Ctip1 Regulates the Balance between Specification of Distinct Projection Neuron Subtypes in Deep Cortical Layers

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Ctip1 regulates the balance between specification of distinct projection neuron subtypes in deep cortical layers

Mollie B. Woodworth¹,²,⁴, Luciano C. Greig¹,²,⁴, Kevin X. Liu¹,², Gregory C. Ippolito³, Haley O. Tucker³, Jeffrey D. Macklis¹,²,∗

¹Department of Stem Cell and Regenerative Biology, Center for Brain Science, and Harvard Stem Cell Institute, Harvard University, Cambridge, MA 02138, USA.
²Harvard Medical School, Boston, MA 02115, USA
³Department of Molecular Biosciences, Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, TX 78712, USA.
⁴These authors contributed equally to this work.
*Correspondence: jeffrey_macklis@harvard.edu
SUMMARY

The molecular linkage between neocortical projection neuron subtype and area development, which enables the establishment of functional areas by projection neuron populations appropriate for specific sensory and motor functions, is poorly understood. Here, we report that Ctip1 controls precision of neocortical development by regulating subtype identity in deep-layer projection neurons. Ctip1 is expressed by postmitotic callosal and corticothalamic projection neurons, but is excluded over embryonic development from corticospinal motor neurons, which instead express its close relative, Ctip2. Loss of Ctip1 function results in a striking bias in favor of subcerebral projection neuron development in sensory cortex at the expense of corticothalamic and deep-layer callosal development, while misexpression of Ctip1 in vivo represses subcerebral gene expression and projections. As we report in a paired paper, Ctip1 also controls acquisition of sensory area identity. Therefore, Ctip1 couples subtype and area specification, enabling specific functional areas to organize precise ratios of appropriate output projections.

RUNNING TITLE

Ctip1 controls deep-layer subtype specification
INTRODUCTION

The remarkable complexity of the neocortex is precisely orchestrated during development, as many millions of neurons are born from progenitors, migrate to birthdate-appropriate layers, adopt subtype-specific gene expression and circuit connectivity, and organize into modality-specific functional areas. Subtype identity is tightly correlated with birthdate, as corticothalamic projection neurons (CThPN) are born around E12.5 in mice, subcerebral projection neurons (SCPN), including corticospinal motor neurons (CSMN), around E13.5, and most callosal projection neurons (CPN) between E14.5-E15.5, with a minority generated and resident with deep-layer subtypes (Woodworth et al., 2012). Nonetheless, functional areas of the cortex vary considerably in their subtype composition; for example, many more SCPN reside in mature motor cortex than in sensory cortex (Greig et al., 2013). This striking reciprocal linkage between subtype and area identity, with functional areas defined in large part by distinctive differences in subtype composition and, therefore, connectivity, has long been appreciated (Brodmann, 1909; Cajal, 1899), but its molecular control remains elusive.

One key transcription factor control over subtype development, Ctip2, is expressed at high levels by SCPN and functions centrally in SCPN terminal differentiation and connectivity (Arlotta et al., 2005). Multiple transcription factors controlling projection neuron specification and postmitotic differentiation operate at least in part by regulating Ctip2 expression. For example, Fezf2 regulates SCPN differentiation partly by promoting expression of Ctip2 (Arlotta et al., 2005; Chen et al., 2008; Molyneaux et al., 2005), and by preventing expression of transcription factors key for specification of alternate subtype fates, including Tbr1 (Bedogni et al., 2010; McKenna et al., 2011) and Satb2 (Alcamo et al., 2008; Britanova et al., 2008). Conversely, repression of Fezf2 and Ctip2 by Sox5 (Kwan et al., 2008; Lai et al., 2008; Shim et al., 2012) and Tbr1 (Bedogni et al., 2010; Han et al., 2011; McKenna et al., 2011) in CThPN, and by Satb2 in CPN (Alcamo et al., 2008; Britanova et al., 2008), is critical for appropriate development of
these subtypes. Although these central controls begin to be expressed immediately after neurons exit the cell cycle, subtype identity continues to be refined as neurons mature (Azim et al., 2009), as, for example, initially promiscuous expression of Ctip2 and Satb2 in newly postmitotic layer V neurons resolves to subtype-specific expression in SCPN and CPN by late embryogenesis (Alcamo et al., 2008; Britanova et al., 2008).

A paralogous zinc finger transcription factor, Ctip1 (also known as Bcl11a/Evi9), is also expressed during development. Ctip2 and Ctip1 are closely related, with approximately 60% identity between their nucleotide sequences (Avram et al., 2000; Satterwhite et al., 2001). In the hematopoietic system, Ctip1 controls the development of B cells (Lee et al., 2013; Liu et al., 2006; 2003) and regulates switching between fetal and adult forms of hemoglobin (Bauer et al., 2013; Canver et al., 2015; Sankaran et al., 2008; 2009; Xu et al., 2011). Ctip1 is expressed during development of many areas of the nervous system, including neocortex, hippocampus, striatum, and cerebellum (Arlotta et al., 2008; John et al., 2012; Kuo and Hsueh, 2007; Leid et al., 2004). Although Ctip1 is known to regulate neuronal migration (Wiegrefe et al., 2015), its functions in neocortical subtype development are not known.

Here, we report that Ctip1 controls projection neuron subtype identity and development of proper proportions of subtypes within a given functional area. Ctip1 is expressed by CPN, CThPN, and subplate neurons, but is progressively excluded from Ctip2-expressing CSMN. In the absence of Ctip1, SCPN expand as a population at the expense of CThPN and deep-layer CPN, with higher expression of genes characteristic of SCPN, more neurons projecting to the cerebral peduncle, and a reduction in CThPN and CPN gene expression and projections. In contrast, Ctip1 overexpression in vivo represses Ctip2 expression and reduces the number of neurons projecting subcerebrally, while increasing the number of neurons projecting through the corpus callosum. In accompanying work, we report that Ctip1 controls the acquisition of sensory area identity and area-specific input/output connectivity (Greig et al., 2016), and thus serves to integrate the dual processes of subtype and area development in neocortex.
RESULTS

CTIP1 is expressed by CPN, CThPN, and subplate neurons, but is largely excluded from CSMN

CTIP1 is first detected by immunocytochemistry at approximately E12.5 in young postmitotic glutamatergic projection neurons in the cortical plate (Figure 1A, Figure S1A). Immunostaining is absent from progenitor zones at mid-gestation (Figure S1D-E), although some expression of Ctip1 mRNA can be observed by in situ hybridization at the same age (Figure S1B-C), suggesting that Ctip1 begins to be transcribed as neurons exit the cell cycle, but detectable amounts of protein are not present until postmitotic neurons migrate to the cortical plate. CTIP1 continues to be expressed in postmitotic cortical neurons throughout embryogenesis (Figure 1B), though expression begins to decrease after the first postnatal week. At postnatal day (P) 4, CTIP1 is highly expressed in all six layers of somatosensory cortex (Figure 1C-C'), while expression in motor cortex is largely restricted to subplate, layer V, and the most superficial aspect of layer II/III.

To investigate the subtype specificity of Ctip1 expression, we retrogradely labeled CThPN, CPN, or CSMN with Alexa fluorophore-conjugated cholera toxin B (CTB) at early postnatal ages (P1-P3) and collected labeled brains at P4. In addition, we performed immunocytochemistry for CTIP1 together with FOG2, SATB2, and CTIP2, markers of CThPN, CPN, and SCPN, respectively (Woodworth et al., 2012). We find that CTIP1 is expressed by CPN and CThPN in sensorimotor cortex, but is essentially excluded from CSMN (Figure 1D-I). CTIP1 is expressed by 90% (±0.9%) of retrogradely labeled CThPN in layer VI, and co-localizes with FOG2 in 62% (±2.7%) of CThPN (Figure 1D, G). Similarly, 68% (±9.1%) of retrogradely labeled CPN express CTIP1, and CTIP1 and SATB2 are co-expressed by 59% (±7.9%) of CPN (Figure 1E, H), with no differences between superficial- and deep-layer CPN (61±7.3% superficial-layer, 57±9.0% deep-layer, not significantly different). Very few neurons labeled
retrogradely from the spinal cord express CTIP1 (4.9±1.1%), and there is very little co-expression of CTIP1 and CTIP2 (4.6±1.4%) in motor cortex (Figure 1F, I).

Having determined that CTIP1 is expressed at high levels by CThPN and CPN, but not by CSMN, we next investigated the temporal course of CTIP1 and CTIP2 expression in embryonic cortex. We find that, at E14.5, nearly all early postmitotic layer V neurons co-express CTIP1 and CTIP2 at high levels, but, by E17.5, CTIP1 and CTIP2 are expressed by two largely distinct populations within layer V (Figure S3A-D). Given the retrograde labeling data above (Figure 1E-I), we reasoned that these populations are likely immature CTIP1-expressing CPN and CTIP2-expressing CSMN.

The early segregation of cortical CTIP1 and CTIP2 expression, together with their functions controlling differentiation of closely-related immune cell types (Liu et al., 2003; Tydell et al., 2007; Wakabayashi et al., 2003), led us to hypothesize that Ctip1 and Ctip2 might interact cross-repressively to control differentiation of deep-layer projection neurons into distinct subtypes. We examined Ctip2 expression in the neocortex of Ctip1−/− mice, and find increased levels of CTIP2 in layers V and VI by qPCR (1.5-fold, p<0.05). Conversely, we find increased CTIP1 expression in layers V and VI of Ctip2−/− neocortex (1.5-fold, p<0.05) (Figure S3E-J). These data indicate that Ctip1 and Ctip2 are genetically cross-repressive, and suggest that these transcription factors might function to sharpen differentiation of distinct neuronal subtypes.

**More projection neurons acquire SCPN identity in the absence of Ctip1 function**

Since Ctip1−/− mice die almost immediately after birth (Liu et al., 2003), we obtained Ctip1fl/fl mice, which survive to adulthood (Lee et al., 2013; Sankaran et al., 2008), to study Ctip1 function as neocortical projections are established and refined. We deleted Ctip1 specifically from cortical neurons using Emx1-Cre, which is expressed beginning in cortical progenitors (Gorski et al., 2002).

Because Ctip1 is expressed by CThPN and CPN, but not by CSMN, we investigated
whether, in the absence of *Ctip1* function, more neocortical neurons differentiate into CSMN, and into SCPN more broadly. We compared wild-type and *Ctip1<sup>fl/fl</sup>;Emx1-Cre* tissue at P4 by Nissl and DAPI stain, and find that conditional nulls exhibit defective cortical layering, with a lack of clearly distinct boundaries between adjacent layers, but that layer V, which contains SCPN, is expanded relative to other cortical layers (*p*<0.01, Figure 2A-D, Figure S2). To investigate further, we performed immunocytochemistry and *in situ* hybridization at P0 for several genes expressed by SCPN, including CTIP2, *Fezf2*, and *Clim1* (Arlotta et al., 2005; Azim et al., 2009; Molyneaux et al., 2005). We find that high-level expression of these genes is expanded radially in *Ctip1<sup>fl/fl</sup>;Emx1-Cre* cortex, indicating increased layer V thickness (Figure 3A-F). This expansion is especially striking in somatosensory cortex, where layer V is normally thinner than in motor cortex, and where there are normally fewer SCPN than in motor cortex. In agreement with our Nissl results, this expansion appears to be primarily in the direction of deeper layers, suggesting that layer VIa CThPN are converted to SCPN.

Indeed, expression of genes specific to CThPN, including TBR1, FOG2, and DARPP-32 (Molyneaux et al., 2007), is markedly reduced in P0 *Ctip1<sup>fl/fl</sup>;Emx1-Cre* cortex, and layer VI is radially thinner (Figure 3G-L). Even within the reduced domain of layer VI, neurons expressing CThPN molecular markers and developmental controls also express inappropriately high levels of CTIP2 and *Fezf2* (asterisks in Figure 3B, 3D), suggesting a mixed SCPN/CThPN identity. TBR1 is expressed by 31% fewer neurons in *Ctip1<sup>fl/fl</sup>;Emx1-Cre* cortex (*p*<0.05; Figure 3T), while 32% more neurons express CTIP2 than in wild-type cortex (*p*<0.01; Figure 3S). These data strongly suggest that, in the absence of *Ctip1* function, many neurons that would normally have differentiated into CThPN have instead become SCPN. Expression of CPN genes SATB2, *LHX2*, and *CUX1* (Molyneaux et al., 2009) is approximately normal in deep layers and superficial layers (Figure 3M-R, U). Similarly, in conditional mutants in which *Ctip1* is deleted postmitotically (*Ctip1<sup>fl/fl</sup>;Nex1-Cre*) (Goebbels et al., 2006), TBR1 expression is reduced relative to wild-type, CTIP2 expression is increased, and SATB2 expression is unchanged in both deep
and superficial layers (Figure S3K-N), indicating that Ctip1 functions postmitotically to specify neuronal subtype. These results demonstrate that, in the absence of Ctip1 function, more projection neurons postmitotically adopt an SCPN molecular identity, and fewer adopt a CThPN identity.

**More neurons project to subcerebral targets in Ctip1<sup>fl/fl</sup>;Emx1-Cre mice, and fewer project to the thalamus**

We next investigated whether, beyond dysregulation of characteristic subtype-specific genes, cortical projection targeting is altered in the absence of Ctip1. We retrogradely labeled SCPN from the cerebral peduncle at P1 (labeling all neurons projecting to targets caudal to cerebrum), revealing that significantly more axons project to subcerebral targets in Ctip1<sup>fl/fl</sup>;Emx1-Cre than in wild-type brains (Figure 4A-C). In particular, the number of SCPN in somatosensory cortex increases 1.4-fold (p<0.01), and the number of SCPN in visual cortex increases 1.2-fold (p<0.05). The number of SCPN in motor cortex, where CTIP1 expression is normally low (Figure 1C), does not change.

In concordance with expression of SCPN-specific genes expanding deeper into layer VI (Figure 3A-F), most additional SCPN in Ctip1<sup>fl/fl</sup>;Emx1-Cre brains are located in the upper portion of layer VI. By combining BrdU birthdating at E12.5 with retrograde labeling from the cerebral peduncle, we find that 23% of Ctip1 conditional null SCPN have incorporated BrdU at E12.5, when CThPN are usually generated, compared with 9% of wild-type SCPN (Figure 4D-F; p<0.01). In the absence of Ctip1 function, newly born layer VI corticofugal neurons, normally destined to differentiate into CThPN, aberrantly differentiate into SCPN.

Because expression of genes characteristic of CThPN is reduced in Ctip1<sup>fl/fl</sup>;Emx1-Cre cortex, and because additional neurons projecting through the cerebral peduncle in Ctip1<sup>fl/fl</sup>;Emx1-Cre cortex are aberrantly differentiated E12.5-born neurons, we investigated CThPN projections in the absence of Ctip1 function. We find that fewer CThPN in Ctip1<sup>fl/fl</sup>;Emx1-
Cre somatosensory cortex are retrogradely labeled by injection of CTB into thalamus than in wild-type cortex (0.5-fold, p<0.05)(Figure 4G-I). Further, we analyzed P0 Ctip1<sup>−/−</sup> mice carrying a Rosa26R-tdTomato reporter allele (Madisen et al., 2010) and either an Rbp4-Cre or an Ntsr1-Cre allele (Gong et al., 2007), which are specific to SCPN and CThPN, respectively. Fewer CThPN in Ctip1<sup>−/−</sup> cortex are labeled by Ntsr1-Cre than in wild-type cortex (0.5-fold, p<0.01), and more SCPN are labeled by Rbp4-Cre (1.6-fold, p<0.01) (Figure 4J-O), indicating that fewer neurons differentiate into mature CThPN in the absence of Ctip1. Taken together, these results indicate that Ctip1 controls the development of corticothalamic projection neurons by suppressing their alternative differentiation into subcerebral projection neurons.

**CPN pathfinding is disrupted in the absence of Ctip1 function**

Ctip1<sup>fl/fl;Emx1-Cre</sup> mice exhibit partial agenesis of the corpus callosum, with prominent Probst bundles containing axons that have failed to cross the midline (asterisk in Figure 2B, D; Richards et al., 2004), and the corpus callosum is thinner in Ctip1<sup>fl/fl;Emx1-Cre</sup> than in wild-type mice (Figure 2A-D). These data indicate that development of the callosal projection is disrupted in the absence of Ctip1. In addition, many superficial-layer neurons in cingulate cortex, normally the earliest-crossing callosal population (Koester and O'Leary, 1994), are instead retrogradely labeled by injection of CTB from the cerebral peduncle (Figure 5A-B), suggesting that pioneering cingulate cortex CPN in Ctip1<sup>fl/fl;Emx1-Cre</sup> brains are fate-converted to SCPN. Consistent with this interpretation, superficial-layer neurons in cingulate cortex fail to express genes typical of cingulate cortex CPN, such as Dkk3 and Lpl (Figure 5C-F; (Molyneaux et al., 2009)), and instead aberrantly express CTIP2 (Figure 5G-H).

Potentially because cingulate CPN fail to pioneer the callosum, fewer deep-layer neurons in somatosensory cortex cross the callosum in Ctip1<sup>fl/fl;Emx1-Cre</sup> cortex than in wild-type mice (Figure 5I-K; p<0.01). Strikingly, layer VI CPN are more severely affected (56% fewer cross; p<0.01) than layer V CPN (19% fewer cross; p<0.05), suggesting that many later-born
CPN are able to follow the few early-born CPN that manage to cross the midline, even in the absence of a fully functional cingulate pioneer population. However, even projections of later-born CPN are cell-autonomously disrupted in the absence of Ctip1, as Cre electroporation into Ctip1<sup>fl/fl</sup> cortex at E15.5 results in 52% fewer axons crossing the callosum (Figure S5I-K; p<0.01). These data indicate that axon pathfinding is disrupted in CPN across all layers of cortex in the absence of Ctip1.

Although the corpus callosum is thinner in Ctip1<sup>fl/fl</sup>;Emx1-Cre mice, the anterior commissure is notably enlarged (arrows in Figure 5L-M). Indeed, many neurons in Ctip1<sup>fl/fl</sup>;Emx1-Cre somatosensory cortex are retrogradely labeled by injection of CTB into the anterior commissure, while retrogradely-labeled neurons are not present in this location in wild-type somatosensory cortex (Figure 5N-R). These data indicate that, in the absence of Ctip1 function, many commissural neurons aberrantly project to the contralateral hemisphere via this abnormal anterior commissure route instead of via the corpus callosum.

**Late-born projection neurons migrate abnormally in Ctip1 mutants**

Although superficial-layer CPN outside cingulate cortex are not fate-converted to SCPN in Ctip1<sup>fl/fl</sup>;Emx1-Cre mice (Figure 4A-B), superficial-layer neurons are improperly laminated (Figure 2A-D, Figure 5I-J) and impaired in their projections (Figure S5I-K). To investigate whether Ctip1<sup>fl/fl</sup>;Emx1-Cre neurons form imprecise layers as a result of abnormal migration, we birthdated cortical neurons in conditional null and wild-type cortex by injecting BrdU into pregnant females at E11.5, E12.5, E13.5, E14.5, and E15.5. We analyzed the laminar position of labeled neurons at P4, after migration is normally complete. We find significant numbers of E14.5- and E15.5-born neurons ectopically located in deep layers in Ctip1<sup>fl/fl</sup>;Emx1-Cre mutants (Figure 6A-G; p<0.05 for neurons located in bins 5, 6, and 7 at both E14.5 and E15.5), indicating that late-born projection neurons fail to migrate appropriately to superficial layers. These neurons express markers of superficial-layer neurons, such as CUX1, appropriate to their
birthdate, but not their laminar position (Figure S5F-H). In contrast, migration of neurons born at E11.5, E12.5, or E13.5 is not significantly affected (Figure S6A-I). These results indicate that many late-born \textit{Ctip1}^{fl/fl};\textit{Emx1-Cre} neurons migrate and become positioned in deep-layer cortex, leading to abnormal lamination of the cortex as a whole.

Migration defects in \textit{Ctip1}^{fl/fl};\textit{Emx1-Cre} animals might be due to defective signaling to migrating neurons from post-migratory neurons in the cortical plate, or they might be due to aberrant interpretation of cues by migrating neurons themselves. To distinguish between these possibilities, we electroporated E14.5 wild-type or \textit{Ctip1}^{fl/fl} embryos with CMV/\textit{β}-actin promoter-driven \textit{Cre} and lox-STOP-lox-\textit{Egfp} constructs, and examined electroporated tissue at E17.5 (Figure 6H). This experiment deletes \textit{Ctip1} from only a small fraction of cortical neurons, allowing sparse deletion of \textit{Ctip1} in an otherwise wild-type context. \textit{Ctip1}^{fl/fl} neurons electroporated with \textit{Cre} at E14.5 are four times less likely than wild-type electroporated neurons to have entered the cortical plate at E17.5 (Figure 6I-K; 13\% \textit{Ctip1}^{fl/fl} vs. 51\% wild-type; p<0.01), indicating that migration abnormalities of superficial-layer neurons lacking \textit{Ctip1} are cell-autonomous. Further, these defects are specific to neurons migrating to the superficial layers, because migration of \textit{Ctip1}^{fl/fl} neurons electroporated with \textit{Cre} at E12.5 and examined at E15.5 is unaffected (not significant; Figure S6J-M).

\textbf{Ctip1 overexpression represses CTIP2 in layer V neurons, preventing them from extending axons subcerebrally}

To further investigate potential functions of \textit{Ctip1} in repression of CSMN and SCPN specification, we tested whether overexpression of \textit{Ctip1} \textit{in vivo} can repress endogenous expression of \textit{Ctip2} by wild-type CSMN. We electroporated CMV/\textit{β}-actin promoter constructs driving expression of either control IRES-\textit{nEgfp} (nuclear EGFP) or \textit{Ctip1}-IRES-\textit{nEgfp} into the ventricular zone of E12.5 wild-type embryos, and examined CTIP2 expression at P4 by immunocytochemistry (Figure 7B-C). Strikingly, while many control E12.5 \textit{Egfp}-electroporated
layer V neurons are CTIP2-positive (39%), as expected, significantly fewer Ctip1-electroporated layer V neurons are CTIP2-positive (7%; p<0.01). These data demonstrate that high levels of CTIP1 are sufficient to repress expression of Ctip2.

Conversely, overexpression of Ctip2 or Fezf2 can repress endogenous Ctip1 in layer V neurons. When we electroporate Ctip2-IRES-nEgfp or Fezf2-IRES-nEgfp at E12.5, we find that fewer layer V neurons express Ctip1 at P4 compared with layer V neurons electroporated with control IRES-nEgfp (nEgfp, 64%; Fezf2, 37%, p<0.01; Ctip2, 8%, p<0.01; Figure S7). High-level expression either of Ctip2 or Fezf2 is therefore sufficient to repress expression of Ctip1, although Ctip2 is more efficient than Fezf2.

This cross-repression between Ctip1 and Ctip2/Fezf2, central transcriptional controls over SCPN development, motivated us to investigate whether Ctip1 is also sufficient to prevent layer V neurons from projecting subcerebrally. We electroporated control IRES-nEgfp or Ctip1-IRES-nEgfp constructs into wild-type embryos at E12.5, then retrogradely labeled CSMN or CPN with CTB (Figure 7A). Strikingly, we find a five-fold reduction in the percentage of Ctip1-electroporated neurons projecting to the spinal cord, compared with control nEgfp-electroporated neurons (Figure 7F-H; 25% vs. 6%; p<0.01). Further, we find a significant increase in the number of Ctip1-electroporated layer V neurons projecting across the corpus callosum (Figure 7I-K; 13% nEgfp vs. 20% Ctip1; p<0.05), indicating that the axons of some Ctip1-electroporated neurons are redirected from subcerebral targets and toward contralateral cortical targets. Taken together, these data demonstrate that Ctip1 overexpression alters the balance of deep-layer projection neuron fate specification against SCPN and in favor of CPN, in both gene expression and axonal projections.
DISCUSSION

Although critical molecular controls over projection neuron subtype development have been identified in recent years, it is clear that additional transcriptional regulators remain to be discovered, particularly those that exert fine control over the final distribution, proportions, and balance of subtypes present in specific functionally specialized areas of the cortex. In this work, we identify that the transcription factor Ctip1 directs the proportional allocation of corticothalamic, subcerebral, and callosal projection neurons in deep cortical layers.

Subtype control over deep-layer neurons

Ctip1 controls the balance between specification of distinct, functionally specialized subtypes of deep-layer cortical projection neurons, and, in the absence of Ctip1 function, more SCPN are generated at the expense of CThPN and deep-layer CPN. In Ctip1\textsuperscript{fl/fl};Emx1-Cre mice, an aberrantly expanded population of neurons with molecular and anatomic characteristics of SCPN occupies an expanded layer V (Figure 3), and more neurons born at E12.5 send axons to the cerebral peduncle instead of to the thalamus, indicating that CThPN located in the upper segment of what would otherwise be layer VI are fate-converted to SCPN (Figure 4).

The population of CThPN located in the most superficial portion of layer VI, near the interface between layer V and layer VI, is particularly vulnerable to switching fate from corticothalamic to subcerebral due to ectopic expression of SCPN genetic controls (Lai et al., 2008; Tomassy et al., 2010). In the absence of Ctip1, all layer VI neurons express higher-than-normal levels of Fezf2 and Ctip2 (asterisks in Figure 3B, 3D); however, only those neurons located in the most superficial portion of layer VI cease to express CThPN controls (Figure 3G-L) and consequently project subcerebrally (Figure 4A-F). The existence of this subpopulation of CThPN, which is generated immediately before SCPN specification begins, and which resides immediately adjacent to layer V, suggests that these closely-related corticofugal neurons require precise transcriptional control by Ctip1 and other genes for correct specification into distinct
projection neuron subtypes, and for allocation of correct proportions CThPN and SCPN depending on cortical area.

Intriguingly, Ctip1 controls subtype specification, but is itself non-subtype-specific, as it is expressed at high levels by CPN, CThPN, and subplate neurons. Although some previously-identified controls are expressed by multiple subtypes, they are generally expressed at high levels by one subtype and low levels by another: Ctip2 and Fezf2 by SCPN (high) and CThPN (low); Tbr1 by CThPN (high) and superficial-layer CPN (low); and Sox5 by CThPN (high) and SCPN (low) (Arlotta et al., 2005; Hevner et al., 2001; Lai et al., 2008; McKenna et al., 2011; Molyneaux et al., 2005; Woodworth et al., 2012). Ctip1, in contrast, is specific only in its exclusion from CSMN (Figure 1D-I). This widespread and non-subtype-specific expression is most likely responsible for Ctip1 not being identified as a candidate in microarray-based screens for genes involved in subtype specification (Arlotta et al., 2005; Chen et al., 2005a; 2005b). The data presented here regarding Ctip1 function suggest that further unidentified controls over subtype development might expand or contract populations of neurons by action in developmentally, and likely evolutionarily, related populations.

Abnormalities of commissural neurons

In the absence of Ctip1 function, fewer deep-layer CPN project through the corpus callosum (Figure 5I-K), and, instead, many deep-layer neurons in somatosensory cortex project to the contralateral hemisphere via the anterior commissure (Figure 5L-R). This re-routing might be necessary because the superficial-layer neurons in cingulate cortex that would normally pioneer the callosal projection instead aberrantly project through the pyramidal tract (Figure 5A-H), impairing pathfinding by deep-layer CPN. Alternatively, more AC-projecting neurons might be specified in the absence of Ctip1 function, reflecting a previously unidentified balance between specification of CC-projecting and AC-projecting commissural neurons controlled, at least in part, by Ctip1. We do not exclude the possibility that Ctip1 might regulate expression of
axon guidance molecules necessary for pathfinding by CPN. \textit{Ctip1} might regulate pathfinding in SCPN and CThPN, as well, particularly in light of aberrant subplate neuron gene expression and axon extension in null mice (Figure S4).

In contrast with these striking abnormalities of deep-layer CPN targeting, \textit{Ctip1}^{fl/fl};\textit{Emx1-Cre} superficial-layer CPN neither express SCPN-specific controls (Figure 3A-F) nor project through the cerebral peduncle (Figure 4A-B), except in cingulate cortex (Figure 5A-H). Although \textit{Ctip1} is expressed at high levels by superficial-layer CPN (Figure 1C), these neurons appear to be specified largely normally in the absence of \textit{Ctip1} (Figure 3M-R), although many of their axons do not successfully cross the corpus callosum (Figure S5I-K). These data are consistent with the hypothesis that superficial-layer and deep-layer CPN are substantially different from each other molecularly and with regard to their developmental origins, perhaps reflecting distinct evolutionary events of projection neuron diversification (Arlotta et al., 2008; Fame et al., 2011; Molyneaux et al., 2009). \textit{Ctip1} is expressed by both superficial-layer and deep-layer CPN, but is necessary for subtype specification only for deep-layer CPN, which appear to bear a closer relationship to concurrently-generated corticofugal projection neurons (Greig et al., 2013; Sohur et al., 2012). Loss of \textit{Ctip1} function contrasts starkly with the loss-of-function phenotype observed for another important negative regulator of SCPN development, \textit{Satb2}, in which large numbers of both superficial-layer and deep-layer neurons aberrantly express SCPN controls such as CTIP2 (Alcamo et al., 2008; Britanova et al., 2008; Srinivasan et al., 2012). This contrast indicates that an increasingly rich set of molecular regulators controls both novel and potentially subtle, but likely functionally critical, aspects of precise projection neuron development.

**\textit{Ctip1} and \textit{Ctip2} interact cross-repressively in developing cortex**

\textit{Ctip1} and \textit{Ctip2}, paralogous and highly similar transcription factors, are initially co-expressed in developing cortex (Figure S3A-B), but later are expressed in a complementary
fashion, with CTIP1 expressed by CPN and CThPN, and CTIP2 expressed by SCPN (Figure 1D-F, Figure S3C-D). Expression of CTIP2 increases in the absence of Ctip1, and expression of CTIP1 increases in the absence of Ctip2 (Figure S3E-J). Furthermore, overexpression of either Ctip1 or Ctip2 at E12.5 in vivo is sufficient to repress expression of the other (Figure 7B-C, Figure S7A, S7C). From these data, we conclude that Ctip1 and Ctip2 cross-repressively interact to control the development of cortical projection neurons. Ctip1 and Ctip2 exhibit similar patterns of exclusive expression in development of other cell types; Ctip1 expression is sharply downregulated as Ctip2 begins to be expressed in maturing T cells (Tydell et al., 2007), and expression of Ctip1 is significantly increased in P0 Ctip2−/− striatal medium-sized spiny neurons (Arlotta et al., 2008).

Our model of Ctip1 function is at odds with that of Cánovas et al. (Cánovas et al., 2015), who recently proposed that CTIP1 is extensively co-expressed with CTIP2 and is a positive control over SCPN development through repression of TBR1. In direct contrast, we demonstrate that expression of TBR1 and other characteristic CThPN genes is reduced in the absence of Ctip1 (Figure 3G-L, Figure 4M-O), and that expression of CTIP2 is increased (Figure 3A-B), leading to more early-born neurons becoming SCPN (Figure 4A-F). These discrepancies likely result from different loss-of-function approaches, as our investigation uses null and conditional null mouse lines, rather than modest knockdown of Ctip1 using mosaic shRNA electroporation in wild-type animals.

**Ctip1 as a candidate human intellectual disability gene**

CTIP1 is highly conserved from mouse to human, with only three amino acid substitutions (99.6% identity) between the longest mouse and human CTIP1 isoforms, and Ctip1 and its promoter are located within one of the most ultraconserved non-coding region-rich stretches of the human genome (Sandelin et al., 2004). CTIP1 is expressed in fetal human brain (Satterwhite et al., 2001), although the subtype specificity of its expression in human brain is not
known. Intriguingly, *Ctip1* is one of three protein-coding genes consistently deleted in 2p15-p16.1 microdeletion syndrome, which is characterized by intellectual disability, microcephaly, and frequently by autistic behavior (Hancarova et al., 2013), and a patient with a *de novo* *Ctip1* microdeletion has been reported with severe speech sound disorder and intellectual delay (Peter et al., 2014). These phenotypes are consistent with functions of *Ctip1* in the subtype-specific differentiation and migration of neocortical projection neurons, suggesting that *Ctip1* also functions critically in organizing the human neocortex.

**Ctip1 couples development of projection neuron subtypes and cortical areas**

Although subtype and area development are typically analyzed as independent developmental vectors in the emergence of the mature neocortex, the two are, in fact, highly interdependent, since the proportion and identity of neuron subtypes varies significantly by cortical area (Brodmann, 1909; Cajal, 1899). Molecular programs controlling subtype and area differentiation likely interact at multiple nodes; here, we identify *Ctip1* as a central node. *Ctip1* directs the proportional allocation of subcerebral and corticothalamic projection neurons in sensory cortex, and, in the absence of *Ctip1*, sensory cortical areas are transformed, with subtype compositions, gene expression, and output connectivity that is usually present in motor areas (Figure 3; Figure 4; Greig et al., 2016). Strikingly, the expression (Figure 1, Figure S1) and functions (Figure S3) of *Ctip1* in postmitotic neurons indicate that this regulation of subtype composition in distinct cortical areas occurs postmitotically. Therefore, the final non-uniform distribution of cortical projection neuron subtypes across cortical areas is based, at least in part, not on differences in subtype production, but in subtype differentiation.

In conclusion, our results indicate that *Ctip1* is a central functional control over the precision of neocortical development. *Ctip1* directs the development of corticothalamic and callosal projection neurons at multiple stages. First, *Ctip1* specifies subtype identity in deep-layer neocortical projection neurons, preventing corticothalamic and callosal projection neurons
from acquiring characteristics of subcerebral projection neurons. Second, Ctip1 directs the development of cingulate cortex neurons, allowing these populations to pioneer callosal projections. Finally, Ctip1 enables the proper migration and laminar positioning of superficial-layer callosal projection neurons, permitting these neurons to populate correct laminar locations. Identification of further fine transcriptional controls over the precise differentiation, diversity, positioning, and connectivity of neocortical projection neurons will increasingly illuminate the staggering organizational and functional complexity of the mature neocortex, and provide insight into overt and subtle dysgenesis that can result in human cognitive, sensory, motor, and integrative disorders.
EXPERIMENTAL PROCEDURES

Animals

All mouse studies were approved by the Harvard University IACUC, and were performed in accordance with institutional and federal guidelines. The date of vaginal plug detection was designated E0.5, and the day of birth as P0. Unless noted otherwise, all experiments with Ctip1^fl/fl;Emx1-Cre were controlled with Ctip1^wt/wt;Emx1-Cre, and Ctip1^−/− with Ctip1^wt/wt, although no abnormal phenotypes were observed in Ctip1^wt/wt or Ctip1^fl/wt;Emx1-Cre heterozygotes. Sources of mouse lines used are described in Supplemental Information.

Immunocytochemistry and in situ hybridization

Mice were transcardially perfused with 4% paraformaldehyde, and brains were dissected and post-fixed at 4°C overnight. Tissue was sectioned at 50µm on a vibrating microtome (Leica). Non-specific binding was blocked by incubating tissue and antibodies in 8% goat serum/0.3% bovine serum albumin in phosphate-buffered saline. For DAPI staining, tissue was mounted in DAPI-Fluoromount-G (SouthernBiotech). Antibodies and in situ probes used are described in Supplemental Information.

BrdU birthdating

Timed pregnant females were intraperitoneally injected with bromodeoxyuridine (50 mg/kg in PBS) at E11.5, E12.5, E13.5, E14.5, or E15.5. Tissue was collected at P4 and processed for BrdU immunocytochemistry (Magavi and Macklis, 2008).

For analysis of BrdU-labeled SCPN, BrdU labeling was performed as above at E12.5, and littermate pairs were retrogradely labeled from the cerebral peduncle as described below. For acute BrdU administration, timed pregnant females were intraperitoneally injected with BrdU (75 mg/kg) at E14.5, and embryos were collected 30 minutes later.
In utero electroporation

For overexpression experiments, a CMV/β-actin promoter plasmid (derived from CBIG; gift of C. Lois) was used to drive expression of IRES-Egfp (control) or Ctip1 XL-IRES-Egfp (experimental; coding sequence from NM_001242934); or IRES-nEgfp (nuclear EGFP) (control) or Ctip1 XL-IRES-nEgfp, Ctip2-IRES-nEgfp, or Fezf2-IRES-nEgfp (experimental) in CD1 timed pregnant females at E12.5. For some experiments, pups were screened for Egfp expression on a fluorescent dissecting microscope at birth, and pups electroporated in somatosensory cortex were retrogradely labeled (as described below). Brains were collected at P4.

For loss-of-function experiments, two CMV/β-actin promoter plasmids were co-electroporated, driving expression of lox-STOP-lox-Egfp and Cre in Ctip1wt/wt (control) or Ctip1fl/fl (experimental) embryos. Plasmids were electroporated at age indicated in figure (E12.5, E14.5, or E15.5) and brains were collected either 72 hours later (Figure 6, Figure S6) or at P4 (Figure S5). Electroporation conditions were described previously (Molyneaux et al., 2005).

Retrograde labeling

Projection neurons were labeled from their axon termini under ultrasound guidance by pressure injection of Alexa fluorophore-conjugated cholera toxin B (Invitrogen). SCPN were labeled by injection into cerebral peduncle at P1, CThPN were labeled by injection into sensory thalamic nuclei (VPM and VPL) at P1, CSMN were labeled by injection into cervical spinal cord at P2, and CPN were labeled by injection into contralateral corpus callosum at P3. Anterior comissure-projecting neurons were labeled by injection into contralateral anterior commissure at P1. Tissue for all injections was collected at P4, and processed as for immunocytochemistry, above.

Quantification and statistics

Littermate pairs of experimental and control mice were collected at age indicated in
figure and processed as for immunocytochemistry, above. Anatomically matched sections from each mouse (generally at the level of the anterior commissure, unless otherwise noted) were selected, and single confocal slices of somatosensory cortex were imaged. Cells positive for indicated marker(s) (retrograde label, protein by immunocytochemistry, BrdU label, GFP by electroporation) were counted within a box of pre-defined size spanning the radial thickness of cortex, with the same size box applied to wild-type and mutant images. Except where specifically noted (Figure 2, Figure S2), data are not corrected for differences in cortical thickness between wild-type and mutant animals (Table S1). Statistical analyses were conducted using unpaired two-tailed t-tests in Microsoft Excel, with a significance threshold of p<0.05.
AUTHOR CONTRIBUTIONS
M.B.W. and J.D.M. conceived the project; M.B.W. and L.C.G. designed and performed all experiments; M.B.W., L.C.G., and J.D.M. analyzed and interpreted the data; and M.B.W. and L.C.G. made figures. K.X.L. performed qPCR experiments. G.C.I. and H.O.T. contributed a critical mouse line. M.B.W., L.C.G., and J.D.M. synthesized and integrated the findings, and wrote and revised the paper. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1. *Ctip1* is expressed by postmitotic corticothalamic and callosal projection neurons, but is excluded from corticospinal motor neurons. (A-C’) CTIP1 immunocytochemistry on coronal brain sections. CTIP1 is expressed by newly-postmitotic projection neurons in the cortical plate beginning around E12.5 (A, arrow) and continuing through E16.5 (B) and P4 (C). At P4, CTIP1 is expressed by neurons in all layers of primary sensory cortex (C’). (D-I) At P4, CTIP1 is expressed by CThPN and CPN, but not by CSMN. CTIP1 co-localizes with FOG2, as well as with cholera toxin B (CTB) retrograde label after injection into thalamus (D). CPN express both CTIP1 and SATB2, and are retrogradely labeled by injection of CTB into the corpus callosum (E). CSMN are retrogradely labeled by CTB injection into the cervical spinal cord, and express CTIP2, but not CTIP1 (F). Quantification, n=4 (G-I).

CC, corpus callosum; CPN, callosal projection neurons; CSMN, corticospinal motor neurons; CThPN, corticothalamic projection neurons; SC, spinal cord; SP, subplate; Th, thalamus. Scale bars: 200um (A-C), 40um (D-F). Data are represented as mean ± SEM.

Figure 2. In the absence of neocortical *Ctip1*, cortical layer V is expanded at the expense of cortical layer VI. (A-H) Nissl staining and DAPI staining of cortex in wild-type (A, C) and *Ctip1*^fl/fl^;*Emx1*-Cre cortical conditional null (B, D) mice at P4. Layer V is expanded in conditional null cortex (F, H) compared with wild-type (E, G). Asterisk in B and D marks the location of a Probst bundle. (I) Quantification of layer thickness performed on Nissl-stained tissue, with each layer presented as a percentage of total cortical thickness (n=5).

Scale bars: 100um. Data are represented as mean ± SEM.
Figure 3. More neurons in Ctip1fl/fl;Emx1-Cre cortex adopt a subcerebral projection neuron identity, and fewer adopt a corticothalamic projection neuron identity.

(A-F) In the absence of Ctip1 function, expression of SCPN marker and control genes CTIP2, Fezf2, and Clim1 increases at P0, especially in somatosensory cortex (arrows in A-F). Dashed lines denote superficial and deep limits of gene expression.

(G-L) In tandem, expression of CThPN marker and control genes TBR1, FOG2, and DARPP-32 is reduced at P0. Dashed lines denote superficial and deep limits of gene expression. Even layer VI neurons that continue to express CThPN identity genes (H, J, L) express aberrantly high levels of CTIP2 and Fezf2 (asterisks in B, D), suggesting mixed CThPN/SCPN identity.

(M-R) CPN marker and control genes SATB2, LHX2, and CUX1 are expressed normally at P0, although boundaries between superficial and deep layers are more difficult to discern. Dashed lines denote superficial and deep limits of gene expression.

(S-U) Quantification of the number of neurons expressing CTIP2 (S), TBR1 (T), and SATB2 (U) in conditional null cortex, presented as a percentage of wild-type neurons, n=3 for each marker. *, p<0.05; **, p<0.01; n.s., not significant

Scale bars: 100um. Data are represented as mean ± SEM.

Figure 4. More neurons in Ctip1fl/fl;Emx1-Cre somatosensory and visual cortex project toward subcerebral targets, and fewer project toward thalamic targets.

(A-B) More neurons in Ctip1 conditional null somatosensory cortex (B, B’) are retrogradely labeled by injection of cholera toxin B (CTB) into the cerebral peduncle than in wild-type cortex (A, A’).

(C) Quantification of retrogradely-labeled SCPN by area, presented as a percentage of wild-type SCPN in each area, n=3.

(D-F) Additional SCPN in Ctip1fl/fl;Emx1-Cre brains are aberrantly differentiated from E12.5-born neurons. More neurons labeled by injection of BrdU at E12.5 are co-labeled by injection of CTB
into the cerebral peduncle in conditional null cortex (E) compared with wild-type cortex (D). Arrowheads mark representative co-labeled neurons. Quantification, n=4 (F).

(G-I) Compared with wild-type (G), fewer CThPN are retrogradely labeled in somatosensory cortex of Ctip1\textsuperscript{fl/fl};Emx1-Cre mutants (H) following CTB injection into sensory thalamic nuclei. Quantification (I).

(J-O) Ctip1 null cortex contains more mature SCPN and fewer mature CThPN than wild-type. More neurons are marked by SCPN-specific Rbp4-Cre in Ctip1\textsuperscript{+/+} P0 cortex (K) compared with wild-type (J) (n=4). In contrast, fewer neurons are marked by CThPN-specific Ntsr1-Cre in null P0 cortex (N) compared with wild-type (M) (n=3). Quantification (L, O). Recombination by Rbp4-Cre and Ntsr1-Cre is reported by a Rosa26R-tdTomato\textsuperscript{+/+} allele.

n.s., not significant; *, p<0.05; **, p<0.01

Scale bars: 100um (A-B, G-H, J-K, M-N), 50um (D-E). Data are represented as mean ± SEM.

Figure 5. Cingulate CPN fail to pioneer the callosum in the absence of Ctip1 function, impairing projections of deep-layer CPN.

(A-H) Superficial-layer neurons in Ctip1\textsuperscript{fl/fl};Emx1-Cre cingulate cortex aberrantly project through the cerebral peduncle (A-B), fail to express cingulate CPN genes Dkk3 (C-D) and Lpl (E-F), and instead express CTIP2 (G-H).

(I-K) Failure of cingulate CPN to pioneer the corpus callosum results in a reduction of retrogradely-labeled deep-layer CPN in Ctip1\textsuperscript{fl/fl};Emx1-Cre cortex. Fewer CPN are retrogradely labeled in P4 Ctip1\textsuperscript{fl/fl};Emx1-Cre layers V and VI (J-J') compared with wild-type (I-I'). Quantification of I-J as a percentage of wild-type deep-layer CPN, n=3 (K). Layer VI CPN are more severely affected than layer V CPN. Schematic of injection site, upper right corner.

(L-R) More neurons in Ctip1 conditional null brains aberrantly project to the contralateral hemisphere via the anterior commissure pathway. The anterior commissure is abnormally enlarged in P4 Ctip1\textsuperscript{fl/fl};Rosa26R-tdTomato\textsuperscript{fl/wt};Emx1-Cre brains (arrow in M) compared with
**Figure 6.** In the absence of neocortical *Ctip1*, superficial-layer projection neurons migrate aberrantly and become inappropriately positioned in cortex.

(A-F) Wild-type neurons labeled by BrdU injection at E14.5 (A-A’) or E15.5 (D-D’) are primarily located in superficial layers, while *Ctip1fl/fl;Emx1-Cre* neurons labeled by BrdU at E14.5 (B-B’) or E15.5 (E-E’) are frequently ectopically located in deep layers. The overall laminar distribution of labeled neurons (marked in red in A’-B’, D’-E’) is strikingly abnormal (C, F). Bin 1 is the most superficial bin, and Bin 10 is the deepest.

(G) Quantification of A-F, with bins 1-4 grouped as “upper” cortical segment, 5-7 as “middle”, and 8-10 as “deep”.

(H-K) Sparse electroporation of Cre at E14.5 causes *Ctip1fl/fl* neurons to be delayed in the intermediate zone rather than migrate into the cortical plate at E17.5. Schematic of experimental approach (H). Wild-type neurons electroporated with Cre (I) are significantly more likely than electroporated *Ctip1fl/fl* neurons (J) to have migrated into the cortical plate by E17.5 (quantification, K).

CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone; *, p<0.05; **, p<0.01

Scale bars: 200um (A-B, D-E), 50um (A’-B’, C’-D’, I-J). Data are represented as mean ± SEM.

**Figure 7.** CTIP1 misexpression *in vivo* represses SCPN identity and projection to the spinal
cord.

(A) Schematic of experimental approach. Wild-type embryos were electroporated at E12.5, and retrograde labeling from spinal cord (F-G) or from the contralateral cortical hemisphere (I-J) was performed on electroporated pups at P2 or P3, respectively. Brains were collected at P4 and somatosensory cortex was imaged and analyzed.

(B-C) Many neurons electroporated with control nuclear Egfp (nEgfp) at E12.5 express CTIP2 (B-B’; 39%), while few neurons electroporated with Ctip1-IRES-nEgfp express CTIP2 (C-C’; 7%), n = 3.

(D-K) Misexpression of Ctip1 causes E12.5-born neurons to redirect their axons away from the brainstem and spinal cord, toward targets on the contralateral cortical hemisphere. Neurons electroporated with Ctip1-IRES-Egfp at E12.5 send few axons to the brainstem (wholemount electroporated brains in D-E, coronal brainstem sections in D’-E’). Fewer neurons electroporated at E12.5 with Ctip1-IRES-nEgfp are retrogradely labeled by spinal cord injection at P2 than those electroporated with control nEgfp (F-G; quantification in H, n = 3), while more Ctip1-electroporated neurons than control nEgfp-electroporated neurons are retrogradely labeled by injection into the corpus callosum (I-J; quantification in K, n = 3).

**, p<0.01

Scale bars: 50um (B-C, F-G, I-J), 1mm (D-E), 100um (D’-E’). Data are represented as mean ± SEM.
### E12.5 IRES-nEgfp → P4

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### E12.5 Ctip1-IRES-nEgfp → P4

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### % nGFP+ CSMN

- nEgfp: **20** ± 2
- Ctip1: **25** ± 2

### % nGFP+ CPN

- nEgfp: **15** ± 2
- Ctip1: **30** ± 2

**Note:** The graphs show the percentage of neurons positive for nGFP in CSMN and CPN, with Ctip1 expression significantly higher compared to nEgfp.
Figure S1. (related to Figure 1) CTIP1 is expressed by postmitotic neurons in the cortical plate.
Figure S2. (related to Figure 2) Defects in lamination, migration, and neuronal positioning in Ctip1<sup>fl/fl</sup>;Emx1-Cre mice persist until adulthood.
Figure S3. (related to Figure 3) *Ctip1* and *Ctip2* are genetically cross-repressive.
Figure S4. (related to Figure 4) Subplate neuron identity and projections are impaired in the global absence of Ctip1 function.
Figure S5. (related to Figure 5) Superficial-layer callosal projection neurons are produced in normal numbers in the absence of Ctip1 function, but are impaired in migration, positioning, and projection.
Figure S6. (related to Figure 6) Migration and laminar positioning of deep-layer neurons is not affected by the absence of Ctip1 function.
Figure S7. (related to Figure 7) Misexpression of either *Ctip2* or *Fezf2* at E12.5 represses expression of CTIP1.
SUPPLEMENTAL FIGURE LEGENDS

**Figure S1.** (related to Figure 1) CTIP1 is expressed by postmitotic neurons in the cortical plate.
(A) High-magnification confocal image from section shown in Figure 1A.
(B-C) Ctip1 mRNA is expressed in proliferative zones at E14.5 (arrow in B), but CTIP1 protein is detected only in postmitotic neurons in an adjacent section (arrow in C).
(D-E) CTIP1 is not co-expressed with TBR2, which marks intermediate progenitors (D-D’’), or with Ki67, which marks actively proliferating progenitors (E-E’’), indicating that it is exclusive to postmitotic neurons in cortex. Arrowheads mark cells expressing proliferative markers, but not CTIP1. CTIP1 expression increases in a graded fashion through the intermediate zone, and reaches highest levels in the cortical plate (D’’’, E’’’). Fluorescence profiles are produced from images shown in D and E, with fluorescence intensity expressed in arbitrary units.
(F) CTIP1 and CTIP2 expression at E16.5. Panel F is reproduced from Figure 1B.
Scale bars: 200um (B-C), 50um (A, D-E).

**Figure S2.** (related to Figure 2) Defects in lamination, migration, and neuronal positioning in Ctip1^fl/fl;Emx1-Cre mice persist until adulthood.
Layer V remains expanded relative to layer VI in P21 Ctip1^fl/fl;Emx1-Cre mice (B) compared with wild-type (A) (quantification in C), and more large, darkly-stained pyramidal neurons are visible in conditional null brains (A’-B’). In addition, cortical layers remain less distinct from each other in conditional null mice, indicating that defects in subtype specification, migration, and neuronal positioning are not resolved later in development. Overall differences between wild-type and conditional null cortex are preserved between P4 and P21, although layer V is proportionally smaller, suggesting that some excess SCPN in conditional null cortex do not persist (D).
Scale bars: 200um (A-B), 100um (A’-B’).
*, p<0.05; **, p<0.01; n.s., not significant; SP, subplate

**Figure S3.** (related to Figure 3) Ctip1 and Ctip2 are genetically cross-repressive.
(A-D) At E14.5, CTIP2 and CTIP1 are extensively co-expressed by newly postmitotic projection neurons (A, quantification in B). By E17.5, most neurons in layer V express either CTIP1 or CTIP2, and only 3.4% express both (C, quantification in D).
(E-J) CTIP2 expression is increased in layers V and VI of P0 Ctip1^+/+;Emx1-Cre cortex (E-F, qPCR quantification in G), and CTIP1 expression is increased in layers V and VI of P0 Ctip2^+/+ cortex (H-I, qPCR quantification in J).
(K-N) Nex1-Cre;Ctip1^fl/fl conditional null mutants recapitulate subtype specification defects observed in Emx1-Cre;Ctip1^fl/fl conditional null mutants, indicating that errors in subtype specification result from postmitotic deletion of Ctip1. TBR1 is expressed by fewer neurons in conditional null cortex, CTIP2 is expressed by more neurons, and SATB2 expression is not significantly changed in either deep or superficial layers (K-L, quantification in M). Nex1-Cre is expressed by postmitotic neurons starting in the intermediate zone, and expression does not overlap with cycling progenitors, labeled by acute BrdU at E14.5 (N-N’’).
Scale bars: 25um (A, C), 100um (E-F, H-I, K-L), 50um (N)
*, p<0.05; **, p<0.01; n.s., not significant; CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; V, layer V

**Figure S4.** (related to Figure 4) Subplate neuron identity and projections are impaired in the global absence of Ctip1 function.
(A-F) Subplate neurons (arrows) normally express Ctgf (A), Pcp4 (C), and NURR1 (E) at P0, but all three are strikingly reduced in Ctip1 null subplate (B, D, F). Expression in other populations is unaffected (for example, arrowheads in C-D, E-F).
(G-H) Subplate projections do not pioneer as far into the internal capsule (IC) by E14.5 in Ctip1^+/+;Rosa26R-tdTomato^fl/wt;Emx1-Cre (null) mice (H) as in Ctip1^+/+;Rosa26R-tdTomato^fl/wt;Emx1-Cre (wild-type) (G). Dotted line indicates axon front.
(I) Quantification of G-H. Axons have extended significantly farther in wild-type than in Ctip1 null internal capsule (left). “Box-and-whiskers” plot of the fifteen sections per brain, three pairs per genotype, used for quantification (right). Boxes extend from first to third quartile measurement, with median plotted as horizontal line within box. “Whiskers” extend from minimum to maximum measurement.
Scale bars: 100um

**Figure S5.** (related to Figure 5) Superficial-layer callosal projection neurons are produced in normal numbers in the absence of Ctip1 function, but are impaired in migration, positioning, and projection.
(A-E) Progenitors cycle in normal numbers in Ctip1\(^{fl/fl}\);Emx1-Cre cortex compared with wild-type at E14.5. Acute BrdU administration (A-B) and Ki67 immunostaining (C-D) label the same number of neurons in wild-type and mutant cortex. Quantification (E).

(F-H) Late-born neurons mispositioned in deep layers express markers of superficial-layer neurons. Few wild-type deep-layer neurons labeled with BrdU at E15.5 express the superficial callosal projection neuron marker CUX1 (arrowheads, F’-F”), but significantly more Ctip1\(^{fl/fl}\);Emx1-Cre deep-layer neurons labeled with BrdU at E15.5 (arrows, G-G”) do. Quantification (H).

(I-K) Fewer late-born neurons project across the corpus callosum in the cell-autonomous absence of Ctip1. Cre electroporation at E15.5 labels fewer Ctip1\(^{fl/fl}\) axons crossing the corpus callosum at P4 (J’-J”) than wild-type axons (I’-I”) (brackets in I’ and J’ are the same size), although electroporations are equivalent (I’”-J’”). Quantification of fluorescence intensity of axons crossing corpus callosum, corrected for fluorescence intensity of electroporation site (K).

n.s., not significant; **, p<0.01

**Figure S6.** (related to Figure 6) Migration and laminar positioning of deep-layer neurons is not affected by the absence of Ctip1 function.

(A-I) When BrdU is administered to pregnant females at E11.5 (A-B), E12.5 (D-E), and E13.5 (G-H), BrdU-labeled neurons in Ctip1\(^{fl/fl}\);Emx1-Cre cortex are equally likely to remain in deep layers compared with wild-type, and the overall laminar distribution of labeled neurons (marked in red) is normal (C, F, I). Comparisons between cortical segments are all non-significant.

(J-M) Sparse electroporation of Cre at E12.5 does not affect migration of Ctip1\(^{fl/fl}\) neurons at E15.5. Schematic of experimental approach (J). Wild-type neurons electroporated with Cre (K) are equally likely as electroporated Ctip1\(^{fl/fl}\) neurons (L) to have migrated into the cortical plate by E15.5 (quantification, M).


**Figure S7.** (related to Figure 7) Misexpression of either Ctip2 or Fezf2 at E12.5 represses expression of CTIP1.

(A-C) Cortical neurons in layer V electroporated at E12.5 with control nEgfp frequently express CTIP1 (arrows in A). Significantly fewer layer V neurons electroporated with Fezf2-IRES-nEgfp express CTIP1 (arrows in B), and most electroporated neurons express no CTIP1 (arrowheads in B). Almost no layer V neurons electroporated with Ctip2-IRES-nEgfp express CTIP1 (arrows in C), and virtually all electroporated neurons express no CTIP1 (arrowheads in C). In both B and C, electroporated neurons that express CTIP1 tend to be weakly electroporated.

(D) Quantification of A-C. Percentage of nEGFP-positive layer V neurons that are also CTIP1-positive. **, p<0.01.

(E) Schematic of experimental approach. Wild-type embryos were electroporated at E12.5, and brains were collected at P4 and analyzed for CTIP1 and nEGFP expression.

Scale bars: 50um.

**Table S1.** (related to Figures 1-S7) Raw values for quantitative measures reported in this study. Average and SEM are presented for each quantitative assessment in the study, organized by figure. Supplemental figure measures are presented with the main figure to which they refer.

**SUPPLEMENTAL MATERIALS AND METHODS**

**Animals**

Ctip1\(^{fl/fl}\) mice were generated by Tucker and colleagues (RRID:MGI_4358088, Sankaran et al., 2009; Lee et al., 2013). Ctip1\(^{-/-}\) mice were generated by Copeland and colleagues (RRID:MGI_2663941, Liu et al., 2003), and were obtained from the RIKEN BioResource Center (stock number RBRC01190). Ctip2\(^{-/-}\) mice were generated by Kominami and colleagues (RRID:MGI_2663971, Wakabayashi et al., 2003; Arlotta et al., 2005). Emx1-Cre (RRID:MGI_4440744, stock number 005628), Rosa26R-tdTomato-Ai9 (RRID: MGI_3809523, stock number 007909), and Rosa26R-NZG (RRID:MGI_3840211, stock number 012429) mice were purchased from Jackson Laboratories. Ntsr1-Cre mice (RRID:MGI_3836636, stock number 030648-UCD) and Rbp4-Cre mice (RRID: MGI_4367067, stock number 031125-UCD) were generated by the GENSAT project (Gong et al., 2007), and were purchased from the MMRRC. Nex1-Cre mice were generated by Nave and colleagues (RRID:MGI_2668659, Goebbel et al., 2006).

**Immunocytochemistry**

Primary antibodies and dilutions used: rat anti-BrdU, 1:500 (Accurate Chemical and Scientific Corporation
Cat# OBT-0030 RRID:AB_2341179; mouse anti-CTIP1 clone 14B5, 1:500 (Abcam Cat# ab19487, RRID:AB_444947); rabbit anti-CTIP2, 1:200 (Abcam Cat# ab28448, RRID:AB_1140055); rat anti-CTIP2, 1:200 (Abcam Cat# ab18465, RRID:AB_2064130); rabbit anti-CDP1/CUX1, 1:200 (Santa Cruz Biotechnology Cat# sc-13024, RRID:AB_2261231); rabbit anti-DARPP-32, 1:250 (Cell Signaling Technology Cat# 2306S, RRID:AB_823479); rabbit anti-FOG2, 1:250 (Santa Cruz Biotechnology Cat# sc-10755, RRID:AB_2218978); chicken anti-GFP, 1:200 (Aves Labs Cat# GFP-1020, RRID:AB_10000240); rabbit anti-GFP, 1:500 (Molecular Probes Cat# A11122, RRID:AB_221565); rabbit anti-Ki67, 1:500 (Abcam Cat# ab15580, RRID:AB_443209); goat anti-LHX2, 1:200 (Santa Cruz Biotechnology Cat# sc-19344, RRID:AB_2135660); goat anti-NURR1, 1:100 (R and D Systems Cat# AF2156, RRID:AB_2153894); mouse anti-SATB2, 1:200 (Abcam Cat# ab51502, RRID:AB_882455); rabbit anti-TBR1, 1:200 (Abcam Cat# ab31940, RRID:AB_2200219); rabbit anti-TBR2, 1:500 (Abcam Cat# ab23345, RRID:AB_778267).

With the exception of rabbit/chicken anti-GFP, staining for all antibodies is improved by a 10-minute antigen retrieval at 95°C in 0.01M citric acid, pH 6.0; rat anti-BrdU requires a 90-minute antigen retrieval at room temperature in 2N HCl (Magavi et al., 2008). Tissue was incubated with primary antibody at 4°C overnight. Secondary antibodies were chosen from the Alexa series (Invitrogen), and used at a dilution of 1:500 for 4 hours at room temperature.

Non-radioactive in situ hybridization was performed as previously described (Arlotta et al., 2005). Probes for Clil1, Ctgf, Dkk3, Fc1f, Lpl, and PcP4 were previously described (Arlotta et al., 2005; Lai et al., 2008; Molyneaux et al., 2009). For Nissl staining, vibratome-sectioned tissue was mounted on gelatin-coated slides and stained with 0.25% cresyl violet, then dehydrated through a graded alcohol series and xylenes. Slides were mounted in DPX.

BrdU birthdating

Timed pregnant females were intraperitoneally injected with bromodeoxyuridine (50 mg/kg) at E11.5, E12.5, E13.5, E14.5, or E15.5. Littermate pairs of Ctip1fl/fl;Emx1-Cre and Ctip1wt/wt;Emx1-Cre pups were collected at P4 and processed for BrdU immunocytochemistry (Magavi et al., 2008). Six anatomically-matched sections from each mouse were selected and single confocal slices were imaged. Cells fully labeled with BrdU were counted by investigators blinded to genotype, dividing cortex into ten equal bins.

For acute BrdU administration, timed pregnant females were intraperitoneally injected with bromodeoxyuridine (75 mg/kg) at E14.5, and embryos were collected 30 minutes later and processed and quantified for BrdU immunocytochemistry, as above.

Quantitative RT-PCR

Total RNA was extracted from whole cortex of P0 Ctip1+/+, Ctip2−/−, and wild-type littermate control mice (n=3 of each genotype for each comparison), and cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen). qRT-PCR was performed using a LightCycler 1.5 system (Roche, Branford, CT). All values obtained were normalized with respect to mRNA expression levels of Gapdh.