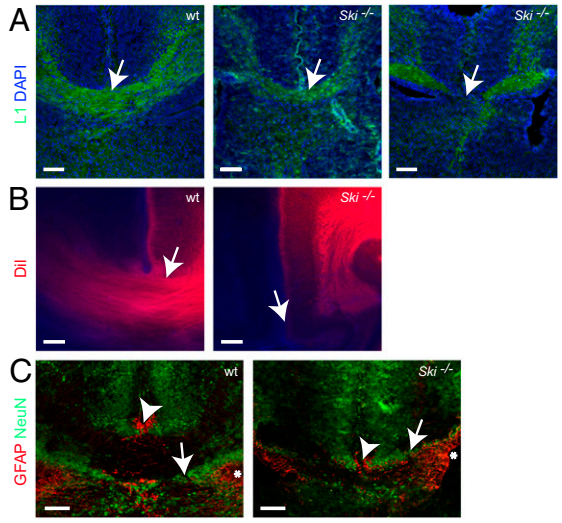

Fig. 3. Ski deletion leads to failure in the formation of the corpus callosum. (*A*) Immunohistochemistry for the axonal marker L1 on E18.5 coronal brain sections depicts axonal projections forming the corpus callosum. In comparison with WT (*Left*, arrow), the population of axons crossing the corpus callosum is largely decreased (*Center*, arrow) or is completely missing (*Right*, arrow) in *Ski*$^{-/-}$ embryos. (*B*) DiI labeling from the neocortex at E18.5 demonstrates that cortical efferent fibers form the corpus callosum in WT but not in *Ski*$^{-/-}$ embryos (arrows). (*C*) Double immunohistochemistry for the neuronal marker NeuN and the glial marker GFAP shows the presence of the glial sling (arrow), the glial wedge (asterisk), and the indusium griseum (arrowhead) in WT and *Ski*$^{-/-}$. Note that these structures are present in *Ski* mutants, but the corpus callosum is missing. (Scale bars: 100 μm.)

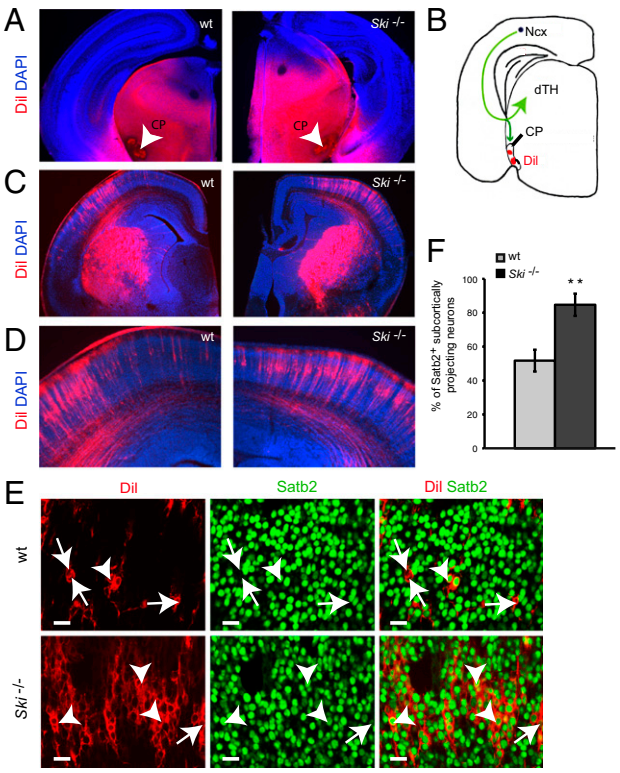

Fig. 4. Callosal neurons redirect their axons toward subcortical targets in *Ski* mutants. (*A*) The placement of DiI crystals in the CP (arrowheads) in WT and *Ski*$^{-/-}$. (*B*) Schematic representation showing the position of DiI crystal placement in the CP. Ncx, neocortex; dTH, dorsal thalamus. (*C*) DiI placed in the CP retrogradely labels subcerebrally projecting cortical neurons in both WT and *Ski*$^{-/-}$. (*D*) Higher magnifications of WT and *Ski*$^{-/-}$ cortical plate shown in *C*. (*E* and *F*) Colocalizing the retrogradely labeled neurons with Satb2 shows that the majority of DiI-labeled neurons are Satb2-positive in the *Ski*$^{-/-}$ mutant (arrowheads in *E*), whereas more DiI-labeled neurons are Satb2-negative in the WT (arrows in *E*). The percentage of subcortically projecting neurons that are Satb2-positive is significantly higher in *Ski* mutants compared with WT (*F*). (Scale bars: 20 μm.) Error bars indicate SEM. Student *t* test, $^{**}P \leq 0.01$.

histochemical staining for the neural cell adhesion molecule L1 revealed striking alterations in axonal connectivity in *Ski* mutants. Axonal tracts either failed to cross the midline, or the population of L1-positive axons forming the corpus callosum was largely decreased (Fig. 3*A*). To investigate the origin of axons arriving at the midline, we performed tract tracing by placing crystals of the lipophilic marker 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) in the neocortex of WT and *Ski*$^{-/-}$ at E18.5, allowing an anterograde labeling of cortical axons traveling to the contralateral hemisphere. Coronal sections of rostral levels showed that in contrast to the WT, DiI-labeled axons in the mutant approached the midline, but did not cross it (Fig. 3*B*). Similar to the findings in the *Satb2* mutant (8), the development of the glial sling, which is required for axons to grow contralaterally (9), appeared normal in the *Ski* mutant (Fig. 3*C*), suggesting that the malformation of the corpus callosum is not solely based on a midline defect.

Lack of Ski in Satb2-Positive Callosal Projection Neurons Causes Them to Project Ectopically to Subcortical Targets. We next examined whether axonal projections of Satb2-positive neurons were altered in the absence of Ski. For this, we placed DiI in the cerebral peduncle at E18.5 (Fig. 4 *A* and *B*) to retrogradely label corticosubcerebral projections (Fig. 4 *C* and *D*). In these experiments, we concentrated on the dorsomedial cortex, and we specifically studied the contribution of Satb2-expressing neurons to corticosubcerebral projections (Fig. 4*E*). We find that ∼55% (214/392) of subcerebrally projecting neurons, retrogradely labeled from the cerebral peduncle, were Satb2-positive in WT mice, whereas the percentage of DiI-positive, Satb2-expressing cells increased to ∼84% (343/408) in the *Ski* mutant. Thus, the percentage of Satb2-expressing neurons that project via the cerebral peduncle was markedly increased in the *Ski* mutants compared with the WT (Fig. 4*F*). Taken together, our data show that callosally projecting neurons redirect their axons to subcortical targets in the absence of Ski, as described previously in *Satb2*$^{-/-}$ mutants (7, 8). These similarities between the *Ski*$^{-/-}$ and *Satb2*$^{-/-}$ phenotypes prompted us to evaluate whether Ski and Satb2 act in a common genetic pathway.

Ski Interacts with Satb2 and Binds to Regulatory Regions of Ctip2 in Cortical Neurons. To investigate whether these two factors interact, we cotransfected HEK293 cells with Ski and Satb2 expression vectors and performed immunoprecipitation experiments using lysates of cotransfected and control untransfected cells (Fig. 5*A* and *B*). Immunoblots with Ski and Satb2-specific antibodies, respectively, showed that Ski and Satb2 coprecipitated (Fig. 5*A*). Because no Ski or Satb2 immunoreactivity was detected when using control antibody, this experiment identifies Satb2 as an intracellular partner of Ski. These results were confirmed using cortical lysates demonstrating the presence of Ski–Satb2 complex formation in vivo (Fig. 5*B*). To examine the location of endogenous Ski and Satb2 complexes in the developing cortex, we applied an antibody-based proximity ligation assay (PLA), which allows individual interacting pairs of protein molecules to be visualized in situ (10) (*Materials and Methods*). Specific Ski–Satb2 complex formation was observed in nuclei of UL neurons (Fig. 5*C*, *Top*, arrows), whereas there was no signal detectable on control *Ski*$^{-/-}$ or *Satb2*$^{-/-}$ sections (Fig. 5*C*, *Middle* and *Bottom*). We next investigated whether Ski targets regulatory sequences of *Ctip2* (Fig. 5 *D* and *E* and Fig. S6 *A*–*C*). Because Ski does not directly interact with DNA, but interacts with Satb2, we asked

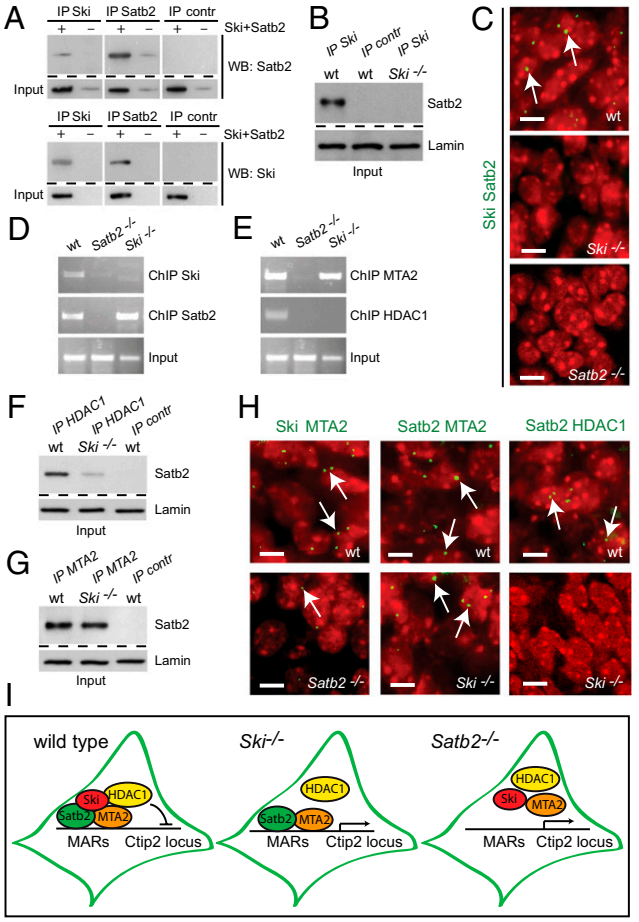

Fig. 5. Ski associates with Satb2 and represses *Ctip2* transcription in cortical neurons. (*A*) Lysates from Ski and Satb2-cotransfected (+) and untransfected (–) HEK cells were analyzed by immunoprecipitation (IP) with anti-Ski Ab (IP Ski), anti-Satb2 Ab (IP Satb2), or an unrelated control Ab (IP contr). Western blotting was subsequently performed using Abs against Satb2 (*Upper*) or Ski (*Lower*). Note that HEK cells endogenously express low levels of Satb2 (input in *Upper*), whereas there is no endogenous Ski detectable (input in *Lower*). (*B*) Lysates of WT cortical tissue were analyzed by IP with anti-Ski Ab (IP Ski) or an unrelated control Ab (IP contr), followed by immunoblotting using Abs against Satb2. An IP with anti-Ski Ab (IP Ski) with lysates of *Ski*$^{-/-}$ cortical tissue served as control to demonstrate the specificity of the anti-Ski antibody. Equal input of protein extracts was controlled by Lamin detection. (*C*) Endogenous Ski–Satb2 complexes were detected in situ in cortical neurons on WT brain sections using PLA. Panels represent magnifications of WT, *Ski*$^{-/-}$, and *Satb2*$^{-/-}$ superficial layers of the CP. The Duolink fluorescent probe 563 (*Materials and Methods*) was used as a hybridization probe (green), and the nuclei were stained with TO-PRO-3 (red). The Ski–Satb2 complex formation in the WT (*Top*, arrow) was specific, because there was no signal detectable in the *Ski*$^{-/-}$ and *Satb2*$^{-/-}$ (*Middle* and *Bottom*). (*D* and *E*) Semiquantitative ChIP assay was performed to detect protein occupancy at the *Ctip2* locus using WT, *Ski*$^{-/-}$, and *Satb2*$^{-/-}$ cortical tissue from E18.5/P0 pups. A 245-bp fragment was amplified from a previously described *Ctip2* regulatory DNA sequence, the matrix attachment region 4 (8) (Fig. S6 *A* and *C*). The samples were immunoprecipitated with anti-Ski and anti-Satb2 antibodies (*D*) or anti-MTA2 and anti-HDAC1 (*E*). (*F* and *G*) Lysates of WT and *Ski*$^{-/-}$ cortical tissue were analyzed by IP with anti-HDAC Ab (IP HDAC) (*F*) or anti-MTA2 Ab (IP MTA2) (*G*) followed by immunoblotting using Abs against Satb2 (*F* and *G*). An unrelated Ab was used as control (IP contr) (*F* and *G*). Equal input of protein extracts was controlled by Lamin detection (*F* and *G*). Note that Satb2–HDAC complex formation is drastically reduced in *Ski*$^{-/-}$ (*F*), whereas Satb2–MTA2 complex formation is unaffected in the absence of Ski (*G*). (*H*) Using PLA, endogenous protein complex formation was detected in UL neurons in situ as indicated on WT, *Ski*$^{-/-}$, and *Satb2*$^{-/-}$ cortical brain sections (arrows). Note that Satb2–HDAC1 complex formation is absent in UL neurons on *Ski*$^{-/-}$ sections. (*I*) Model for Ski function at the *Ctip2* locus in callosal projection neurons. Ski is required to assemble a functional NuRD repressor complex containing Satb2, MTA2, and HDAC1 at MAR sites in the *Ctip2* locus. In the absence of Ski, Satb2 still binds the regulatory DNA sequences together with MTA2, but recruitment of HDAC1 is impaired. In the absence of Satb2, the NuRD complex is not assembled. Thus, Satb2 and Ski play specific roles in the formation of a functional NuRD complex, and individual loss of these factors prevents transcriptional repression of *Ctip2* in callosal projection neurons. (Scale bars: *C* and *H*, 5 μm.)

whether Ski is recruited to the previously identified Satb2 binding sites in the *Ctip2* gene locus in vivo (8) (Fig. S6*A*). We performed ChIP with mouse E18.5/P0 cortical tissue using a Ski antibody and previously published primer pairs for targeting sequences of matrix attachment regions (MARs) within the *Ctip2* locus (amplicons within five *Ctip2* MAR regions: A1–A6) (8) (Fig. S6*A*). The analysis using cortical lysates revealed an enrichment of five MAR sequences (A1–A5), suggesting that Ski was targeted to complexes at multiple MAR sequences within the *Ctip2* locus (Fig. S6*B*). Because the enrichment of the A4 amplicon was most prominent, we next examined Ski–Satb2 complex formation at this site. Semiquantitative and quantitative PCR with primer pairs amplifying the A4 site disclosed a specific Ski protein–Ctip2 DNA complex in WT, which was substantially reduced in the absence of Satb2 protein and was undetectable in the *Ski*$^{-/-}$ negative control (Fig. 5*D* and Fig. S6*C*). In contrast, ChIP experiments with Satb2 antibody revealed that Satb2 binding to the A4 site was as efficient in the absence of Ski as in WT (Fig. 5*D* and Fig. S6*C*). Taken together, we demonstrate that in the presence of Satb2, Ski is recruited to the *Ctip2* locus in vivo, but Satb2 binding to the *Ctip2 cis*-regulatory region is independent of Ski. As previously reported, Satb2 down-regulates Ctip2 expression by interacting with two members of the nucleosome remodeling and deacetylase (NuRD) complex, the histone deacetylases HDAC1 and MTA2 (7). Thus, to further evaluate the role of Ski in the formation of repressor complexes on the *Ctip2* gene, we asked whether the NuRD–Ctip2 DNA complex was affected by *Ski* deletion. ChIP experiments with MTA2 and HDAC1 revealed that only MTA2, but not HDAC1, interacted with the *Ctip2* locus in the absence of Ski (Fig. 5*E* and Fig. S6*C*). To add further evidence that Ski is required for the recruitment of HDAC1 to the Satb2-containing repressor complex, we performed coimmunoprecipitation studies for Satb2, MTA2, and HDAC1 on cortical cell lysates (Fig. 5 *F* and *G*). Our data demonstrate that Satb2 and MTA2 interactions were largely unaffected in the *Ski* mutant compared with WT (Fig. 5*G*), whereas Satb2–HDAC1 complex formation was markedly reduced in the absence of Ski (Fig. 5*F*). Further, in situ PLA assays showed that in the absence of Satb2, Ski–MTA2 interactions were reduced compared with WT (Fig. 5*H*, *Left*), whereas MTA2–Satb2 interactions were readily found in the absence of Ski (Fig. 5*H*, *Center*), and no Satb2–HDAC1 complexes were detectable upon loss of Ski (Fig. 5*H*, *Right*). To further assess the role of Ski in transcription of *Ctip2,* we performed transient transfection assays with a *fos* luciferase reporter containing MAR region A4. As shown in Fig. S6*D*, concomitant expression of Satb2 and Ski in transfected COS cells was necessary to reduce the activity of the A4-*fos* luciferase reporter ~twofold, whereas no change in activity was detected upon expression of either protein alone. These results demonstrate that Ski is necessary for the Satb2-mediated A4 transcriptional repression in vitro, and further support our in vivo finding that both proteins are required to repress *Ctip2* expression. Alcamo et al. (8) previously demonstrated that forced expression of Satb2 alone led to a substantial decrease of the A4 sequence activity in mouse EL4 lymphoma cells. These cells are likely to express endogenous Ski, as previously reported for numerous cancer cell lines (3); this may explain the observed cell type-related differences. Taken together, our findings demonstrate that *Ctip2* is a direct target of Ski and Satb2 in cortical neurons, and that both proteins are required for efficient recruitment of members of the

NuRD complex to the *Ctip2* locus. Whereas Satb2 directly binds MAR sequences in the *Ctip2* locus and recruits MTA2 to the site, Ski is required for attracting HDAC1, thereby allowing the NuRD complex to form appropriately (Fig. 5*I*).

Discussion

In this report, we identify Ski as a determinant for the specification of callosal neurons, and provide evidence for a molecular mechanism underlying its mode of action in these cells. Previous in vitro studies demonstrated that Ski is a component of the complex containing N-CoR/SMRT, mSin3A, and HDAC1, and interacts with other HDAC1-associated proteins (3). However, the biological significance of these interactions is still largely unclear. In view of these facts, our findings are of particular relevance, because they provide insights into the molecular functions of Ski in vivo.

On the basis of numerous studies in normal and cancer cell lines, it becomes evident that expression levels of Ski strongly impact the molecular machinery of distinct cell types (3). In agreement, our findings show that Ski protein is selectively present in distinct subtypes of progenitor cells and projection neurons in the developing dorsal telencephalon, where it exerts cell type-specific functions. Thus, it is conceivable that Ski is not only specifying callosal neurons, but is also essential for the specification of subcerebrally projecting neurons as suggested by the misregulation of DL-specific genes. This finding is in line with previous reports describing the activities of Ski as cell and tissue dependent, insofar as Ski regulates differentiation of glia cells (6), as well as muscle and hematopoietic cells, through interactions with lineage-specific transcription factors (3).

In UL neurons, Ski and Satb2 are largely coexpressed and form repressor complexes in situ. In deep layers, however, the percentage of Ski-positive cells that coexpress Satb2 is lower. Accordingly, ectopic expression of Ctip2 is less distinct in deep compared with upper layers upon loss of Ski. It is known that subcortical and corticocortical projection neurons can coexist within a layer (11), and that Satb2-expressing DL neurons comprise a heterogeneous population of projection neurons (7). Thus, Ski might be selectively expressed in Satb2-positive callosal but not subcortical projection neurons within the deep layers. Alternatively, high expression of Ski might be only transiently required for initiating proper callosal neuron identity in young neurons, which would explain the higher percentage of Ski–Satb2 double-positive cells in later-born, UL callosal neurons at E17.5. Some neurons in *Satb2* mutants exhibit a delayed migration into the CP, a phenotype that is not observed in *Ski* mutants. Considering that Ski and Satb2 are coexpressed only in subtypes of projection neurons, it is conceivable that individual loss of these factors will only result in partially overlapping phenotypes. Indeed, the in situ hybridization data demonstrate that Ski-deficient neurons experience changes in gene expression that are only partially overlapping with the changes described in Satb2-deficient cortices. Thus, it appears that the role of Satb2 in radial migration is independent of Ski function.

Our data demonstrate that Ski function in Satb2-expressing callosal neurons is essential for maintenance of their identity. Similar to Ski (3), Satb2 has been shown to function both as transcriptional activator (12) and repressor (7, 8). The present work demonstrates that Ski enables the Satb2-containing protein assembly to act as an inhibitory complex. Satb2 regulates gene expression by binding MARs and promoting higher-order chromatin organization (13). Alcamo et al. (8) identified several MAR binding sites in the promoter of *Ctip2*, and demonstrated that Satb2 interacts differentially with each of these regions. We now provide evidence that these interactions are independent of Ski, and that Ski is essential for recruiting HDAC1 to the protein assembly. Previous work has shown that methylation at K4 of histone H3 activates transcription (14). Studies in differentiated Satb2-deficient neural stem cells showed preferential H3K4 dimethylation at site A4 (8), indicating that chromatin at this site is transcriptionally active in the absence of Satb2. In support of this finding, our ChIP experiments reveal that binding of Ski is strongest to the A4 site in WT cortical tissue, whereas binding to the other identified Satb2 binding sites (amplicons A1–A3, A5, and A6) was weaker or not detectable. This observation suggests that in UL neurons, not all MAR sites within the *Ctip2* locus are equally targeted by Ski, which is reminiscent of the results obtained from cultured neural stem cells upon forced Satb2 expression (8).

Our findings indicate that Ski and Satb2 might cooperate in repressing additional DL-specific genes besides *Ctip2*, but that they differently influence the expression levels of CPN-specific molecules. Alcamo et al. (8) observed reductions in the expression of CPN-specific factors upon loss of Satb2,and suggested that Satb2 might directly regulate their transcription. In support of this assumption, expression levels of CPN-specific genes remain normal or are elevated in Ski-deficient callosal neurons, where Satb2 is still expressed. It is conceivable that Ski acts as a transcriptional inhibitor of distinct CPN-specific genes, possibly by negatively regulating transcriptional activity of Satb2. Based on these results, we suggest that callosal neurons acquire characteristics of subcortical projection neurons through ectopic expression of DL-specific genes rather than through selective up- or down-regulation of CPN-specific molecules.

Materials and Methods

All mouse studies were approved by the veterinary office of the Canton of Basel-Stadt. Ski mutant mice were generated and genotyped as described previously (5), and maintained on a pure C57BL/6 background. We considered the day of vaginal plug as E0.5. *Satb2*$^{-/-}$ P0 pups (7) were provided by V. Tarabykin (Charité, Berlin).

Experimental details can be found in *SI Materials and Methods*.

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