Transcriptional control of the establishment of neocortical projections in the mammalian telencephalon

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DECLARATION

This thesis has been written independently and with no other sources and aids than quoted.

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TABLE OF CONTENTS

ABSTRACT	1
1. INTRODUCTION.....	3
1.1 Structure of the neocortex.....	3
1.2 Neocortical projections.....	5
1.3 The molecular mechanism of axon guidance	7
1.4 The corpus callosum.....	8
1.5 Role of Netrin1, Unc5C and DCC interactions in cortical axon guidance	9
1.6 Axon guidance at the midline	10
1.7 Axonal branching.....	12
1.8 Microtubule assembly and function in neurons.....	14
1.9 <i>Ninein</i> – Organization and function	16
1.10 Transcriptional control over establishment of neocortical projections.	17
1.11 Role of <i>Satb2</i> and <i>Ctip2</i> in controlling axonal trajectory choice	18
1.12 Transcriptional role of <i>Sip1</i>	18
1.13 Role of <i>Sip1</i> in CNS development.....	19
1.14 <i>SIP1</i> and Mowat-Wilson syndrome	21
1.15 Scope of the study: Transcriptional role of <i>Sip1</i> , <i>Satb2</i> and <i>Ctip2</i> in axon guidance and corpus callosum formation.....	21
2. MATERIALS AND METHODS.....	23
2.1 Mouse mutants used	23
2.1.1 <i>Sip1</i> conditional knockout	23
2.1.2 <i>Satb2</i> null mice	23
2.1.3 <i>Ctip2</i> null mice	24
2.1.4 <i>Netrin1</i> and <i>Unc5C</i> mutants.....	24
2.2 Genotyping	24
2.2.1 <i>Sip1</i> -floxed and wild type alleles:	25
2.2.2 <i>Cre</i> allele (for <i>Nex-Cre</i>).....	25
2.2.3 <i>Satb2</i> null allele	25

2.3 Tissue processing	26
2.3.1 Embryonic and early postnatal tissue	26
2.3.2 Perfusion	26
2.3.3 Cryo-sections	27
2.3.4 Paraffin sections	27
2.3.5 Vibratome sections	28
2.4 Histological staining	28
2.4.1 Nissl Staining	28
2.4.2 Immunohistochemistry using fluorescent primary and secondary antibodies	29
2.4.3 Immunohistochemistry using alkaline phosphatase based chromogenic reaction	30
2.4.4 Immunocytochemistry	31
2.4.5 Antibodies used	32
2.4.6 <i>In-situ</i> hybridization	32
2.4.7 Immunofluorescence – Fluorescence <i>in situ</i> hybridization (IF-FISH)	36
2.5 DiI labeling	36
2.6 Image acquisition	37
2.7 <i>In utero</i> electroporation	37
2.8 Plasmids used	38
2.9 DCC knockdown assay using <i>shRNA</i>	39
2.10 Dissociated cortical neuron culture	40
2.11 Nocodazole assay	41
2.12 GFP-EB3 +TIPs live imaging	41
2.13 <i>In vitro</i> cortical explant outgrowth assay	42
2.14 Organotypic slice culture and bead implantation assay	43
2.15 Chromatin-Immunoprecipitation (ChIP)	43
2.16 Luciferase Assay	44
2.17 Data analysis and Statistics	46
3. RESULTS	47

3.1 Commissural projections are severely altered in the <i>Sip1</i> conditional knockout.....	47
3.2 Cortico-fugal projections are affected in the <i>Sip1</i> conditional knockout	48
3.3 Retrograde DiI labeling reconfirms the absence of the corticospinal tract and the presence of the corticothalamic tract.	50
3.4 The corticospinal tract is present at early developmental stages in the <i>Sip1</i> mutant	50
3.5 Loss of corticospinal tract is not a due to cell death of Layer V neurons	51
3.6 Midline glia is almost completely lost in the <i>Sip1</i> conditional mutant ...	53
3.7 Subcallosal sling is not formed in the <i>Sip1</i> conditional mutant	54
3.8 <i>Slit1</i> , <i>Sema3C</i> and other axon guidance molecules show altered expression levels in the <i>Sip1</i> mutant	56
3.9 The lack of <i>Sema3C</i> expression in the <i>Sip1</i> mutant is a non-cell autonomous effect.....	59
3.10 <i>Sip1</i> mutant shows altered midline morphology	60
3.11 Altered distribution of neurons at the midline is a non- cell autonomous effect of <i>Sip1</i> deletion	61
3.12 Cell autonomous defects also contribute to the acallosal phenotype in the <i>Sip1</i> mutant.....	64
3.13 Mosaic deletion of <i>Sip1</i> is achieved by electroporation of <i>Cre recombinase</i>	65
3.14 Cell intrinsic deletion of <i>Sip1</i> also affects CC formation.....	66
3.15 Callosally projecting neurons within the cingulate cortex are also affected by <i>Sip1</i> deletion	68
3.16 Cell autonomous deletion of <i>Sip1</i> slows the rate of axon extension	69
3.17 <i>Sip1</i> deficiency affects ipsilateral axon-collateral formation	70
3.18 Microtubule associated protein – ninein, is downregulated in the <i>Sip1</i> mutant.....	73
3.19 <i>Sip1</i> regulates <i>ninein</i> expression through direct binding.....	74
3.20 <i>Sip1</i> binding to the <i>ninein</i> enhancer is functional	76
3.21 Ninein can be detected in the axons, axonal branches and the axonal growth cone of developing neurons	77

3.22 Axonal growth is retarded in <i>Sip1</i> deficient neurons <i>in vitro</i>	80
3.23 Restoration of <i>ninein</i> expression in <i>Sip1</i> deficient neurons rescues the corpus callosum.....	81
3.24 Restoration of <i>ninein</i> expression in <i>Sip1</i> deficient neurons restores ipsilateral axonal branching of layer II and III projection neurons	83
3.25 Retarded microtubule growth contributes to the stunted growth of <i>Sip1</i> deficient axons and is controlled by <i>ninein</i>	85
3.26 Ninein influences the stability of axonal microtubules.....	86
3.27 <i>Unc5C</i> expression requires <i>Satb2</i> and is restricted to callosal neurons.	89
3.28 <i>Ctip2</i> directly represses <i>Unc5C</i> expression	92
3.29 Partial Restoration of the CC in <i>Satb2</i> ^{-/-} ; <i>Ctip2</i> ^{-/-} compound mutants	96
3.30 <i>Satb2</i> deficient callosal neurons have defects in axon targeting and not outgrowth or midline guidance	97
3.31 Restored expression of <i>Unc5C</i> in <i>Satb2</i> ^{-/-} mutants results in partial rescue of CC	98
3.32 Neocortical callosal axons are repelled by <i>Netrin1</i>	100
3.33 Misrouting of <i>Satb2</i> positive axons in both <i>Netrin1</i> and <i>Unc5C</i> mutants.	102
3.34 <i>Satb2</i> is a direct repressor of <i>DCC</i> transcription.....	104
3.35 Downregulation of <i>DCC</i> in <i>Satb2</i> mutants results in partial rescue of the CC	108
3.36 <i>Netrin1</i> / <i>DCC</i> / <i>Unc5C</i> interactions regulate callosal projections of only deep layer neurons	111
3.37 <i>Satb2</i> has limited capacity to restore the corpus callosum through cell intrinsic mechanisms	112
4. DISCUSSION	116
4.1 Commissural and sub-cortical projections are disrupted in the <i>Sip1</i> mutant.....	117
4.2 Dual role of <i>Sip1</i> in CC formation	120
4.3 Non-cell autonomous role of <i>Sip1</i> at the cortical midline	121
4.4 Cell autonomous regulation of axon development by <i>Sip1</i>	123

4.5 Mechanism involved in cell intrinsic control of <i>Sip1</i> over axon growth and guidance	123
4.6 Ninein localization and function	125
4.7 Mode of action: Ninein stabilizes microtubules	126
4.8 Ninein function in axon collateral formation.....	127
4.9 Microtubule stability influencing microtubule growth.....	128
4.10 New insights gained: The role of <i>Sip1</i> in axon development	130
4.11 <i>Satb2</i> and <i>Ctip2</i> control the formation of divergent axonal paths.....	132
4.12 Lack of <i>Ctip2</i> repression in the absence of <i>Satb2</i> contributes to the acallosal phenotype in <i>Satb2</i> ^{-/-} mice	133
4.13 <i>Unc5C</i> is under the direct transcriptional control of <i>Ctip2</i> and indirect control of <i>Satb2</i>	133
4.14 <i>Unc5C</i> mutants also show corpus callosum defects	134
4.15 <i>Unc5C</i> aids in corpus callosum formation in response to <i>Netrin1</i> expression.....	135
4.16 <i>Unc5C</i> and <i>Netrin1</i> mutants show similar axonal behavior albeit dissimilar callosal phenotype.....	136
4.17 Together with <i>Netrin1</i> - <i>Unc5C</i> interaction, <i>Netrin1</i> - <i>DCC</i> interaction regulates medial vs. lateral axonal trajectory choice	137
4.18 <i>Satb2</i> ^{-/-} , <i>DCC</i> ^{-/-} and <i>Netrin1</i> mutants show disparate accallosal phenotypes.....	138
4.19 Cell intrinsic control of <i>Satb2</i> / <i>Ctip2</i> and hence <i>DCC</i> / <i>Unc5C</i> in the formation of commissural vs. corticofugal projections is restricted to a sub-population of deep layer neurons	138
4.20 New insights gained : Understanding the molecular basis of axonal trajectory choice.....	139
REFERENCES	141
ACKNOWLEDGEMENTS.....	158

LIST OF FIGURES

<i>Figure 1. Cortical projections are severely altered in the Sip1 mutant.....</i>	<i>47</i>
<i>Figure 2. Commissural and corticofugal projections are affected in the Sip1 mutant.....</i>	<i>48</i>
<i>Figure 3. Corticospinal tract is present at birth (P0) in the Sip1 mutant.....</i>	<i>51</i>
<i>Figure 4. No apparent cell death of Sip1 deficient layer V neurons.....</i>	<i>52</i>
<i>Figure 5. Midline glia is absent in the Sip1 mutant.....</i>	<i>53</i>
<i>Figure 6. Calretinin positive sub-callosal sling neurons are mislocalized.....</i>	<i>55</i>
<i>Figure 7. Many axon guidance molecules including Slit1 and Sema3C show altered expression pattern in the Sip1 mutant.....</i>	<i>56</i>
<i>Figure 8. Loss of Sema3C expression in the Sip1 mutant is a non-cell autonomous effect.....</i>	<i>59</i>
<i>Figure 9. Sip1 mutant midline is narrower and shows an altered distribution of cell density</i>	<i>60</i>
<i>Figure 10. Change in relative distribution of cell density at the midline is a non-cell autonomous effect of Sip1 deletion.....</i>	<i>62</i>
<i>Figure 11. Acallosal phenotype in Sip1 mutant is due to both cell-intrinsic as well as cell-extrinsic effects.....</i>	<i>64</i>
<i>Figure 12. Cell autonomous deletion of Sip1 through in utero electroporation of Cre.....</i>	<i>66</i>
<i>Figure 13. Callosal fibers fail to cross the midline on cell autonomous deletion of Sip1</i>	<i>67</i>
<i>Figure 14. Projections of cingulate callosal axons are also affected due to cell intrinsic deletion of Sip1</i>	<i>68</i>
<i>Figure 15. Lack of corpus callosum crossing with cell autonomous deletion of Sip1 is due to delay in axon extension</i>	<i>70</i>
<i>Figure 16. Upper layer neurons commence the formation of ipsilateral axonal collaterals at the level of layer V neurons at P4.....</i>	<i>71</i>
<i>Figure 17. Sip1 deficient upper layer neurons do not form ipsilateral axon collaterals.....</i>	<i>72</i>
<i>Figure 18. Ninein is downregulated in the Sip1 mutant cortex</i>	<i>74</i>
<i>Figure 19. Sip1 directly binds to ninein promoter region and regulates its expression.....</i>	<i>75</i>
<i>Figure 20. Luciferase assay confirms functional binding of Sip1 to ninein upstream region.....</i>	<i>76</i>
<i>Figure 21. Ninein localizes to the soma, dendrites, axon and axonal growth cone.....</i>	<i>78</i>
<i>Figure 22. Ninein localizes to axonal and dendritic branches in neurons.....</i>	<i>79</i>
<i>Figure 23. Sip1 negative neurons show stunted axonal growth in vitro, which is rescued by restoring ninein expression</i>	<i>80</i>
<i>Figure 24. Restoring ninein expression partially rescues the corpus callosum in brains with cell autonomous deletion of Sip1</i>	<i>82</i>
<i>Figure 25. Axon collateral formation by upper layer neurons is restored by expression of ninein in Sip1 deficient neurons.....</i>	<i>84</i>
<i>Figure 26. Microtubules grow slower in Sip1 deficient cells and the growth rate is restored in the presence of ninein.....</i>	<i>85</i>
<i>Figure 27. Ninein stabilizes microtubules in the axons</i>	<i>87</i>

<i>Figure 28. Unc5C is expressed by callosally projecting Satb2 positive neurons and Satb2 regulates Unc5C expression.</i>	90
<i>Figure 29. Ctip2 is up-regulated in the deep layers of the cortex upon Satb2 deletion</i>	92
<i>Figure 30. Unc5C expression is restored in Satb2^{-/-};Ctip2^{-/-} double mutants</i>	93
<i>Figure 31. Ctip2 directly represses Unc5C expression.</i>	94
<i>Figure 32. The corpus callosum is partially restored in Satb2^{-/-}; Ctip2^{-/-} compound mutants</i>	96
<i>Figure 33. Satb2^{-/-} neurons do not show defects in neurite outgrowth and midline guidepost cells are not altered in Satb2^{-/-}, Ctip2^{-/-} or Satb2^{-/-}Ctip2^{-/-} mutants</i>	98
<i>Figure 34. The corpus callosum is partially restored by over-expressing Unc5C in Satb2^{-/-} mutant cortex.</i>	99
<i>Figure 35. Netrin1 repels Unc5C positive neocortical axons.</i>	101
<i>Figure 36. Satb2 cells are similarly misrouted to subcortical targets in Netrin1 and Unc5C mutants</i>	103
<i>Figure 37. DCC is up-regulated in the Satb2^{-/-} mutant and Satb2^{-/-};Ctip2^{-/-} double mutant cortex.</i>	105
<i>Figure 38. Satb2 is a direct transcriptional repressor of DCC expression</i>	107
<i>Figure 39. Down-regulating DCC in the Satb2^{-/-} cortex leads to a partial rescue of the corpus callosum.</i>	109
<i>Figure 40. Over-expression of Unc5C with the simultaneous down-regulation of DCC does not rescue the corpus callosum.</i>	110
<i>Figure 41. Over-expression of Unc5C or down-regulation of DCC at E14.5 does not rescue the CC in Satb2^{-/-} mutants</i>	111
<i>Figure 42. Satb2 electroporation in Satb2^{-/-} cortex partially rescues the corpus callosum.</i>	113
<i>Figure 43. Quantification of CC with respect to efficiency of electroporation in wild type and Satb2^{-/-} mutant</i>	114
<i>Figure 44. Connectivity defects observed in the Sip1 mutant forebrain</i>	118
<i>Figure 45. Non-cell autonomous defects in the Sip1 mutant midline</i>	121
<i>Figure 46. Ninein localizes to neocortical axons and influences microtubule growth and stability downstream of Sip1</i>	130
<i>Figure 47. Satb2 Ctip2 dependent establishment of cortical connections</i>	132

LIST OF TABLES

<i>Table 1. Axon guidance molecules tested by ISH in the Sip1 mutant</i>	58
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ABSTRACT

Neuronal connections in the neocortex that relay and integrate information form the basis of our higher cognitive abilities. Transcription factors play an important role in controlling the trajectory of neuronal projections. They act as molecular switches capable of modulating downstream targets that in turn alter growth cone behavior. In this study we have investigated the role of three different transcription factors, namely *Sip1*, *Satb2* and *Ctip2* and their downstream mechanisms that influence the establishment of neocortical projections.

Sip1 is an important transcription factor that regulates many different aspects of CNS development. We show that, *Sip1* also plays an important role in the development of neocortical projections. In the absence of *Sip1* from the neocortex, many axonal tracts within the forebrain are severely affected. While the commissural projections like the corpus callosum (CC) and anterior commissure are not formed in the *Sip1* mutant, corticofugal connections like the cortico-spinal tract is also affected.

Sip1 exerts both cell autonomous as well as non-cell autonomous control over CC formation. The non-cell autonomous effects of *Sip1* deletion alter the cortical midline, making it unfavorable for commissural axons to cross. Fusion of the dorsal midline does not occur, different populations of GFAP positive midline glia are either reduced or absent and subcallosal sling neurons are mislocalized.

Cell autonomous deletion of *Sip1* also results in the lack of corpus callosum formation and lack of ipsilateral axon-collateral formation. *Sip1* mediates these effects through its direct downstream effector *ninein*, a microtubule binding protein. We further show that *ninein* in turn influences axon growth and branching by affecting microtubule stability and dynamics.

The transcription factors *Satb2* and *Ctip2*, are critical regulators of neuronal cell fate that control commissural versus corticofugal projections respectively. In this study, we have investigated the axon guidance molecules downstream of *Satb2* and *Ctip2* that govern the trajectory choice made by neocortical neurons. We show that *Satb2* and *Ctip2* transcriptionally regulate the expression of two Netrin1 receptors - *DCC* and *Unc5C* respectively. Within deep layer neurons, Netrin1- *Unc5C*/*DCC* interactions are involved in controlling the commissural versus corticofugal trajectory choice made by these axons.

1. INTRODUCTION

The neocortex, which forms the outer most cover of the brain, is arguably the crowning achievement of mammalian evolution (Hill & Walsh, 2005). It forms the biological substrate of our higher cognitive capacity that includes language, problem solving, reasoning, decision making etc., abilities that distinguish humans from other less-evolved species. As a first step in understanding these processes and how the brain computes seemingly complex tasks with tremendous ease, one must first understand the development of the neocortex (Geschwind & Rakic, 2013).

1.1 Structure of the neocortex

The mammalian neocortex is a highly organized structure comprising of a vast array of neurons that have different cellular identity, structure and function (Fishell & Hanashima, 2008a). This includes projection neurons, that extend their axons much further than their point of origin and are responsible for relaying information both within the cortex and to other sub-cortical structures, and interneurons that project their axons more locally and are involved in local circuitry. They have a modulatory function. The neocortex arises from a relatively smaller population of precursors or progenitors called radial glial cells that line the dorsal lining of the lateral ventricles called the ventricular zone (VZ) (Anthony et al., 2004; Malatesta, Hartfuss, & Götz, 2000a, 2000b; McConnell, 1995; Noctor et al., 2001; Noctor et al., 2002). Apart from producing neurons and glia, the radial glial cells also known as the apical progenitors give rise to basal progenitors that occupy the sub-ventricular zone (SVZ). The basal progenitors act as an additional step of amplification during neurogenesis. Sequential symmetric and asymmetric divisions of the progenitors give rise to the diverse set of projection neurons and glia that populate the neocortex (Fishell & Kriegstein, 2003; Miyata et al., 2001; Noctor et al., 2004). Within the cortex the projection neurons are

arranged in six different layers. The neocortex shows a characteristic inside-out manner of generation wherein the first neuronal layer produced (layer VI) occupies the inner most region of the cortex and every subsequent layer produced, then occupies the next higher position. Neurons occupying the deeper layers of the neocortex are generated earlier by apical progenitors and the neurons occupying the upper layers of the cortex are born later from basal or intermediate progenitors (Desai & McConnell, 2000; Frantz & McConnell, 1996; Haubensak et al., 2004; Molyneaux et al., 2007; Rakic, 1974; Takahashi, 1995; Tarabykin et al., 2001). Every neuron, cell intrinsically, possesses the ability to respond to the immediate cellular environment to migrate through the cortex, passing previously born neurons and occupying its respective position within the cortical plate (Angevine & Sidman, 1961; Huang, 2009; Kriegstein & Noctor, 2004a, 2004b; Nadarajah et al., 2001; Nadarajah & Parnavelas, 2002). Within a layer, neurons share to a large extent a common morphology, function and molecular identity (Fishell & Hanashima, 2008b; Greig et al., 2013; Guo et al., 2013; Kmet et al., 2013; Rouaux & Arlotta, 2013).

- **Layer VI** is comprised of the corticothalamic projection neurons that connect different regions of the cortex to specific nuclei within the thalamus.
- **Layer V** is comprised of pyramidal neurons that extend their axons all the way to the spinal cord relaying information out of the brain to subcerebral structures and hence are called the corticospinal neurons. These neurons also project to the optic tectum, striatum and pons. Layer V and VI are together referred to as deep layer (DL) neurons.
- **Layer IV** neurons also referred to as spiny-stellate neurons because of their morphology, play the critical function of receiving inputs from the thalamus. Hence axons of the thalamic neurons that convey information to the cortex innervate layer IV.
- **Layer III and II** comprise of projection neurons that primarily project within the cortex either within the same hemisphere or to the other hemisphere hence conveying and integrating information from

different parts of the cortex. Together they are referred to as upper layer neurons.

- **Layer I** is also called the molecular layer and contains the apical dendrites of the neocortical neurons and a population of cells called the Cajal retzius cells.

1.2 Neocortical projections

Figure (i). Major axonal pathways of the forebrain.

The figure shows a schematic representation of the major axonal tracts within the forebrain. Commissural projections that connect the two hemispheres with each other consist of the Corpus callosum (CC), Hippocampal commissure (HC) and Anterior commissure (AC). The corticofugal projections that connect cortical neurons to subcortical targets comprises of the Corticothalamic tract (CT) and Corticospinal tract (CST).

As neurons migrate to their final position within the different layers of the cortex they simultaneously project their axons to different targets (Noctor et al., 2004). The projections that arise from the different layers of the cortex fasciculate into axonal bundles forming the white matter of the brain (Innocenti & Price, 2005). Based on where they originate and where they project to, they can be broadly classified as

- Cortico-cortical connections
- Cortico-fugal connections

Cortico-cortical projections comprise of the intra-hemispheric projections, which connect neurons within the same hemisphere and inter-hemispheric commissural projections that connect one neocortical hemisphere with the other. The commissural projections of the cortex include the corpus callosum (CC) and the anterior commissure (AC). In the mouse, mainly axons from layer II and layer III and to a lesser extent from layer V project through the commissural path (Aboitiz & Montiel, 2003; Fame, MacDonald, & Macklis, 2011; Robin, 2006; Schüz & Preißl, 1996). The corticofugal projections are formed by axons that leave the neocortex and project to various subcerebral structures. These include the corticothalamic tract (CT) and the corticospinal tract (CST). Projections to other structures like the midbrain, pons etc. emanate as branches from the CST during development and are pruned when synaptic targets are recognized (Aronoff et al., 2010; Fame et al., 2011; Kennedy & Dehay, 1993; Molnár et al., 2007).

Apart from these projections that arise from within the neocortex, the thalamocortical axons (TC) project from the thalamus into the neocortex and the hippocampal commissure (HC) interconnect the two hippocampi. Since all of these cortical projections act as conduits for the passage of information to and from the cerebral cortex to other regions of the central nervous system, understanding how these connections are established forms an integral part of understanding brain function (Innocenti & Price, 2005).

1.3 The molecular mechanism of axon guidance

Observations of early development of axonal trajectory *in vivo* have revealed that the process of path finding is highly directed, precise and stereotyped. The axonal growth cone extends towards the target making apparently very few errors on the way. Axons sense a variety of molecules present in their immediate environment that help direct their trajectory. The axon in turn carries the machinery that responds to these guidance cues by extension, growth cone collapse or axonal turning. Apart from molecular-cue based axon guidance there also exists activity dependent axon guidance, which helps in axonal pruning and establishing the right synaptic contacts. Hence, molecular-cue based axon guidance seems to be an initial mode of navigation adopted by the axon for target finding and the activity dependent mode is involved in pruning these connections (Goodman & Shatz, 1993; Tessier-Lavigne & Goodman, 1996).

Work from over three decades in the field of axon guidance and path finding has led to the discovery of a plethora of ligands and their cognate receptors, which act as guidance cues and help in directing the axon towards its target and away from non-target locations. The guidance cues comprise of molecules that are secreted (and hence act over long-range) or membrane bound (acting over a short- range). They are also classified as chemo/contact - attractant or repellent based on the response that they illicit. These ligand-receptor pairs among others include Netrins - UNC5s/DCC, Robo-Slit, Semaphorins - Plexins and Neuropillins, Ephrins and Eph-receptors (Bagnard et al., 1998; Brose & Tessier-Lavigne, 2000; Kennedy et al., 1994; Kidd et al., 1998; Killeen & Sybingco, 2008; Kullander & Klein, 2002; Long et al., 2004; Nakamura, Kalb, & Strittmatter, 2000; Seeger et al., 1993; Serafini et al., 1996). Although one set of ligand - receptor pair might elicit one kind of response from an axon, it is a combination of these attractive and repulsive forces that defines the trajectory of the axon.

Furthermore, it is very interesting to see how the mechanism of guiding axons to their target has evolved so as to make the process more precise and time efficient. For example, many structures called ‘guideposts’ can be found within the axonal trajectories that act as intermediate targets. Axons are hence able to traverse long distances with relative ease as they respond to these intermediate cues that breakdown the entire journey of a neuron into smaller parts (Colón-Ramos & Shen, 2008). For example, commissural axons found in the brain and the spinal cord respond to groups of specialized cells present in the midline that are essential for directing the axons to the contralateral side (Colamarino & Tessier-Lavigne, 1995b; Klämbt, Jacobs, & Goodman, 1991). Another beneficial mechanism that neurons have adopted is the ‘pioneer and follower’ mechanism (McConnell, Ghosh, & Shatz, 1989). Pioneer axons are the very first set of axons that project from the place of origin towards the target. These show a much slower rate of growth and more elaborate growth-cone structure and dynamics. The follower axons fasciculate with the pioneers and hence show relatively simpler growth cone structure and extend much faster. If these axons need to deviate from the path they can in some cases develop the characteristics of a pioneer by elaborating their growth cone and consequently slowing down their growth rate (Bak & Fraser, 2003; Kim et al, 1991).

1.4 The corpus callosum

The corpus callosum (CC) is the largest axonal tract in the brain and consequently the most important commissural connection. It comprises of about 80 percent of the commissural fibers in the brain and helps in the coordinated exchange of information between the two cerebral hemispheres. In the mouse, a majority of the axons forming the CC originate in layer II and III, but few neurons of layer V also project medially through the corpus callosum (Aboitiz & Montiel, 2003; Fame et al., 2011; Robin, 2006; Schüz & Preißl, 1996). Partial or complete loss of the corpus callosum is called agenesis of the corpus callosum (ACC) and is associated with over 50 human

pathologies, suggesting that defects in a variety of developmental paradigms ranging from cell proliferation, migration to failure in axon guidance can result in abnormalities in this structure (Paul et al., 2007; Richards, Plachez, & Ren, 2004). Individuals with ACC present a range of behavioral as well as neuropsychiatric abnormalities such as learning deficits, sleep disorders, language and social communication disorders. The CC is pioneered by axonal projections of neurons from the medial most part of the cortex called the cingulate cortex (deAzevedo et al., 1997; Koester & O'Leary, 1994). The first set of fibers seen crossing the midline in case of the mouse is seen as early as E16.5, following which callosal axons from other regions of the cortex fasciculate and form the thick commissural bundle (Rash & Richards, 2001).

1.5 Role of Netrin1, Unc5C and DCC interactions in cortical axon guidance

Netrin1, the first identified diffusible long-range guidance cue, is expressed at the ventral midline of the hindbrain and spinal cord as well as ventral structures of the forebrain (Colamarino & Tessier-Lavigne, 1995; Kennedy et al., 1994; Métin et al., 1997; Serafini et al., 1996). It mediates both chemoattractive as well as chemorepulsive effects based on the receptor(s) expressed on the axonal growth cone (Colamarino & Tessier-Lavigne, 1995a). DCC is a receptor of Netrin1 and mediates an attractive response on binding to Netrin1 (Deiner et al., 1997; Keino-Masu et al., 1996). Unc5C is another known receptor of Netrin1 (Ackerman et al., 1997). Unc5C, either independently or together with DCC, mediates a repulsive response to Netrin1 signals (Chan et al., 1996; Finger et al., 2002; Gitai et al., 2003; Hong et al., 1999; Kim & Ackerman, 2011). In the forebrain, Netrin 1 is expressed in the ventral telencephalon within the ganglionic eminence, which gives rise to the basal ganglia (Hamasaki et al., 2001; Métin et al., 1997). It is also expressed in the ventral region of the dorsal midline during early embryonic stages till around E15.5 (Barallobre et al., 2000; T Fothergill et al., 2013). Unc5C and DCC, on the other hand, are expressed by cortical projection neurons. DCC is

more strongly expressed by neurons present in the cingulate cortex and Unc5C shows a stronger expression within the lateral cortex. The interaction between these molecules is crucial for CC development. While Netrin1 and DCC mutant brains lack a corpus callosum (Fazeli et al., 1997; Ren et al., 2007; Serafini et al., 1996), Unc5C deficient brains show the presence of callosal crossing (Finger et al., 2002).

1.6 Axon guidance at the midline

Figure (ii). Midline guidance structures that aid in corpus callosum formation

The figure shows a schematic representation of the midline glia as well as the sub callosal sling neurons that act as guidepost cells for the formation of the corpus callosum. The indusium griseum (IG), glial wedge cells (GW) and the midline zipper glia (MZG) form the midline glia that express different guidance cues like Slit 1, 2, 3, Wnt5a, Draxins etc. The subcallosal sling neurons, which are a mixed population of glia, glutamatergic and GABAergic cells also act as accessory guidance structures. A population of sling neurons expresses Sema3C. The cortical neurons in turn express cognate receptors or respective ligands that make them responsive to these midline cues, for example Robo1 and Robo2.

It has been shown that the midline of the central nervous system is a very critical organizing center with respect to axon guidance and establishment of commissural connections (Colamarino & Tessier-Lavigne, 1995b; Dickson & Zou, 2010; Evans & Bashaw, 2010; Kaprielian, Imondi, & Runko, 2000). Morphologically the dorsal midline has to fuse in order to allow for axons to navigate on a permissible substrate. At early embryonic stages when the birth of callosal neurons has commenced (E14.5- E15.5), the cerebral hemispheres are fused ventrally. Dorsally, they are separated mid-sagittally by a connective tissue filled fissure. The process of midline fusion has been suggested to involve the fusion of the basal lamina while simultaneously eliminating the fibroblast from the fissure, which is then replaced by neural tissue. This process is thought to be carried out by glial cells found in the midline that essentially 'zips up' the cortical midline. This population is hence termed as the midline zipper glia (MZG) (Silver et al, 1993).

The cortical midline, apart from acting as a substrate for axons to pass through, also acts as a locus that expresses a variety of physical and molecular guidance cues. These cues mediate a delicate balance between attractive forces, which help in bringing the axons from one hemisphere towards the midline, and repulsive forces, that allow these axons to leave the vicinity of the midline and move towards their target on the contralateral side. These signals have to be appropriately regulated to prevent the already crossed axons from re-entering the midline and re-crossing towards the ipsilateral cortex (Chen et al, 2008; Richards et al., 2004; Sabatier et al., 2004). The accessory midline structures that aid in CC formation can be grouped into two different populations. The first group of cells is the midline glia, which includes the glial wedge (GW) and the indusium griseum glia (IG), and the second group of cells is called the midline sling neurons. The GW cells arise from the dorso-medial edge of the lateral ventricle and are differentiated radial glial progenitors. They are GFAP positive and are present as wedge shaped structures in the ventro-lateral aspect of the CC crossing. The IG is also GFAP positive but has been proposed to originate from the midline zipper glia. It is

positioned dorsal to the CC crossing. Both these glial populations have been shown to express slit-2 and hence repel Robo-1 and Robo-2 expressing cortical axons *in vitro*. It is hence thought that the midline glial structures help in axonal turning of the CC at the midline and restricts the pathway of these axons so that they form the characteristic U-shaped turn (T Shu & Richards, 2001). For example, it has been shown that if the midline glial structure are severed, the CC fails to cross the midline and instead forms Probst bundles on either sides of the midline (Silver & Ogawa, 1983).

The midline glial population act in concert with the subcallosal sling neurons. The sling consists of immature neurons born between E15.5 and P2 in the dorsal SVZ that migrate towards the midline and occupy the ventral aspects of the CC. They have been found to express neuronal markers like NeuN and calretinin (Shu et al, 2003). They also play an important role in CC guidance as they express *Sema3C* that functions as an attractant molecule for the pioneering axons expressing *Nrp1*. Apart from the glutamatergic neurons, GABAergic interneurons are also found within the CC white matter as a part of the subcallosal sling (Niquille et al., 2009).

1.7 Axonal branching

Axons form the principal output structure of neurons as they transduce electrical impulses from the cell body to the synapse. In order for an individual neuron to connect to multiple targets the primary axon of the neuron frequently branches into collaterals. Axonal branch formation is a very dynamic process, which involves rapid extension and retraction of axonal projections resulting in a net enlargement of arborization. Three different processes have been used to describe branch formation (Acebes & Ferrús, 2000; Portera-Cailliau et al., 2005; Schmidt & Rathjen, 2010);

- **Splitting mode**, where the growth cone bifurcates and the two growth cones extend in divergent directions.

- **Delayed mode**, where a region of the axon with unstable cytoskeletal structure and filopodial activity is left in the wake of a growth cone, and an axonal branch sprouts from that point soon after the primary growth cone extends further.
- **Interstitial mode**, where axon collaterals emerge as a single outgrowth of the main axon shaft orthogonal to the primary axon. The branch initiation can take place days after the growth cone has passed the corresponding area (O'Leary & Terashima, 1988; Szebenyi et al., 1998; Yamamoto et al., 1997).

Delayed interstitial branching is not a very well understood mechanism and has garnered a lot of interest over the years. One possible mechanism proposed for collateral branching from specific regions of the axon refers to target derived signals that induce branching behavior in axons (Kennedy & Tessier-Lavigne, 1995) . Alternatively, it has also been shown that the axonal growth cone recognizes the target and exhibits a pausing behavior that involves extension, collapse and retraction in this region during axon extension. This growth cone behavior reorganizes the cytoskeleton in this region and demarcates the axonal position for future branch formation (Szebenyi et al., 1998).

Within the neocortex, the different classes of neurons have a characteristic axonal and dendritic branching pattern specific to the neuronal subtype and function. For example axons of layer II and III neurons extensively ramify within the layer and the descending axon also ramifies at layer V. The axon passes through layer IV without branching (Burkhalter, 1989; Kritzer & Goldman-Rakic, 1995; Thomson & Lamy, 2007). Axon guidance molecules that are implicated in influencing axonal trajectories have also shown to be involved in initiating axonal collateral formation (Harwell et al., 2012; Heffner, Lumsden, & O'Leary, 1990; Kennedy & Tessier-Lavigne, 1995; Leary, 1996). These guidance signals elicit molecular pathways within the neuron that finally translates into cytoskeletal modifications. Branch formation is

initiated by modulation of actin cytoskeleton and is then stabilized when a more rigid microtubule structure is formed.

1.8 Microtubule assembly and function in neurons

In responses to the different guidance cues discussed above, on ligand activation the receptors on the growing axon elicit downstream signaling cascades, which finally converge in remodeling the axonal cytoskeleton. These cytoskeletal elements are the ultimate effectors of movement and guidance (Dent, Gupton, & Gertler, 2011). The cytoskeleton of a cell acts as a scaffold that decides the shape of the cell, organization of its inner organelles and to a large extent the functioning of the cell. The cytoskeletal polymers are primarily actin-filaments and microtubules (MTs). MTs are tubular macromolecules formed by polymerized tubulin dimers namely α - and β -tubulin. MTs have a distinct polarity that governs their biological function. The slow-growing minus-end corresponds to the end of the MT which has an α - subunit exposed and the fast-growing plus-end corresponds to the region with the β - subunit is exposed (Baas & Ahmad, 1992). A characteristic property of MTs is that it exists in a state of dynamic instability, i.e. a constant state of growth (polymerization) and breakdown (catastrophe). This intrinsic property of MTs allows it to rapidly reorganize within a cell thus modifying the cell with respect to its environment or generate a pushing and pulling forces within a cell, which is essential for processes like cellular migration and growth (Inoué & Salmon, 1995; Kirschner & Mitchison, 1986). The centrosome is the cellular organelle predominantly associated with nucleation or birth of MTs. More generically it is referred to as the microtubule organizing center (MTOC) which includes the pair of centrioles surrounded by the centrosomal matrix, also called the pericentriolar material (PCM) from which MTs emanate (Kellogg, Moritz, & Alberts, 1994). In a neuron MTs are found throughout the cytoplasm and hence can be seen within the soma, axon and dendrites. MTs also show specialized organization within these structures. Within an axon the MTs are arranged such that all the minus-ends

are towards the soma and the plus-end or the growing end is directed towards the growth cone (Heidemann, Landers, & Hamborg, 1981). In case of dendrites, the MTs show mixed polarity (Baas, 1998; Keating et al., 1997). Although MTs are generated or nucleated at the centrosome they are released from the centrosome by MT cleaving enzymes like katanin and are then transported into the neurites by MT associated motor proteins like dynein. (Ahmad et al., 1999; Yu et al., 1993). These free MTs are essential for neurons to carry out their specialized function (Keating & Borisy, 1999). For example, since the length of an axon has been found to vary from a few millimeters to more than a meter long, a single MT molecule for the entire length of the axon would not provide stability or functionality to the axon. Hence a series of short overlapping microtubules can be found in the axon that help as tracks for the cargo carrying machinery in addition to acting as structural scaffolds (Ahmad & Baas, 1995; Baas & Yu, 1996; Bray & Bunge, 1981).

MTs are essential not only for polarizing neurons and for axonal and dendritic growth but also for neurite branching. It has been shown that a large degree of microtubule reorganization occurs at interstitial branch point of axons and dendrites (Dent et al., 1999). It has been suggested that similar to generating free microtubules in the axon, severing enzymes like katanin and spastin are involved in fragmenting microtubules within an axon, which are then transported into the developing axonal branches with the help of motor proteins such as dynein (Dent et al., 1999; Yu et al., 1994; Yu et al., 2008). Although a plethora of proteins that behave as MT associated proteins or MAPs have been discovered, not all of them have been assigned to a physiological or molecular function with respect to axonogenesis. Different MAPs have been shown to help in the assembly, growth or maintenance of the dynamic state of MTs (Conde & Cáceres, 2009). MT capping proteins form one class of MAPs. These bind to the minus-end of MTs and assist in nucleation and/or provide stability to the growing MT (Keating & Borisy, 1999). One such molecule that we have studied in the work presented here is *ninein*.

1.9 Ninein – Organization and function

Ninein, a 235 kDa coil-coil protein, was discovered as a centrosome associated protein localized to the pericentriolar matrix of the MTOC. It was shown to be associated with the centrosome throughout cell cycle and with mitotic spindles. It consists of a putative GTP binding site, four leucine zipper domains and a potential EF-hand-like domain and may exist in macromolecular complexes (Bouckson-Castaing et al., 1996). Interestingly, the human isoform of ninein has been shown to interact with the phosphorylating enzyme GSK3- β (Y. R. Hong et al., 2000), which has shown to be extensively involved in microtubule assembly and dynamics (Galjart, 2005; Hur et al., 2011; Kuijpers & Hoogenraad, 2011; Neukirchen & Bradke, 2011; Sakakibara et al., 2013; Schmidt & Rathjen, 2010). It has been suggested that the MTOC houses two distinct set of protein complexes, one that is involved in MT nucleation and the other that is involved in MT capping, anchoring and stabilization (Bornens, 2002; Mogensen et al., 2000; Mogensen, 1999). In cells that present the classical radial microtubule array, ninein was found to primarily localize to the mother centriole, which forms the center from where the radial microtubules emanate. Although, only the mother centriole has been shown to contain ninein, both the centrioles posses the same capacity to nucleate microtubules (Piel et al., 2000). Additionally, in polarized cells like the cochlear epithelial cells, where non-centrosomal microtubules are found, ninein was also shown to be associated with the minus-end of these MTs (Mogensen et al., 2000). γ -tubulin and pericentrin, molecules important for MT nucleation have been shown to be absent from these non-centrosomal MTs. It has hence been suggested that ninein plays a role in stabilizing, positioning and anchoring the minus-end of the MTs, centrosomal or released, and is probably not involved in nucleation. There is still considerable debate about the role of ninein with respect to nucleation of MTs, as work by Delgehyr et al. has shown that ninein acts as an accessory molecule in promoting MT nucleation by docking the γ -TuRC complex at the centrosome (Delgehyr, Sillibourne, & Bornens, 2005).

Much like MTs that show both centrosomal as well as non centrosomal localization in neurons, ninein is also found to be localized in the centrosome as well as dispersed within the cellular matrix associated with the minus-end of these non centrosomal MTs (Baird et al, 2004; Ohama & Hayashi, 2009). In the developing neocortex, *ninein* has been shown to be present in the progenitor rich ventricular zone as well as in differentiated neurons within the cortical plate. Ninein is localized to the centrosome in progenitors and is found bound to non-centrosomal microtubules of dendrites in differentiated neurons (Ohama & Hayashi, 2009; Wang et al., 2009). So far all the functional studies with respect to the role of *ninein* in the developing neocortex have pertained to the centrosomal ninein (Shinohara et al., 2013; Wang et al., 2009). The functional role of non-centrosomal ninein found in mature neurons is yet to be revealed and forms an essential part of this study.

1.10 Transcriptional control over establishment of neocortical projections

As pointed out earlier for the correct establishment of neuronal circuitry as well as to propel neurons to extend axons towards the right target, a repertoire of molecules in the form of ligands and their cognate receptors, down-stream signaling molecules, cytoskeleton binding and modifying proteins are involved. Needless to say, these different molecules need to be tightly regulated so that the right molecules are expressed at the right time in both the neurons as well as in the environment. Transcription factors act as molecular switches that control the expression of these molecules both spatially and temporally. For example, *Satb2* is expressed in the callosal neurons of the cortex and is required for the formation of the CC (Alcamo et al., 2008; Britanova et al., 2008). *Fezf2* and *Ctip2* transcription factors are expressed in deep layer neurons and are indispensable for the establishment and targeting of the CST (Arlotta et al., 2005; B. Chen et al., 2008; B. Chen, Schaevitz, & McConnell, 2005). Similarly, other transcription factors such as *Sox5*, *Thr1* and *Otx1* have also been shown to influence cortical projection

neuron identity (Bedogni et al., 2010; Han et al., 2011; Hevner et al., 2002; Kwan et al., 2008; Lai et al., 2008; McKenna et al., 2011; Weimann et al., 1999). In this study we have investigated the role of three such transcription factors *Sip1*, *Satb2* and *Ctip2* that control and coordinate the establishment of the cortical axonal trajectories.

1.11 Role of *Satb2* and *Ctip2* in controlling axonal trajectory choice

Two genes, *Satb2* and *Ctip2* have been shown in recent years to orchestrate important and mutually exclusive genetic programs that establish cortico-cortical versus corticofugal projections respectively (Alcamo et al., 2008; Arlotta et al., 2005; Britanova et al., 2008). While *Satb2*, a matrix attachment region (MAR) interacting protein has been shown to be indispensable for the formation of the CC, *Ctip2* on the other hand is required for the fasciculation and pathfinding of sub-cortically projecting neurons (Alcamo et al., 2008; Arlotta et al., 2005; Britanova et al., 2008; Szemes et al., 2006). Interestingly, *Satb2* regulates the expression of *Ctip2* as it binds to the upstream region of the *Ctip2* locus and brings about transcriptional repression of the gene. Thus, the deletion of *Satb2* in the cortex leads to an ectopic upregulation of *Ctip2* in upper layer neurons, with a corresponding misrouting of callosally projecting neurons to sub-cortical targets (Alcamo et al., 2008; Britanova et al., 2008). Thus, in normal development, *Satb2* overrides the *Ctip2* driven molecular pathway in order to establish inter-hemispheric projections versus corticofugal projections.

1.12 Transcriptional role of *Sip1*

Sip1 (Smad interacting protein 1) also called ZFHX1b or ZEB2 is a 140kDa nuclear protein that was identified using the yeast two -hybrid system to bind to the SMAD proteins (Postigo & Dean, 2000; Verschueren, 1999). SMADs are intracellular proteins that help in the signal transduction of

extracellular signals to the nucleus as a result of Tgfb β – serine/threonine kinase receptor complex activation (Heldin et al., 1997; Kretzschmar & Massague, 1998; Whitman, 1998). Sip1 shares a high degree of resemblance with the zinc finger homeodomain protein - ∂ EF1 (delta-crystallin enhancer binding factor 1). Similar to ∂ EF1, Sip1 also has conserved N and C terminal zinc finger motifs called NZF and CZF respectively that flank the homeodomain region (HD). This two-handed zinc finger domain confers DNA binding ability to Sip1. Sip1 has been shown to transcriptionally regulate gene expression by binding to a spaced bipartite CACCT(G) sequence on the promoters of target genes through its zinc finger domains (Remacle et al., 1999). *Sip1* has been shown to be an important regulator of the TGF β as well as BMP pathway. It has been shown to regulate gene expression by repressing transcription of many target genes in the TGF β and BMP pathway.

Sip1 requires the presence of activated R-SMADs i.e phosphorylated R-SMADs for binding to the SMAD protein (Postigo, 2003; Verschueren, 1999). Sip1 has also been shown to bind to the corepressor CtBP (C-terminal-binding protein) to bring about repression of various genes involved in the TGF β and BMP pathway (Postigo et al., 2003). Interaction of Sip1 with the NuRD complex has also been shown to be an important interaction in influencing target gene repression (Verstappen et al., 2008). Although the role of Sip1 has been largely characterized as a transcriptional repressor (Comijn et al., 2001; Grooteclaes & Frisch, 2000; Postigo, 2003), in certain cell contexts and with respect to certain genes, Sip1 has been shown to augment transcription in the presence and in the absence of SMADs (Yoshimoto et al., 2005), thus conferring a more global regulatory role to *Sip1*.

1.13 Role of *Sip1* in CNS development

Sip1 has been shown to be involved in neurogenesis in *Xenopus laevis* (Eisaki et al., 2000; van Grunsven et al., 2000). In the mouse, at early embryonic stages, around E8.5, *Sip1* mRNA expression can be detected in the developing

neural epithelium and in the neural crest cells. Homozygous *Sip1* mutants show a failure in neural tube closure, severely retarded growth, do not undergo embryonic turning and are not viable beyond E9.5 (Van de Putte et al., 2003).

Previous work from our lab has shown that *Sip1* expression can be detected in the developing cortical plate at E12.5 in postmitotic cells. As the population of postmitotic cells grows within the cortex, the expression domain of *Sip1* correspondingly expands. Expression of *Sip1* mRNA can also be detected in the developing basal ganglia and in the thalamus. (Miquelajauregui et al., 2007; Seuntjens et al., 2009). Interestingly the human isoform of *SIP1* shows a comparable expression pattern in the human CNS. *SIP1* mRNA is present in the cerebral cortex, cerebellum, corpus callosum as well as the thalamus (Cacheux et al., 2001).

In mice, *Sip1* has been shown to play a very important role in hippocampal development by regulating *Wnt* signaling, wherein the absence of *Sip1* the hippocampus is extremely reduced in size due to both cell death as well as decreased proliferation (Miquelajauregui et al., 2007). Further, seminal work from our lab has also shown that *Sip1* plays a very important role in cortical feed back signaling, wherein cortical post-mitotic neurons send signals back to progenitors and alter progenitor cell fate. In the absence of *Sip1* from the neocortex, there is a temporal shift in corticogenesis wherein during neurogenesis, upper layer neurons are generated earlier at the cost of deep layer neurons and at later developmental stages, glial cells are precociously generated at the cost of neurons. In the spinal cord, *Sip1* plays a central role in thermal pain sensation where it controls the transduction properties of the thermal nociceptive neurons by coordinating changes in the DRG-neuron voltage gated ion channels (Jeub et al., 2011). *Sip1* has also been shown to play an important role in myelination in the CNS (Weng et al., 2012). *Sip1* is also highly expressed in the neural retina and in the lens placode, where in the absence of *Sip1* severe deficiencies in ocular lens development could be

noticed (Grabitz & Duncan, 2012; Yoshimoto et al., 2005). Recently, two independent groups demonstrated the role of *Sip1* in interneuron differentiation and migration (McKinsey et al., 2013; van den Berghe et al., 2013). As seen from the above examples, the role of *Sip1* as a modulator of different developmental paradigms in the CNS has been well studied, but the role of *Sip1* in establishing cortical projections has not been investigated. Studying the role of *Sip1* in axon guidance and establishing correct cortical circuitry forms a principal aim of the study presented in this thesis.

1.14 SIP1 and Mowat-Wilson syndrome

Haploinsufficiency of *SIP1* due to nonsense or frame shift mutations in one of the alleles of *SIP1* gene has been found to cause the Mowat-Wilson syndrome (MWS). Patients suffering from this syndrome show severe mental retardation, motor deficits, epilepsy and craniofacial abnormalities etc. (Cacheux et al., 2001; Wakamatsu et al., 2001; K. Yamada et al., 2001). These effects are caused by a number of developmental disorders in the patients which frequently include microcephaly and agenesis of the corpus callosum (ACC) (Mowat, Wilson, 2003; Garavelli & Mainardi, 2007; Moal et al., 2007; Wilson et al., 2003). Thus *Sip1* is a very important molecule to be studied from a developmental as well as a clinical perspective. Since most MWS patients show ACC, studying the role of *Sip1* in CC formation deserves special attention.

1.15 Scope of the study: Transcriptional role of *Sip1*, *Satb2* and *Ctip2* in axon guidance and corpus callosum formation

In this study we have, in a broader sense, studied different molecular programs involved in the formation of axonal tracts in the forebrain, paying special attention to the corpus callosum. More specifically, we have studied the transcriptional role of *Sip1*, *Satb2* and *Ctip2* that modulate the expression of multiple axon guidance cues, guidepost structures and cytoskeletal

molecules. We have shown that mice lacking *Sip1* in the cortex show many anomalies in cortical projections. We have further dissected out the cell autonomous and non-cell autonomous role of *Sip1* in the formation of the CC. We have also studied at the role of transcription factors *Satb2* and *Ctip2* in controlling the trajectory choice made by deep layer neurons between taking a medial route forming the CC or a lateral trajectory forming the corticofugal tract. Here, we show that *Ctip2* and *Satb2* transcriptionally regulate a set of axon guidance molecules that help in deciding the axonal trajectory taken.

2. MATERIALS AND METHODS

2.1 Mouse mutants used

All mouse experiments were carried out in compliance with the German law and were approved by the Bezirksregierung Braunschweig, the Landesamt für Gesundheit und Soziales Berlin and the University of Queensland animal ethics committee.

2.1.1 *Sip1* conditional knockout

The *Sip1* floxed mice generated by Higashi et al., was used for the work presented in this thesis (Higashi et al., 2002). Lox-p sites were inserted flanking the exon 7 of the *Sip1* allele, which renders the protein inactive after *Cre* mediate excision. In order to generate *Sip1* conditional mutant, where *Sip1* has been deleted from postmitotic cells within the cortex, *Sip1^{fl/fl}* mice were mated with *Nex^{Cre}* mice (Goebbels et al., 2006). In these mice, *Cre recombinase* is expressed under the *Nex* promoter, which ensures expression only within the postmitotic population of the neocortex. *Sip1^{fl/wt} Nex^{Cre}* animals obtained from this cross were used as parents to generate the *Sip1* conditional knockouts designated as *Sip1^{fl/fl} Nex^{Cre}* mice. Since *Sip1^{fl/wt} Nex^{wt/wt}*, *Sip1^{fl/f} Nex^{wt/wt}*, *Sip1^{fl/wt} Nex^{Cre}*, littermates did not show any deviation from the wild type phenotype, they were interchangeably used as controls for the various experiments performed.

2.1.2 *Satb2* null mice

The *Satb2* null mice described in (Britanova et al., 2006) have been used in this work. Here, the *Satb2* null allele was generated through homologous recombination by elimination of the second exon in the protein-coding region and replacement with a *Cre* recombinase-coding sequence. *Satb2^{+/-}* mice were treated as wild type.

2.1.3 *Ctip2* null mice

Ctip2 null mice used in the experiments here were produced by conventional gene targeting described in (Wakabayashi et al., 2003). The animals were maintained either as *Ctip2*^{+/-} heterozygous parents or *Satb2*^{+/-}; *Ctip2*^{+/-} double heterozygous parents.

2.1.4 *Netrin1* and *Unc5C* mutants

The *Netrin1* hypomorphs and the *Unc5C* null mutants used were generated as described in (Ackerman et al., 1997; Serafini et al., 1996).

2.2 Genotyping

Tail tip of 3 weeks old mice was used for genotyping. Tail tissue was digested in 0.3ml Lysis buffer (100mM Tris- HCl pH8.5, 5mM EDTA, 200mM NaCl, 0.2% SDS, 100µg/ml Proteinase K) at 55°C for 2hrs to overnight. The samples were centrifuged at 9000 rpm for 10 mins to remove un-lysed tissue and residual hair. The DNA in the supernatant was precipitated by adding an equal volume of isopropanol, followed by gently mixing and centrifugation at 13000 rpm for 15mins. The DNA precipitate was washed twice in 80% ethanol, air dried and resuspended in 50 to 100µl sterile distilled water.

All PCR reactions were done in a final volume of 20µl in a mixture prepared according to the following protocol-

5x Buffer GoTaq (Promega) - 4µl

10mM dNTPs (Invitrogen) - 0.4µl (20pmol/ml)

Forward/Reverse primers (IBA) - 1µl (10pmol/ml) each

GoTaq polymerase (Promega) - 0.2µl (0.5 units)

Template DNA -1µl

ddH₂O -12.4µl

The following primer sequences were used to detect the amplified product:

2.2.1 *Sip1*-floxed and wild type alleles:

Forward- 5' TGGACAGGAACTTGCATATGCT 3'

Reverse- 5' GTGGACTCTACATTCTAGATGC 3'

Amplification program-

94°C- 10''

59°C- 20''

72°C- 40''

for 31 cycles;

Wild type allele yields a product of ~450bp and the floxed *Sip1* allele yields a product of ~600bp.

2.2.2 *Cre* allele (for *Nex-Cre*)

Forward- 5' TCGATGCAACGAGTGATGAG 3'

Reverse- 5' TTCGGCTATACGTAACAGGG 3'

Amplification program-

94°C- 10''

55°C- 30''

72°C- 40''

30 cycles;

Presence of at least one *Cre* allele yields a product of ~500bp.

2.2.3 *Satb2* null allele

To detect *wt* and *Satb2* KO alleles

5'- CAAGAGAGCCATCCAACCTGC- 3'

a reverse primer that recognizes *Cre* 5'- CCAGACCGCGCGCCTGAAGA- 3'

and 5'- AACCATCAGGCTCAACC3'

Amplification program-

94°C- 10''

55°C- 30''

72°C- 40''

30 cycles;

In *wt* mice, the PCR generated a fragment of ~400 bp where as mutant alleles generated a fragment of ~200 bp.

All PCR products were analyzed by electrophoresis on a 1.2-1.4% agarose gel at 90-120V. The products were separated in TAE buffer ((40mMTris- acetate, 1mMEDTA, pH 8) containing 1: 1x10⁵ Serva green (Serva Electrophoresis), and were visualized under UV illumination in a INTAS gel documentation system. The size of the PCR products was determined by comparing with standard DNA ladders (Bioline) loaded at 200ng/μl.

2.3 Tissue processing

2.3.1 Embryonic and early postnatal tissue

The day of the vaginal plug was considered as embryonic day 0.5 and the required embryonic stage for experiments was accordingly calculated. Pregnant females at the appropriate stage were sacrificed by administration of an overdose of Avertin, followed by cervical dislocation. The embryos were dissected out of the uterus into ice-cold 1X PBS and brains were isolated. The brains were fixed in 4% PFA in 1X PBS at 4°C and the duration was decided based on the embryonic age.

E12.5 to E13.5: 4 to 6 hours

E16.5 to E18.5: 8 to 10 hours

P0 to P4: 10 to 14 hours

P4 and above: perfusion followed by overnight (o/n) incubation

The fixed tissue was then washed twice or thrice with 1X PBS. The brains were further processed based on the type of histological sections required.

2.3.2 Perfusion

Mice older than P5 were fixed using perfusion with 4% PFA before immersing in the same solution overnight in order to ensure proper fixation of the tissue.

The mice were anesthetized by intraperitoneal administration of Avertin (100-300µl per animal, depending on the age). Subsequently, they were dissected open to expose the chest-cavity, rib cage was cut, followed by the diaphragm. A fine needle connected to a syringe with cooled PBS was inserted into the left ventricle and held in position. A small nick was made in the right auricle to expel the blood. This ensured circulation of PBS through the entire circulatory system, facilitated initially by the beating of the heart. As a sign of successful perfusion, visceral organs such as the liver can be seen to turn pale in color. The PBS was then replaced with 4% PFA and the perfusion was continued for a few minutes until certain prominent signs of penetration of PFA into tissues was detected. The signs would include, movement of the head (in case of young pups), movement of the tail (in juvenile and adult mice) etc. After this, the brain was dissected out (should appear pale and devoid of any blood in the vessels), and immersed in 4% PFA overnight.

2.3.3 Cryo-sections

The brains were taken through a series of sucrose gradients starting from 7% Sucrose in 1X PBS, followed by 15% sucrose and finally in 30% Sucrose. The brains were allowed to equilibrate in each solution till the tissue sunk to the bottom of the well. The brains were then washed one time in OCT (TissueTek) and placed in an embedding mould immersed in fresh OCT. The mould was then placed on a slab of dry ice to freeze the sample. The sample was then stored at -20°C or -80°C. Coronal sections with a thickness of 12µm to 20µm were cut using a Cryotome (Leica). Sections were collected on Superfrost plus slides (Menzel- Gläser) and stored at -20°C or -80°C till use.

2.3.4 Paraffin sections

The fixed brains were incubated overnight in 0.9% NaCl, followed by progressive dehydration through a series of increasing strength of ethanol (50% to 100%), followed by isopropanol. They were then incubated in toluol

for at least 6hrs, followed by Paraplast wax (maintained at 60°C) (twice, overnight). Finally they were embedded in wax within plastic chambers and sectioned into 10µm thick frontal sections using a Microtome (Leica). The sections were mounted on silane- coated slides (Marienfeld Histobond).

2.3.5 Vibratome sections

The fixed brains were embedded in melted 4% Agarose in 1X PBS and allowed to solidify. The block was then sectioned using a Vibratome (Leica) with a thickness varying between 60 to 100 µm. The floating sections were collected with a brush in 1X PBS with 0.1% Sodium Azide used as an antifungal agent.

2.4 Histological staining

2.4.1 Nissl Staining

Paraffin section of the brains were used for Nissl staining. The slides were firstly de-paraffinized by immersing them in Xylol for 10 minutes with 1 change. The sections were then re-hydrated by taking them through a gradient of decreasing concentration of ethanol from 100% ethanol to 30% ethanol for 2 minutes each ending in two washes in 1X PBS. The slides were then washed in double distilled H₂O and then incubated in 50% potassium disulfite for 15mins. They were washed again, and stained in Cresyl violet (1.5% in acetate buffer) for approx. 20mins. The sections were then washed twice in acetate buffer (10mM sodium acetate, 10mM acetic acid in H₂O) for 2 mins each to remove excess stain. This was followed by a very quick dip in Differentiation buffer (0.14% glacial acetic acid) until the desired color/intensity was obtained. The slides were then dehydrated again in 70% ethanol, followed by two-2 minute incubations in 100% ethanol. The slides were then immersed in Xylol for 10 minutes with 1 change. Finally, the

sections were mounted using a Xylol- based mounting medium (Eukitt) and dried before imaging.

2.4.2 Immunohistochemistry using fluorescent primary and secondary antibodies

The pre-treatment protocol for immunohistochemistry was slightly modified depending on the type of sections used. For paraffin sections, the slides were first re-hydrated in decreasing concentration of ethanol in H₂O from 100% to 30% for 2 minutes each followed by two 5 minute washes in 1 X PBS. The sections were then subjected to antigen retrieval by boiling the slides for 3mins in a preheated 0.96% antigen unmasking solution (Vector Labs), followed by cooling to 50°C, heating again for 3mins in the unmasking solution, and finally cooling to room temperature. The slides were then rinsed in PBS for 10mins. In case of cryosections, the slides were brought to room temperature and washed in 1X PBS for 10 minutes to remove the dried OCT surrounding the sections. In case of vibratome sections, no pre-treatment was required. The sections were then blocked to prevent background staining in 2% BSA/0.3% TritonX-100 in 1X PBS for 1 hour at room temperature (RT). Primary antibody cocktail at the appropriate dilution was made in the same blocking solution and sections were incubated in it overnight at 4°C. Three rounds of 15min washes in PBS at RT followed this. The sections were then incubated in secondary antibody cocktail made in blocking solution for 1 hour at RT. The secondary antibodies used were AlexaFluor- tagged secondary antibodies (1:500, Molecular Probes) or DyLight conjugated secondary antibodies (1:500, Jackson Labs). In some cases nuclear stain DRAQ5 was used along with the secondary antibodies (1:1000, eBioscience). The slides were finally mounted in a fluorescent mounting medium (DAKO Cytomation).

2.4.3 Immunohistochemistry using alkaline phosphatase based chromogenic reaction

The sections were pre-processed as mentioned above. In this case all types of sections including cryosection and vibratome sections were subjected to antigen retrieval or incubation at 65°C for at least 1 hour before blocking. This was done to inactivate endogenous alkaline phosphatase (AP). The blocking solution was made in TBS and all washes were also performed using 1X TBS. After overnight incubation at 4°C with primary antibody the sections were washed 3 times for 15 minutes with 1X TBS. Secondary antibody conjugated to AP (Promega) was used at 1:500 dilution in blocking solution for 1 hour incubation at RT followed by at least two 10 minute washes with 1X TBS and one wash with NTMT, pH 9.5. The AP substrate NBT/BCIP (Roche) in NTT (1:50) was added to the sections and incubated till the purple precipitate developed to the required intensity. The enzymatic reaction was stopped with two washes in 1X PBS and the sections were mounted using a fluorescent mounting medium (DAKO Cytomation).

1X TBS (Tris Buffer Saline)

50 mM Tris-Cl, pH 7.5

150 mM NaCl

To prepare, dissolve 6.05 g Tris and 8.76 g NaCl in 800 mL of H₂O. Adjust pH to 7.5 with 1 M HCl and make volume up to 1 L with H₂O.

NTMT (Alkaline Phosphatase buffer)- 200ml

100mM NaCl (4ml of 5M NaCl)

100mM Tris-Cl, pH 9.5 (20ml of 1 M Tris-Cl, pH 9.5)

50mM MgCl₂ (10ml of 1M MgCl₂)

1% Tween 20 (2ml)

H₂O to 200 ml

NTT (Alkaline phosphate buffer without Magnesium salt) – 200 ml

100mM NaCl (4ml of 5M NaCl)

100mM Tris-Cl, pH 9.5 (20ml of 1 M Tris-Cl, pH 9.5)

1% Tween 20 (2ml)

H₂O to 200 ml

2.4.4 Immunocytochemistry

Coverslips covered with cultured cells were first fixed in 4% PFA with 4% Sucrose in 1X PBS for 20 min at RT. They were then washed twice in PBS for 10min per wash, and left overnight in the same solution at 4°C. The cells were firstly permeabilised in 0.1% Tween20 in PBS for 15min at RT, followed by blocking of non-specific binding sites with the blocking solution (2% BSA in 0.1% Tween20 in PBS) for 1hr at RT in a humid chamber. The coverslips were then treated with the primary antibodies prepared in blocking solution overnight at 4°C. They were then washed three times in PBS for 15 min per wash, and incubated for in 1:500 diluted secondary antibody solution for 1hr at RT. Finally, excess secondary antibodies were washed off twice in PBS (10min each) and the coverslips were mounted in fluorescent mounting medium (DAKO Cytomation).

2.4.5 Antibodies used

Name	Species	Company	Dilution
Ac-tubulin	Rabbit	Sigma	1:3000
Calretinin (CR)	Rabbit	Swant	1:1000
Cre	Mouse	Sigma	1:500
Ctip2	Rat	Abcam	1:250
DCC	Rabbit	H.Cooper	1:100
dsRed	Rabbit	Clonotech	1:250
GFAP	Mouse	Sigma	1:500
GFP (1)	Goat	Rockland	1:500
GFP (2)	Chicken	Abcam	1:1000
L1	Rat	Millipore	1:200
Myc	Goat	Abcam	1:2000 (W.B)
Ninein	Rabbit	K. Hayashi	1:500
RFP (1)	Rabbit	Abcam	1:250
Satb2	Rabbit	Self Generated	1:1000
Sip1	Rabbit	Self generated	1:500
Tau-1	Mouse	Millipore	1:400
Unc5C	Rabbit	S. Ackerman	1:300

2.4.6 *In-situ* hybridization***Cloning and transformation***

800bp to 1000bp sequence within the coding region of the gene of interest was chosen and forward and reverse primers were designed accordingly. Using E16.5 mouse cDNA as template the selected region was amplified through PCR. GoTaq polymerase was used for the PCR amplification as it provides a 3' deoxyadenosine 'A' over hang in the amplicon. The amplicon was ligated into pGEMT plasmid vector (Promega) through TA ligation. The ligated

plasmid was transformed into DH5 α , XL-10G or XL-10blue competent bacteria. For this the plasmid mix was incubated with 100 μ l of competent cells on ice for 10 minutes followed by a heat shock at 42°C for 60 to 90 seconds and immediate cooling on ice for 5 minutes. The cells were then grown for 1 hour at 37°C in 1 ml LB media without antibiotic in a shaker. The cells were then pelleted down, resuspended in 100 μ l LB and plated on LB agar plates with ampicillin. For blue white selection of the colonies the LB agar plated were also coated with X-gal/IPTG before plating out the cells. The plates were incubated at 37°C over night. Colonies were picked the next day and grown in 5 ml LB media with ampicillin followed by plasmid extraction using Qiagen's mini-prep kit and columns. The presence of the DNA sequence of interest and the orientation was verified using restriction enzyme digestion and appropriate clones were selected and glycerol stocks of the culture was made and stored at -80°C. All *in situ* probes used in this study were obtained from Kuo Yan, Institute for Cell and Neurobiology, Charité-Berlin.

Plasmid linearization

The probe sequences (cloned into plasmids) were flanked by two different types of promoters (usually T3, T7 or Sp6) oriented in such a way that they could be used to synthesize either the antisense or the sense RNA. Therefore, depending on the requirement, the purified plasmids were linearised using a specific restriction enzyme (New England Biolabs) that would cleave the cDNA sequence at the end opposite to the promoter that will be used subsequently for probe synthesis. 1-5 μ g of plasmid DNA was digested per reaction. 10units of restriction enzyme was used for linearising 1 μ g plasmid DNA. Reactions were carried out at 37°C for 5hrs until the linearization was completed, which in turn was tested by gel electrophoresis. The linearized DNA was extracted from the gel by cutting out the portion of the gel with the linearized DNA band and processed using Qiagen- DNA gel extraction kit as per manufacturers instructions.

Synthesizing ribo-probe

Linearized plasmid DNA	1µl
DIG RNA labeling mix (Roche)	2µl
10x transcription buffer (Roche)	2µl
RNase inhibitor (Roche)	1µl (20 U)
RNA polymerase (T7, T3, Sp6) (Roche)	1µl
DEPC treated H ₂ O	13µl

Incubate for 2 hours at 37°C and purify using RNA cleanup kit (QIAGEN).

In-situ hybridization: Tissue processing and probe application (Day 1)

The embryos at the required stage were sacrificed and the brains were dissected in ice cold 1X PBS treated with DEPC to make it RNase free. The brains were then fixed overnight in 4% PFA-7% Sucrose in 1X DEPC treated PBS followed by incubation in 15% Sucrose and 30% Sucrose and finally cryo-embedding in OCT. 14 to 16 µm sections were cut using a cryotome and the sections were collected and dried on Superfrost slides for about 30 minutes. The sections were then post fixed in 4% PFA for 15 minutes followed by two washes with PBS. All solutions used on day 1 were treated with DEPC to make them RNase free. The sections were then treated with ProtienaseK (at 20µg/ml) for 3 minutes at RT to sufficiently permeablize the tissue. Following this step, the slides were immediately immersed in 0.2% Glycine in 1 X PBS for 5 minutes followed by two washes with 1x PBS. The sections were then treated with pre-hybridisation mix (50% formamide, 5x SSC pH7.0, 2.5M EDTA, 0.1% Tween-20, 0.15% CHAPS, 0.1mg/ml Heparin, 100µg/ml yeast tRNA, 50µg/ml salmon sperm DNA, 1x Denhardt's solution) for two hours at 65°C followed by incubation with RNA probe diluted in the hybridization mix, and incubated overnight at 65°C.

In situ hybridization: Blocking and antibody application (Day 2)

On the second day the tissue was treated with RNase A in RNase buffer for 30 minutes at 37°C followed by 3 washes with 2x SSC, pH 4.5 in 50% formamide at 65°C for 30 minutes each. The slides were then immersed in KTBT solution

for 5 minutes and then the tissue was blocked using 20% sheep serum. Sheep anti-DIG antibody (Roche, 1:1000) was applied overnight at 4°C.

In situ hybridization: washes and developing (Day 3)

On the third day, the tissue was washed in 6 times in KTBT and 4 times in NTMT. The reaction was developed by adding substrate NBT/BCIP (Roche, chromogenic) at 1:50, diluted in NTT solution. Upon reaching appropriate signal intensity, the reaction was stopped with at least two washes in PBS. The sections were dehydrated with increasing concentration of ethanol and mounted using Eukitt mounting media.

Solutions used

1X PBS- DEPC

1 ml of DEPC is added to 1l 1XPBS, Shake o/n at 37°C, autoclave

1X PBST

1l 1XPBS

1ml Triton X-100 (0.1%)

Proteinase K buffer

30mM Tris pH 8.0 (30ml of 1M Tris pH 8.0)

10mM EDTA pH 8 (50ml of 0.2M EDTA)

H₂O to 1000 ml

KTBT (1l)

150mM NaCl (30ml of 5M NaCl)

50mM Tris-Cl, pH 7.5 (50ml of 1 M Tris-Cl, pH 7.5)

10mM KCl (10ml of 1M KCl)

1% TritonX-1000 (10ml)

H₂O to 1000 ml

NT(M)T (Alkaline Phosphatase buffer)- 500ml

100mM NaCl (10ml of 5M NaCl)

100mM Tris-Cl, pH 9.5 (50ml of 1 M Tris-Cl, pH 9.5)

50mM MgCl₂ (25ml of 1M MgCl₂)

0.05% Tween 20 (250μl)

H₂O to 500 ml

RNase Buffer

15 ml 5M NaCl

1.5 ml 1M Tris pH 7.5

H₂O upto 150 ml

2.4.7 Immunofluorescence – Fluorescence *in situ* hybridization (IF-FISH)

The IF-FISH protocol was similar to the *in situ* hybridization protocol with certain specific modifications. The ProtienaseK step on day 1 was omitted for the IF-FISH protocol and was instead substituted with a 25-minute incubation in 2X SSC pH7.0. On day 2, for the IF-FISH, antibodies against Satb2 and Ctip2 were included together with anti DIG antibody. On the third day, the reaction was developed by adding substrate Fast Red (Abcam). Upon reaching appropriate signal intensity, for the IF-FISH, the sections were washed in PBS and stained with appropriate secondary antibodies.

2.5 DiI labeling

For anterograde cortical axon tracing, after fixing the isolated brains in 4%PFA, crystals of DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine-perchlorate) (Molecular probes) were placed with the help of a tungsten electrode used to make a fine incision into the somato-sensory, motor and visual cortical areas of the brain. After DiI placement, brains were kept for 2 weeks in case of P0 brains and 3-4 weeks in case of P5 brains at 37°C immersed in PBS with 0.1% Sodium Azide in the dark.

Alternatively, for retrograde tracing of cortico-thalamic or cortico-spinal tract, DiI crystals were placed in the thalamus or the cerebral peduncle. For this the brain was firstly cut coronally at the appropriate rostro-caudal level and then the dye was placed in the respective structures using the tungsten electrode. Methyl green solution was added onto the brains to increase the contrast and aid in crystal placement at the correct position.

After incubation for the stipulated time, brains were embedded in 4% Agarose (Applichem) and sectioned into 100 μ m thick sections using a vibratome (Leica). The sections obtained were counterstained with Hoechst (1:1000, Sigma) and mounted in PBS. Alternatively sections were processed for immunostaining with Satb2. No detergent was used for staining DiI labeled sections. Images were procured on a Leica SL/SP2 confocal microscope and counting was done manually. Statistics was done using R and Microsoft excel. Graphs were made on GraphPad prism 5.

2.6 Image acquisition

Bright field and fluorescence images were acquired using Olympus BX51 and BX60. Confocal images were acquired using Leica Confocal System-SL.

2.7 In utero electroporation

This procedure was carried out as described before (Saito, 2006). Pregnant and staged female was anesthetized in a chamber using isofluran. The animal was then placed on its back on a heating pad while constantly inhaling isofluran to stay anesthetized. Analgesic was administered subcutaneously. A vertical incision was made in the abdominal area and the uterine horns were carefully retrieved from the female without damaging the blood vessels and other visceral organs. The plasmid solution together with fast green dye used for visualization was filled into a fine capillary that was pulled using HEKA-

PIP6 capillary puller. The sharp end of the capillary was inserted into the head of the embryo so as to penetrate the skull and enter the lateral ventricles of the brain. Using a foot pedal pump, the plasmid was injected into the lateral ventricle. A square wave pulse consisting of 35mV current in 6 pulses was administered using an electrode and BTX electroporator. The uterus was constantly kept moist by addition of 1X PBS with penicillin streptomycin maintained at 37°C. This was then repeated for all the embryos and the uterus was re-inserted into the body cavity of the mouse. The body wall was sutured using sterilized sutures and the skin was clipped together using wound clips (Autoclip). The female was allowed to recover and put back into her cage. The female was monitored over the next days to ensure that the animal was normal. The embryos were then harvested at the chosen embryonic or postnatal stage.

2.8 Plasmids used

Plasmid name	Promoter	Protein expressed	Source
<i>pFUGW-Cre</i>	Ubiquitin	GFP IRES Cre	Dr. Hiroshi Kawabe
<i>pCAG-Cre</i>	CAG	Cre	Generated in the lab
<i>pCAG eGFP</i>	CAG	eGFP	Generated in the lab
<i>pCAG dsRed</i>	CAG	dsRed	Dr. Gregory Wulczyn
<i>pCAG FMF eGFP</i>	CAG	eGFP in the presence of Cre	Self generated
<i>pCAG FMF Sip1-myc</i>	CAG	Sip1-myc in the presence of Cre	Generated in the lab
<i>pCAG FMF eGFP-Ninein</i>	CAG	eGFP-Ninein in the presence of Cre	Generated in the lab
<i>pCMV eGFP-Ninein</i>	CMV	eGFP-Ninein	Dr. Michel Bornens
<i>pCAG Ninein</i>	CAG	Ninein	Generated in the lab

<i>pCMV GFP-EB3</i>	CMV	GFP-EB3	Dr. Judith Stegmüller
<i>pCAG GFP-EB3</i>	CAG	GFP-EB3	Generated in the lab
<i>pCβA-FLE_x</i>	β -actin	tdTomato when Cre is absent and eGFP in the presence of Cre	Dr. Ulrich Müller
<i>pCMV Ctip2</i>	CMV	Ctip2	Dr. Leid
<i>pCAG Ctip2</i>	CAG	Ctip2	Generated in the lab
<i>pCAG Venus</i>	CAG	Venus GFP	Dr. Handjantonakis
<i>pCAG Satb2</i>	CAG	Satb2	Dr. Nenad Sestan
<i>pCAG Ski</i>	CAG	Ski	Dr. Suzana Atanasoski
<i>pCAG Unc5C</i>	CAG	Unc5C	Dr. Franck Polleux
<i>pCAG DCC-myc</i>	CAG	DCC-myc	Dr. Helen Cooper
<i>pU6 DCCshRNA2</i>	U6	DCCshRNA	Genecopoeia
<i>pU6 DCCshRNA4</i>	U6	DCCshRNA	Genecopoeia

All the plasmids generated in the lab were cloned by Srinivas Parthasarathy, Institute for Cell and Neurobiology, Charité, Berlin.

2.9 DCC knockdown assay using *shRNA*

DCC shRNA clones were ordered from GeneCopoeia with the following target sequences

DCC shRNA 1: aagccggatgaaggacttt

DCC shRNA 2: agagaccatccaacgtaat

DCC shRNA 3: ttgtcgctacaatgagtg

DCC shRNA 4: agcagcgggtactattccat

The *DCC shRNA* plasmids were expressed in HEK-293T cells together with a plasmid coding for *DCC-myc* using Lipofectamine 2000 (Invitrogen). An unrelated RNA sequence was used for the scrambled *shRNA*. The cells were lysed after 2 days in lysis buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1mM

EDTA, 1% Triton X-100, 1x protease inhibitor cocktail (Sigma), pepstatin). Lysates were centrifuged for 20 minutes at 12000g, 4°C. Protein concentration was measured using BCA assay (Thermo scientific) and 30µg protein was loaded on a SDS-PAGE gel and transferred onto a PVDF membrane. The membrane was subsequently blocked for one hour in 3% BSA in TBST (TBS + 0.5% Tween-20), followed by overnight incubation in anti-Myc (1:2000) and anti-tubulin (1:50000) antibodies at 4°C. The membranes were washed in TBST on the next day and incubated for one hour in peroxidase coupled secondary antibodies (Jackson Laboratory). Chemiluminescence (ECL western blotting detection reagent, Perkin Elmer) was used for visualizing the protein bands. The chemiluminescence was captured using the Image lab software on a ChemiDoc XRS+ detector (Bio-Rad). The efficiency of knockdown was calculated using ImageJ.

2.10 Dissociated cortical neuron culture

On the previous day, sterile coverslips were placed in a 24 well plate and coated with poly-L-lysine at 0.1 mg/ml concentration and kept at 37°C overnight. The next day the plates were washed with water or PBS three times and the plates were left to dry within the cell culture hood. On the day of culture E16.5/E17.5 embryos were harvested and brains were isolated on ice cold 1X HBSS with Calcium and Magnesium - HBSS [++] (GIBCO). The two cerebral hemispheres were separated and the meninges were removed. The basal ganglia and the hippocampus were dissected out and only the cortex was harvested. If the brains were previously electroporated then the cortices were visualized under a fluorescent binocular and only the fluorescent tissue was dissected out. The tissue was pooled from the entire litter and collected in a 15ml falcon in HBSS [++]. The tissue was washed three times with 10 ml HBSS without Calcium and Magnesium HBSS - - (GIBCO). After the last wash 3.5 ml of HBSS [- -] was left in the falcon and 500µl of 2.5% Trypsin (GIBCO) was added to it and incubated at 37°C for 30 minutes. 2 ml of heat inactivated

horse serum was added to the tissue in order to inactivate the Trypsin. This was then followed by three 10 ml washes with cold HBSS [++]. After the last wash with minimum media around the tissue, 100 μ l of 1mg/ml DNase (Worthington) was added and incubated for 10 to 30 seconds. The tissue was then washed three times with 10 ml Neurobasal media (GIBCO). With the last wash, 2 ml of Neurobasal media was left behind in the falcon and the tissue was dissociated by using a 1 ml pipette. After all the cell clumps were broken 8 ml of Neurobasal media was added to this and centrifuged at 300 rpm, 4°C for 5 minutes. The supernatant was again centrifuged at 1200 rpm, 4°C for 5 minutes. The cell pellet was resuspended in warm (37°C) Neurobasal media supplemented with 1X Glutamax (GIBCO), 1% Penicillin- Streptomycin (GIBCO) and 1X B27 (GIBCO). The cell density was counted using a Neubauer chamber cell counter. The cells were plated on PLL coated coverslips at 50,000 cells per well density in a 24 well plate.

2.11 Nocodazole assay

Wild type mice belonging to the NMRI strain were electroporated with either *pCAG-FMF-eGFP* + *pCAG-Cre* plasmid or *pCAG-FMF-eGFP* + *pCAG-Cre* + *pCAG-FMF-eGFP-Ninein* plasmid cocktail at E14.5 and the embryos were harvested at E17.5. The electroporated region of the cortex was isolated, dissociated and cultured *in vitro* as described before. On DIV3 5mM nocodazole in Neurobasal complete medium was added to the neurons. Coverslips were harvested after 2 hours, 4 hours, 6 hours and 8 hours after treatment. The coverslips were fixed, stained for ac-tubulin and GFP and analyzed.

2.12 GFP-EB3 +TIPs live imaging

Sip1^{fl/wt} and *Sip1*^{fl/fl} embryos were electroporated with *pCAG-Cre* + *pCAG-GFP-EB3* plasmid at E14.5. *Sip1*^{fl/fl} embryos were also electroporated with

pCAG-Cre + pCAG-Ninein + pCAG GFP-EB3 plasmid cocktail. The embryos were harvested at E16.5 and the electroporated region was isolated, neurons were dissociated and cultured on FluroDish tissue culture dish. At DIV2 time series of GFP-EB3 comets were recorded using a Zeiss Spinning Disc system CXU-S1 at 1 frame per second for 200 seconds. The data was analyzed using the Kymograph ImageJ plugin.

2.13 In vitro cortical explant outgrowth assay

These experiments were performed in the lab of Dr. Linda Richards, Queensland Brain Institute, Australia. *Satb2Cre;Rosa flox stop lacZ* mice were time-mated (E13 or E17) and pregnant dams were anesthetized with 100mg/kg sodium pentobarbitone, and placed on a heating pad. Embryos were removed and placed in Leibovitz's L-15 medium (Invitrogen), the brain was removed, and the neocortex dissected. Explants and collagen gels were prepared and cultured exactly as described earlier (Thomas Fothergill et al., 2013). Explants displaying poor growth (those below one standard deviation of the mean level of outgrowth for all experiments across all conditions) were eliminated from the experiment. Each explant was stained with TuJ1 immunohistochemistry as previously described (Thomas Fothergill et al., 2013) and imaged using a Zeiss upright microscope equipped with an Apotome optical sectioning device. Digital images were analysed using a Matlab (The Mathworks Inc.) program to define a line through the center of the explant (center of mass of pixels representing the explant body). The number of pixels representing neurites on either side of this line was then counted (values U and D respectively). "Outgrowth" was defined as total neurite pixels (U+D) divided by the total number of pixels representing the neurite body (Mortimer et al., 2009; W.J.Rosoff et al., 2004). 46-139 explants per condition were pooled across four experiments and the amount of outgrowth analyzed using an unpaired two-tailed Student's t-test. Data are presented as average values $\pm$ standard error of the mean (SEM).

2.14 Organotypic slice culture and bead implantation assay

We cut 200–250- μ m-thick sections of E16.5 *Unc5C* or *EGFP* electroporated brains using a vibratome and cultured them on cell-culture inserts (1- μ m pore size, BD Biosciences) according to the protocol described previously (Polleux & Ghosh, 2002; E Seuntjens et al., 2009). The slices were cultured in a medium consisting of complete Hank's Balanced Salt Solution, basal medium Eagle, 20 mM D-glucose, 1 mM L-glutamine, penicillin (100 U ml⁻¹), streptomycin (0.1 mg ml⁻¹), N2 supplement (100 μ l per 12.5 ml) and 10% heat-inactivated horse serum (vol/vol). Agarose beads (Affi-gel Blue gel, Bio-Rad Laboratories) were washed three times with sterile water and four times with PBS. For coating the beads with Netrin1, 20 μ l of bead solution was mixed with 10 μ l of a Netrin1 solution (250 ng μ l⁻¹, R&D Biosciences) and incubated overnight at 4°C. BSA coated beads were used as a control for which 20 μ l of bead solution was mixed with 20 μ l of BSA (1 mg ml⁻¹). Before placing the beads on the slices, they were washed with PBS and then resuspended in 100 μ l of slice culture medium. Using a mouth pipette, the beads of the desired size were picked and placed over the midline in the region where the CC crosses. The cultured slices were immunostained (see above) after three days.

2.15 Chromatin-Immunoprecipitation (ChIP)

The ChIP-IT express kit (Active Motif) was used for performing the ChIP assay, according to the manufacture's instructions. Rat monoclonal anti-Ctip2 (Abcam), Rabbit anti- Satb2 (self generated), Rabbit anti- Sip1 (self generated) and polyclonal anti-rabbit IgG (Active Motif) antibodies were used. The following primers were used for qRT-PCR:

NIN-P1 fwd: 5' CTGGTGGTGAATGTGTCCTG 3'

NIN-P1 rev: 5' TGGGCTTCATATCCATCTCC 3'

NIN-P2 fwd: 5' ATGCATCCAGGTGTTGGTTT 3'

NIN-P2 rev: 5' GAGGTCAAGGCCATTCTCAG 3'
NIN-P3 fwd: 5' GGGCTAAAGAGCAGGAGGAA 3'
NIN-P3 rev: 5' CTGTCAATCTGCCAAACCAG 3'
NIN-P4 fwd: 5' ACAGCCCTGATGAAAACCAG 3'
NIN-P4 rev: 5' CTGAGCACAAGTGTGGGAGA 3'
NIN-P5 fwd: 5' TGCAGCCTGTTCTGATTGAC 3'
NIN-P5 rev: 5' CCTTTCTTGGTCCTGTGCAT 3'

Unc5C E1 fwd: 5' ATCAAGCGCAACTCCCTAAA 3'
Unc5C E1 rev: 5' CTTGCTCACTTGCTCACTCG 3'
Unc5C E2 fwd: 5' CCCTTGGAGAAAGTGGAGTG 3'
Unc5C E2 rev: 5' GTGTACGGGGAAGGGAAAC 3'

DCC MAR1 fwd: 5' TGCACAGCACCTATGATCTTG 3'
DCC MAR1 rev: 5' AACAGAGGAGTCAGAGCGAAA 3'
DCC MAR2 fwd: 5' CGCACACACATTATTCTTTTGG 3'
DCC MAR2 rev: 5' ACTGCCTGGCTCTGTACTCC 3'

Data are presented as average values $\pm$ standard deviation (SD).

2.16 Luciferase Assay

The luciferase assay for Sip1 binding to Ninein promoter was done in HEK293T cells. The following primers were used for cloning the Nin-P1 region and Nin-P4 regions. The PCR products were initially cloned into pGEMT (promega) and sequence verified. Subsequently, the inserts were sub-cloned into pMCS-GL (Thermoscientific).

NIN-P1 fwd: 5' AGCTGAATTCCTTCTGAAGACATCTTGATAATACAGC 3'
NIN-P1 rev: 5' AGCTGGATCCAGGAGGAGGTAGGTCACCAGGAACA 3'
NIN-P4 fwd: 5' AGCTACTAGTGGGGAGAGACTACACCTGCTCACA 3'
NIN-P4 rev: 5' AGCTGGATCCGGATGTCCCCCGTCTCTTAAAAGC 3'

Fragments cloned into pMCS-GL were transfected along with pCMV-Alkaline Phosphatase in HEK293T cells using lipofectamine 2000. 48 hours post transfection, cell media was collected and used for assaying using the Secrete-Pair Dual Luminescence assay kit (Genecopoeia). Glomax (promega) was used for measuring the luminescence. Values are represented as Ratio of Gaussia luciferase to Alkaline phosphatase luminescence ratio $\pm$ standard deviation. Values were collected from three independent experiments, with atleast three replicates for each experiment.

The luciferase assay for Ctip2 binding *Unc5C* promoter was done in HEK293T cells. Putative Ctip2 binding promoter regions in the *Unc5C* promoter region were amplified with the following primers:

E1Fw: 5'TTAAGAATTCTTGCCTTCTTTCCCATCT 3'

E1Rv: 5'TATTGAGGATCCAGTGCTGGGAGGTGTAGCGC 3'

E2Fw: 5'TTAAGAATTCTCCTCCTCGGAGGCTC 3'

E2Rv: 5'TATTGAGGATCCTGAGACGCGCAAACAGCCGA 3'

The fragments were cloned into pC1-TK-luciferase vector (Evrogen). E1 and E2 fragments included -842 to -124 nt and -60 to +65 nt of the *Unc5C* 5'-flanking promoter region, respectively. Lipofectamine2000 (Invitrogen) was used for transfecting cells, according to the manufacture's instructions. Transfected cells were analyzed 24 hours after transfection. Luciferase assays were done using the Dual-Luciferase kit (Promega). A mixture of plasmids coding for renilla-luciferase (RL) were used (CMV-RL, TK-RL and SV40-RL; 1:2:10 molar proportions, respectively) to account for variable transfection efficiency. Lumat LB96V reader (Berthold Technologies) was used for reading the assays. Results are shown as Renilla-normalized relative luciferase units (RLUs) $\pm$ standard deviation (SD). Data shown is atleast from three independent experiments. All p values were $\leq 0,05$.

For the luciferase assay to test Satb2 binding to *DCC* promoter, the following primers were used for cloning the *DCC* MAR1 and MAR2 regions. The PCR products were initially cloned into pGEMT (promega) and sequence verified.

Subsequently, the MAR containing inserts were sub-cloned into pMCS-GL (Thermoscientific).

DCC MAR1 fwd: 5' ACTACCTAGTAGAGGCAGCCACAAG 3'

DCC MAR1 rev: 5' AGGTGAAATTTCCACCAATGTGAAT 3'

DCC MAR2 fwd: 5' AAAATGCTACCCATGTTCCAGAAG 3'

DCC MAR2 rev: 5' ACTGCCTGGCTCTGTACTCCAAATT 3'

Fragments cloned into pMCS-GL were transfected along with pCMV-Alkaline Phosphatase in COS7 cells using lipofectamine 2000. 48 hours post transfection, cell media was collected and used for assaying using the secreta-Pair Dual Luminescence assay kit (Genecopoeia). Glomax (promega) was used for reading the plates. Values are represented as Ratio of Gaussia luciferase to Alkaline phosphatase luminescence ratio $\pm$ standard deviation. Values were collected from three independent experiments, with atleast three replicates for each experiment.

2.17 Data analysis and Statistics

All the experiments that have been quantified have been analyzed by Student's t test unless otherwise mentioned using GraphPad Prism 5.0a or Microsoft Excel software. All the graphs have been plotted using GraphPad Prism 5.0a.

3. RESULTS

PART I

Non-cell autonomous role of *Sip1* in Corpus Callosum formation

3.1 Commissural projections are severely altered in the *Sip1* conditional knockout

Figure 1. Cortical projections are severely altered in the *Sip1* mutant

Histological analysis of the *Sip1*^{fl/fl} *Nex*^{Cre} mutant at E18.5 through Nissl staining shows that the corpus callosum (CC) and the anterior commissure (AC) are absent, while the hippocampal commissure (HC) is unaffected. The callosal axons in the *Sip1*^{fl/fl} *Nex*^{Cre} mutant form Probst bundles. Higher magnification images correspond to the boxed area in the lower magnification image.

Morphological and anatomical analysis of the *Sip1* mutant embryos revealed that the corpus callosum (CC) and anterior commissure (AC) were absent in the *Sip1* mutant. The callosal axons failed to cross the midline and formed loose-ended bundles called Probst bundles. The AC on the other hand was completely absent. Interestingly, the hippocampal commissure (HC), which inter-connects the two hippocampi, was unaffected in the *Sip1* mutant (Figure 1).

3.2 Cortico-fugal projections are affected in the *Sip1* conditional knockout

Figure 2. Commissural and corticofugal projections are affected in the *Sip1* mutant

Anterograde (A, B) and retrograde (C, D) Dil labeling in the *Sip1^{fl/fl} Nex^{Cre}* mutant and *Sip1^{fl/wt} Nex^{Cre}* control, P5 brains reveals further connectivity defects in the *Sip1* mutant. (A) Anterograde Dil labeling from the neocortex (NCx) confirms the absence of the corpus callosum (CC) in the *Sip1* mutant. The corticothalamic tract (CT) that connects the neocortex (NCx) to the thalamus (Th) is unaffected. (B) The corticospinal tract (CST) is absent in the *Sip1^{fl/fl} Nex^{Cre}* brains with almost no fibers passing through the cerebral peduncle (CP). (C) Retrograde Dil labeling from the thalamus shows that thalamocortical axons are unaffected in the *Sip1^{fl/fl} Nex^{Cre}* brains and neurons in layer V and VI of the neocortex are back-labeled in the

mutant and the control. (D) Retrograde DiI labeling from the cerebral peduncle confirms the absence of the CST. Very few layer V neurons are back-labeled in the *Sip1^{fl/fl} Nex^{Cre}* brain when compared to *Sip1^{fl/wt} Nex^{Cre}* brain. Inset boxes in C and D show the position of DiI crystal placement. Data from Master's Thesis of Srinivas Parthasarathy, IMPRS-Neuroscience Göttingen.

To further understand and characterize the changes in cortical projections as a consequence of deletion of *Sip1* from the neocortex, we analyzed *Sip1^{fl/fl} Nex^{Cre}* and *Sip1^{fl/wt} Nex^{Cre}* brains at P5 using anterograde and retrograde DiI labeling. DiI (1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate) is a lipophilic dye that is taken up by the neuronal membrane at the site of application and is transported passively through the lipid bi-layer along the axon. Placing the DiI crystals at the cell body can trace the axonal projection of the neuron and dye placement at the axonal terminal or the axonal trajectory can back-label the cell body from which the axon originates. To study the various projections from the cortex, crystals of DiI were placed in the sensory, motor and the visual cortex of fixed brains. After dye diffusion, the brains were sectioned and the axonal trajectories were analyzed. As observed earlier, the CC was absent in the *Sip1* mutant. The callosal axons stopped prematurely in the ipsilateral cortex and formed Probst bundles (Figure 2A). Similar to the control brains, in the *Sip1* conditional mutant, axonal bundles could be seen taking the corticofugal path and passing through the internal capsule. At the region where the corticofugal tract branches, only the dorsal branch going to the thalamus called the corticothalamic tract (CT) was present in the mutant. Axons could be followed all the way into the thalamus into the various thalamic nuclei. The presence of the thalamocortical axons (TC) could also be confirmed, as thalamic neurons were back-labeled from the cortex (Figure 2A). The ventral branch of the corticofugal tract projecting to the spinal cord called the corticospinal tract (CST) was absent in the *Sip1* mutant. Examining further caudal sections revealed that no DiI labeling could be observed within the cerebral peduncle in the mutant, thus reconfirming that the CST was absent (Figure 2B). Thus in the *Sip1^{fl/fl} Nex^{Cre}* mice, although the CT was present the CST was missing.

3.3 Retrograde DiI labeling reconfirms the absence of the corticospinal tract and the presence of the corticothalamic tract.

In order to confirm the presence of the CT tract, crystals of DiI were placed in the various nuclei of the thalamus. After dye diffusion, back-labeled cells in the cortex were studied. We observed that in both the control and *Sip1^{fl/fl} Nex^{Cre}* brains, layer VI neurons, which are known to project to the thalamus, were back labeled (Figure 2C). Similarly, to study the CST, crystals of DiI were placed in the cerebral peduncle. After dye diffusion, back-labeled cells in the cortex were studied. While in the control, layer V neurons were specifically labeled, in the *Sip1^{fl/fl} Nex^{Cre}* brains very few cells were back-labeled, thus confirming the absence of the CST in the *Sip1* mutant (Figure 2D).

3.4 The corticospinal tract is present at early developmental stages in the *Sip1* mutant

In order to understand the developmental progression of the cortical projections in the *Sip1* mutant we decided to study these axonal trajectories at P0. Anterograde DiI labeling from the cortex re-confirmed the absence of the CC and AC in the *Sip1^{fl/fl} Nex^{Cre}* brain at P0. It was interesting to note that axons that project from the external capsule, which in the wild type scenario, would turn into the AC and project to the contralateral hemisphere, were defasciculated in the *Sip1^{fl/fl} Nex^{Cre}* brain (double arrowheads). They projected ventrally from the external capsule and could also be seen in further caudal sections of the brain unlike in the controls. Interestingly at P0, unlike at P5, the CST was present in the *Sip1* mutant. Axonal projections of the CST could be traced all the way to the cerebral peduncle in the *Sip1^{fl/fl} Nex^{Cre}* brains (arrowhead). Therefore, the CST is present at an early developmental stage (P0) in the *Sip1* mutant but is lost as development progresses to P5 (Figure 3).

Figure 3. Corticospinal tract is present at birth (P0) in the *Sip1* mutant

Anterograde cortical DiI labeling in *Sip1^{fl/wt} Nex^{Cre}* and *Sip1^{fl/fl} Nex^{Cre}* brains at P0 shows that the anterior commissure (AC) is misrouted in the *Sip1* mutant. The axons misproject ventrally instead of crossing horizontally to the contralateral hemisphere (double arrow heads). Unlike P5, at P0 the corticospinal tract (CST) was present in the *Sip1^{fl/fl} Nex^{Cre}* mutant (arrow head). External capsule (EC), Internal capsule (IC) Corticothalamic tract (CT), Neocortex (NCx), Thalamus (Th).

3.5 Loss of corticospinal tract is not a due to cell death of Layer V neurons

The loss of CST could be attributed to two possible causes. Either cell death of layer V neurons as a result of which their axonal projections are lost or that these axons are withdrawn due to reasons like misprojection or defasciculation. In order to verify if layer V neurons in the *Sip1^{fl/fl} Nex^{Cre}* brain were dying prematurely, we performed immunohistochemistry with anti-cleaved caspase3 antibody on sections of control and *Sip1^{fl/fl} Nex^{Cre}* brains at P2. Cleaved caspase3 is a marker for cell death. We additionally stained for Ctip2, which is a marker for layer V cortical neurons. Draq5 was used to label all the cell nuclei. The developmental stage P2 was chosen as the loss of the CST occurs between P0 and P5.

Figure 4. No apparent cell death of *Sip1* deficient layer V neurons

Cleaved caspase3 staining at P2 in *Sip1^{fl/wt} Nex^{Cre}* and *Sip1^{fl/fl} Nex^{Cre}* cortex shows that there is no apparent difference in cleaved caspase3 signal at layer V, demarcated by Ctip2 immuno labeling (arrowhead). Although, an increase in cleaved caspase3 staining in the upper layers of *Sip1* deficient cortex can be seen (double arrowheads). Draq5 labels all the nuclei.

We observed that in the *Sip1^{fl/fl} Nex^{Cre}* and control brains there was no apparent difference in cleaved caspase3 staining in layer V hence ruling out the possibility of cell death of corticospinal neurons (Figure 4). Thus the loss of layer V projections to the spinal cord was not due to cell death but probably due to other causes. It was noted though, that there was a significant increase in cell death within the upper layers of *Sip1* mutant cortex compared to the controls.

3.6 Midline glia is almost completely lost in the *Sip1* conditional mutant

Figure 5. Midline glia is absent in the *Sip1* mutant

(A) *Sip1* is expressed in the GFAP positive midline glial cells in the indusium griseum (IG) and in the midline zipper glia (MZG). *Sip1* is also expressed in the sub-callosal sling neurons (double arrow heads). (B) In the *Sip1^{fl/fl} Nex^{Cre}* brains the cortical midline is not fused. GFAP positive IG glia is absent and the MZG is severely reduced. Subcallosal sling neurons are absent (double arrows in A). Higher magnification images in both panels correspond to the boxed areas in the merged images.

GFAP positive midline glial cells have been shown to be important for guiding callosal axons. They secrete various guidance cues that bind to cognate receptors on the axons to influence axonal turning (Richards et al., 2004; Shu & Richards, 2001; Shu et al., 2003; Silver & Ogawa, 1983). To determine if the midline glia are intact in the *Sip1* mutant, we performed immunohistological staining on control and *Sip1^{fl/fl} Nex^{Cre}* brain sections at E17.5 using antibodies against GFAP and Sip1. In the control brains, GFAP positive glial cells could be seen lining the cortical midline. Dorsal to the CC, GFAP positive midline glia forming the Indusium griseum (IG) could be seen. Ventral to the CC, another cluster of GFAP cells called the midline zipper glia (MZG) was present. Glial cells lining the lateral aspects of the CC called the glial wedge (GW) were also visible. Sip1 was expressed in all these cell populations (Figure 5A). In contrast to the control, in the *Sip1* mutant brains, the IG glia was completely lost. Although the GW and MZG glia were present, they were severely reduced. Additionally, the dorsal midline in the *Sip1* mutant was not fused (Figure 5B).

3.7 Subcallosal sling is not formed in the *Sip1* conditional mutant

Apart from the midline glial structures that help in the guidance of callosal neurons during midline crossing, previous studies have also shown the importance of subcallosal sling neurons, which can act as a source of physical as well as chemical guidance cues for callosal axons (Figure 5A- double arrowheads, Figure 6A). One population of these sling neurons includes calretinin (CR) expressing cells (Niquille et al., 2009; Shu et al., 2003). The subcallosal sling neurons were absent in the *Sip1* mutant; a sling like structure could not be discerned at the midline (Figure 5B). To hence study this population of cells further, sections of E15.5 and E17.5 *Sip1^{fl/wt} Nex^{Cre}* and *Sip1^{fl/fl} Nex^{Cre}* brains were stained with an antibody against CR. At E15.5, a stage before midline fusion and midline crossing of CC occurs; the distribution of CR positive neurons in the *Sip1* mutants matched the distribution in the control brains. The CR positive cells were found to line the

two edges of the cortical midline (Figure 6B). By E17.5 in the control brains, a stage when callosal axons have usually crossed the midline in wild type brains, CR positive sling neurons were found to line the ventral trajectory of the corpus callosum. They were also found as dispersed cells within the white matter. On the other hand, in *Sip1* mutants, the CR positive neurons did not form a midline sling but instead were found as aggregates of cells lining the two edges of the midline (Figure 6C). Therefore, apart from the midline glia, another accessory guidance structure- the sling neurons, was also disrupted in the *Sip1* mutant possibly contributing to the acallosal phenotype.

Figure 6. Calretinin positive sub-callosal sling neurons are mislocalized

(A) Calretinin (CR) positive cells form one population of sub-callosal sling neurons and are found in the cortical midline below the corpus callosum. (B) CR positive cells are similarly distributed in *Sip1^{fl/wt} Nex^{Cre}* and *Sip1^{fl/fl} Nex^{Cre}* midline at E15.5, a stage before CC formation. (C) At E17.5, when callosal fibers have crossed the midline, CR positive neurons can be seen forming a subcallosal sling in *Sip1^{fl/wt} Nex^{Cre}* brain but not in *Sip1^{fl/fl} Nex^{Cre}* mutant.

3.8 *Slit1*, *Sema3C* and other axon guidance molecules show altered expression levels in the *Sip1* mutant

Figure 7. Many axon guidance molecules including *Slit1* and *Sema3C* show altered expression pattern in the *Sip1* mutant

In situ hybridization for different axon guidance molecules showed altered expression pattern in E16.5 *Sip1* mutant sections when compared to controls. Arrowheads highlight the region of altered expression. Expression of *Slit1* and *Sema3C* is lost at the midline. *Cntn2* and *Robo2* show a downregulation in the *Sip1* mutant while *ephrinA4* and *PlexinA4* show an upregulation. *Nrp1*, which is expressed in the cingulate cortex, is marginally upregulated while *Nrp2*, which is also expressed at the midline, is downregulated in the mutant midline.

Slit family of guidance factors are expressed by the midline glia forming the GW, IG and MZG. The callosal axons in turn express the *Robo* family receptors that respond to *Slit* family ligands, aiding in axon guidance (Shu & Richards, 2001). Similarly the CR positive sling neurons express *Sema3C* that attracts *Neuropillin1* (*Nrp1*) expressing cortical neurons (Niquille et al., 2009). Since in the *Sip1^{fl/fl} Nex^{Cre}* mice both the midline glia as well as the midline sling neurons were affected we wanted to investigate if the expression of *Slit* family molecules and *Sema3C* was also affected. *In situ* hybridization using probes against *Slit 1,2,3* and *Sema3C* showed that there was a visible down regulation of *Slit1* and *Sema3C* at the midline in the *Sip1* mutant (Figure 7). The lack of these chemical guidance cues in addition to the change in the midline morphology could explain the absence of midline crossing in the *Sip1* mutant.

Apart from the *Slit* family and *Sema3C*, we tested for changes in expression levels and patterns of other axon guidance molecules as well (Table 1). Of the many different molecules tested we found changes in expression levels of *Contactin2* (*Cntn2*), *Robo2*, *EphrinA4*, *PlexinA4*, *Neuropillin1* (*Nrp1*) and *Neuropillin2* (*Nrp2*) in the *Sip1* mutant (Figure 7). In wild type E16.5 embryos *Cntn2* showed a high lateral-low medial expression gradient in the cortex. In the *Sip1* mutant this gradient was reversed such that the expression level of *Cntn2* was higher medially and lower laterally. While *Robo2* was downregulated in the *Sip1* mutant cortex, *EphrinA4* was upregulated. The expression domain of *PlexinA4* seemed to be expanded in case of the *Sip1* mutant. *Nrp1* and *Nrp2* are expressed in the cingulate cortex in the wild type brain. In the *Sip1* mutant while the expression of *Nrp1* was marginally upregulated, the expression of *Nrp2* was considerably downregulated. Thus apart from crucial midline-guidance molecules other axon guidance molecules that have been shown to be important for influencing axonal trajectory were also altered in the *Sip1* mutant.

Table 1. Axon guidance molecules tested by ISH in the *Sip1* mutant

Nr.	Gene	ISH result	Nr.	Gene	ISH result
1	<i>Contactin 1</i>	No change	16	<i>PlexinA1</i>	No change
2	<i>Contactin2</i>	Down-laterally and up-medially	17	<i>PlexinA2</i>	No change
3	<i>Ephrin A1</i>	No change	18	<i>PlexinA3</i>	No change
4	<i>Ephrin A2</i>	No change	19	<i>PlexinA4</i>	Up-regulated
5	<i>Ephrin A4</i>	Up-regulated	20	<i>PlexinB4</i>	No change
6	<i>Ephrin A5</i>	No change	21	<i>Robo1</i>	No change
7	<i>Ephrin B1</i>	No change	22	<i>Robo2</i>	Down-regulated
8	<i>Ephrin B2</i>	No change	23	<i>Robo3</i>	No change
9	<i>EphrinB3</i>	No change	24	<i>Slit1</i>	Down-regulated
10	<i>EphA2</i>	No change	25	<i>Slit2</i>	Inconclusive
11	<i>EphA4</i>	No change	26	<i>Slit3</i>	Inconclusive
12	<i>EphB1</i>	No change	27	<i>Sema3A</i>	No change
13	<i>EphB2</i>	No change	28	<i>Sema3C</i>	Down-regulated
14	<i>Neuropilin 1</i>	Marginally up-regulated	26	<i>Sema3F</i>	No change
15	<i>Neuropilin 2</i>	Down-regulated	27	<i>Sema3G</i>	No change

3.9 The lack of *Sema3C* expression in the *Sip1* mutant is a non-cell autonomous effect

Figure 8. Loss of *Sema3C* expression in the *Sip1* mutant is a non-cell autonomous effect

(A) *In utero* electroporation of GFP-IRES-Cre at the cortical midline of *Sip1^{fl/fl}* and *Sip1^{fl/wt}* brains does not alter the position of calretinin (CR) positive subcallosal sling neurons. (B) *In situ* hybridization of the adjacent sections of the electroporated brains show that the expression of *Sema3C* is also unaltered.

To further understand if the change in *Sema3C* expression levels in the *Sip1* mutant is due to cell intrinsic loss of *Sip1* or due to cell extrinsic effects we electroporated GFP-IRES-Cre plasmid into the cortical midline of *Sip1^{fl/wt}* and *Sip1^{fl/fl}* embryos at E12.5. This ensured a mosaic deletion of *Sip1* specifically in the electroporated cells at the midline while not affecting the rest of the brain. The embryos were harvested at E16.5; alternate sections were stained for CR and GFP (Figure 8A), while subsequent sections were processed for *in situ* hybridization for *Sema3C* (Figure 8B). We observed that despite the deletion of *Sip1* from the electroporated cells in the midline, no visible changes could be seen in the distribution of CR positive cells in the *Sip1^{fl/fl}* brain (Figure 8A). *Sema3C* probe signal was also comparable to the control electroporation (Figure 8B). Hence the loss of *Sema3C* expression in the *Sip1* mutant midline is a non- cell autonomous effect of *Sip1* deletion.

3.10 *Sip1* mutant shows altered midline morphology

Figure 9. *Sip1* mutant midline is narrower and shows an altered distribution of cell density
(A) *In utero* electroporation of pBLBP-GFP together with pCAG-dsRed and immuno staining for calretinin shows that the relatively cell free region in the midline that corresponds to the corpus callosum in *Sip1^{fl/wt} Nex^{Cre}* is replaced by a cell dense midline in *Sip1^{fl/fl} Nex^{Cre}* brains. Calretinin (CR) positive neurons populate these cell dense regions in the *Sip1* mutant instead of forming a sub-callosal sling. **(B)** The cortical midline of *Sip1^{fl/fl} Nex^{Cre}* brains is significantly narrower than control brains (arrow) and shows altered distribution of cell density within the three equally sized bins.

To allow for callosal axons to traverse through the midline, elaborate reorganization of the midline takes place (Smith et al., 2006). The cortical

midline of wild type brains were electroporated at E14.5 with pBLBP-GFP plasmid, which labels radial glial cells and pCAG-dsRed, which labels the progenitor and postmitotic cells and analyzed at E17.5. As described previously (Shu et al., 2003; Smith et al., 2006), this experiment revealed that the BLBP positive cells present at the midline either retracted their apical process and migrated towards the pia forming the IG glia or retracted their basal process and migrated towards the ventricle forming the GW. This essentially left a cell free region in between the two populations for the callosal axons to pass through. The pattern formed by the dsRed positive cells also followed a similar arrangement (Figure 9A). On the other hand, in the *Sip1* mutant brains, at the midline neither the BLBP-GFP positive glial cells nor dsRed cells separate into two populations (Figure 9A). Quantifying the relative distribution of dsRed cells at the midline within three equally spaced bins in the wild type and the mutant further reflected this difference (Figure 9B). Apart from the relative distribution of cells at the midline it was also apparent that the *Sip1* mutant midline was much narrower than the wild type (Figure 9B).

3.11 Altered distribution of neurons at the midline is a non-cell autonomous effect of *Sip1* deletion

To further establish non-cell autonomous dependence of neurons on *Sip1* for the distribution at the midline, we electroporated *Sip1^{fl/fl}* embryos at E14.5 with a plasmid called pC β A-FLE $\times$ together with a plasmid encoding *Cre* and harvested the embryos at E16.5. The pC β A-FLE $\times$ plasmid uses the Cre-loxP system to transcribe *tdTomato* in the absence of Cre and transcribe *eGFP* on Cre mediated recombination (Figure 10B). Hence using this plasmid and varying the concentration of the *Cre* plasmid used, we could, in parallel, study Cre positive, hence *Sip1* negative- GFP positive cells as well as Cre negative, hence *Sip1* positive - tdTomato positive cells within the same cortical section (Figure 10A). The tdTomato positive cells thus served as an internal control for this experiment. To compare the distribution of

electroporated cells at the midline, the cortical midline was divided into three equally sized bins and the relative distribution of the two different cell populations within each of the bins was examined. We observed that there was no difference in the relative distribution of the two kinds of cell populations, suggesting a non-cell autonomous dependence on *Sip1* (Figure 10C).

Figure 10. Change in relative distribution of cell density at the midline is a non-cell autonomous effect of *Sip1* deletion

(A) *In utero* electroporation of pC β A-FLEX plasmid and Cre at the midline of *Sip1^{fl/fl}* embryos (B) from E14.5 to E16.5 resulted in a mosaic pattern of tdTomato positive/ Cre negative/ Sip1 positive cells, interspersed with eGFP positive/ Cre positive/ Sip1 negative cells. (C) Relative distribution of Sip1 positive and Sip1 negative cells within three equally sized bins drawn at the midline was measured. Both cell populations show a similar distribution at the midline.

Taken together these experiments indicate that, the cortical midline in the *Sip1^{fl/fl} Nex^{Cre}* mutant mice is severely altered when compared to the wild type. The midline glia as well as the midline sling neurons that help in callosal axon guidance are absent and do not express the required guidance cues. The dorsal midline does not fuse and shows an altered morphology. These changes in the midline are probably due to non-cell autonomous effects of *Sip1* deletion, as the cell intrinsic deletion of *Sip1* from these cells does not recapitulate the phenotype.

PART II

Cell autonomous role of *Sip1* in establishing cortical projections

3.12 Cell autonomous defects also contribute to the acallosal phenotype in the *Sip1* mutant

Figure 11. Acallosal phenotype in *Sip1* mutant is due to both cell-intrinsic as well as cell-extrinsic effects

In utero electroporation of pCAG eGFP from E12.5 to E18.5 in (A) *Sip1*^{fl/wt} Nex^{Cre} embryos and in (B) *Sip1*^{fl/fl} Nex^{Cre} embryos shows that the GFP positive axons in *Sip1*^{fl/fl} Nex^{Cre} brains do not form a corpus callosum. The fibers stall medially before reaching the midline (arrowhead). In utero electroporation of pCAG eGFP + pCAG-FMF-Sip1myc expressing plasmids from E12.5 to E18.5 in (C) *Sip1*^{fl/wt} Nex^{Cre} embryos and in (D) *Sip1*^{fl/fl} Nex^{Cre} embryos. The Sip1 positive/ GFP positive axons within the *Sip1* mutant, do not cross the midline but yet project much further till the midline fissure, when compared to eGFP electroporation in *Sip1*^{fl/fl} Nex^{Cre} embryos (arrowheads in B, D). (A, C) show normal corpus callosum formation.

Although the midline of the *Sip1* mutant had numerous defects that prevent midline crossing of callosal axons we wanted to determine if these non-cell autonomous defects in the *Sip1* mutant were the only cause for the lack of corpus callosum formation. If this were the case then when *Sip1* is re-expressed in a subset of callosal neurons in the *Sip1* mutant brain, they should continue to form Probst bundles and not form the CC. To test this we electroporated a plasmid expressing *Sip1* together with *eGFP* into *Sip1^{fl/wt} Nex^{Cre}* and *Sip1^{fl/fl} Nex^{Cre}* embryos at E12.5 and also electroporated *eGFP* alone into *Sip1^{fl/wt} Nex^{Cre}* and *Sip1^{fl/fl} Nex^{Cre}* embryos at E12.5 as controls. The brains were harvested at E18.5. *eGFP* expressing neurons in the *Sip1^{fl/wt} Nex^{Cre}* brains projected axons to the contralateral cortex forming a CC (Figure 11A), whereas *eGFP*-expressing neurons in the *Sip1^{fl/fl} Nex^{Cre}* brains ended prematurely in the ipsilateral hemisphere (Figure 11B). Electroporation of *Sip1* together with *eGFP* in *Sip1^{fl/fl} Nex^{Cre}* brains on the other hand did not look similar to the *Sip1^{fl/fl} Nex^{Cre}* brains where *eGFP* alone was expressed. Here, although axons did not completely cross the midline to reach the contralateral hemisphere, they were still able to navigate further, till the midline fissure (Figure 11D). Expressing *Sip1* and *eGFP* in *Sip1^{fl/wt} Nex^{Cre}* brains resulted in a normally formed CC (Figure 11C). Thus these experiment put together showed that although the defects in the *Sip1* mutant at the midline were definitely responsible for the lack of formation of the CC, there might be other cell intrinsic defects in the *Sip1* mutant, which contribute towards the acallosal phenotype.

3.13 Mosaic deletion of *Sip1* is achieved by electroporation of *Cre recombinase*

To test the cell-autonomous role of *Sip1* in axonal extension and CC formation it was important to study *Sip1* deficient neurons and their axons within a wild type background. We hence used a plasmid expressing *Cre* recombinase under a ubiquitously expressed promoter following a *GFP-IRES* sequence. The *GFP-IRES* ensured that all the cells expressing *Cre* would also express

GFP. The GFP-IRES-Cre plasmid was electroporated into *Sip1^{fl/wt}* and *Sip1^{fl/fl}* embryos at E12.5 and the brains were harvested at E18.5. The sections were stained for GFP and Sip1. All the cells expressing GFP and hence Cre in the *Sip1^{fl/fl}* embryos were negative for Sip1 expression while the electroporated cells in the *Sip1^{fl/wt}* brain were unaffected (Figure 12). Hence mosaic deletion of *Sip1* could be achieved with the expression of a GFP-IRES-Cre plasmid.

Figure 12. Cell autonomous deletion of *Sip1* through *in utero* electroporation of Cre
In utero electroporation of a GFP-IRES-Cre plasmid at E12.5 and immunohistochemistry with anti-GFP and anti-Sip1 antibody at E18.5 in *Sip1^{fl/wt}* and *Sip1^{fl/fl}* embryos demonstrates the mosaic deletion of *Sip1* in *Sip1^{fl/fl}* embryos expressing GFP/Cre (arrowheads and outlines).

3.14 Cell intrinsic deletion of *Sip1* also affects CC formation

A GFP-IRES-Cre plasmid was electroporated into the sensory- motor cortex of *Sip1^{fl/wt}* and *Sip1^{fl/fl}* embryos at E12.5 and analyzed at E18.5. The sections were stained of GFP and L1 (anti-neuronal cell adhesion factor that labels axons). While the Cre/GFP positive axons in the *Sip1^{fl/wt}* sections could be traced forming the CC and projecting all the way into the contralateral hemisphere, the Cre/GFP positive, hence *Sip1* negative, axons in the *Sip1^{fl/fl}* embryos did

not form the CC Almost no GFP positive fiber could be detected at the midline crossing (Figure 13A).

Figure 13. Callosal fibers fail to cross the midline on cell autonomous deletion of *Sip1*

(A) *In utero* electroporation of GFP-IRES-Cre at E12.5 to E18.5 in *Sip1*^{fl/wt} and *Sip1*^{fl/fl} embryos shows that GFP positive/Cre positive /*Sip1* positive axons in *Sip1*^{fl/wt} embryos cross the midline while GFP positive/Cre positive /*Sip1* negative axons in *Sip1*^{fl/fl} embryos do not cross the midline. (B) Quantification of the corpus callosum thickness at the midline relative to the electroporated cells shows severe loss of callosal fibers in *Sip1*^{fl/fl} embryos when compared to *Sip1*^{fl/wt} embryos. *Sip1*^{fl/wt} = 0.2656 ± 0.06457, *Sip1*^{fl/fl} = 0.0025 ± 0.0005, p value = 0.002, n = 3.

Quantifying the thickness of the callosal axons crossing the midline, relative to the number of cells electroporated showed a 98% decrease in *Sip1*^{fl/fl} embryos when compared to *Sip1*^{fl/wt} (Figure 13B). All values represent the mean of the fluorescent area of the callosal midline relative to the fluorescent

area of the cells electroporated in the cortex $\pm$ standard deviation. $Sip1^{fl/wt} = 0.2656 \pm 0.06457$, $Sip1^{fl/fl} = 0.0025 \pm 0.0005$, $p\text{-value} = 0.002$, $n=3$.

3.15 Callosally projecting neurons within the cingulate cortex are also affected by *Sip1* deletion

Figure 14. Projections of cingulate callosal axons are also affected due to cell intrinsic deletion of *Sip1*

In utero electroporation of plasmids expressing *Cre* and *dsRed* at E14.5 in *Sip1^{fl/wt}* and *Sip1^{fl/fl}* embryos at the midline show that axons projecting from the cingulate cortex do not cross the cortical midline in the *Sip1^{fl/fl}* brains at E17.5 unlike *Sip1^{fl/wt}* brains where midline crossing can be seen (arrowheads).

The cingulate cortex, which is the medial most region of the neocortex, houses neurons that form the pioneers of the CC. They are the first set of neurons to cross the midline and aid in the crossing of the follower axons to the contralateral cortex. They take a dorsal path in crossing the midline, and the axons from the more laterally placed neurons in the cortex take a ventral path (deAzevedo et al., 1997; Koester & O'Leary, 1994). The cingulate pioneer neurons hence have very different growth cone dynamics, speeds of axon

extension and sometimes respond to a different set of guidance cues (G. J. Kim et al., 1991). In order to understand if these cingulate pioneer neurons also require *Sip1* for correct axon extension and CC formation we injected *Cre-recombinase* together with *dsRed* into the lateral ventricles of E14.5 *Sip1^{fl/fl}* and *Sip1^{fl/wt}* embryos and electroporated the cingulate cortex. The brains were harvested at E17.5. We observed that axons of the electroporated neurons in the *Sip1^{fl/wt}* projected into the contralateral hemisphere. *Sip1* deficient neurons, on the other hand were incapable of extending their axons much beyond the ipsilateral white matter with very few axons managing to reach the midline (Figure 14). Hence both the pioneers as well as the follower axons require *Sip1* cell intrinsically in order to form the CC.

3.16 Cell autonomous deletion of *Sip1* slows the rate of axon extension

In order to determine if the lack of CC formation at E18.5 in the *Sip1^{fl/fl}* brains with *Cre* electroporation was due to a delay in axon extension, we electroporated *GFP-IRES-Cre* plasmid into *Sip1^{fl/wt}* and *Sip1^{fl/fl}* embryos at E12.5 and harvested the brains from postnatal day 7 pups. We observed that callosal axons could be seen forming the CC and crossing over to the contralateral hemisphere in the *Sip1^{fl/wt}* as well as in the *Sip1^{fl/fl}* brains at P7 (Figure 15). Thus *Sip1* deficient neurons show an axon extension defect wherein axons grow at a much slower rate when compared to their wild type counterparts.

Figure 15. Lack of corpus callosum crossing with cell autonomous deletion of *Sip1* is due to delay in axon extension

In-utero electroporation of GFP-IRES-Cre in *Sip1*^{fl/wt} and *Sip1*^{fl/fl} brains at E12.5 and analyzed at P7. Immunofluorescence labeling with GFP and L1. The GFP positive axons form the corpus callosum in both *Sip1*^{fl/wt} and *Sip1*^{fl/fl} brains with axons crossing the midline (arrowheads).

3.17 *Sip1* deficiency affects ipsilateral axon-collateral formation

Upper layer neurons, apart from projecting to the contralateral cortex through the CC and connecting with contralateral neurons, also form axonal branches and hence synaptic connections with ipsilateral neurons. One such characteristic ipsilateral connection is formed by the axons of the upper layer

neurons on ipsilateral layer V neurons through the formation of axonal collaterals (Burkhalter, 1989; Harwell et al., 2012; Thomson & Lamy, 2007).

Figure 16. Upper layer neurons commence the formation of ipsilateral axonal collaterals at the level of layer V neurons at P4

In utero electroporation of *eGFP* at E15.5 in wild type embryos and subsequent immuno staining of cortical sections at P0, P2, P3, P4, P5, and P7 with Ctip2. Axons of upper layer neurons do not form branches till P3. Collateral branches start to form at P4 at the level of layer V neurons in the cortical plate and continue to increase at later stages (boxed region).

Since the lack of *Sip1* affected axonal growth we wanted to test whether *Sip1* deficiency also affects the formation of ipsilateral axonal collaterals. In order to investigate the normal course of collateral formation, we electroporated *eGFP* expressing plasmid at E15.5 in wild type embryos and harvested the embryos at subsequent postnatal stages (P0 to P7). In these experiments we could specifically visualize the UL neurons and their axonal branches in the deep layers of the cortex. The sections were also stained for Ctip2, which labels layer V neurons. We observed that between P0 and P3 as the UL neurons migrate and take up their final position in the cortical plate, they in parallel project axons to the white matter with no visible axonal collaterals. By P4, the first branches of ipsilateral axonal collaterals started to appear at the level of layer V neurons in the cortex, demarcated by Ctip2 positive cells

(Figure 16). Between P4 and P7, there was a steady increase in the density of these axonal branches. Hence, ipsilateral branching of UL axons commences at P4 and is very prominent at P7 (Figure 16). These experiments also indicated that the axonal collaterals are most probably formed by the interstitial mode of branch formation, as the branches appear from the main axon shaft many days after initial axon extension.

Figure 17. *Sip1* deficient upper layer neurons do not form ipsilateral axon collaterals

(A) *In utero* electroporation of *GFP-IRES-Cre* at E15.5 to P7 in *Sip1*^{fl/wt} and *Sip1*^{fl/fl} embryos shows that *Sip1* negative neurons do not form ipsilateral axon collaterals at the level of layer V neurons at P7, while the control brains show robust branching. (B) Quantification of the extent of branching at layer V between *Sip1*^{fl/wt} and *Sip1*^{fl/fl} sections confirms the absence of branching in the *Sip1* deficient scenario. Relative fluorescence area in layer V: *Sip1*^{fl/wt} = 850.78 ± 78.598, *Sip1*^{fl/fl} = 147.05 ± 57.909, p value = 0.0003

To study the ipsilateral axon collateral formation in the presence and absence of *Sip1*, we electroporated *GFP-IRES-Cre* plasmid into *Sip1*^{fl/fl} and *Sip1*^{fl/wt}

embryos at E15.5 and harvested the pups at P7, a stage when ipsilateral axonal collaterals are well established. We observed that while the neurons in the *Sip1^{fl/wt}* cortex projected axons that form a dense meshwork of branches at layer V, *Sip1* lacking upper layer neurons in the *Sip1^{fl/fl}* brains did not form any branches at all (Figure 17A). To quantify this effect we calculated the fluorescent area of the axonal collateral at layer V within a radial unit relative to the number of electroporated neurons. There was an approximate 6-fold decrease in relative fluorescence area at layer V in *Sip1^{fl/fl}* brains when compared to *Sip1^{fl/wt}* brains (Figure 17B). All values are depicted as mean $\pm$ standard deviation, *Sip1^{fl/wt}* = 850.78 $\pm$ 78.598, *Sip1^{fl/fl}* = 147.05 $\pm$ 57.909, *p-value*= 0.0003, n=3.

3.18 Microtubule associated protein - ninein, is downregulated in the *Sip1* mutant

Sip1 deficient neurons show defects in axonal extension and axonal branching. In order to identify *Sip1* targets that control axonal growth and collateral formation we performed microarray experiments at E16.5 searching for genes down-regulated in the *Sip1* deficient cortex. This analysis revealed that *ninein*, a centrosome and MT minus-end binding protein was downregulated in the absence of *Sip1*. We verified these findings by performing immunohistological staining with anti-ninein antibody on E16.5, *Sip1^{fl/wt} Nex^{Cre}* and *Sip1^{fl/fl} Nex^{Cre}* embryos. Within the *Sip1^{fl/wt} Nex^{Cre}* neocortex, ninein was strongly expressed in the cortical plate and the ventricular zone. Expression of ninein was also detected within the white matter. In the *Sip1^{fl/fl} Nex^{Cre}* mutant, a severe downregulation of ninein expression in the cortical plate as well as in the white matter of the cortex could be observed. The expression of ninein was unaltered in the progenitor zone (Figure 18).

Figure 18. Ninein is downregulated in the *Sip1* mutant cortex

Immunohistochemistry shows that ninein is expressed in the cortical plate (CP), white matter (WM) and the ventricular zone (VZ) in the *Sip1^{fl/wt} Nex^{Cre}* brains at E16.5, but is down regulated in the CP as well as WM of *Sip1^{fl/fl} Nex^{Cre}* embryos. The expression of ninein at the VZ is unaffected in the *Sip1^{fl/fl} Nex^{Cre}* embryos.

3.19 *Sip1* regulates *ninein* expression through direct binding

To test whether *Sip1* directly binds to the *ninein* promoter region to regulate its transcription, we performed Chromatin immunoprecipitation (ChIP) assay on E16.5 wild type cortices. We used E16.5 *Sip1^{fl/fl} Nex^{Cre}* mutant cortices as control. The immuno precipitated DNA was analyzed through quantitative real time PCR for 5 different regions within 10 Kb upstream of the *ninein* transcription start site (TSS), denoted as *Nin-P1* to *Nin-P5* that were found to have the bipartite *Sip1* consensus binding motif: CACCT(G) (Figure 19A). The ChIP assay showed 7 to 10-fold (*Sip1*:IgG) relative enrichment in all five regions tested. *Sip1^{fl/fl} Nex^{Cre}* mutant cortex showed no relative enrichment (Figure 19B).

Figure 19. Sip1 directly binds to *ninein* promoter region and regulates its expression

(A) Five different regions with Sip1 consensus binding sequence within 10Kb upstream region of *ninein* transcription start site were selected; *Nin-P1* to *Nin-P5*. Primers to amplify 150-200 bp regions within the selected regions were designated for the ChIP assay. (B) ChIP data shows significant enrichment in all five regions tested in wild type tissue when compared to *Sip1* mutant tissue. Wild type, n=4 (where each sample (n) contained pooled cortices from two brains): *Nin-P1* = 9.7335 ± 4.3579, *Nin-P2* = 7.4594 ± 3.5617, *Nin-P3* = 7.6909 ± 0.8916, *Nin-P4* = 7.4790 ± 3.4918, *Nin-P5* = 7.2495 ± 3.2511; *Sip1^{fl/fl} Nex^{Cre}*, n=3 (where each sample (n) contained pooled cortices from two brains): *Nin-P1* = 0.8060 ± 0.2880, *Nin-P2* = 0.76255 ± 0.4252, *Nin-P3* = 0.9289 ± 0.2925, *Nin-P4* = 0.9332 ± 0.3278, *Nin-P5* = 0.7662 ± 0.2036; *p-values* *Nin-P1* = 0.0259, *Nin-P2* = 0.0316, *Nin-P3* = 0.0001, *Nin-P4* = 0.0323, *Nin-P5* = 0.0278.

All values are represented as mean of ratio of Sip1 normalized to IgG values ± standard deviation. Wild type, n=4 (where each sample (n) contained pooled cortices from two brains): *Nin-P1* = 9.7335 ± 4.3579, *Nin-P2* = 7.4594 ± 3.5617, *Nin-P3* = 7.6909 ± 0.8916, *Nin-P4* = 7.4790 ± 3.4918, *Nin-P5* = 7.2495 ± 3.2511; *Sip1^{fl/fl} Nex^{Cre}*, n=3 (where each sample (n) contained pooled cortices from two brains): *Nin-P1* = 0.8060 ± 0.2880, *Nin-P2* = 0.76255 ± 0.4252, *Nin-P3* = 0.9289 ± 0.2925, *Nin-P4* = 0.9332 ± 0.3278, *Nin-P5* = 0.7662 ± 0.2036; *p-values* *Nin-P1* = 0.0259, *Nin-P2* = 0.0316, *Nin-P3* = 0.0001, *Nin-P4* = 0.0323, *Nin-P5* = 0.0278.

3.20 Sip1 binding to the *ninein* enhancer is functional

Figure 20. Luciferase assay confirms functional binding of Sip1 to *ninein* upstream region

(A) Two of the five promoter elements upstream of *ninein* coding region tested in the ChIP assay were selected and cloned into a plasmid upstream of Gaussia luciferase called, *GLuc-Nin-P1* and *GLuc-Nin-P2*. (B) There is a significant decrease in relative luminescence on Sip1 binding to *Nin-P1* but not to *Nin-P4*. *GLuc* = 1.6353 ± 0.037 , *GLuc* + *Sip1* = 0.0908 ± 0.002 , *p* - value = 0.078; *GLuc-Nin-P1* = 0.7269 ± 0.0944 , *GLuc-Nin-P1* + *Sip1* = 0.1393 ± 0.0450 , *p* - value = 0.0437; *GLuc-Nin-P4* = 0.055 ± 0.0005 , *GLuc-Nin-P4* + *Sip1* = 0.0651 ± 0.0285 ; *p* - value = 0.6259.

To test the functionality of the binding of Sip1 to the *ninein* enhancer regions, we performed an *in vitro* luciferase assay. Two of the five different promoter regions upstream of the *ninein* transcription start site that were tested for the ChIP assay were chosen, namely *Nin-P1* and *Nin-P4* (Figure 20A). They were cloned upstream of *Gaussia luciferase* and expressed in HEK293T cells either with or without *Sip1*. Luminescence from the *Gaussia luciferase* was measured relative to the luminescence from alkaline phosphatase used as a control. We observed a significant decrease in transcriptional activity in *Nin-*

P1 enhancer by 5.2 fold when *Sip1* was co-transfected. *Nin-P4* as well as the control plasmid did not show a change in relative luminescence (Figure 20B). *In vivo*, *ninein* expression is downregulated with the loss of *Sip1* (Figure 18), thus attributing the role of a transcriptional activator to *Sip1*, whereas in the heterologous system used for the luciferase assay, *Sip1* behaves as a transcriptional repressor. This maybe due to the absence of essential cofactors in the heterologous system used, which might be required for *Sip1* to behave as a transcriptional activator. All values have been indicated as relative luminescence ratio between Gaussia luciferase and alkaline phosphatase $\pm$ standard deviation, averaged over three independent experiments; $G-Luc = 0.1635 \pm 0.0376$, $G-Luc + Sip1 = 0.0908 \pm 0.0022$; $GLuc-Nin-P1 = 0.7269 \pm 0.0944$, $GLuc-Nin-P1 + Sip1 = 0.139 \pm 0.0450$; $GLuc-Nin-P4 = 0.0557 \pm 0.0005$, $GLuc-Nin-P4 + Sip1 = 0.0651 \pm 0.0285$. Comparing with and without *Sip1* co-transfection: $GLuc-NinP1$ *p-value* = 0.0437, $GLuc-Nin-P4$ *p-value* = 0.6259, *n*=3.

3.21 Ninein can be detected in the axons, axonal branches and the axonal growth cone of developing neurons

Ninein has been shown to bind to the minus-end of both centrosomal and non-centrosomal microtubules (Mogensen et al., 2000). It has been previously shown that in cultured neurons, ninein is present in the somato-dendritic compartment (Ohama & Hayashi, 2009). Immunohistochemistry with an anti-ninein antibody in E16.5 wild type cortex showed that apart from the cortical plate, ninein expression was also present in the cortical white matter (Figure 18). Hence, in order to verify if ninein is localized within the axon and the axonal growth cone of neurons, we transfected *eGFP* tagged *ninein* in DIV 1 cultured cortical neurons and analyzed them at DIV 2 and DIV 7. We co-transfected a *dsRed* expressing plasmid to be able to visualize the entire cellular morphology. At DIV 2 GFP-ninein puncta could be seen in the cell soma, in the axon and also within the growth cone. As expected no GFP could be seen within the nucleus (Figure 21). Analyzing the neurons at DIV 7 also showed similar localization. GFP-ninein puncta were visible in the dendrites,

dendritic branches, axon, axonal branches and in the growth cone (Figure 22). It can hence be hypothesized that ninein might play an important role in modulating the cytoskeletal architecture and dynamics of these different cellular compartments.

Figure 21. Ninein localizes to the soma, dendrites, axon and axonal growth cone

DIV 2 cortical dissociated neurons transfected at DIV 1 with *eGFP* tagged *ninein* (to help localize ninein) and pCAG-*dsRed* (to visualize the complete neuronal morphology) shows that GFP-ninein puncta are localized to the soma, dendrites, axon and the axonal growth cone. No GFP-ninein puncta are visible within the nucleus (nu). The higher magnification images correspond to the boxed regions.

Figure 22. Ninein localizes to axonal and dendritic branches in neurons

DIV 7 cortical dissociated neurons transfected at DIV 1 with *eGFP* tagged *ninein* (to help localize ninein) and pCAG-*dsRed* (to visualize the complete neuronal morphology) reveal that *GFP-ninein* puncta also localize to axonal and dendritic branches that develop as the neurons mature, indicating that *GFP-ninein* is present in all cellular compartment of cortical neurons. Higher magnification images correspond to the boxed regions.

3.22 Axonal growth is retarded in *Sip1* deficient neurons *in vitro*

Figure 23. *Sip1* negative neurons show stunted axonal growth *in vitro*, which is rescued by restoring ninein expression

(A) GFP-IRES-Cre expressing *Sip1*^{fl/fl} and *Sip1*^{fl/wt} cortical dissociated neurons were cultured *in vitro*. (B) GFP-IRES-Cre expressing *Sip1*^{fl/fl} cortical dissociated neurons at DIV 2 had shorter axons when compared to *Sip1*^{fl/wt} neurons. (A, B) The axon length was rescued by co expression of *pCAG-FMFeGFP-ninein* together with *GFP-IRES-Cre* in *Sip1*^{fl/fl} cortical dissociated neurons. *Sip1*^{fl/wt} + Cre (n= 258) = 379.43 ± 89.09, *Sip1*^{fl/fl} + Cre (n= 233) = 241.36 ± 75.51, *Sip1*^{fl/fl} + Cre + ninein (n=166) = 322.41 ± 72.82; *p*-values < 0.001.

We wanted to test if *ninein*, being a MT binding protein, that localizes to the axon and is down regulated in the *Sip1* mutant, could influence axonal length. First we tested if *Sip1* deletion from neocortical neurons would affect axonal length *in vitro*. *Sip1^{fl/wt}* and *Sip1^{fl/fl}* embryos were electroporated with GFP-IRES-Cre plasmid at E14.5 and brains were isolated at E17.5. The electroporated region of the cortex was isolated, dissociated and neurons cultured for 2 days *in vitro* and fixed. *In utero* electroporation was used instead of plasmid transfection in order to delete *Sip1* as early as possible. The length of the axons of GFP positive neurons was measured. We observed that the average length of the axon of *Sip1* deficient neurons was significantly shorter when compared to wild type neurons, thus reconfirming the cell autonomous requirement of *Sip1* for axon extension (Figure 23A, B). To address whether *ninein* acts downstream of *Sip1* to control axonal length, we performed similar experiments where *ninein* expression was restored in *Sip1* deficient neurons. Expressing *ninein* in *Sip1* deficient cells significantly increased the average length of the axon when compared to *Sip1* negative neurons, although it was not restored to wild type levels (Figure 23A, B). All values depicted as average length $\pm$ standard deviation. *Sip1^{fl/wt}* + Cre (n= 258) = 379.43 ± 89.09 , *Sip1^{fl/fl}* + Cre (n= 233) = 241.36 ± 75.51 , *Sip1^{fl/fl}* + Cre + *ninein* (n=166) = 322.41 ± 72.82 ; *p-values* < 0.001.

3.23 Restoration of *ninein* expression in *Sip1* deficient neurons rescues the corpus callosum

Since overexpression of *ninein* could increase the axonal length of *Sip1* deficient neurons *in vitro* we wanted to test if *ninein* could also potentially influence axonal growth *in vivo*. We hence electroporated a plasmid expressing eGFP tagged *ninein* that is preceded by a floxed *mCherry-polyA* cassette, designated as *pCAG-FMF-eGFP Ninein*, together with the GFP-IRES-Cre plasmid into *Sip1^{fl/fl}* embryos at E12.5. Together the plasmid cocktail ensured onset of *ninein* expression only in the Cre positive and hence *Sip1*

negative cells. The brains were analyzed at E18.5 and compared with *Sip1^{fl/fl}* and *Sip1^{fl/wt}* brains electroporated with only *GFP-IRES-Cre* plasmid.

Figure 24. Restoring *ninein* expression partially rescues the corpus callosum in brains with cell autonomous deletion of *Sip1*

(A) *pCAG-FMFeGFP Ninein* co-expressed with *GFP-IRES-Cre* in *Sip1^{fl/fl}* embryos at E12.5 and analyzed at E18.5. The corpus callosum is compared with that formed with *GFP-IRES-Cre* electroporation in *Sip1^{fl/wt}* and *Sip1^{fl/fl}* embryos (arrowheads). Sections are immunostained for GFP and Draq5. (B) Quantifying the thickness of the corpus callosum at the midline shows a partial rescue of callosal fibers in *Sip1^{fl/fl}* embryos on co-electroporation of *pCAG-FMFeGFP Ninein* with *GFP-IRES-Cre*. *Sip1^{fl/fl}* + *Cre* and *ninein* = 0.0972 ± 0.0215 , when compared to *Sip1^{fl/fl}* + *Cre* = 0.0025 ± 0.0005 , *p-value* = 0.016

We observed that while in *Cre-IRES-GFP* alone electroporated *Sip1^{fl/fl}* embryos, the GFP positive axons did not form the CC, the neurons electroporated with *Cre-IRES-GFP* as well as *ninein* were able to extend their axons medially and cross the callosal midline into the contralateral hemisphere (Figure 24A).

Quantifying the thickness of the CC at the midline showed that similar to the *in vitro* rescue of axonal length, the *in vivo* rescue of the CC was partial, as both the proportion of GFP positive axons crossing the midline and maximal axonal length at E18.5 were not restored to wild type levels (Figure 24B). The values are expressed as mean $\pm$ standard deviation, $n=3$, $Sip1^{fl/fl} + Cre$ and $ninein = 0.0972 \pm 0.0215$, when compared to $Sip1^{fl/fl} + Cre = 0.0025 \pm 0.0005$, $p\text{-value} = 0.016$, $Sip1^{fl/wt} + Cre = 0.2656 \pm 0.06457$.

3.24 Restoration of *ninein* expression in *Sip1* deficient neurons restores ipsilateral axonal branching of layer II and III projection neurons

We electroporated *ninein* together with *Cre-IRES-GFP* in $Sip1^{fl/fl}$ embryos at E15.5 and harvested the brains at P7. *Cre-IRES-GFP* electroporation in $Sip1^{fl/fl}$ embryos as well as $Sip1^{fl/wt}$ embryos served as positive and negative controls. We observed that, on restoring *ninein* expression in *Sip1* negative upper layer neurons, they were able to form ipsilateral axonal collaterals at the level of layer V neurons in the cortical plate (Figure 25A, B). The extent of arborization measured in terms of relative fluorescent intensity was comparable to the wild type (Figure 25B, C). Values expressed as mean $\pm$ standard deviation, $n=3$, $Sip1^{fl/fl} + Cre$ and $ninein = 845.90 \pm 14.598$, when compared to $Sip1^{fl/fl} + Cre = 147.05 \pm 57.909$, $p\text{-value} = 0.0011$, $Sip1^{fl/wt} + Cre = 850.78 \pm 78.598$.

Figure 25. Axon collateral formation by upper layer neurons is restored by expression of *ninein* in *Sip1* deficient neurons

(A) *pCAG-FMF-eGFP Ninein* was co-expressed with *GFP-IRES-Cre* in *Sip1*^{fl/fl} embryos at E15.5 and analyzed at P7. The ipsilateral axonal collaterals formed at layer V in these brains was comparable with that formed with *GFP-IRES-Cre* electroporation in *Sip1*^{fl/wt}. *GFP-IRES-Cre* electroporation *Sip1*^{fl/fl} embryos shows no branching. (B) Higher magnification images of boxed area in panel (A). (C) Quantifying the axonal branching at layer V shows a rescue of collaterals in *Sip1*^{fl/fl} embryos on co-electroporation of *pCAG-FMF-eGFP Ninein* with *GFP-IRES-Cre*. *Sip1*^{fl/fl} + *Cre* and *ninein* = 845.90 ± 14.598, when compared to *Sip1*^{fl/fl} + *Cre* = 147.05 ± 57.909, *p*-value = 0.0011, *Sip1*^{fl/wt} + *Cre* = 850.78 ± 78.598.

3.25 Retarded microtubule growth contributes to the stunted growth of *Sip1* deficient axons and is controlled by *ninein*

Figure 26. Microtubules grow slower in *Sip1* deficient cells and the growth rate is restored in the presence of *ninein*

(A) Example of time series tracking the movement of GFP-EB3 +TIPs over 10 seconds in DIV 2, *Sip1*^{fl/wt} + Cre expressing, *Sip1*^{fl/fl} + Cre expressing and *Sip1*^{fl/fl} + *ninein* + Cre expressing cortical neurons. (B) Kymographs tracking the movement of GFP-EB3 comets over time, in all three experimental conditions. Slope of the graph indicates the speed of GFP-EB3 comets, which in turn correlates to the growth rate of microtubules. (C) Graph representing the average velocity of GFP-EB3 comets. Average velocity is represented as pixels traveled per second. *Sip1*^{fl/wt} + Cre expressing neurons = 1.65 ± 0.42 , *Sip1*^{fl/fl} + Cre expressing neurons = 1.40 ± 0.369 , *Sip1*^{fl/fl} + *ninein* + Cre expressing neurons = 1.59 ± 0.50 , *p* values < 0.001.

Since *ninein* is a MT binding protein that was able to rescue the axon length of *Sip1* deficient neurons, we hypothesized that *Sip1* through *ninein* may control the growth rate of axonal MTs. To assess this we used microtubule plus-end binding protein EB3 fused with GFP and tracked EB3-GFP comets through live imaging experiments (Stepanova et al., 2003). We firstly compared EB3-GFP comet velocity in control neurons (*Sip1*^{fl/wt} neurons expressing Cre + EB3-GFP) and in *Sip1* deficient neurons (*Sip1*^{fl/fl} neurons expressing Cre + EB3-GFP) at DIV 2. The average anterograde velocity of EB3-GFP comets was significantly reduced in *Sip1* deficient neurons probably contributing to the stunted axonal growth. When we examined axons of *Sip1* deficient neurons

where *ninein* expression was restored (*Sip*^{fl/fl} neurons expressing *Cre* + *Ninein* + *EB3-GFP*), we observed that the average anterograde velocity of EB3-GFP comets was significantly higher than in the axons of *Sip1* deficient neurons and was almost restored to wild type levels (Figure 26). EB3-GFP comets from 13 to 16 neurons were analyzed per condition, only the comets that could be tracked for 10 seconds or more were included. Data is represented as average velocity $\pm$ standard deviation: Controls = 1.65 ± 0.42 , *Sip1* deficient neurons = 1.40 ± 0.369 , *Sip1* deficient neurons + *ninein* = 1.59 ± 0.50 , *p* values < 0.001.

3.26 Ninein influences the stability of axonal microtubules

We observed that re-expressing *ninein* in *Sip1* deficient neurons could increase the rate of axonal extension as well as restore axonal branching *in vivo*. Further, we observed that *ninein* influences axonal growth by affecting MT growth. To understand whether *ninein*, being a MT binding protein also contributes to the stability of axonal microtubules, we performed a timed Nocodazole induced microtubule breakdown assay with cultured DIV 3, wild type cortical neurons expressing either *eGFP* or *ninein* + *eGFP*. The neurons were fixed and stained for acetylated tubulin (ac-tub) and GFP after 2 hrs, 4 hours, 6 hours and 8 hours of 5mM nocodazole exposure. We observed that at 2 hours both *eGFP* expressing control neurons and *eGFP* + *ninein* expressing neurons showed the presence of stable microtubules labeled by ac-tubulin along the length of the axon and other neurites of the cell (Figure 27A). By 4 and 6 hours of nocodazole exposure a higher percentage of control neurons showed the absence of ac-tubulin staining within the axon while *eGFP* + *ninein* expressing neurons seemed to be more resistant to microtubule breakdown (Figure 27C). Therefore, we observed that control neurons showed a steep decline in the proportion of cells with stable microtubules in the axonal compartments with increasing exposure time to nocodazole, falling to 22% at 8 hours. On the other hand *ninein* expressing cells were resistant to nocodazole breakdown and 85% of the cells contained stable microtubules within the axon at 8 hours (Figure 27B, C)

Figure 27. Ninein stabilizes microtubules in the axons

(A) Figure panel shown four example images of *eGFP* expressing DIV 3 cortical neurons as well as *ninein* + *eGFP* expressing DIV 3 neurons after 2 hours of Nocodazole exposure, immunostained for GFP and acetylated tubulin. Ac-tubulin staining can be seen in the axons of both types of neurons (arrowheads). (B) Figure panel shown four example images of *eGFP* expressing DIV 3 neurons as well as *ninein* + *eGFP* expressing DIV 3 neurons after 8 hours of Nocodazole exposure, immunostained for GFP and acetylated tubulin. Ac-tubulin staining can be seen in the axons of as *ninein* + *eGFP* expressing neurons but not in the *eGFP* alone expressing neurons (arrowheads). (C) Graph comparing the resistance of microtubule breakdown against increasing duration of Nocodazole exposure between *eGFP* expressing DIV 3 neurons as well as *ninein* + *eGFP* expressing DIV 3 neurons. 2 hours: *eGFP* = 89.56% ± 2.27, *eGFP* + *ninein* = 96.92% ± 1.35; 4 hours: *eGFP* = 75.77% ± 5.00, *eGFP* + *ninein* = 94.29% ± 0.76; 6 hours: *eGFP* = 64.09% ± 2.60, *eGFP* + *ninein* = 93.02% ± 2.88, 8 hours: *eGFP* = 22.17% ± 5.41, *eGFP* + *ninein* = 85.04% ± 4.35. *p*-value < 0.001 (two way ANOVA).

Hence the presence of ninein resists break down of microtubules induced by nocodazole suggesting that ninein acts as a microtubule-stabilizing molecule within the growing axon. The proportion of cells with stable microtubules

was calculated by averaging the percentage obtained from three different cover slips for each condition and time point, 100 to 380 neurons were counted per cover slip. Data is represented as proportion of cells with stable microtubules $\pm$ standard deviation. 2 hours: eGFP = 89.56% $\pm$ 2.27, eGFP + ninein = 96.92% $\pm$ 1.35; 4 hours: eGFP = 75.77% $\pm$ 5.00, eGFP + ninein = 94.29% $\pm$ 0.76; 6 hours: eGFP = 64.09% $\pm$ 2.60, eGFP + ninein = 93.02% $\pm$ 2.88, 8 hours: eGFP = 22.17% $\pm$ 5.41, eGFP + ninein = 85.04% $\pm$ 4.35. *p-value* < 0.001 (two way ANOVA).

Taken together these experiments describe the cell autonomous role of *Sip1* in cortical callosal neurons, where *Sip1* influences the rate of axonal growth as well as axonal collateral formation *in vivo* through its direct downstream effector *ninein*. Ninein, in turn exerts its influence on these process by regulating microtubule growth and stability.

PART III

Transcriptional control of *Satb2* and *Ctip2* over corpus callosum formation

(Data presented in this part of the thesis are the results from the work performed along with **Srinivas Parthasarathy**, Charité-Berlin with **equal contribution**. Pilot experiments were performed by Paraskevi Sgourdou and have not been shown)

3.27 *Unc5C* expression requires *Satb2* and is restricted to callosal neurons.

Satb2^{-/-} mutants lack a corpus callosum suggesting the involvement of *Satb2* in controlling, at least in part, the molecular program involved in CC formation (Alcamo et al., 2008; Britanova et al., 2008). In order to identify molecules down-stream of *Satb2* that control CC formation we investigated changes in the expression of a variety of axon guidance molecules in the *Satb2*^{-/-} mutants. We assumed that such molecules should be downregulated in the *Satb2* mutant cortex and that their expression should be confined to callosal neurons (*Satb2* positive), while being excluded from corticofugal neurons (*Ctip2* positive). Among others tested, the expression of the Netrin1 receptor - *Unc5C* satisfied these two criteria. *Unc5C* expression was previously shown to be down-regulated in *Satb2*^{-/-} mutants (Alcamo et al., 2008; Britanova et al., 2006; Srinivasan et al., 2012), where *in situ* hybridization against the *Unc5C* mRNA showed a complete loss of *Unc5C* from the *Satb2*^{-/-} cortical plate, with only expression in the subplate remaining (Figure 28A). Further, *Unc5C* has a very dynamic expression pattern during cortical development. At E14.5 *Unc5C* is predominantly expressed in the subplate. At E18.5 the expression of *Unc5C* in the cortical plate reaches a very high level and demonstrates a high-lateral-low-medial gradient (Figure 28A, B).

To test whether ectopic and premature expression of *Satb2* can induce *Unc5C* expression, we performed *in utero* electroporation of *Satb2* and *Ski* expressing plasmids into the lateral ventricle at E12.5 and analyzed the brains at E14.5. *Satb2* requires *Ski* as a co-factor for recruiting chromatin remodeling complexes and thus silencing its targets (Baranek et al., 2012).

Figure 28. *Unc5C* is expressed by callosally projecting *Satb2* positive neurons and *Satb2* regulates *Unc5C* expression.

(A) *Unc5C* mRNA expression in wild type and *Satb2*^{-/-} mutant mice at E18.5. In sections of *Satb2*^{-/-} mutant brains, *Unc5C* continues to be expressed in only the subplate, but is absent in other cortical layers. (B) *Unc5C* expression in E14.5 wild type embryos in cells that were electroporated at E12.5 with *Satb2/Ski/EGFP* or *EGFP* control. GFP immunohistochemistry depicts the region electroporated. *Unc5C* *in situ* hybridization shows additional ectopic expression of *Unc5C* within cells electroporated with *Satb2/Ski* apart from the expression in

the subplate seen in the contralateral hemisphere or in the control electroporation. (C) *Unc5C* mRNA expression with respect to *Satb2* and *Ctip2* expressing cells through immunofluorescence/fluorescence *in situ* hybridization in P0 wild type cortex. High magnification image of P0 wild type cortex shows that *Unc5C* is expressed primarily in the upper layers but also in a subset of cells in the deep layers – layer V (boxed regions) *Unc5C* mRNA expression coincides with the expression of *Satb2* and is excluded from *Ctip2* expressing cells. Layer V expression of *Unc5C* is restricted to cells expressing *Satb2* (arrows) and absent in *Ctip2* positive cells (double arrowheads). Scale bar=100µm, unless specified differently in the figure.

This experiment induced ectopic expression of *Unc5C* in the cells electroporated with *Satb2* compared to a control construct (Figure 28B). To test our hypothesis that *Unc5C* is expressed by callosally projecting *Satb2*-positive cells and absent in *Ctip2*-positive cells, we combined *in situ* hybridization (ISH) using a probe against *Unc5C* and immunohistochemistry (IHC) using antibodies against *Satb2* and *Ctip2*. *Unc5C* mRNA is expressed predominantly in upper layers and weakly in deeper layers. Since combining ISH and IHC requires the omission of proteinase-K from the protocol, a physical limitation to the extent of probe penetration is an unavoidable drawback. Thus, even though in conventional ISH, *Unc5C* expression is seen in both upper and deeper layers, with FISH such a dual domain of expression was detected in separate experiments. We observed that *Unc5C* and *Ctip2* were not expressed in the same cells in most cases whereas *Unc5C* and *Satb2* were usually co-expressed. Even in layer V, *Unc5C* and *Satb2*-were co-expressed but neither was expressed in *Ctip2*-positive cells (Figure 28C).

The layer V expression of *Unc5C* specifically in cells negative for *Ctip2* and positive for *Satb2* indicated that *Satb2* might also be able to repress *Ctip2* in deep layer neurons, in addition to the previously reported repression in upper layer neurons (Britanova et al., 2008). In order to confirm this we counted the proportion of *Ctip2* and *Satb2* double positive cells within the deep layers of the cortex of wild type embryos and compared it with the proportion of *Ctip2* and Cre double positive cells within the deep layers of the *Satb2* mutant cortex (Figure 29A). Here Cre expression indicated the *Satb2* deleted population of cells. We observed that there was a 70% increase in the

number of Ctip2/Cre double positive cells within the deeper layers of the cortex in the *Satb2* mutant (Figure 29B).

Figure 29. Ctip2 is up-regulated in the deep layers of the cortex upon *Satb2* deletion (A) Immunohistochemical staining using anti-Cre and anti-Ctip2 antibody was performed in *Satb2*^{+/+} and *Satb2*^{-/-} brains. The proportion of deep layer Ctip2-positive cells that were also *Satb2* (Cre) double positive in *Satb2*^{-/-} brains as compared to *Satb2*^{+/+} was plotted. (B) 8-fold increase was observed in the number of Ctip2-Cre double positive cells in *Satb2*^{-/-} mutant when compared to the heterozygote (n=3, p-value=0.0006). Scale bar=100μm. Data are presented as average values ± standard deviation (SD).

3.28 Ctip2 directly represses *Unc5C* expression

Since *Unc5C* and Ctip2 were not co-expressed, we hypothesized that *Unc5C* expression could be negatively regulated by Ctip2. To investigate this, we examined whether *Unc5C* expression would be restored in *Satb2*^{-/-}; *Ctip2*^{-/-} double mutants. We observed an increase of *Unc5C* expression in *Satb2*^{-/-}; *Ctip2*^{-/-} mutant cortex as compared to *Satb2*^{-/-} mutants. In Ctip2 single mutants we did not detect significant changes in *Unc5C* expression compared to controls (Figure 30).

This result indicated that in *Satb2* mutants, the down-regulation of *Unc5C* could be the result of the ectopic upregulation of Ctip2. To test this hypothesis we over-expressed Ctip2 in the cortex. Ctip2 expression was validated in HEK293T cells and a confocal maximum Z-projection image for the electroporated region also confirmed the expression of Ctip2 in a large population of cortical cells (Figure 31A). An EGFP-expression plasmid was electroporated either alone or with the Ctip2 expression plasmid in wild type

E12.5 embryos and the brains were harvested at E17.5. Subsequent sections were labeled with a probe against *Unc5C* mRNA. A decrease in *Unc5C* expression was observed in *Ctip2* over-expressing cells within the electroporated cortical region, as compared to the contralateral hemisphere (n=3) or *EGFP*-alone electroporated brains (n=2, Figure 31B). In all further experiments wild type, *Satb2* heterozygous, *Ctip2* heterozygous and *Satb2/Ctip2* compound heterozygous embryos have all been referred to interchangeably as controls as none of these genotypes show any deviation from the wild type phenotype for the pathway in question.

Figure 30. *Unc5C* expression is restored in *Satb2*^{-/-};*Ctip2*^{-/-} double mutants

Immunohistological staining against *Unc5C* and *L1* in wild type, *Satb2*^{-/-}, *Ctip2*^{-/-} and *Satb2*^{-/-};*Ctip2*^{-/-} double mutant brains. *Unc5C* expression is absent in *Satb2*^{-/-} brains when compared to wild type and *Ctip2*^{-/-} brains. The expression of *Unc5C* is restored in *Satb2*^{-/-};*Ctip2*^{-/-} double mutant brains.

Figure 31. Ctip2 directly represses *Unc5C* expression.

(A) Plasmid expressing *Ctip2* under the ubiquitous CAG promoter was transfected into HEK 293T cells. Immunohistochemistry of *Ctip2* together with nuclear marker Draq5 confirmed the expression of *Ctip2*. Plasmid expressing *Ctip2* expressed in cortical neurons by *in utero* electroporation at E12.5 and analyzed at E17.5. Immunohistochemistry of *Ctip2* confirmed the expression of *Ctip2* *in vivo* as well. Scale bar=100 μ m. (B) *Unc5C* expression in E17.5 wild type embryos in cells electroporated at E12.5 with *Ctip2*/EGFP or EGFP control within the electroporated as well as contralateral hemisphere. Subsequent section stained for GFP, *Ctip2*,

Draq5 (a nuclear marker) immunohistochemistry. *Unc5C* mRNA expression is lost in the region electroporated with *Ctip2* when compared to either the control electroporation or the corresponding region in the contralateral hemisphere. (C) Scheme showing the two regions in the *Unc5C* promoter where *Ctip2* was predicted to bind. (D) Chromatin immunoprecipitation from P0 cortex of wild type and *Satb2*^{-/-} mutants show that *Ctip2* binds to the *Unc5C* promoter region. 2.78 ± 0.2722 fold enrichment for E2 compared to 1.23 ± 0.73 for E1 in *Satb2*^{-/-}, n=3, *p*-value=0.027 and 1.67 ± 0.42 fold enrichment for E2 compared to 1.18 ± 0.14 for E1 in wild type n=4, n.s (E) Luciferase assay to demonstrate that *Ctip2* binds to *Unc5C* genomic region and represses its expression. In the presence of full length *Ctip2*, 1.9 and 1.7 fold decrease in luminescence ratio was observed in case of E1 and E2 respectively. E1 and E2 being two putative *Ctip2* binding sites. All *p* values were ≤ 0.05 . Scale bar=100 μ m. The luciferase assay was performed by Olga Britanova, IBCh - Russian academy of Science, Russia

Next, we investigated whether *Ctip2* directly represses *Unc5C* expression. An *in silico* search revealed two putative *Ctip2* binding sites in the *Unc5C* promoter region, that we designated E1 and E2 (-842 to -142 and -60 to +100) upstream of the transcription initiation site (Figure 31C). Chromatin-immunoprecipitation (ChIP) from wild type and *Satb2*^{-/-} cortices followed by qRT-PCR, showed that *Ctip2* binds strongly to the E2 but not the E1 *Unc5C* promoter region. Since the wild type cortex consists of a relatively low *Ctip2* population, *Satb2*^{-/-} cortices were used as a means of enriching for *Ctip2* positive cells in the cortex (Figure 31D, 2.78 ± 0.2722 fold enrichment for E2 compared to 1.23 ± 0.73 for E1 in *Satb2*^{-/-}, n=3, *p*-value=0.027 (Student's t-test) and 1.67 ± 0.42 fold enrichment for E2 compared to 1.18 ± 0.14 for E1 in wild type, n=4, n.s).

To confirm this result we performed an *in vitro* luciferase assay. In these experiments the same two *Unc5C* upstream genomic regions E1 and E2 were inserted into a plasmid encoding the luciferase gene and transfected into cells with a plasmid encoding *Ctip2*. We observed a decrease in the activity of the *Unc5C* enhancer by approximately 1.9-fold and 1.7-fold, in the case of E1 and E2 respectively, when *Ctip2* was co-transfected (Figure 31E, both *p*-values < 0.05). These results indicate that *Ctip2* can directly repress *Unc5C* transcription.

3.29 Partial Restoration of the CC in *Satb2*^{-/-};*Ctip2*^{-/-} compound mutants

Figure 32. The corpus callosum is partially restored in *Satb2*^{-/-};*Ctip2*^{-/-} compound mutants

The panel of images with higher magnification of the midline shows a series of experiments, wherein *Venus GFP* was electroporated into the developing cortex at E12.5 and the brains harvested and analyzed at E18.5. Electroporation of *Venus GFP* shows a normal CC in wild type and *Ctip2*^{-/-} embryos. *Satb2*^{-/-} mutant brains clearly demonstrate a complete absence of callosal fibers, wherein no fibers could be seen approaching the midline. Partial rescue of the CC could be seen in *Satb2*^{-/-};*Ctip2*^{-/-} compound mutants. Fibers manage to reach and cross the midline (arrows). The sections have been subjected to immunohistochemical staining for the neural cell adhesion molecule L1 and for GFP. Scale bar= 450μm for low magnification images and 100μm for high magnification images.

Others and we have previously shown that the deletion of *Satb2* results in the ectopic up-regulation of *Ctip2* and misprojection of callosal axons laterally (Alcamo et al., 2008; Britanova et al., 2008). Since *Satb2* and *Ctip2* regulate divergent genetic programs, that control the formation of interhemispheric versus corticofugal projections (Alcamo et al., 2008; Arlotta et al., 2005; Britanova et al., 2008), we investigated whether down-regulating *Ctip2* in the *Satb2*^{-/-} cortex could restore CC formation. We analyzed *Satb2*^{-/-};*Ctip2*^{-/-} double mutant animals for the presence of the CC. To visualize callosal axons, we

introduced a plasmid coding for *Venus-GFP* in neocortical cells by *in utero* electroporation at E12.5 and harvested the brains at E18.5. We found that while in *Satb2*^{-/-} animals, no cortical axons approached or even turned towards the dorsal midline, in *Satb2*^{-/-};*Ctip2*^{-/-} compound mutants, GFP positive axons approached, crossed the midline and invaded the contralateral cortex. It is noteworthy though that the number of crossing axons that projected into the contralateral hemisphere were fewer when compared to that seen in wild type and *Ctip2*^{-/-} littermates (Figure 32). The results were quantified by measuring the fluorescent area of the fibers projecting medially with respect to the fluorescent area of the electroporated region in the cortex (Figure 43).

3.30 *Satb2* deficient callosal neurons have defects in axon targeting and not outgrowth or midline guidance

To rule out the possibility of axonal outgrowth defects in *Satb2* negative neurons as a cause for the lack of extension of the axons contralaterally, we performed an *in vitro* axon-outgrowth assay using wild type and *Satb2* mutant cortical explants, but did not observe an axonal outgrowth deficit in *Satb2* deficient neurons (Figure 33A). Another possibility was that the axons were indirectly affected due to changes in the formation of midline guidepost structures in the *Satb2* mutant and *Satb2*^{-/-};*Ctip2*^{-/-} double mutants (Niquille et al., 2009; Shu & Richards, 2001). To examine this, we stained for midline guidepost cells: Sling neurons- with anti-calretinin (CR) and midline glia: with anti-GFAP. We did not detect any differences in the size or morphology of midline structures between the different genotypes suggesting that *Satb2* deficient neurons are defective in axonal targeting and not outgrowth or midline crossing (Figure 33B).

Figure 33. *Satb2*^{-/-} neurons do not show defects in neurite outgrowth and midline guidepost cells are not altered in *Satb2*^{-/-}, *Ctip2*^{-/-} or *Satb2*^{-/-}*Ctip2*^{-/-} mutants

(A) Outgrowth ratio of axons from cortical explants of wild type, *Satb2*^{+/-} and *Satb2*^{-/-} were compared. *Satb2* deficient neurons did not show impairment in axonal growth, instead showed a slight increase in outgrowth ratio when compared to the controls (p-value= 0.0037 Student's t-test). (B) Midline guidepost cells were stained for using antibodies against Calretinin and GFAP in wild type, *Satb2*^{-/-}, *Ctip2*^{-/-} and *Satb2*^{-/-}*Ctip2*^{-/-} brains, showing comparable expression patterns and levels in all four genotypes. Scale bar=100µm.

3.31 Restored expression of *Unc5C* in *Satb2*^{-/-} mutants results in partial rescue of CC

Since the *Satb2*^{-/-}*Ctip2*^{-/-} double mutants showed a partial rescue of callosal crossing fibers and an increased *Unc5C* expression compared to single *Satb2*^{-/-} mutants, we tested whether we could rescue callosal fibers in *Satb2*^{-/-} mutants by over-expressing *Unc5C*.

Figure 34. The corpus callosum is partially restored by over-expressing *Unc5C* in *Satb2*^{-/-} mutant cortex.

Images show the result of *Unc5C/EGFP* over-expression in wild type and *Satb2*^{-/-} mutant cortex. Over-expression of *Unc5C* in *Satb2*^{-/-} partially restores the CC where axons of electroporated neurons reach and cross the midline (arrow). Normally projecting callosal

axons were observed in the control cortex. Scale bar= 450µm for low magnification images and 100µm for high magnification images.

An *Unc5C* expressing plasmid was co-expressed with *Venus-GFP* at E12.5 and compared with *Venus-GFP* expression alone in *Satb2*^{-/-} as well as control brains. We observed that axons of the *Unc5C* electroporated neurons in the *Satb2*^{-/-} cortex extended medially, and crossed the midline (n=3). This rescue was again only partial as fewer axons crossed the midline compared to that in the control animals (Figure 34). Electroporation of only *Venus-GFP* in *Satb2*^{-/-} embryos served as a negative control where no callosally extending fibers were observed (Figure 32). The extent of callosal midline crossing with electroporation of *Unc5C* was comparable to the partial rescue obtained in *Satb2*^{-/-};*Ctip2*^{-/-} double mutants at E12.5 (Figure 32, 34, Quantification of CC rescue - Figure 43).

3.32 Neocortical callosal axons are repelled by Netrin1.

Unc5C has been shown to be a receptor for the secreted ligand Netrin1, mediating long range repulsive signaling (K. Hong et al., 1999; Serafini et al., 1996). Since we observed that the formation of callosal projections depended upon the presence of *Unc5C* expression, we tested whether these *Unc5C*-positive callosally projecting neurons were also responsive to Netrin1. To test this we devised a slice culture system where wild type embryos were co-electroporated with either *Unc5C* and *EGFP* (Figure 35A) or only *EGFP* (Figure 35B) at E12.5 and harvested at E16.5. Slices prepared from these brains were cultured for 3 days *in vitro* and Netrin1 soaked agarose beads were placed in the midline at the position where callosal fibers cross. If the *Unc5C* positive callosal axons were responsive to the repulsive Netrin1 signal, then they would be prevented from crossing the midline upon encountering the ligand in their trajectory. When *Unc5C* positive fibers encountered Netrin1 soaked beads in the midline, they were repelled and did not cross the

midline. Instead they misprojected ventrally or dorsally within the ipsilateral hemisphere showing an avoidance behavior towards the beads.

Figure 35. Netrin1 repels Unc5C positive neocortical axons.

Images showing the axonal trajectory of neurons electroporated with *Unc5C* (A) or *EGFP* (B) on encountering neutral BSA coated beads (arrows) or Netrin1 coated beads (arrows). *Unc5C*-positive fibers are unaffected by BSA coated beads, placed along the midline, and continue along their initial trajectory to project into the contralateral hemisphere. *Unc5C*-positive fibers, upon encountering a Netrin1 source in the midline show clear repulsive behavior with fibers projecting either dorsal or ventral to their initial path, but none continuing into the contralateral hemisphere. Wild type axons electroporated with only *EGFP* (with endogenous *Unc5C*) are unaffected by the BSA beads and are repelled by the Netrin1 beads similar to the *Unc5C* over-expressing axons. Scale bar specified in the figure.

Conversely, when these *Unc5C* positive axon fibers encountered BSA soaked beads, they were unaffected and continued in their trajectory, crossing the midline and entering the contralateral hemisphere (Figure 35A). This experiment indicated that *Unc5C* expressing cortical neurons are indeed repelled by a Netrin1 source. By expressing only *EGFP* in cortical neurons, we

tested our hypothesis that if callosal fibers express *Unc5C* endogenously, then they would also be repelled from a Netrin1 source. Again, these fibers failed to cross the midline upon encountering Netrin1, whereas BSA coated beads posed no hindrance to the midline crossing (Figure 35B).

3.33 Misrouting of Satb2 positive axons in both *Netrin1* and *Unc5C* mutants.

Since *Unc5C* is expressed in *Satb2* positive neurons (Figure 28), and their axons are repelled by Netrin1 *in vitro* (Figure 35), we asked whether Netrin1 deletion would have a similar effect on callosal axons *in vivo*. In the developing telencephalon *Netrin1* is expressed in the basal ganglia and ventral midline (Barallobre et al., 2000; Métin et al., 1997). We reasoned that the source of Netrin1 in the basal ganglia might normally repel the axons of *Satb2* positive/*Unc5C* positive neurons from the internal capsule. We hypothesized that *Satb2* positive neurons would misproject to sub-cortical targets in *Netrin1* deficient brains. To test this hypothesis, we placed crystals of the lipophilic tracer DiI at the cerebral peduncle of E17.5 *Netrin1* hypomorph and wild type brains to back trace the neurons that project sub-cortically. After staining these sections with an antibody against *Satb2*, we counted the proportion of DiI-positive retrograde-labeled neurons that were also *Satb2* positive (Figure 36A). We observed that the proportion of *Satb2* positive- DiI labeled cells representing the proportion of *Satb2*-positive, sub-cortically projecting neurons, almost doubled in *Netrin1* hypomorph brains compared to their wild type littermates (Figure 36B, 15.36% in wild type compared to 27.33% in *Netrin1* hypomorph, p-value=0.011, n=3).

Figure 36. Satb2 cells are similarly misrouted to subcortical targets in *Netrin1* and *Unc5C* mutants

(A) Retrograde labeling of subcerebrally projecting neurons by placing crystals of DiI in the cerebral peduncle of wild type and *Netrin1*^{-/-} brains. The smaller inset image show the respective position of the crystal placement. Back-labeled DiI-positive cells were co-localized with Satb2 in wild type and *Netrin1* mutant brains. Arrows point towards double positive cells while double arrows highlight single DiI-positive cells. (B) Quantification of the percentage of DiI-positive cells that are Satb2-positive; 15.36% in wild type versus 27.33% in E17.5 *Netrin1* mutant, p-value= 0.011, n=3. Error bars represent s.e.m. (C) Crystals of the

lipophilic tracer DiI were placed in the cerebral peduncle of wild type and *Unc5C*^{-/-} brains to retrogradely label cortical neurons projecting sub-cerebrally. The smaller inset images show the location of the crystals placed in the cerebral peduncle. Higher magnification image of the boxed region show layer V neurons back-labeled in the cortex. **(D)** Co-localization of DiI-positive and Satb2-positive cells in the neocortex. Arrows indicate double positive cells, while double arrows indicate single DiI-positive cells. Scale bar=100μm. **(E)** Quantification of the proportion of sub-cortically projecting neurons that are Satb2 positive; 49.10% in wild type versus 64.98% in E18.5 *Unc5C*^{-/-}; *p*-value=0.0286, n=3. Error bars represent s.e.m.

We also tested whether *Unc5C* ablation in neocortical neurons would cause a similar effect. In contrast to the acallosal *Netrin1* mutant, *Unc5C* mutants display callosal midline crossing fibers. We therefore tested whether some axons that would normally project to the CC are redirected to the internal capsule in the *Unc5C* mutant. We performed similar DiI retrograde-labeling experiments in *Unc5C*^{-/-} brains and counted the number of Satb2 positive cells projecting to the internal capsule (Figure 36C, D). We found that the proportion of Satb2 positive, sub-cortically projecting cells was 16% higher in the *Unc5C* mutants compared to wild type littermate brains (Figure 36E; 49.1% in wild type versus 64.98% in *Unc5C*^{-/-} brains, *p*-value=0.0286, n=3). Therefore, although callosal axons cross the midline in the *Unc5C* mutant, more Satb2-positive axons project to the internal capsule in both these mutants. This finding indicates that a *Netrin1*-*Unc5C* interaction plays an important role in determining the choice of axonal target projection (medially versus laterally), in a cell-autonomous manner.

3.34 Satb2 is a direct repressor of DCC transcription.

Since restoring the expression of *Unc5C* in *Satb2*^{-/-} mutants lead to only a partial rescue of the CC, we tested whether additional axon guidance molecules in this pathway that are important in CC formation displayed altered expression in *Satb2*^{-/-} brains. Like *Unc5C*, DCC is also a receptor for *Netrin1*, but has been shown to mediate axonal attraction (Chan et al., 1996b; Deiner et al., 1997; Fazeli et al., 1997).

Figure 37. DCC is up-regulated in the *Satb2*^{-/-} mutant and *Satb2*^{-/-};*Ctip2*^{-/-} double mutant cortex

(A) Immunohistochemical staining for DCC in rostral and caudal sections show an up-regulation of DCC expression in *Satb2*^{-/-} mutant brains as compared to wild type littermates. Arrows point to the region of altered DCC expression between wild type and *Satb2*^{-/-} sections. (B) *In situ* hybridization reflects an up-regulation of DCC in E18.5 *Satb2*^{-/-} cortex when compared to *Satb2* heterozygous brains. *Ctip2*^{-/-} mutant did not show any alteration in DCC expression when compared to controls. *Satb2*^{-/-};*Ctip2*^{-/-} double mutant showed comparable expression to the *Satb2*^{-/-} mutant. Scale bar=100μm.

We performed immunohistochemical staining and *in situ* hybridization for *DCC* in wild type as well as *Satb2*^{-/-} cortices at E18.5. Both, *DCC* protein and mRNA expression was upregulated in *Satb2*^{-/-} brains as compared to wild type littermates (Figure 37A, B). In contrast, no changes in *DCC* expression were observed between the control and *Ctip2*^{-/-} or between the *Satb2*^{-/-};*Ctip2*^{-/-} double mutant and *Satb2*^{-/-} single mutant indicating that *Ctip2* did not effect *DCC* expression (Figure 37B).

In the wild type cortex, *DCC* demonstrated a very dynamic pattern of expression. In contrast to *Unc5C* expression, *DCC* mRNA expression is high in the cortical plate at E14.5 (Figure 38A) and below detection levels at E18.5. Only a low level of *DCC* protein expression was observed in the white matter at E18.5 (Figure 37B). In order to investigate if *Satb2* could regulate *DCC* expression, we over-expressed *Satb2* and *Ski* prematurely in wild type cortex at E11.5 and analyzed the brains for *DCC* expression at E14.5. Again, *Ski* was co-electroporated to enable *Satb2* to recruit the transcription repressor complex (Baranek & Atanasoski, 2012; Baranek et al., 2012). At E14.5, since there are few endogenous *Satb2* positive cells in the cortex and since *DCC* expression is high in the cortical plate, we assumed that any alteration in *DCC* expression would be observable. In this experiment *DCC* expression was significantly lower in the electroporated region compared to the non-electroporated hemisphere (n=2). In contrast, *EGFP* electroporation alone did not alter the expression of *DCC* (n=2) (Figure 38A).

To investigate whether *Satb2* could directly control *DCC* expression, we performed ChIP assays for two putative *Satb2* binding sites (MAR1 (matrix attachment region1) and MAR2) upstream of the *DCC* transcription initiation site (Figure 38B). These regions are AT rich and could therefore serve as *Satb2* binding sites. We observed a significant enrichment of the region more proximal to the TSS (MAR1) in wild type tissue as compared to *Satb2* mutant tissue, which served as the negative control (5.27 ± 2.75 fold enrichment in n=6 wild type compared to 1.72 ± 0.015 in n=2 *Satb2*^{-/-} mutants, *p*-value=0.025).

Figure 38. *Satb2* is a direct transcriptional repressor of *DCC* expression

(A) Series of images show *DCC* expression in E14.5 wild type embryos in cells electroporated with either *Satb2/Ski/EGFP* at E11.5 or with *EGFP* alone at E12.5. GFP immunohistochemistry delineates the region electroporated. *DCC* *in situ* hybridization demonstrates a down regulation of *DCC* mRNA within cells electroporated with *Satb2/Ski* compared to *DCC* expression in the contralateral hemisphere, or in the control electroporation. (B) Schematic diagram showing two putative binding MAR sites for *Satb2* upstream of the *DCC* transcription initiation site. Chromatin-immunoprecipitation from P0 cortices showed a 5.27 ± 2.75 fold enrichment in $n=6$ wild type compared to 1.72 ± 0.015 in $n=2$ *Satb2*^{-/-} mutants for *DCC* MAR1, p -value= 0.025 Student's t -test and for *DCC* MAR2, $n=7$ wild type showed a range of 0.91-10.83 fold enrichment with an average of 4.69 ± 4.38 , as compared to 1.47 ± 0.39 in $n=2$ *Satb2*^{-/-} mutants, p -value= 0.10 Student's t -test (C) Luciferase assay to demonstrate that *Satb2* binds to the *DCC* genomic region and represses its expression. In the presence of full length *Satb2*, *DCC* MAR1 showed a 1.5 fold decrease in luminescence ratio while *DCC* MAR2

did not show any change (Student's t-test, p-value = 0.0035). Scale bar=100 μ m, unless specified differently in the figure.

In contrast, enrichment for the second MAR region (MAR2) was highly variable indicating that *Satb2* might bind very weakly to this promoter region (n=7 wild type showed a range of 0.91-10.83 fold enrichment with an average of 4.69 ± 4.38 , as compared to 1.47 ± 0.39 in n=2 *Satb2*^{-/-} mutants, p-value=0.10). To further investigate the functional significance of this MAR binding, we cloned the two genomic regions, *DCC MAR1* and *DCC MAR2*, into a Gaussia Luciferase vector and transfected them into COS cells either in the presence or absence of *Satb2*. *DCC MAR1* (chromosome 18 72356779-72357851) showed a 1.5 fold decrease in transcription levels when co-expressed with *Satb2* (Figure 38C, n=3, p-value=0.0035). *DCC MAR2* (chromosome 18 72362066-72363664) did not show any change in transcription levels upon *Satb2* co-expression (Figure 38C, n=3, p-value=0.742). Taken together, these findings suggest that *Satb2* can directly repress *DCC* expression and might be another factor influencing cortico-cortical versus cortico-subcortical axon targeting choice.

3.35 Downregulation of DCC in *Satb2* mutants results in partial rescue of the CC

To test the hypothesis that *DCC* up-regulation contributed to the misrouting of callosal axons in *Satb2* deficient brains, we tested whether downregulating *DCC* in the *Satb2* mutant could restore callosal projections. We tested the downregulation efficiency of four different shRNA constructs by transfecting *DCC shRNA* or the scrambled shRNA together with a *DCC* expression plasmid in HEK293T cells and checking protein levels by Western blot (Figure 39A). Based on these experiments we selected two *DCC shRNA* constructs (clone 2 and 4) and co-electroporated them with *Venus-GFP* at E12.5 in *Satb2*^{-/-} and wild type cortices.

Figure 39. Down-regulating *DCC* in the *Satb2*^{-/-} cortex leads to a partial rescue of the corpus callosum.

(A) The efficiency of *DCC* knockdown was verified in HEK293T cells. Four different shRNAs were tested for their efficiency in *DCC* knockdown. A scrambled shRNA was used as a control. Western blot analysis shows a clear reduction in *DCC* protein levels in all four shRNAs compared to the scrambled shRNA. Quantification shows relative protein levels of *DCC* to tubulin. *DCC shRNA 2* and *4* proved most efficient in knocking down *DCC*, and were thus used in the experiments. (B) Images show a series of experiments, wherein *in utero* electroporation was done at E12.5 and the brains harvested and analyzed at E18.5. *In utero* electroporation of *DCC shRNA* and *Venus GFP* do not seem to have a major effect on the formation of the CC in wild type embryos. *In utero* electroporation of two different *DCC shRNAs* (shRNA2 and shRNA4) along with *Venus GFP* show a reproducible partial rescue of

the CC in *Satb2*^{-/-} embryos. *In utero* electroporation of a scrambled shRNA and *Venus GFP* cannot rescue the CC in *Satb2*^{-/-} embryos. Arrows point towards the dorsal midline. Scale bar= 450µm for low magnification images and 100µm for high magnification images.

While callosal axons crossed the midline in wild type brains electroporated with either the *DCC shRNA* or the *scrambled shRNA*, no midline crossing fibers were observed when *scrambled shRNA* was electroporated in *Satb2*^{-/-} brains (n=2). In contrast, neurons electroporated with *DCC shRNA* in the *Satb2*^{-/-} cortex now extended towards and crossed the midline (n=3, Figure 39B). The rescue was however again only partial (Quantification of CC rescue- Figure 43). Together, these results indicate that *Satb2* directly represses the expression of *DCC* and that an up-regulation of *DCC* in the *Satb2*^{-/-} cortex could contribute to the lack of callosal axons crossing the midline.

Figure 40. Over-expression of *Unc5C* with the simultaneous down-regulation of *DCC* does not rescue the corpus callosum.

Images show experiments, wherein *Unc5C*, *DCC shRNA* and *Venus GFP* were co-electroporated at E12.5 and the brains harvested and analyzed at E18.5. Wild type embryos display a normal CC at E18.5. The CC was not rescued when *Unc5C* and *DCC shRNA* were co-electroporated in *Satb2*^{-/-} embryos. Inset images show higher magnification of the midline. Scale bar= 450µm.

In order to test whether *Unc5C* and *DCC* act as parallel pathways and the extent of CC restoration in *Satb2* mutants could be enhanced by simultaneously down-regulation *DCC* and up-regulating *Unc5C* in the *Satb2* mutant, we coexpressed *DCC shRNA* and *Unc5C* overexpression construct

together with *eGFP* in *Satb2*^{-/-} mutants. Surprisingly, in these experiments we did not detect any CC restoration (Figure 40).

3.36 Netrin1/DCC/Unc5C interactions regulate callosal projections of only deep layer neurons

Figure 41. Over-expression of *Unc5C* or down-regulation of *DCC* at E14.5 does not rescue the CC in *Satb2*^{-/-} mutants

(A) Over-expression of *Unc5C* at E14.5 by *in utero* electroporation in *Satb2*^{-/-} mutants does not rescue the CC, whereas wild type littermates show normal callosum. (B) Down regulation of *DCC* by shRNA electroporation at E14.5 does not rescue the CC in *Satb2*^{-/-} mutants, whereas wild type littermates show normal callosum. Inset images show higher magnification of the midline. Scale bar=450μm.

Callosal neurons are a heterogeneous population of neurons (B J Molyneaux et al., 2009). The majority of callosal axons are from upper layer neurons,

hence in order to test if upper layer neurons also show dependency on *Satb2/Ctip2* and hence *DCC/Unc5C* for making the choice between taking a medial or a corticofugal trajectory, we electroporated *Unc5C* (Figure 41A) or *DCC shRNA* (Figure 41B) in *Satb2*^{-/-} cortex at E14.5 and harvested the brains at E18.5. The later stage of electroporation ensures targeting of mainly the upper layer neurons. We observed that both the experiments did not yield in a rescue of the CC, and no fibers could be observed taking the medial path. Hence, with respect to the molecular mechanisms described here, it could be concluded that deep neocortical layers, born before E14.5, are dependent upon the Netrin1/Unc5C/DCC pathway. However, upper layer neurons, born after E14.5 probably require pathway(s) other than Netrin1/Unc5C/DCC.

3.37 *Satb2* has limited capacity to restore the corpus callosum through cell intrinsic mechanisms

The lack of a complete restoration of the CC in *Satb2* mutants after *Unc5C* up-regulation or *DCC* down-regulation, questioned the extent of the cell autonomous contribution of *Satb2* to CC formation. We therefore electroporated a *Satb2* expression plasmid together with *EGFP* into *Satb2*^{-/-} brains at E12.5. At E18.5 we tested the ability of the electroporated *Satb2* in repressing *Ctip2* and found an absence of *Ctip2* from the electroporated cells (Figure 42A). In the electroporated brains we observed that although the electroporated neurons in the *Satb2*^{-/-} mutants were able to form a CC, the rescue of the CC was again only partial, similar to the rescues obtained in case of either the *Satb2*^{-/-};*Ctip2*^{-/-} double mutants, *Unc5C* over-expression or *DCC* down-regulation in the *Satb2*^{-/-} mutants (n=2, Figure 42B, Quantification of CC rescue - Figure 43). A similar principal seems to be underlying all of these scenarios as *Satb2* upon ectopic over-expression in wild type cortices can lead to *Unc5C* over expression (Figure 28B) and *DCC* downregulation (Figure 38A). Again, we did not observe any rescue of the CC upon over-expression of *Satb2* at later stages (E14.5, Figure 42C).

Figure 42. *Satb2* electroporation in *Satb2*^{-/-} cortex partially rescues the corpus callosum.

(A) *In utero* electroporation of *Satb2* in *Satb2*^{-/-} cortex results in a down regulation of *Ctip2* expression in the electroporated cells, verifying the activity of *Satb2* in these cells. Scale bar=100μm. (B) *In utero* electroporation of plasmids encoding for *Satb2* and *EGFP* in wild type and *Satb2*^{-/-} embryos at E12.5, harvested at E18.5 followed by immunohistochemical staining for the neuronal adhesion molecule L1, show a partial rescue of the CC in *Satb2*^{-/-} mutants. Alternatively, sections were counter stained with the far-red nuclear marker Draq5. Arrows

indicate fibers crossing the midline. Scale bar= 450μm for low magnification images and 100μm for high magnification images. (C) Over-expression of *Satb2* at E14.5 does not rescue the CC in *Satb2*^{-/-} mutants, whereas wild type littermates show normal CC. Inset images show higher magnification of the midline. Scale bar=450μm. Inset images show higher magnification of the midline.

Figure 43. Quantification of CC with respect to efficiency of electroporation in wild type and *Satb2*^{-/-} mutant

(A) In order to quantify the extent of rescue of medial projections in the *in utero* experiments performed, ratio of the fluorescent area of the medially projecting fibers in the ipsilateral hemisphere (within box a) to the fluorescent area of the cells electroporated in the cortical plate (within box b) was measured. The size of box 'b' varied depending upon the extent of electroporation. The size of box 'a' was adjusted so as to enclose the extent of medially projecting fibers after they make the dorsal turn within the ipsilateral cortex till the midline. (B) The *in utero*-experiments with GFP in wild type, GFP in *Satb2*^{-/-}, GFP in *Satb2*^{-/-};*Ctip2*^{-/-}, *Unc5C* in *Satb2*^{-/-}, *Satb2* in *Satb2*^{-/-}, DCC shRNA in *Satb2*^{-/-} and Scrambled shRNA in *Satb2*^{-/-} were quantified. All values are represented as mean ± SEM. All p values are calculated by student's t test. GFP in wild type (n=3) = 0.199±0.0415, GFP in *Satb2*^{-/-} (n=3) = 0.00003±8.26x10⁻⁶, p value = 0.0086, GFP in *Satb2*^{-/-};*Ctip2*^{-/-} (n=2) = 0.1419±0.027, p value = 0.0058, *Unc5C* over-expression in *Satb2*^{-/-} (n=3) = 0.0498±0.009, p value = 0.0058, *Satb2* electroporation in *Satb2*^{-/-} (n=2) = 0.0606±0.003, p value = 0.00012, DCC shRNA in *Satb2*^{-/-} (n=3) = 0.0489±0.003, compared to scrambled electroporation in the *Satb2*^{-/-} (n=2) = 0.00017± 7.83x10⁻⁶, p value = 0.00023.

Together, the above experiments suggest that *Satb2* and *Ctip2* play a crucial role in the decision of axonal projections to take a medial route and form the corpus callosum or take a sub/cortical route. The axons make this choice with the help of the axon guidance molecules DCC and Unc5C that respond to Netrin1, where *Satb2* transcriptionally regulates *DCC* and *Ctip2* regulates *Unc5C* expression.

4. DISCUSSION

Evolution of the brain has witnessed a consistent growth in both the number of neocortical neurons as well as in the connections that these neurons make. This robust increase in size and circuitry has tremendously augmented the variety as well as the complexity of the tasks the brain is capable of executing. Understanding the developmental mechanisms involved in establishing these connections hence forms an integral part of understanding the functions of the brain as a whole (Geschwind & Rakic, 2013). To understand how neurons extend their axons, elongate at a particular rate, branch out at particular regions and finally stop growing at a critical time is essential not only for understanding the development of the nervous system but also to understand the mechanisms involved in regrowth of axons after injury (Goldberg, 2003). In this study we have investigated the contribution of three different transcription factors, namely *Sip1*, *Satb2* and *Ctip2* and the regulatory mechanisms downstream of these factors involved in establishing cortical projections.

Sip1 is a SMAD binding protein, which together with a cohort of cofactors has been shown to behave as both a transcriptional repressor as well as a transcriptional activator depending on the cellular and genomic context (Comijn et al., 2001; Postigo & Dean, 2000; Postigo et al., 2003; Verschueren et al., 1999; Yoshimoto et al., 2005). In the neocortex *Sip1* is expressed exclusively in the postmitotic compartment of the cortex and the expression of the protein is detectable when the first set of neurons populate the cortical plate and the expression expands as the cortical plate grows (Seuntjens et al., 2009). Almost all neocortical neurons express *Sip1* indicating that it could potentially play a global role with respect to development of the different types of neocortical neurons and their connections. In this study we have investigated the various cortical projection defects in the *Sip1^{fl/fl}Nex^{Cre}* conditional mutant and

dissected out the cell intrinsic and cell extrinsic mechanisms with which *Sip1* exerts its control over establishing cortical circuitry.

Unlike *Sip1*, *Satb2* and *Ctip2* are expressed in very specific sub sets of neocortical neurons. While *Satb2* is expressed mostly in callosally projecting neurons in layer II, III and layer V, *Ctip2* is expressed in neurons that project through the CST that form the majority of layer V neurons (Alcamo et al., 2008; Arlotta et al., 2005; Britanova et al., 2008). In the mouse brain, though the majority of callosally projecting neurons lie in layers II, and III, some of the layer V neurons also project through the CC (Ivy & Killackey, 1981). Hence, in the mouse, layer V neurons consist of a heterogenous population of cells wherein some neurons express *Ctip2* and project subcortically and others express *Satb2* and project callosally. We have investigated the molecular mechanisms downstream of *Satb2* and *Ctip2* in deep layer neurons, which enables a population of cells born around the same developmental time point, occupying the same laminar position in the cortex, make opposite decisions with respect to their projections.

4.1 Commissural and sub-cortical projections are disrupted in the *Sip1* mutant

Many different cortical axon tracts are severely altered in the absence of *Sip1* (Figure 44). The CC, which forms the most important commissural projection in the brain, is absent in the *Sip1* mutant. While the callosal neurons are able to project towards the cortical midline, these axons fail to cross the midline and form Probst bundles. The AC, which is an evolutionarily more primitive commissural projection in the brain is also absent in the *Sip1* mutant. This makes the *Sip1* mutant a very interesting model to study not only from a developmental point of view but also its behavioral aspects, as all forms of interhemispheric cortical projections are absent making each neocortex an independent unit. While the two commissural projections that originate from within the cortex are absent in the *Sip1* mutant, the hippocampal commissure,

which is a commissural tract that interconnects the two hippocampi is unaffected. This is an interesting finding as it provides evidence that *Sip1* control over the formation of commissural projections is specific to cortical neurons and does not extend to hippocampal neurons, even though *Sip1* is expressed in hippocampal neurons.

Figure 44. Connectivity defects observed in the *Sip1* mutant forebrain

A summary of the connectivity defects observed in the *Sip1^{fl/fl} Nex^{Cre}* conditional mutant when compared to the wild type. The corpus callosum (CC), anterior commissure (AC) and corticospinal tract (CST) are absent. The cortico-thalamic tract (CT), thalamo-cortical tract (TC) and hippocampal commissure (HC) are unaffected by *Sip1* deletion.

A possible explanation for the presence of the HC in the *Sip1* mutant while the CC does not form could be because the HC forms further ventral to the CC. In the *Sip1* mutant, since the fusion of the dorsal midline does not occur, CC axons cannot cross, but since midline fusion does occur ventrally, HC axons have the required scaffold to navigate across to the contralateral side. At the outset, this argument does not hold true for the AC, since these axons traverse further ventral to the HC in the wild type forebrain, but do not form in the *Sip1* mutant although midline fusion has occurred ventrally. DiI labeling at P0 in the *Sip1* mutant brain showed that AC axons misproject

ventrally from the external capsule instead of projecting towards the contralateral hemisphere. We hence, believe that the absence of the AC axons in the *Sip1* mutant is due to misprojection of the axons rather than midline defects.

Cortical connections projecting to subcortical targets are also affected to varying degrees in the *Sip1* mutant (Figure 44). While the CT tract projecting to the thalamus is present in the *Sip1* mutant, the CST is absent. Although initial projections of the CST can be seen at the level of the cerebral peduncle of *Sip1* mutants at P0, these projections disappear by P5. This loss of CST in the *Sip1* mutant at early postnatal stages is not due to cell death of layer V neurons as confirmed by cleaved caspase 3 staining in the neocortex at P2. This indicates that the loss of CST in the *Sip1* mutant is due to axon guidance or axon growth defects.

It is interesting to note that of all the axonal tracts originating from within the neocortex it is only the CT tract that is unaffected in the *Sip1* mutant. This might be correlated to the fact that the CT is the only tract, which depends on an external (non-cortical) source of axons to function as pioneer neurons (Koester & O'Leary, 1994; McConnell et al., 1989; Molnar, Adams, & Blakemore, 1998; Rash & Richards, 2001). It is probable that like all other neocortical tracts the neocortical pioneer axons are also affected in the *Sip1* mutant which in turn influence the formation of the commissural projections and the corticospinal tract. In case of the CT, since the incoming thalamic axons are unaffected, the CT might be affected to a lesser degree. Interestingly, the CST, which follows the CT in its initial trajectory till the internal capsule, is also present in the *Sip1* mutant at early stages. Its disappearance after P0 might be due to the absence of guidance cues further ahead in the trajectory or deficiency in internal axonal machinery of the neuron.

An alternate possibility for the presence of CT but none of the other cortical tracts could be that, the trajectory of corticothalamic axons does not cross the

midline. While the CC crosses the midline at the dorsal forebrain and AC at the ventral forebrain, the CST crosses the midline at the pyramidal decussation in the brainstem. As the midline is a very important intermediate target that involves a complex interplay between attractive and repulsive cues; specific receptors need to be expressed by the crossing axons at specific time points in order to cross the midline (Chen et al., 2008; Colamarino & Tessier-Lavigne, 1995; Dickson & Zou, 2010; Evans & Bashaw, 2010; Kaprielian et al., 2000; Kidd et al., 1998; Klämbt, Jacobs, & Goodman, 1991; Long et al., 2004), which in turn may be under *Sip1* control. For example, Robo-Slit interactions have been shown to be extremely important for midline crossing. Our *in situ* hybridization experiments in the *Sip1* mutant show that *Robo2* is severely downregulated in the absence of *Sip1*.

4.2 Dual role of *Sip1* in CC formation

Heterozygous mutation in *SIP1* gene cause a disease known as the Mowat-Wilson syndrome in humans which is characterized by severe craniofacial defects, microcephaly and severe mental retardation. In most cases, it is also associated with agenesis of the CC (Cacheux et al., 2001; D R Mowat, M J Wilson, 2003; Garavelli & Mainardi, 2007; Moal et al., 2007; Verstappen et al., 2008; Wakamatsu et al., 2001; Wilson et al., 2003; K. Yamada et al., 2001), which makes studying the role of *Sip1* in CC formation essential, also from a clinical point of view. We firstly dissected out the cell autonomous as well as the non-cell autonomous function of *Sip1* in aiding callosum formation. Re-expression of *Sip1* in the *Sip1* mutant neocortex, so as to express the molecule in a mosaic manner in a mutant background, showed that unlike *Sip1* deficient neurons that stall within the ipsilateral neocortex much before the midline fissure, the *Sip1* over-expressing neurons were capable of extending their axons all the way till the midline fissure and stopped short of crossing the midline. This experiment goes to show that *Sip1* plays a dual role with respect to CC formation. It cell intrinsically controls certain factors, at least within callosal neurons, that help in extension of the axons towards the

midline, and additionally, the lack of midline crossing shows that there are cell extrinsic mechanisms involved as well.

4.3 Non-cell autonomous role of *Sip1* at the cortical midline

Figure 45. Non-cell autonomous defects in the *Sip1* mutant midline

A schematic representation of the wild type and *Sip1^{fl/fl} Nex^{Cre}* mutant midline, summarizing the non-cell autonomous effects of *Sip1* deletion on the morphology of the cortical midline and its effect on callosal projections. In the *Sip1* mutant the dorsal midline does not fuse, no indusium griseum glia (IG) is present, glial wedge (GW) and midline zipper glia (MZG) are highly reduced, sub callosal sling neurons (SS) are mislocalized. As a consequence callosal axons (CC) do not pass the mutant midline and instead form Probst bundles.

The midline forms a very critical intermediate guidepost structure for the formation of commissural projections. In case of the CC, the cortical midline possesses various physical and chemical cues, which act as short and long range signals to help direct axons towards the contralateral hemisphere (Niquille et al., 2009; Shu & Richards, 2001; Shu et al., 2003; Shu et al., 2003; Silver et al., 1993). In the *Sip1* mutant, the cortical midline shows a variety of developmental defects (Figure 45). Firstly, unlike in the wild type neocortex, the dorsal midline in the *Sip1* mutant is not fused, thus not providing a pathway for axons to pass through. The process of midline fusion is thought to be performed by midline zipper glia (MZG) that replace the fibroblasts present in the midline fissure with CNS tissue (Richards, Plachez, & Ren, 2004; Shu & Richards, 2001; Silver et al., 1993). The MZG is severely reduced in the *Sip1* mutant. Secondly, midline glia that act as guidepost cells namely the indusium griseum cells (IG) and the glial wedge cells (GW) are not

formed in the *Sip1* mutant. These structures express Slit family of proteins, which provide a repulsive environment for *Robo* expressing callosal neurons (Shu & Richards, 2001). Concurrent with the loss of midline glia, *Slit1* expression is also lost in the *Sip1* mutant midline. Since *Sip1* expression is restricted to the post-mitotic population of the neocortex, the lack of midline glial cells is probably not due to a proliferation defect at the midline, but most-likely due to defects in the differentiation process of the radial glia into the midline glia. Thirdly, sub-callosal sling neurons (SS) that form another group of guidance structures for the formation of the CC (Niquille et al., 2009; Shu et al., 2003), are also absent in the *Sip1* mutant. They also do not express *Sema3C*, which acts as an attractive guidance cue for the *Nrp1* expressing pioneer axons (Niquille et al., 2009). The contribution of *Sip1* to the formation of these midline guidance structures is a non-cell autonomous effect, as deleting *Sip1* by Cre electroporation in a small population of cells within the cortical midline of *Sip1^{fl/fl}* embryos does not result in a phenotype similar to the *Sip1^{fl/fl} Nex^{Cre}* mutant.

It has not been clearly understood whether the formation of sling neurons is independent of the formation of the CC and precedes the crossing of the first callosal fibers or if the pioneering fibers of the CC assist in the migration of these sling neurons hence exhibiting a more synergistic interdependent relationship. The *Sip1* mutant can be used as an interesting model for studying such a relationship. For example, since re-expression of *Sip1* in the mutant neocortex causes further extension of the callosal fibers at the midline, using live imaging experiments, one could follow the difference in behavior of the sling neurons between a mutant scenario and *Sip1* re-expression scenario. This would help to shed further light on the accessory role of the cortical midline in assisting CC formation.

4.4 Cell autonomous regulation of axon development by *Sip1*

We observed that on cell autonomous deletion of *Sip1* within a subset of cortical projection neurons, callosal fibers were unable to cross the midline by P0. While at E18.5 these axons did not make it to the contralateral hemisphere, by P8 at least most of the fibers could cross over. This together with the *in vitro* axon length assay showed that *Sip1* deletion results in shortened axons due to a stunted growth rate. Additionally, cell intrinsic deletion of *Sip1* in UL neurons, also affects the formation of interstitial axonal collaterals, which help in establishing synaptic contacts with the dendrites of Layer V pyramidal neurons. These findings hence suggest that apart from the non-cell autonomous role of *Sip1*, that regulates the architecture and cell specification of the cortical midline, *Sip1* controls many cell intrinsic aspects in cortical neurons that are important for axon growth, guidance and branching, making it an essential transcription factor for the development of neocortical circuitry.

4.5 Mechanism involved in cell intrinsic control of *Sip1* over axon growth and guidance

The formation of axonal projections that connect specific neurons to specific targets is a multi-step process that involves a plethora of axon guidance molecules that help the axonal growth cone navigate through a permissive environment while avoiding repulsive cues (Tessier-Lavigne & Goodman, 1996). While one form of molecular control over axon guidance regulates the time and expression pattern of guidance cues, regulation of various cytoskeletal structures that are at the core of growth cone behavior in response to these extracellular cues, is another critical step (Conde & Cáceres, 2009; Erik W Dent et al., 2011; Erik W. Dent, Tang, & Kalil, 2003; Quinn & Wadsworth, 2008). Our findings show that *Sip1* exerts a regulatory role on both these critical aspects of axon guidance - regulating the expression of axon guidance cues as well as influencing cytoskeletal modifications.

The expression of many different axon guidance molecules expressed by cortical projection neurons like *Cntn2*, *EphrinA4*, *Robo2* and *Nrp2* were affected in the *Sip1* mutant. While it remains to be investigated if *Sip1* regulates the expression of these molecules directly or indirectly, it can indeed be inferred that alterations in these many important guidance signals could be contributing to the vast number of guidance defects in the *Sip1* mutant.

Loss of *Sip1* in the neocortex also leads to a severe downregulation of *ninein*, a minus-end MT binding protein. In addition, as confirmed by ChIP assay, *Sip1* directly regulates the transcription of *ninein* by binding to its promoter region (Figure 46A). Although *in vivo* *Sip1* acts as a transcriptional activator of *ninein*, when luciferase assay was performed *in vitro* in HEK293T cells, there was a significant downregulation of transcription driven by the *ninein* enhancer region in the presence of *Sip1*. This discrepancy between the *in vivo* and *in vitro* function could probably be attributed to the fact that a heterologous system was used for the luciferase assay, and probable cofactors that might be required for *Sip1* to behave as an activator were absent like activated R-SMADs and SMAD4 (Yoshimoto et al., 2005). In the heterologous system used for the luciferase assay, such co-factors might not be present. It is also possible that *Sip1* activation of *ninein* is specific to neurons as it might be binding to neuron specific co-factors that aid in the transcriptional activation of *ninein*. The role of *Sip1* as a transcriptional repressor has been well established (Comijn et al., 2001; Postigo & Dean, 2000; Postigo et al., 2003; Postigo, 2003; Remacle et al., 1999; Verschueren, 1999), with few well-characterized instances where *Sip1* has been shown to play the role of an activator (Yoshimoto et al., 2005). The direct interaction between *Sip1* and *ninein* enhancer in this study further strengthens the evidence that *Sip1* can not only behave as a repressor but also as an activator and can thus perform a broader regulatory role depending on the cellular context.

4.6 Ninein localization and function

Ninein was initially discovered to be a centrosome-associated protein localized to the pericentriolar matrix of cells that possess a radial array of MTs. In these cells, ninein acts as a MT minus-end binding protein that helps to anchor the MTs to the centrosome (Bouckson-Castaing et al., 1996). In cells that are polarized and show the presence of non-centrosomal MTs, ninein is also shown to be present in non-centrosomal locations in association with the MT minus-end, thus conferring a more general 'MT minus-end capping' role to ninein that is independent of the centrosome (Mogensen et al., 2000). Additionally Moss et al., have shown that ninein granules are transported in a MT dependent manner from the centrosome to peripheral locations supporting a non-centrosomal role for ninein (Moss et al., 2007).

In neurons, the presence of ninein has previously been shown in the somato-dendritic compartments of the cell (Baird et al., 2004; Ohama & Hayashi, 2009). In this study we have presented evidence using direct immunohistochemical labeling and expression of GFP tagged *ninein*, that ninein is also present in the axonal compartment of neurons (Figure 46B). *In vivo*, ninein is expressed in the white matter of the neocortex that corresponds to the fasciculate axons of pyramidal projection neurons. *In vitro*, in cultured cortical neurons at DIV 2 and DIV 7, GFP-ninein could be observed in the somato-dendritic as well as axonal compartments of the cell including the axonal collaterals. These results hence suggest that ninein could be playing a more extensive role in neurons that is not restricted to the somato-dendritic compartments.

Overexpression of *ninein* in *Sip1* deficient neurons could rescue the axon extension defect both *in vitro* and *in vivo*. *In vivo*, although the axons of the electroporated neurons could extend across the midline into the contralateral hemisphere, the extent of midline crossing and invasion into the contralateral hemisphere was lesser than in the wild type. One possible explanation for such a partial rescue of the CC could be attributed to the fact that many axon

guidance molecules also showed altered expression patterns and levels in the *Sip1* mutant. These axon guidance molecules, apart from dictating the course of the axonal trajectory also act as signals that initiate axonal outgrowth and regulate the rate of axon extension (Quinn & Wadsworth, 2008).

4.7 Mode of action: Ninein stabilizes microtubules

Neurons are extremely polarized cells and the bulk of MTs in neurons are present as free MTs in axons and dendrites (Ahmad & Baas, 1995; Keating et al., 1997). Presence of non-centrosomal MT arrays allows for a more diverse set of MT arrangement and hence a diverse set of cellular morphology. It has been proposed that although the free MTs are not attached to the centrosome at the minus end, in order to remain stable, these MTs bind to other minus end stabilizing molecules (Keating & Borisy, 1999; Murphy, Vallee, & Borisy, 1977; Stearns & Kirschner, 1994; Zheng, Wong, Alberts, & Mitchison, 1995). In the absence of such a minus end stabilization, free MTs can form but do not persist (Keating & Borisy, 1999). Experiments by Rodinov et al., have shown that in the absence of minus end capping, the minus end of MTs are very unstable and continually shorten. Hence regulating the levels and distribution of these minus-end capping/stabilizing proteins acts as a way to control MT distribution and turnover (Rodionov, Nadezhdina, & Borisy, 1999).

The rescue of axonal length of *Sip1* deficient neurons on re-expression of *ninein* together with the finding that that ninein is expressed in all cellular compartments, in addition to the previous knowledge that ninein binds MT minus-ends, makes it a very suitable candidate for a minus end capping and MT stabilizing protein. Evidence from previously published work has shown that in cells with radial MT arrays (where only centrosomal MTs are present), MTs associated with the ninein expressing mother centriole, are more resistant to MT destabilizing agents (Mogensen et al., 2000) and the knockdown of ninein from the pericentriolar matrix of such cells results in severe MT disorganization (Dammermann & Merdes, 2002) . Although these

findings indicate that centrosome-associated ninein plays an important role as a stabilizing molecule for the radial MT asters, the role of non-centrosomal ninein has not been investigated so far. To our knowledge we have for the first time, shown that ninein plays a similar MT stabilizing role in neurons and possibly in other cells exhibiting non-centrosomal MTs as well (Figure 46C). Multiple pieces of evidence support this idea. Firstly, most cell types where non-centrosomal MTs have been studied, non-centrosomal ninein has also been found (Mogensen et al., 2000; Ohama & Hayashi, 2009). Secondly, work by Baird et al., showed that in migrating cells where non-centrosomal MTs are required, non-centrosomal ninein might be responsible for capturing the minus end of released MTs so as to prevent re-capture by the centrosome (Baird et al., 2004). Thirdly, in the present study we have shown that in neurons, ninein localizes to all cellular compartments where MTs are present including axons and axonal branches. And most importantly, we have also shown, for the first time that non-centrosomal ninein can also act as a MT-stabilizing agent in the axons and most likely dendrites of neurons. Neurons in culture over-expressing *ninein* show a very strong resistance to MT depolymerization in the axonal as well as dendritic compartments in the presence of Nocodazole- a microtubule destabilizing agent (De Brabander et al., 1976; Friedman & Platzer, 1978; Hoebeke et al., 1976; Ireland et al., 1979; Lee et al., 1980).

4.8 Ninein function in axon collateral formation

During growth and navigation of axons, MTs reorganize and re-orient themselves towards the direction of axon growth. Similarly at the vicinity of the target region of an axon from which interstitial branches emerge, MTs undergo extensive re-organization (Dent et al., 1999; Dent et al., 2003; Gallo, 2011; Kalil et al., 2000; Schmidt & Rathjen, 2010). Overexpression of *ninein* in *Sip1* deficient upper layer neurons could also rescue the axonal collaterals formed by these axons at layer V. Similar mechanism might be involved downstream of *Sip1* in axon growth and branch initiation, mediated by

ninein. For example, just as MTs are severed and transported into the growing axon from the centrosome with the help of enzymes like katanin and spastin (Ahmad et al., 1999; Baas, 1998; Sharp & Ross, 2012), at the interstitial branch sites too, MTs are cleaved by these enzymes (Yu et al., 1994; Yu et al., 2008). This generates smaller MTs, while simultaneously generating many free minus ends that need to be stabilized. We propose that similar to its role in the main axonal shaft, ninein might be required to re-capture the free MT minus-ends generated at the interstitial branch points to stabilize the newly formed MTs. A second possibility is that the defect in axon branch formation in the *Sip1* mutant is secondary to the retarded axon growth. For example, target-derived signals from the contralateral neocortex might be required for callosal upper layer neurons to start forming collaterals at the ipsilateral layer V. Since *Sip1* deficient axons are much slower than their wild type counterparts, they show a delay in reaching the target and in turn a delay in branch formation. In the presence of ninein, since the rate of extension of the axon is partially restored, the branching process is also rescued. Further experiments where the *GFP-IRES-Cre* electroporated *Sip1^{fl/fl}* brains are taken at sequential stages beyond P7 will help to deduce if collateral development in the *Sip1* deficient scenario is completely abolished or is only retarded similar to the axon growth defect.

4.9 Microtubule stability influencing microtubule growth

MTs exist in a state of dynamic instability, where the macromolecules oscillate between a state of growth or polymerization and break down or catastrophe, which may or may not be interspersed with a pause period (Mitchison & Kirschner, 1984a, 1984b; Walker et al., 1988). This dynamic instability enables rapid changes in the cytoskeleton that in turn influences axon growth, guidance and branching (Conde & Cáceres, 2009). Since MTs form the basic internal architecture of any cell, the growth rate of the MTs dictates the growth rate of the axon (Daniels, 1973; K. M. Yamada, Spooner, & Wessells, 1970). As the growth rate of MTs at the plus-end is much faster than the

minus-end, MT plus-end dynamics serves as a good indicator of net MT growth rate (Akhmanova & Steinmetz, 2008). Several MT binding proteins have been shown to bind specifically to the plus-end, thus making it possible to track MT dynamics by tracking the movement of these plus-end associated molecules (Galjart, 2005). One such molecule that has been successfully used for studying MT growth is EB3 (Stepanova et al. 2003). Examining GFP tagged EB3 dynamics in *Sip1* deficient neurons revealed that the plus end growth of the MTs was significantly slower when compared to controls, which also co-related with the finding that *Sip1* negative neurons have slow growing axons.

Many different factors control and influence the rate of MT growth (Cassirneris, 1993; Erickson & Brien, 1992), one such factor being the presence of stabilizing or destabilizing molecules associated with MTs. For example, in the presence of nanomolar concentration of Nocodazole, a MT destabilizing agent, the dynamic instability of MTs is disrupted and the MTs show very slow growth rate at the plus-end (Vasquez et al., 1997). Since *ninein* overexpressing neurons showed resistance to nocodazole treatment indicating a more stable MT framework in addition to the finding that the rate of axonal growth in *Sip1* mutants could be rescued by *ninein*, we hypothesized that ninein might contribute to the MT growth in these cells (Figure 46C). When GFP-EB3 dynamics was tested, we observed the rate of growth of MTs returned to wild type levels on expressing *ninein* in *Sip1* negative cells, hence indicating that ninein influences the dynamic instability of MTs thereby regulating axonal growth. It is still unclear as to how ninein being a MT minus-end binding protein could influence plus-end dynamics. We suggest two possibilities. By binding to the MT, ninein serves to primarily stabilize the growing MT polymer to which new tubulin dimmers are added, and in the absence of ninein, this MT lattice might be weak hence reducing the rate at which polymerization takes place. Alternatively, ninein might have additional role in MT dynamics other than binding to the minus-end. For example, in dendritic growth cones it has been shown that about 40% of

ninein granules observed were not associated with MTs (Ohama & Hayashi, 2009). These free ninein molecules might intermittently associate with other MAPs or regions other than the MT minus-end and in turn directly influence the plus-end dynamics. Such a dual role for a minus-end binding protein to directly regulate the dynamics of the plus end has been shown in case of the γ -TurC complex in *Drosophila* S2-cells (Bouissou et al., 2009).

4.10 New insights gained: The role of *Sip1* in axon development

Figure 46. Ninein localizes to neocortical axons and influences microtubule growth and stability downstream of *Sip1*

The schematic representation summarizes the cell autonomous role of *Sip1* in axon growth and branching. (A) Transcription factor *Sip1* binds to the *ninein* promoter and activates its expression in cortical postmitotic neurons. (B) *Ninein* protein localizes to the minus end of the non-centrosomal MTs in the soma, dendrites, axon and axonal branches. (C) *Ninein* influences the rate of axon growth and the formation of axonal branches by stabilizing MTs and influencing MT growth rate.

In this study, we provide evidence to show that *Sip1* transcriptionally activates the expression of *ninein*. *Ninein* binds to the minus end of free MTs and is present in the soma dendrites as well as the axons of neurons. By stabilizing the MT and regulating the growth of MTs, *ninein* influences axonal growth as well as branch formation. Although, the work presented here

concentrates on the formation of the CC, it is quite probable that such a *Sip1* – *ninein* dependent mechanism for axon growth is not specific to callosal neurons but extends to other cortical neurons as well. This idea is supported by the findings that in the *Sip1* mutant almost all cortical projections are affected. Additionally, *ninein* does not show a layer specific pattern of expression in the wild type cerebral cortex. On these lines we could speculate that the loss of CST in the *Sip1* mutant could be due to slow growing axons that do not reach a specific intermediate target at an appropriate developmental time point. It is hence withdrawn and does not exist at later developmental stages. Further experiments, specifically looking at the formation of other cortical tracts in the *Sip1* mutant need to be performed in order to understand the role of *ninein* in these cells.

4.11 *Satb2* and *Ctip2* control the formation of divergent axonal paths

Figure 47. *Satb2* *Ctip2* dependent establishment of cortical connections

(A) During early corticogenesis cingulate neurons with high *DCC* expression form the pioneer axons for the CC. These axons respond to midline *Netrin1* and are attracted towards the *Netrin1* source (B) Later, when Layer V cells start projecting medially, *Satb2* represses *Ctip2*, thereby promoting *Unc5C* expression. These *Unc5C* positive axons are repelled by the *Netrin1* source in the internal capsule and thus turn towards the midline. Corticofugally projecting neurons however, express high levels of *Ctip2* and thus repress *Unc5C*. Additionally the lack of repression of *DCC* by *Satb2*, promotes high *DCC* levels in these neurons. Thus, these axons are attracted towards the Internal capsule (C). Despite expressing high levels of *Unc5C* and low levels of *DCC*, upper layer neurons are not dependent on these molecules and instead either follow the deep layer callosal pioneer axons or are dependent on other axon guidance molecules. (D-F) Represent the scenario in the *Satb2*, *Netrin1* or *Unc5C* mutants, respectively. In each of the mutants, the lack of an *Unc5C*-*Netrin1* interaction causes a mis-routing of deep layer callosal axons to sub-cortical targets.

The expression of *Satb2* or *Ctip2* in cortical neurons confers mutually exclusive genetic programs with respect to their targeting (Alcamo et al., 2008; Arlotta et al., 2005; Britanova et al., 2008; Lickiss et al., 2012). *Ctip2* dictates a corticofugal fate and is required for the fasciculation and appropriate targeting of these fibers (Arlotta et al., 2005). In contrast, deletion of *Satb2* from the neocortex results in the absence of the CC and misrouting of callosal axons towards the internal capsule. Deletion of *Satb2* also causes an ectopic up-regulation of *Ctip2* in callosal neurons as *Satb2* acts upstream of *Ctip2* and represses it (Alcamo et al., 2008; Britanova et al., 2008). Although, a

generalization can be made that *Satb2* marks callosal neurons, it must be noted that not all callosal neurons express *Satb2* (Britanova et al., 2008). The complete absence of the CC in *Satb2* mutants suggests that callosal axons may require *Satb2* both cell intrinsically and cell extrinsically. At the same time, some *Satb2* positive neurons within the deep cortical layers, do not down-regulate *Ctip2*, and project to the internal capsule (Britanova et al., 2008). *Satb2* was shown to require the transcriptional co-factor Ski in order to repress *Ctip2*, therefore, these *Satb2*-positive/*Ctip2*-positive/Ski-negative cells are apparently non-callosal neurons (Baranek & Atanasoski, 2012; Baranek et al., 2012).

4.12 Lack of *Ctip2* repression in the absence of *Satb2* contributes to the acallosal phenotype in *Satb2*^{-/-} mice

In this study we have shown that the ectopic up-regulation of *Ctip2* in callosal neurons is one of the reason that contributes to the lack of a CC in *Satb2*^{-/-} mutants. Upon deletion of *Ctip2* from the *Satb2*^{-/-} cortex using *Satb2*^{-/-};*Ctip2*^{-/-} double mutants, the CC was partially restored. Similar results were also recently reported (Alcamo et al., 2008; Britanova et al., 2008; Srinivasan et al., 2012). As the rescue was only partial, other factors in addition to the upregulation of *Ctip2*, could be responsible for the lack of a CC in *Satb2*^{-/-} mutants. Since the up-regulation of *Ctip2* in *Satb2*^{-/-} mutants is a cell intrinsic effect, the rescue we observed in the *Satb2*^{-/-}*Ctip2*^{-/-} brains is likely to reveal the cell intrinsic ability of *Satb2*-positive cells to form the CC (Britanova et al., 2008).

4.13 *Unc5C* is under the direct transcriptional control of *Ctip2* and indirect control of *Satb2*

Unc5C and *DCC* act as receptors for the secreted ligand *Netrin1*, mediating either a chemo-repulsive (*Unc5C*-*DCC* together) or a chemo-attractive (*DCC* alone) response (Colamarino & Tessier-Lavigne, 1995; Fazeli et al., 1997;

Hamelin et al., 1993; Hedgecock, Culotti, & Hall, 1990). DCC independent role for *Unc5C* has also been shown. Expression patterns of both these receptor molecules in the developing neocortex are very dynamic. *DCC* is expressed at a high level in the early cortical plate at E14.5 and it is down regulated in later born neurons between E16.5-E18.5. In contrast, the highest levels of *Unc5C* expression are detected in later born neurons at E16.5-E18.5 and its expression is low in the early cortical plate. The expression of these two genes is also different in the neocortex of *Satb2* mutants: while *Unc5C* is down-regulated in the absence of *Satb2*, *DCC* is upregulated (Figure 47D).

Ctip2 acts as a transcriptional repressor of *Unc5C*. Multiple pieces of evidence support this finding. Firstly, *Unc5C* expression is regained in *Satb2*^{-/-}*Ctip2*^{-/-} compound mutants. Secondly, we demonstrated through ChIP and luciferase assay that *Ctip2* is a direct transcriptional repressor of *Unc5C*. Thirdly, electroporation of *Satb2* could ectopically induce *Unc5C* expression in cells when *Ctip2* was downregulated. Thus, *Satb2* controls the expression of *Unc5C* in the neocortex indirectly by repressing *Ctip2* expression. Overexpression of *Unc5C* in *Satb2*^{-/-} mutants, similar to the *Satb2*^{-/-}*Ctip2*^{-/-} double mutant, also leads to a partial restoration of the CC, demonstrating a cell autonomous ability for *Unc5C* to promote callosal targeting. This result was independently confirmed in a recent publication (Srinivasan et al., 2012).

4.14 *Unc5C* mutants also show corpus callosum defects

It has been previously reported that *Unc5C* mutants show a normal CC, with cortical fibers crossing the midline (Ackerman et al., 1997). Contrarily, our experiments indicate that *Unc5C* contributes cell autonomously to the formation of the CC. The question then arises that if *Unc5C* is required for CC formation then why are there no obvious defects of the CC observed in *Unc5C*^{-/-} mutants? Retrograde labeling of sub-cortically projecting neurons in *Unc5C*^{-/-} mutants showed that these mutants display an increase in the number of *Satb2* positive neurons that project sub-cortically (Figure 47F).

These misprojected axons that would have projected callosally in the wild type situation probably go undetected on histological and morphological analysis of the *Unc5C* null brains, as they constitute only a small percentage of the total axonal bundle. Thus, *Unc5C* is probably required for CC formation only in those neurons that rely on *Satb2* cell intrinsically to project callosally. Since late electroporation of *Unc5C* does not rescue the callosum in *Satb2*^{-/-} mutants, these *Unc5C* dependent neurons seem to be early born deep layer neurons but not later born upper layer neurons.

4.15 *Unc5C* aids in corpus callosum formation in response to Netrin1 expression

Netrin1 belongs to a family of phylogenetically conserved proteins and acts as a long-range diffusible guidance cue (Hedgecock et al., 1990; Ishii, Wadsworth, Stern, Culotti, & Hedgecock, 1992; Serafini et al., 1996; Wadsworth, Bhatt, & Hedgecock, 1996). In the wild type forebrain, there are two major sources of Netrin1 in the developing telencephalon; one in the septum, at the cortical midline (in the vicinity of the CC) and the other in the basal ganglia (in the vicinity of the initial segment of the corticofugal tracts in the internal capsule) (Barallobre et al., 2000; Metin et al., 1997). With the help of an *in vitro* cortical slice culture model we showed that, *Unc5C* expressing cortical neurons were responsive to Netrin1 signals and were repelled by it. We further showed that similar to the *Unc5C* mutants, *Netrin1* mutants had a higher proportion of *Satb2* positive cells projecting sub-cortically compared to wild type brains (Figure 47E). The differences in the proportion of *Satb2* positive cells that we observed projecting subcortically in the *Unc5C* mutant when compared to *Netrin1* mutants might be due to the different genetic backgrounds of these strains and/or because of the involvement of other Netrin1 receptors such as DCC and Draxin (Ahmed et al., 2011; Islam et al., 2009). Both these findings support the hypothesis that *Unc5C* mediated CC formation is dependent on *Unc5C*-Netrin1 repulsion.

4.16 *Unc5C* and *Netrin1* mutants show similar axonal behavior albeit dissimilar callosal phenotype

Although based on the theory put forward by this study, *Unc5C* and *Netrin1* control the same pathway for CC formation, an apparent discrepancy arises between the *Netrin1* mutant which is acallosal and the *Unc5C* mutant which is not. We propose that *Netrin1* mutant and the *Unc5C* mutant show a similar behavior with respect to cortical axons projecting medially. A probable explanation for this could be as follows. The *Netrin1* mutants lack the expression of *Netrin1* at the basal ganglia as well as in the cortical midline. Midline *Netrin1* expression is important for CC formation as it is responsible for attracting DCC positive cingulate axons that pioneer the CC. In the *Netrin1* mutant, since midline *Netrin1* expression is lost, the pioneering axons of the CC do not cross (Thomas Fothergill et al., 2013)(Figure 47A). Although the *Netrin1* mutant does not show callosal crossing, the majority of callosal fibers nonetheless project medially and arrive at the midline. These fibers are unable to cross due to the absence of pioneering callosal axons forming Probst bundles instead (Ren et al., 2007). On the other hand, in *Unc5C* mutants, since the midline *Netrin1* interaction with DCC-positive cingulate axons is not disrupted, callosal axons cross the midline. Therefore, we suggest that in *Netrin1*^{-/-} and *Unc5C*^{-/-} mutant mice, only the callosal fibers that depend on *Netrin1*-*Unc5C* interaction misproject to sub-cortical targets, whereas neurons which depend on other signaling pathways, continue to project medially towards the midline. In the *Unc5C*^{-/-} brains they cross the midline but in the *Netrin1*^{-/-} brains they stall at the midline.

It is further interesting to note that *Unc5C* expressing callosal neurons do not respond to midline *Netrin1*. This is probably because, at late embryonic stages (E16.5 onwards) the expression level of *Netrin1* in the midline is very low as compared to the expression in the basal ganglia (Barallobre et al., 2000; Thomas Fothergill et al., 2013) and secondly, it has been shown that axons of later born cortical neurons do not require midline *Netrin1* to form the CC (Thomas Fothergill et al., 2013) (Figure 47B).

4.17 Together with Netrin1 - Unc5C interaction, Netrin1 - DCC interaction regulates medial vs. lateral axonal trajectory choice

In addition to the changes in *Unc5C* expression levels in the *Satb2* mutant, we also observed that *DCC* was upregulated in the absence of *Satb2* (Figure 47D). We demonstrated *in vivo*, through ChIP and *in vitro* luciferase assay that *DCC* is under direct transcriptional control of *Satb2*. We hence hypothesized that in the *Satb2* mutant due to the upregulation of *DCC* in callosal neurons there might be an increase in DCC-Netrin1 attraction towards the internal capsule. Additionally, the downregulation of *Unc5C* results in reduced Unc5C-Netrin1 mediated repulsion. This hence forces callosal fibers, which are normally repelled by Netrin1 from the internal capsule in the wild type, to be attracted to the Netrin1 source in the *Satb2* mutant. In support of our hypothesis, we found that down-regulation of DCC by electroporation of *DCC shRNA* in *Satb2*^{-/-} mice, resulted in a partial rescue of the CC.

We hence propose that tempering down the DCC signal helps the axons make a medial choice and hence project callosally. At the outset, this theory seems to contradict the fact that *DCC* mutants display an acallosal phenotype (Fazeli et al., 1997). The question then arises that if the downregulation of *DCC* helps in CC formation, why does the *DCC* mutant show an acallosal phenotype. This apparent discrepancy could probably again be explained by the fact that the acallosal phenotype in the *DCC* mutant is characterized by callosal axons projecting medially but not crossing the midline (Fazeli et al., 1997). The lack of midline crossing is probably due to a secondary defect, the absence of *DCC* expression in the cingulate pioneer neurons (Figure 47A). Therefore, similar to the *DCC shRNA* electroporation in the *Satb2* mutant, *DCC* mutant brains also show medial projections. A simultaneous co-electroporation of *Unc5C* together with *DCC shRNA* did not result in a more profound rescue of the CC. Instead we observed that there was no rescue at all. This is a surprising result indicating that the dosage of Unc5C/DCC in each cell might be critical in deciding the projection fate of cortical projection neurons (Muramatsu et al., 2010).

4.18 *Satb2*^{-/-}, *DCC*^{-/-} and *Netrin1* mutants show disparate accallosal phenotypes

Although the *Satb2* mutant, *DCC* mutant and the *Netrin1* mutant are all acallosal, they differ with respect to the trajectory of these callosal fibers. While in both the *Netrin1* and *DCC* mutants callosal fibers project medially but fail to cross the midline and form Probst bundles, in the *Satb2* mutant, the acallosal phenotype is not restricted to the absence of callosal midline crossing, but occurs because of the absence of any neocortical axons navigating medially. *Satb2* mutants do not display Probst bundles. The callosal axons are misrouted towards the internal capsule and the anterior commissure (Alcamo et al., 2008; Britanova et al., 2008). Overexpression of *Unc5C* or downregulation of *DCC*, in *Satb2*^{-/-} brains results in a subset of neurons projecting medially, which can be seen as a clear bundle of axons leaving the electroporated region and approaching the midline. Hence, it is not only the midline crossing of these axons that is of significance, but more so the choice to project interhemispherically (medially) and not in the corticofugal direction (laterally). Although we have not been able to detect any neocortical axons forming the CC in our *Satb2* mutant, Alcamo et al. reported that their *Satb2* mutant has a severely reduced callosum. These few fibers that remain in the *Satb2* mutant could be attributed to the presence of *DCC* positive cingulate axons that project interhemispherically through the CC that maybe unaffected in the *Satb2* mutant (Alcamo et al., 2008).

4.19 Cell intrinsic control of *Satb2*/*Ctip2* and hence *DCC*/*Unc5C* in the formation of commissural vs. corticofugal projections is restricted to a sup-population of deep layer neurons

Our data strongly indicates that the control of *Satb2* and *Ctip2* over callosal and corticofugal fate specification is dependent on *Unc5C* and *DCC* only with respect to deep layer neurons (Figure 47B) as none of the rescue experiments performed at E14.5 lead to the formation of a CC. Secondly, the DiI

experiments on *Unc5C* and *Netrin1* mutants predominantly labeled deep layer neurons and thus the differences that we report here are most likely a reflection of the misrouting of deep layer *Satb2* positive neurons. Electroporation of *Satb2* into the *Satb2*^{-/-} brain could as well not rescue the entire acallosal phenotype in these mice, and was effective in rescuing only early born, deep layer, callosal projections. This clearly shows that the potential of each individual *Satb2* positive cell to project callosally is highly limited and requires a supportive environment. Hence, it is likely that deep layer neurons act in a cell autonomous manner in deciding between callosal and sub-cortical fates and that later born *Satb2* positive cells are dependent on other mechanisms for correct path-finding (Figure 47B, C).

4.20 New insights gained : Understanding the molecular basis of axonal trajectory choice

Together the experiments performed in this study indicate an important role for Netrin1, DCC and Unc5C interaction in the trajectory choice made by deep layer cortical axons. We suggest a scenario where the source of Netrin1 in the internal capsule attracts Unc5C-negative/DCC-positive axons while repelling Unc5C-positive/DCC-negative axons. Hence, callosally projecting deep layer neurons require higher levels of Unc5C and lower levels of DCC while on the other hand, low levels of Unc5C and high levels of DCC instruct neurons to project subcortically (Figure 47A, B, C). It is still not clear how this Netrin1-DCC/Unc5C interaction mechanism functions during the initial steps of axon navigation. We suggest two alternative scenarios. One possibility is that cortical neurons send two axonal branches in two opposite directions, one towards CC and the other towards the internal capsule. The presence of such dual projections in early neocortical development has been reported previously (Garcez et al., 2007; Lickiss et al., 2012). Here, Unc5C-positive/DCC-negative neurons would retract the lateral branch after arrival at the Netrin1 source in the internal capsule and stabilize the callosal branch. Unc5C-negative/DCC-positive neurons would retract the callosal branch and

stabilize their subcortical branch. A second alternative scenario is that all early cortical neurons send their axons towards the internal capsule, but upon reaching the vicinity of a Netrin1 source, Unc5C-positive/DCC-negative neurons would retract these axons and develop an independent callosal branch. Further experiments using advanced live-imaging techniques can help answer these questions and shed further light on how the complex yet highly organized system of cortical connections is established.

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