The neocortex contains hundreds to thousands of distinct subtypes of precisely connected neurons, allowing it to perform remarkably complex tasks of high-level cognition. Callosal projection neurons (CPN) connect the cerebral hemispheres via the corpus callosum, integrating cortical information and playing key roles in associative cognition. CPN are a strikingly diverse set of neuronal subpopulations, and development of this diversity requires precise control by a complex, interactive set of molecular effectors. We have found that the transcriptional coregulator Cited2 regulates and refines two stages of CPN development. Cited2 is expressed broadly by progenitors in the embryonic day 15.5 subventricular zone, during the peak of superficial layer CPN birth, with a progressive postmitotic refinement in expression, becoming restricted to CPN of the somatosensory cortex postnatally. We generated progenitor-stage and postmitotic forebrain-specific Cited2 conditional knock-out mice, using the Emx1-Cre and NEX-Cre mouse lines, respectively. We demonstrate that Cited2 functions in progenitors, but is not necessary postmitotically, to regulate both (1) broad generation of layer II/III CPN and (2) acquisition of precise area-specific molecular identity and axonal/dendritic connectivity of somatosensory CPN. This novel CPN subtype-specific and area-specific control from progenitor action of Cited2 adds yet another layer of complexity to the multistage developmental regulation of neocortical development.

Key words: arealization; callosum; neocortex; neuronal differentiation; somatosensory

Significance Statement
This study identifies Cited2 as a novel subtype-specific and area-specific control over development of distinct subpopulations within the broad population of callosal projection neurons (CPN), whose axons connect the two cerebral hemispheres via the corpus callosum (CC). Currently, how the remarkable diversity of CPN subtypes is specified, and how they differentiate to form highly precise and specific circuits, are largely unknown. We found that Cited2 functions within subventricular zone progenitors to both broadly regulate generation of superficial layer CPN throughout the neocortex, and to refine precise area-specific development and connectivity of somatosensory CPN. Gaining insight into molecular development and heterogeneity of CPN will advance understanding of both diverse functions of CPN and of the remarkable range of neurodevelopmental deficits correlated with CPN/CC development.

Introduction
The neocortex contains hundreds to thousands of distinct neuronal subtypes that enable it to perform remarkably complex tasks. Callosal projection neurons (CPN) are the broad population of commissural neurons whose axons connect the two cerebral hemispheres via the corpus callosum (CC), the largest axonal
tract in the mammalian brain. CPN are excitatory pyramidal projection neurons whose cell bodies reside in neocortical layers II/III (~80% in mouse), V (~20%), and VI (a few percent; Catanpano et al., 2001; Fame et al., 2011; Greig et al., 2013), and play key, diverse roles in complex associative and integrative cognition. CPN in the four primary functional neocortical areas are molecularly, morphologically, and functionally diverse (Lomber et al., 1994; Olivares et al., 2001; Grove and Fukushima-Shimogori, 2003; Benavides-Piccione et al., 2006; O’Leary et al., 2007). Further, subpopulations of CPN maintain other noncallosal projections to the contralateral or ipsilateral striatum, the primary somatosensory cortex, or frontal areas (Wilson; Mitchell and Macklis, 2005; Fame et al., 2011). CPN are thus a strikingly heterogeneous set of neuronal subpopulations, requiring a complex and interactive set of molecular controls to precisely regulate development of their distinct subpopulations.

Currently, how the remarkable diversity of CPN subtypes and connectivity is specified, and how they differentiate to form highly precise and specific circuits, are largely unknown. We previously identified a combinatorially expressed set of genes that both define CPN as a broad population and identify novel subpopulations of CPN during development (Molyneaux et al., 2009). Cited2 encodes a transcriptional coregulator that is significantly enriched in CPN over other cortical projection neuron subpopulations, with particularly high expression at early stages of CPN development (Molyneaux et al., 2009). CITED2 functions as a transcriptional coactivator by interacting with CBP/p300 (Bhattacharya et al., 1999; Freedman et al., 2003), or as a transcriptional corepressor by competing with transcription factors for binding to CBP/p300 (Freedman et al., 2003; Lou et al., 2011). Cited2 is critical for proper development of multiple systems, including the heart, lung, lens, placenta, and blood, in addition to neural tube closure (Bamforth et al., 2001, 2004; Barbera et al., 2002; Weninger et al., 2005; Withington et al., 2006; Chen et al., 2008, 2009; Xu et al., 2008; Kranc et al., 2009). Although Cited2 function has not been investigated in cortical development, in these other systems CITED2 interacts with or regulates transcription factors known to function critically in cortical specification and development, including LHX2 (Glenn and Maurer, 1999), PAX6 (Chen et al., 2008, 2009), and AP2γ (Bamforth et al., 2001).

Here, we demonstrate that Cited2 regulates and refines two stages of CPN development. Cited2 is expressed broadly by progenitors of the embryonic day (E) 15.5 subventricular zone (SVZ) during the peak of superficial layer CPN birth, with a progressive postmitotic refinement in expression to CPN of the somatosensory cortex postnatally. We generated progenitor-stage and postmitotic forebrain-specific Cited2 conditional-null (cKO) mice, using the Emx1-Cre and NEX-Cre mouse lines, respectively. In progenitor-stage cKO, we identify broad reduction of TBR2-positive progenitors at E15.5 across the neocortex, resulting postnatally in both reduced thickness of superficial layers and a highly area-specific reduction of layer II/III somatosensory neocortical length. Importantly, loss of Cited2 function does not disrupt the barrel field, resulting instead in an unprecedented misalignment of molecular areal identity between layers II/III and IV. Further, we identify area-specific disruption of dendritic complexity and precise axonal connectivity of somatosensory CPN. Cited2 is not required postmitotically for these functions, even though some processes, such as arealization and dendritic arborization, are completed postmitotically. Together, our results demonstrate that Cited2 functions differently from previously described mechanisms to regulate two stages of precise CPN development, acting in neocortical progenitors to both broadly regulate generation of superficial layer CPN throughout the neocortex and, in an areally restricted manner to refine the distinct identity and precise connectivity of somatosensory CPN. This novel subtype-specific and area-specific control from progenitor action adds yet another layer of complexity to the multistage development of the neocortex.

Materials and Methods
Mice. C57BL/6 wild-type (WT) mice were obtained from Charles River Laboratories for retrograde labeling, Western blotting, and determining gene expression. Cited2 conditional floxed mice (Preis et al., 2006), Lm04 conditional floxed mice (Deng et al., 2010), and NEX-Cre mice (Geobbel et al., 2006) were previously described. Emx1-Cre mice were generated by Guo et al. (2000) and obtained from The Jackson Laboratory (strain number 005628). To avoid nonspecific CRE recombinase activity in oocytes (Hayashi et al., 2003), all cKOs were generated by crossing fl females with fl cre + males, and no offspring from fl cre + dams were analyzed. The morning of the day of the appearance of the vaginal plug was defined as E0.5. The day of birth was designated postnatal day (P) 0. All animal procedures were approved by the Massachusetts General Hospital and/or Harvard University Institutional Animal Care and Use Committees.

In situ hybridization and histology. Postnatal tissue was fixed overnight in 4% paraformaldehyde (PFA)/PBS at 4°C; for flatmount analysis, cortices were flattened and fixed 3 d in 4% PFA/PBS at 4°C. Fixed tissue was sectioned on a vibrating microtome for in situ hybridization. Embryonic tissue was flash frozen in 2-methyl butane, embedded in TBS, and cryosectioned. In situ hybridization was performed as previously described (Cederquist et al., 2013). The probes were synthesized as described previously: Cited2, Molyneaux et al. (2009); RORβ, ephrinA5, Allen Brain Atlas Resources (http://www.brain-map.org); EphA7, Mori et al. (1995); Cadh6, Joshi et al. (2008).

Immunocytochemistry and Western blotting. Brains were postfixed overnight in 4% PFA/PBS at 4°C, then were sectioned on a VT1000S vibrating microtome (Leica Microsystems). Sections were incubated in primary antibody dilutions at 4°C overnight, and appropriate secondary antibodies were selected from the Invitrogen Alexa series (Invitrogen). Antigen retrieval methods were required to expose antigens for some of the primary antibodies. Sections were incubated in 0.1% citric acid, pH 6.0, for 10 min at 95–98°C. Primary antibodies were used as follows: goat anti-LMO4 (Santa Cruz Biotechnology SC-11122), rat anti-TBR2 (eBioscience 14-4875), rabbit anti-TBR2 (Abcam ab23345), rabbit anti-PAx6 (Millipore Ab2237), goat anti-BHLHB5 (Santa Cruz Biotechnology SC-6045), goat anti-CUX1 (Santa Cruz Biotechnology SC-13024), mouse anti-phosphorylated histone H3 (pH3; Abcam ab14955), rabbit anti-pH3 (Millipore 06-570), mouse anti-βIII tubulin (Covance mms-435P), mouse anti-PCNA (Sigma-Aldrich WH000511M2), rat anti-Ctip2 (Abcam ab18465), rabbit anti-Ki67 (Abcam ab15580), rabbit anti-RORβ (a generous gift from the Stunnenberg laboratory), rabbit anti-SHT (Immunostar 20080), mouse anti-myelin basic protein (MBP; Millipore Bioscience Research Reagents MAB387), and rabbit anti-GFP (Invitrogen A-11122). Immunocytochemistry was performed as previously described (Cederquist et al., 2013).

Immunoblotting was performed as previously described (Macdonald et al., 2010). Briefly, neocortical tissue was isolated, and protein homogenates were separated by 4–20% SDS-PAGE, and transferred to nitrocel-
lulose membrane (Bio-Рad Trans-Blot). Membranes were incubated for 12–20 h at 4°C in rabbit anti-CITED2 (Abcam ab108345) primary antibody diluted in 2% milk/TBS, and developed with goat anti-rabbit HRP IgG (Bio-Rad) diluted in 2% milk/TBS, and signals were detected with chemiluminescence (Pierce).

Quantification of neocortical length and thickness. All length and thickness measurements were performed with images of matched sagittal 50 μm sections using ImageJ to trace the curvature of the neocortical surface. Areas were delineated using the noted marker gene expression. Deep layers (V, VI) were identified as those including cells expressing high levels of CTP2 and deeper. Superficial layers (II–IV) were identified as those superficial to high level CTP2 expression. P values were calculated using the unpaired two-tailed Student’s t test using GraphPad Prism for Mac (GraphPad Software, www.graphpad.com). A robust regression outliers (ROUT) test (Q = 0.5%) was performed for all datasets with N ≤ 10 (Motulsky and Brown, 2006). No outliers were discovered.

Golgiz staining and dendritic complexity analysis. P22 mouse brains were freshly immersed in prepreparation impregnation solution (FD Rapid GolgiStain kit, FD Neurosciences), and processed according to the protocol provided by the company. Neurons were imaged, and then traced blinded to genotype. Dendritic complexity was quantified using Sholl analysis (Sholl, 1953) using ImageJ (W. S. Rasband, ImageJ, National Institutes of Health, Bethesda, Maryland) with the Sholl Analysis Plugin (v1.0; Ghosh Laboratory, http://labs.biology.ucsd.edu/ghosh/software/).

The following parameters were used for dendrite analysis: step, 10 μm; beginning radius, 20 μm; final radius, 200 μm.

Results

Cited2 is expressed in the SVZ at E15.5 and, postnatally, by a restricted population of somatosensory CPN

The transcriptional coregulator Cited2 [CBP/p300-interacting transactivator with glutamic acid/aspartic acid-rich C-terminal domain 2] is more highly expressed by CPN relative to other cortical projection neuron subpopulations [in particular, corticospinal motor neurons (CSMN)] in the developing neocortex, with peak differential expression at E18.5, the earliest time point at which CPN and CSMN can be purified by retrograde labeling (Molynieux et al., 2009; Fig. 1A). We confirmed and more broadly investigated the neocortical expression of CITED2 protein by Western blotting from the developing neocortex, revealing high CITED2 expression beginning at E15.5, and decreasing postnatally (Fig. 1B). In situ hybridization reveals that there is minimal pallial expression of Cited2 at E13.5, but Cited2 is highly expressed in progenitor regions throughout the SVZ of the developing neocortex during superficial layer CPN generation at E15.5 (Fig. 1CD), with expression decreasing as differentiation proceeds. Postnatally, total expression levels of Cited2 decrease, and its neocortical expression strikingly refines to layers II/III, V, and VI of somatosensory areas, mirroring the laminar distribution of CPN (Fig. 1E,F). To further investigate this refinement of Cited2 expression, we examined Cited2 expression in sagittal preparations (Fig. 1G,H). Embryonically, Cited2 is expressed across the rostrocaudal extent of the neocortex (Fig. 1G). Postnatally, Cited2 expression becomes progressively restricted to CPN (in layers II/III, V, and VI) of the somatosensory neocortex by P3, in keeping with the known boundary...
ies (Fig. 1H). This broad early SVZ progenitor expression across areas, with postnatal areal refinement to somatosensory CPN, led us to hypothesize two stages of function for Cited2 in neocortical, and specifically CPN, development: broadly, in generation of superficial layers, and area-specifically, in maturation of somatosensory CPN.

The SVZ is largely composed of intermediate progenitor cells (IPCs; or basal progenitors), which are transit-amplifying progenitors that arise from asymmetric divisions of radial glial cells (RGCs) of the VZ, and which undergo a limited number of symmetric divisions before generating pairs of postmitotic neurons (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). Although IPCs give rise to projection neurons of all laminae, at E15.5, the peak of Cited2 expression, they largely generate superficial layer projection neurons (TBR2+) of the VZ, and which undergo asymmetric divisions of radial glial cells (RGCs) of the VZ, and which undergo a limited number of symmetric divisions before generating pairs of postmitotic neurons (Sessa et al., 2008; Kowalczyk et al., 2009), of which CPN are the predominant population.

The high Cited2 expression throughout the SVZ suggests a potential Cited2 function in IPCs at E15.5. At E15.5, Cited2 is most highly expressed in the laminar domain that overlaps with TBR2-expressing (TBR2+) IPCs of the SVZ, extending into the early postmitotic neurons of the intermediate zone, but it is mostly excluded from the region containing PAX6+ RGCs of the VZ (Fig. 1J). The SVZ region expressing Cited2 includes both proliferating and nonproliferating populations, as assessed by the mitotic markers Ki67 and pH3 (Fig. 1J,K). Together, these data suggest that CITED2 functions in SVZ IPCs as they transition from cycling progenitors to postmitotic neurons across all areal anlagen at E15.5, when superficial neocortical neurons are being generated.

**Cited2 controls TBR2+ IPC number and proliferation in the E15.5 neocortex**

To investigate developmental requirements for Cited2 in neocortical generation and precise CPN maturation, we generated mice null for Cited2 in the neocortex.

To bypass a set of early patterning defects in homozygous Cited2-null mutants (Bamforth et al., 2001), and any confounding role Cited2 might have in the subpallial domain, we generated pallial cortex-specific cKO's using Emx1-promoter driven cre-recombinase (Emx1-Cre; Guo et al., 2000; Jin et al., 2000). In all experiments, we compared Cited2Δcre; Emx1-Cre+ cKO mice to littermate controls, both Cited2Δcre−; Emx1-Cre− and Cited2Δcre+/Emx1-Cre+. Because no significant differences were observed between the two control genotypes, they were combined as Cited2 WT in analyses. We verified that these cKO mutants are viable and healthy, and confirmed that early cortical neurogenesis, preceding onset of pallial Cited2 expression, is not affected in Cited2 cKO brains, as assessed by VZ (Cited2 WT, 2694 ± 36 μm; Cited2 cKO, 2683 ± 56 μm; N = 10 WT and 5 cKO, p = 0.87) and cortical plate length (Cited2 WT, 3163 ± 33 μm; Cited2 cKO, 3093 ± 75 μm; p = 0.33) and thickness at E15.5 across the rostrocaudal axis (medial: Cited2 WT, 317 ± 6 μm; Cited2 cKO, 297 ± 12 μm; p = 0.12).

**Figure 1. Cited2 is expressed broadly by CPN progenitors at E15.5, with expression refining to CPN of the somatosensory cortex by P3.** A, Cited2 is highly expressed by CPN (red) relative to CSMN (blue) at critical times during development, as detected by microarray analysis of FACS-purified CPN and CSMN. Error bars denote SEM (Molyneaux et al., 2009). B, Western blot analysis showing that CITED2 protein is highly expressed as early as E15.5 in the neocortex, with expression decreasing postnatally, relative to a β-actin loading control. C–F, Expression of Cited2 is largely restricted to subpallial progenitors at E13.5 (C), but Cited2 is highly expressed in the cortical SVZ at E15.5 (D), the peak of superficial layer CPN birth, with expression maintained in layers II/III and V postnatally (E,F). G, Embryonically, Cited2 is expressed uniformly across the neocortex, detected across the SVZ at E18.5 (arrowheads) and across the cortical plate (CP). H, In the first days postnatally, however, its expression refines and becomes restricted to somatosensory cortex (arrows) by P3. I, At E15.5, Cited2 is highly expressed in the SVZ, extending into the intermediate zone (IZ). J, Cited2 (blue) is largely excluded from Pax6+ (green) radial glial progenitors of the VZ, but is highly expressed by TBR2+ (red) IPCs of the SVZ; K, Cited2 is largely excluded from the highly proliferative Ki67+ (green) VZ and apical mitotic cells, as indicated by ph3 (red), but is expressed by basally proliferating IPCs of the SVZ and IZ. Scale bars: C–F, 500 μm; G–I, 1 mm; J–H’, 100 μm; I’, 200 μm. Dotted lines in J and K indicate apical, ventricular surface.
Because Cited2 is highly expressed throughout the SVZ at E15.5, we specifically investigated whether the population of TBR2+/IPCs is altered in the absence of Cited2 function. In the setting of a broadly well patterned and laminated E15.5 neocortex, there is a highly specific 20% reduction of TBR2+/IPCs in Cited2 cKO mice \((N= 11\text{ WT}, 6\text{ cKO})\), as indicated by position of pH3-positive mitotic cells \((N= 10\text{ WT, 5\text{ cKO}})\); and/or increased cell death \((E)\), as indicated by expression of aC3. There is increased apoptotic cell death in the Cited2 cKO neocortex, both within the progenitor population and postmitotically \((N= 11\text{ WT, 6\text{ cKO}})\). To directly investigate whether Cited2 cell-autonomously regulates proliferation of IPCs, we electroporated Cre recombinase and GFP into VZ progenitors of Cited2\(^{fl/fl}\) and Cited2\(^{fl/wt}\) littermates at E14.5 to excise Cited2 in a small subpopulation of neocortical progenitors. We used a BrdU pulse at E15.5, and immunocytochemistry for Ki67 at E16.5 to identify progenitors that continued to proliferate. There is a significant reduction in the number of Cited2-null \((N= 10\text{ WT, 5\text{ cKO}})\); progenitors that incorporate BrdU at E15.5, or express Ki67 at E16.5. We directly investigated the population of actively proliferating IPCs as basal mitotic pH3+/PCNA- progenitors (Fig. 2D). While there is no general disruption in mitotic progenitors, there is a specific reduction in basally located mitotic progenitors, indicating a specific requirement for Cited2 in IPC proliferation, but not in RGC proliferation.

In addition to perturbed proliferation, a reduction in TBR2+/IPCs might also result from cell death. We directly investigated this possibility by assessing apoptosis at E15.5 using activated caspase 3 (aC3+). We identified a significant increase in aC3+ cells in the Cited2 cKO neocortex, in both proliferating progeni-
tors (PCNA+) and postmitotic neurons (PCNA−) (Fig. 2E). This significant increase in apoptosis among progenitors likely contributes to the substantial decrease in TBR2+ progenitors in the Cited2 cKO neocortex.

The reduction in PCNA+/TBR2+ IPCs and basally located mitotic progenitors in the Cited2 cKO neocortex suggests that Cited2 is necessary for expansion of the IPC population. To directly investigate whether Cited2 cell-autonomously regulates proliferation of IPCs, we electroporated Cre recombinase into progenitors of Cited2fl/fl and Cited2fl/−/fl littermates at E14.5, to excise Cited2 in a small subpopulation of neocortical progenitors. We coelectroporated GFP to identify progenitors that were cycling at E14.5 and were electroporated, and we used a BrdU pulse at E15.5 and immunocytochemistry for Ki67 at E16.5 to identify progenitors that continued to proliferate (Fig. 2F–P”). We identified a significant reduction both in the number of Cited2-null (Cited2fl/−/fl; Cre+) progenitors that incorporated BrdU at E15.5 and in the number of Cited2-null (Cited2fl/−/fl; Cre+) progenitors that were Ki67+ at E16.5. Further, there was a significant reduction in the number of cells proliferating at E15.5 that were still cycling at E16.5 (BrdU/Ki67 double positive). Additionally, we used immunocytochemistry for aC3 at E16.5, and did not identify a significant change in the number of Cited2-null (Cited2fl/−/fl; Cre+) progenitors that were dying at E16.5. These analyses demonstrate that Cited2-null IPCs are less likely to re-enter the cell cycle than their heterozygous counterparts, further supporting the conclusion that Cited2 cell-autonomously contributes to regulation of IPC proliferation, and that this reduction in basal progenitor proliferation contributes to the reduction in TBR2+ progenitors in the Cited2 cKO neocortex at E15.5.

The later areal refinement of Cited2 expression raises the hypothesis that CITED2 function in the SVZ is areally restricted based on overlapping, intersectional expression of coregulators. For example, the known CITED2 interactor AP2γ (Bamforth et al., 2001) is broadly expressed across the VZ; however, it regulates specification of TBR2+ IPCs and generation of superficial layers only in the occipital cortex (Pinto et al., 2009), presumably through an area-restricted coregulator. To investigate whether Cited2 functions in an areally restricted manner within SVZ progenitors, we analyzed IPC numbers in three presumptive areal regions, and determined that the progenitor abnormality is uniform across the extent of the developing neocortex (Tbr2+ cells/100 μm: rostral: WT, 76.7 ± 1.6; cKO, 61.7 ± 3.4, p = 0.0004; medial: WT, 69.5 ± 1.9; cKO, 52.2 ± 3.7, p = 0.0003; caudal: WT, 63.7 ± 2.9; cKO, 47.3 ± 5.5, N = 10 WT and 6 cKO, p = 0.01 Student’s t test), indicating that the function of Cited2 in IPC proliferation and survival is not areally restricted.

Cited2 regulates neocortical size, including superficial layer thickness and neocortical surface length

Superficial layer CPN arise predominantly from IPCs (Sessa et al., 2008; Kowalczyk et al., 2009); therefore, we investigated whether this significant, broad reduction of IPCs in the Cited2-null neocortex at E15.5 causes a reduction in superficial layer CPN, presenting as a change either radially in thickness of the superficial layers and/or tangentially in the cortical length. Cited2 is not required for gross neocortical development or laminar organization. At P6, the Cited2 cKO neocortex is smaller than those of WT littermate controls, but both CPN (SATB2+ and CSMN (CTIP2+)) are present and appropriately positioned (Fig. 3A). Anterograde labeling with DiI and retrograde labeling with fluoro-rescently conjugated CTB demonstrate that CPN are present and are appropriately targeting axons to the contralateral hemisphere in the Cited2 cKO neocortex (Fig. 3B,C). However, both the distribution of retrogradely labeled CPN (Fig. 3C) and CUX1 and CTIP2 immunocytochemistry (Fig. 3D) indicate that superficial layers are thinner in the Cited2 cKO cortex, while the thickness of deep layers is unchanged.

Quantitative analysis of neocortical layer thickness at P3 reveals a significant ~20% reduction in superficial layer thickness (layers II/III and IV, as delineated as cells superficial to CTIP2 expression) across multiple neocortical areas, including the rostral motor cortex, the primary somatosensory area, and the caudal visual cortex. There is no significant change in deep layer thickness (layers V and VI) in any region where CPN account for only a minority of projection neurons (Fig. 3D). To directly investigate whether the reduced superficial layer thickness identified in the Cited2 cKO neocortex is due to reduced cell number and/or increased cell packing, we quantified cell density within the reduced superficial layers. There is no significant difference in cell density in layers II/III of the motor or visual cortices, and a modest, but significant, increase in cell density in layer II/III of the somatosensory cortex, where the greatest reduction in thickness is observed (motor: Cited2 WT, 369 ± 128 cells/100 μm2; Cited2 cKO, 462 ± 30 cells/100 μm2; p = 0.44; somatosensory: Cited2 WT, 652 ± 66 cells/100 μm2; Cited2 cKO, 803 ± 26 cells/100 μm2; p = 0.01; visual: Cited2 WT, 590 ± 35 cells/100 μm2; Cited2 cKO, 608 ± 17 cells/100 μm2; p = 0.55, N = 6 WT and 3 cKO). Additionally, the reduction in superficial laminar thickness in the Cited2 cKO neocortex persists at P6 (Fig. 3D) and into adulthood. These data indicate that the reduction in the number of TBR2+ IPCs observed early in the development of the Cited2-null neocortex results in a significant reduction in the number of superficial layer CPN throughout the neocortex. Interestingly, the findings regarding cell density indicate a further area-specific requirement for Cited2 in development of layer II/III neurons of the somatosensory neocortex.

In addition to the observed reduction in radial thickness in the Cited2 cKO neocortex, early loss of TBR2+ progenitors might also result in an overall decrease in neocortical length on the tangential axis (Sessa et al., 2008; Kowalczyk et al., 2009; Tuoc et al., 2013). We measured cortical surface length at P3, identifying an ~5% smaller diagonal cortical length, and an ~10% reduction in rostrocaudal neocortical surface length in the P3 Cited2 cKO neocortex, measured across multiple mediolateral sagittal sections (Fig. 3F,G). These data indicate that there is both a significant reduction in cortical thickness as a result of Cited2 loss of function and a significant reduction in neocortical tangential length. This reduction of cortical length does not normalize over time; at P21, the Cited2 cKO neocortex is still significantly shorter than that of WT littermates (Cited2 cKO normalized to Cited2 WT littermates, 90.9 ± 3% neocortical length; p = 0.005, N = 6 WT and 3 cKO), indicating that the reduction is not simply a delay in maturation, but rather a persistent reduction of ~10% of the neocortical rostrocaudal surface length.

Cited2 refines the boundary of areal molecular identity of layer II/III somatosensory CPN

Progressive postmitotic refinement of Cited2 expression to the somatosensory cortex led us to hypothesize that CITED2 might have a second phase of function in areal specification of CPN. Dual functions of Cited2 have previously been identified in eye development, in which Cited2 acts upstream of Pax6 to regulate lens morphogenesis, and negatively regulates HIF-1 signaling to regulate hyaloid vasculature formation (Chen et al., 2008). Therefore, we assessed whether specification and development of somatosensory CPN are specifically disrupted in the Cited2 cKO
neocortex, beyond the broad reduction in IPCs and superficial layer generation. First, we investigated whether all neocortical neocortex, beyond the broad reduction in IPCs and superficial layer generation. First, we investigated whether all neocortical

neocortex is smaller than in WT littermate controls, but both CPN (SATB2+) and CSMN (CTIP2+) are present and appropriately positioned. B, C, Anterograde labeling with DiI (B) and retrograde labeling with CTB (C) demonstrate that CPN are present and are targeting the contralateral hemisphere in the Cited2 cKO neocortex. However, both the distribution of retrogradely labeled CPN (C) and CUX1 (red, superficial layers) and CTIP2 (green, deep layers) immunocytochemistry (D) indicate that superficial layers are thinner in the Cited2 cKO neocortex, while the thickness of deep layers is unchanged (N = 4–5 per genotype for A–D). E, Quantitative analysis of neocortical layer thickness at P3 reveals that superficial layers (II–IV; LMO4, red) are significantly thinner cKO neocortex, while the thickness of deep layers is unchanged (N = 4–5 per genotype for A–D). E, Quantitative analysis of neocortical layer thickness at P3 reveals that superficial layers (II–IV; LMO4, red) are significantly thinner than WT. Strikingly, analysis based on three broad cortical areas distinguished by LMO4 expression at P3 (Joshi et al., 2008) indi-

cates that there is a highly specific and substantial (~35%) reduction in the rostrocaudal length of the somatosensory cortex in Cited2 cKO mice versus no significant change in the motor or caudal cortex (Fig. 4A–C). A similar reduction in the somatosensory cortex is observed by expression of BHLHB5 (Fig. 5F, H), which has a largely complementary expression to LMO4, and is highly expressed by CPN of the somatosensory neocortex (Joshi et al., 2008; Cederquist et al., 2013). Thus, the entire 10% neocortical tangential length reduction occurs in the somatosensory region in which Cited2 is normally expressed postnatally.

Because Cited2 expression progressively refines to CPN of the somatosensory cortex during the first postnatal days, we investigated when the area-specific function of Cited2 arises. At P0, before motor and somatosensory cortical areas are fully refined, there is a reduction in both motor and somatosensory cortical lengths, as delineated by LMO4 immunostaining (cKO relative length: motor, 0.89 ± 0.04, p = 0.037; somatosensory, 0.83 ± 0.047, p = 0.018; visual, 0.99 ± 0.046, N = 6 WT and 3 cKO, p = 0.89, Student’s t test). The reduction in Cited2 cKO cortical length progressively becomes restricted to the somatosensory cortex by P3 (cKO relative length: motor, 0.94 ± 0.046, p = 0.13; somatosensory, 0.64 ± 0.13, p = 0.005; visual, 1.02 ± 0.029, N = 8 WT and 4 cKO, p = 0.32, Student’s t test) as WT expression of Cited2 becomes restricted to the somatosensory cortex; this specificity for Cited2 function in the somatosensory cortex is maintained at P8 (cKO relative length: somatosensory, 0.73 ± 0.06; motor, 0.93 ± 0.89; visual, 1.00 ± 0.99, N = 10 WT and 5 cKO, p < 0.0001, Student’s t test).

We confirmed the somatosensory-specific reduction of cortical length in P3 Cited2 cKO mice using the expression of multiple overlapping and/or complementary molecular markers that, in combination, delineate cortical areas at P3 (Dye et al., 2011). Strikingly, layer II/III measurements of somatosensory cortex length by Cadh8, EphrinA5, and EphA7 expression confirm the reduced length observed with LMO4 measurements (Fig. 4D–F). Consistent with the CPN subtype-specific expression of Cited2, this area-specific reduction is also layer specific, with no significant difference in length of acallosal layer IV, by expression of EphrinA5 and Ror6 in somatosensory cortex in Cited2 cKO mice (Fig. 4F, G). In addition, loss of Cited2 function does not disrupt somatosensory barrel morphology, size, or placement (data not shown).
These results indicate highly specific and significant disruption of layer II/III somatosensory cortex in the absence of Cited2 function. Together, these data suggest that Cited2 is selectively necessary for acquisition of molecular areal identity of layer II/III somatosensory CPN, but not generally for somatosensory neocortical arealization. In the P3 WT neocortex, molecular markers of the layer IV primary somatosensory cortex (e.g., Rorβ) and the layer II/III somatosensory neocortex (e.g., BHLHB5) align to indicate the boundary between motor and somatosensory cortices (Fig. 4H–I*). Notably, upon loss of Cited2 function, the rostral boundary of Rorβ expression is no longer aligned with BHLHB5 expression, (Fig. 4I–I*), but rather extends further in the rostral direction than the molecularly identified somatosensory region in layer II/III, indicating an unprecedented misalignment of molecular areal identity between layer II/III and layer IV in the Cited2 cKO neocortex. This neuronal subtype-specific areal misalignment could result in columnar wiring irregularities, the functional consequences of which would be intriguing to investigate.

Cited2 and Lmo4 cooperatively control CPN areal identity

The above evidence indicates functions for Cited2 in specific acquisition of somatosensory CPN identity, leading to the hypothesis that Cited2 might act as part of a molecular network to generate neuronal areal identity; additional molecular controls might function in acquisition of other CPN areal identities, interacting with Cited2 at the boundaries to define these subpopulations. Lmo4 is a particularly compelling candidate to function in CPN of the motor cortex, and to interact with Cited2 at the motor–somatosensory cortex boundary. Lmo4 has reciprocal areal expression in the neocortex compared with Cited2, and has been shown to have areally restricted roles in neocortical projection neuron subtype identities and connectivity (Kashani et al., 2006; Joshi et al., 2008; Lee et al., 2008; Azim et al., 2009; Huang et al., 2009; Cederquist et al., 2013). Cited2 interacts genetically with Lmo4, and Lmo4 can partially functionally compensate for Cited2 in thymus development (Michell et al., 2010). This knowledge led us to hypothesize that Lmo4 might be performing a parallel, areal-specific function in motor cortex CPN, intersecting with Cited2 in areal boundary regions. Its maintained expression in the motor cortex might underlie some of the CPN areal differences observed with Cited2 loss of function.

Figure 4. Neocortical surface length reduction is restricted to layers II/III of somatosensory cortex in the P3 Cited2 cKO neocortex. A–C, Analysis of three broad neocortical areas identified by LMO4 expression at P3 indicates a highly specific and substantial reduction (~30%) in rostrocaudal surface length of the somatosensory area (blue) in the Cited2 cKO neocortex, entirely accounting for the total cortical surface length reduction (N = 8 WT, 4 cKO). D–G, Reduced somatosensory cortex length (black arrowheads) was confirmed via expression of multiple genes either excluded from superficial layers of the somatosensory cortex (D, E, Cadhi, EphA5), or specifically expressed in the somatosensory cortex (F, ephrinA5). Measurements of the acallosal layer IV somatosensory cortex (black arrows), by contrast (F, G, ephrinA5, Rorβ), reveals that there is no significant difference in non-CPN somatosensory cortex length in Cited2 cKO mice compared with that in WT mice (N = 8 WT, 4 cKO). H–I*, In the P3 Cited2 WT neocortex, molecular markers of layer IV (green, Rorβ; bracket) and the somatosensory cortex in layers II/III (red, BHLHB5; white line) align at the motor/somatosensory border, shown in sagittal view (rostal to left). I–I*, In the P3 Cited2 cKO neocortex, by contrast, the boundary of layer II/III expression of BHLHB5 (white line, with additional low-level expression indicated by dashed line) is located caudal to layer IV Rorβ expression (bracelet), resulting in a misalignment of molecular areal boundaries between CPN of layer II/III and acallosal layer IV (H, I, P = 0.05; N = 4–5 per genotype). Scale bars: D–G, 500 μm; H–I, 200 μm. Error bars denote SEM. *p < 0.05; **p < 0.001 (Student’s t-test).

To test this hypothesis, we generated cortex-specific Lmo4/Cited2 double cKO (dcKO) mice using Emx1-Cre recombinase. While LMO4 plays broader roles in defining neocortical areas and the barrel cortex (Kashani et al., 2006; Huang et al., 2009; Cederquist et al., 2013), we focused our analyses on motor and somatosensory superficial layer CPN. Because the Lmo4 floxed line is maintained on a mixed (C57BL/6 and S129S6) genetic background, we confirmed that Cited2 cKO on this background
demonstrates the same radial and tangential reductions in neocortical size as identified on a pure C57BL/6 background. In all analyses, we compared control (Emx1-Cre negative), Lmo4 cKO (Cited2\(^{-/-}\); Lmo4\(^{fl/fl}\); Emx1\(^{Cre^{+/+}}\)), Cited2 cKO (Cited2\(^{-/-}\); Lmo4\(^{+/+}\); Emx1\(^{Cre^{+/+}}\)), and dcKO (Cited2\(^{-/-}\); Lmo4\(^{fl/fl}\); Emx1\(^{Cre^{+/+}}\)) littermates.

We identified that the additional loss of Lmo4 function does not alter the laminar thickness reduction of the Cited2 cKO motor or visual cortex, but does lead to an increase in superficial layer thickness in the somatosensory cortex (Fig. 5A–C). Further, additional loss of Lmo4 function does not alter the overall reduced neocortical length (data not shown).

Figure 5. Additional loss of Lmo4 function does not alter Cited2 cKO neocortical thickness, but does reestablish layer II/III somatosensory neocortical length at the expense of the motor cortex. A–C, Additional loss of Lmo4 function in the Cited2-null neocortex (Cited2\(^{-/-}\); Lmo4\(^{fl/fl}\); Emx1\(^{Cre^{+/+}}\)) does not alter the reduced reduction in superficial layer thickness of the Cited2-null motor or visual cortex, but does increase superficial layer thickness in the somatosensory cortex. Additional loss of Lmo4 function does not alter the overall reduction in total neocortical length (data not shown). D–G, Additional loss of Lmo4 function does, however, re-establish layer II/III somatosensory neocortical length (as measured by Bhlhb5 expression) to normal control length, at the expense of the layer II/III motor cortex. H, Length of motor (rostral to Bhlhb5 layer II/III expression), somatosensory (Bhlhb5 layer II/III positive), and visual (caudal to layer II/III Bhlhb5 expression) cortical areas was measured in control (Emx1-Cre negative), Lmo4 cKO (Cited2\(^{-/-}\); Lmo4\(^{+/+}\); Emx1\(^{Cre^{+/+}}\)), Cited2 cKO (Cited2\(^{-/-}\); Lmo4\(^{+/+}\); Emx1\(^{Cre^{+/+}}\)), and dcKO (Cited2\(^{-/-}\); Lmo4\(^{fl/fl}\); Emx1\(^{Cre^{+/+}}\)) littermates. I–L, In the context of the shortened neocortical surface length in Cited2 cKO mice, additional loss of Lmo4 function re-establishes the length of the somatosensory area boundary, at the expense of the motor cortex length. By contrast, loss of Lmo4 function has no effect on the overall reduced neocortical length or layer II/III thickness of the Cited2 cKO neocortex. Scale bars: C, 100 \(\mu m\); D–G, 1 mm. For each neocortical area, data were analyzed by a one-way ANOVA with Tukey’s post-test. For all experiments, \(N = 14\) controls, 7 Lmo4 cKO, 7 Cited2 cKO, and 8 double cKO mice. Error bars denote SEM. *\(p < 0.05\); **\(p < 0.001\); ***\(p < 0.0001\).
Cited2 do not genetically interact in the neocortex to control neocortical size. Additionally, misexpression of Cited2 does not repress expression of Lmo4 in the motor cortex, nor induce Lmo4 expression in the somatosensory cortex (data not shown). Because Lmo4 is not expressed in neocortical progenitor regions, the maintenance of the reduced neocortical size of the Cited2 cKO cortex in the Lmo4/Cited2 dcKO cortex suggests that the reduction in neocortical size in the Cited2 cKO neocortex results from broad Cited2 function within the SVZ progenitors.

Strikingly, the Lmo4/Cited2 dcKO neocortex, while exhibiting the same reduction in overall length as the Cited2 cKO cortex, has the same somatosensory cortex size as control, exhibiting neither reduction in somatosensory length as exhibited by Cited2 cKO mice, nor increase in somatosensory length as seen in Lmo4 cKO mice (Kashani et al., 2006; Huang et al., 2009; Fig. 5D–I). Importantly, the shifts in areal boundaries that occur with single and double loss of Cited2 and Lmo4 function are restricted to the motor–somatosensory boundary, with the length of the visual cortex remaining the same across all genotypes. In the context of reduced total neocortical length with loss of Cited2 function, additional loss of Lmo4 function results in a rostral shift of the motor–somatosensory boundary (to restore somatosensory cortex length), and reduction in motor cortex length. Together, these data indicate that Cited2 and Lmo4 participate in a network of compensatory and opposing molecular controls over subtype and specific areal identity within superficial layer CPN of the somatosensory and motor cortices. Loss of Lmo4 function can rescue the superficial layer CPN arealization phenotype found in the Emx1-Cre-driven Cited2 cKO neocortex.

#### Cited2 is not required postmitotically for CPN areal identity

Somatosensory-specific postnatal phenotypes of Cited2-null CPN might be dependent on progenitor function of Cited2, or they could be fully independent phenotypes, indicating a biphasic function for Cited2 during CPN development. To investigate potential postmitotic requirements for Cited2 in acquisition of superficial layer CPN areal identity, we used NEX-promoter-driven cre-recombinase (NEX-Cre) to generate mice null for Cited2 in early postmitotic neocortical pyramidal neurons. In this mouse line, Cre recombinase is expressed specifically in postmitotic pyramidal neurons of the neocortex and is absent from progenitors, interneurons, oligodendroglia, and astrocytes (Goebbels et al., 2006). Using a highly sensitive cre recombinase reporter (adenovirus GFP), Goebbels et al. (2006) reported that a small number of proliferating cortical cells were observed at E15.5, indicating that there is a very small window of time, if any, between cell cycle exit and onset of NEX-Cre expression. In Cited2;NEX-Cre cKO mice, there is highly efficient expression of a β-gal reporter across the postnatal neocortex, indicating highly efficient Cited2 excision (data not shown).

We find that postmitotic loss of Cited2 function in the neocortex (with NEX-Cre excision) does not overly disrupt neocortical development or laminar organization (Fig. 6A). Further, there is no reduction in neocortical surface length or laminar thickness in Cited2;NEX-Cre cKO mice (Fig. 6B–C′), indicating that the reduction in superficial layer CPN observed in the postnatal Cited2; Emx1-Cre cKO results entirely from Cited2 function in IPCs. We next investigated whether refinement of layer II/III somatosensory areal identity is disrupted following postmitotic Cited2 loss of function, as it is following Emx1-mediated Cited2 excision. Interestingly, there is no disruption in somatosensory length in the P3 Cited2; NEX-Cre cKO neocortex (Fig. 6D, E). Together, these results indicate that the reduction in acquisition of layer II/III somatosensory areal identity results from Cited2 function in neocortical progenitors, and it is not a fully independent postmitotic function of Cited2.
Figure 7. Loss of Cited2 function results in aberrant dendritic complexity of superficial layer somatosensory CPN. A, A’. Neuronal soma size (NeuN area) is not affected by loss of Cited2. Increased neuronal density is evident here, consistent with the modest, but significant, increase in cell density in layer II/III of the somatosensory cortex (23% increase, \( p = 0.01 \)) quantified at P6 (see Results). \( N = 3 \) WT, 3 cKO. B–D. Dendritic complexity of layer II/III pyramidal neurons (primarily CPN) was analyzed at P22 by Golgi staining and Sholl analysis in the (Figure legend continues.)
Disruption of area-specific CPN dendritic complexity following excision of Cited2 in progenitors

Cited2 functions broadly in early IPCs to both regulate generation of neocortical superficial layer CPN broadly, and to control acquisition of somatosensory molecular layer identity by layer II/III somatosensory CPN specifically. Somatosensory CPN have unique connectivity features, both in their axonal and efferent connections (Innocenti and Price, 2005; Benavides-Piccione et al., 2006). We therefore directly investigated potential functions of Cited2 in central properties of their specific connectivity that govern the unique functionality of somatosensory CPN. We first examined neuronal soma size across all lamina and areas of Cited2; Emx1-Cre cKO mice to determine whether Cited2 loss of function disrupts overall size or growth of cortical projection neurons. Using NeuN to mark neuronal somata, we found no change in neuronal soma size (average soma cross-sectional area) at P21 in either deep or superficial layers (Fig. 7A). These results indicate that the reduced superficial layer thickness (Fig. 3) is not due to decreased CPN soma size.

We then investigated dendritic complexity of layer II/III CPN by Golgi staining and Sholl analysis in multiple neocortical areas (Fig. 7). While there is no significant change in layer II/III CPN dendritic complexity within the motor or visual cortex in the Cited2; Emx1-Cre cKO mice, there is a significant increase in CPN dendritic complexity in the somatosensory cortex (Fig. 7B–D). In the Cited2 WT neocortex, dendritic complexity of layer II/III CPN is distinct across different neocortical areas (Fig. 7E), with decreasing complexity rostral to caudal, as has been shown previously (Benavides-Piccione et al., 2006). Interestingly, there is a shift in total dendritic arbor distribution in Cited2; Emx1-Cre cKO somatosensory cortex CPN to more closely resemble CPN in the motor cortex, with more dendrites close to neuronal cell bodies (Fig. 7F). Together these results indicate that dendritic complexity is specifically increased in somatosensory CPN in the Cited2; Emx1-Cre cKO cortex, perhaps suggesting that Cited2-null CPN might be partially “motorized” with respect to dendritic complexity.

Because dendritic arborization occurs postmitotically, we investigated whether dendritic development of somatosensory CPN is also perturbed following postmitotic-specific Cited2 loss of function, or whether it is dependent on the reduction in acquisition of layer II/III somatosensory areal identity that results from Cited2 function in neocortical progenitors. We find that NEX-cre-mediated excision of Cited2 does not alter CPN soma size (data not shown) or CPN dendritic complexity in any area (Fig. 7G–I). Together, these data indicate that continuous postmitotic expression of Cited2 in somatosensory CPN is not required for development of areal subpopulation-appropriate CPN size and dendritic complexity; rather, alterations in CPN generation and areal identity acquisition arising from Cited2 function in progenitors disrupts development of somatosensory-specific CPN dendritic complexity.

Cited2 is required for precise, homotopic CPN axonal connectivity

In addition to area-specific dendritic complexity, we investigated potential Cited2 function in CPN axonal connectivity, both broadly and in an area-specific manner. HARDI tractography based on MRI reveals that there are fewer correlated pathways passing throughout the CC of adult Cited2 cKO mouse brains compared with control (Fig. 8A, B), which is consistent with the overall reduction in superficial layer thickness and CPN number. The reduced CC is confirmed by examining myelinated fibers in midsagittal sections labeled with MBP, revealing a significantly smaller CC area (Fig. 8C, D). Overall forebrain and midbrain area is not reduced in Cited2 cKO mice, as measured on these midsagittal sections (WT, 38.6 ± 0.7 μm²; cKO, 37.0 ± 0.8 μm²; N = 3 WT, 4 cKO; p = 0.18), and Cited2 cKO CC area is reduced relative to brain area (p = 0.04). Neocortical area, on the other hand, is reduced in Cited2 cKO mice (WT, 11.5 ± 0.3 μm²; cKO, 10.1 ± 0.3 μm²; N = 3 WT, 4 cKO; p = 0.02), suggesting that the reduced CC area in Cited2 cKO mice results from the reduced number of CPN. Of particular note, HARDI reveals an apparent additional disruption in callosal fibers in mid-CC, even though the CC is overall present, but reduced, by MBP staining, suggesting localized axonal disorganization in addition to fewer fibers (Fig. 8A’, B’).

We directly investigated precision of CPN projections at the border between motor and somatosensory cortical areas, which is especially molecularly disrupted in Cited2; Emx1 cKO mouse cortex during development. Precisely matched, focal AAV-GFP anterograde labeling demonstrates that Cited2; Emx1 cKO somatosensory CPN project contralateral axons imprecisely, with a bimodal distribution of axonal projections covering a more expansive target area on the contralateral hemisphere than the tightly delineated homotopic areas targeted by WT CPN (Fig. 8E–K). Together, these data indicate that Cited2 is required for precise areal-specific connectivity of somatosensory CPN, both afferent and efferent.

Discussion

CPN are a remarkably diverse set of neuronal subpopulations, requiring precise control over development of these subpopulations for proper organization and function. This study demonstrates that the transcriptional coregulator CITED2 regulates two aspects of precise CPN development in mice. Cited2 functions broadly in embryonic progenitors of the SVZ to regulate generation of superficial layer CPN throughout the neocortex. Cited2 also functions within progenitors to establish the distinct identity and development of somatosensory CPN, in an areally restricted manner. Understanding molecular controls over development of CPN subpopulations, such as CITED2 control over somatosensory layer II/III CPN, will advance understanding of diverse system functions of CPN, and the broad range of neurodevelopmental disorders with associated abnormalities of CPN/CC development—both overt and subtle.
Cited2 regulates the precise number of TBR2+ IPCs generating layer II/III CPN

The SVZ has expanded concomitantly with the expansion of the cerebral cortex during mammalian evolution, suggesting that an increase in IPCs contributes substantially to the evolutionary expansion of the neocortex (Kriegstein et al., 2006; Martinez-Cerdeno et al., 2006; Molnár et al., 2006; Noctor et al., 2008; Betizeau et al., 2013). Disrupting the IPC population results in reduced cortical thickness of all layers and in reduced cortical surface area (Sessa et al., 2008; Kowalczyk et al., 2009; Tuoc et al., 2013), with superficial layers most significantly affected (Pontious et al., 2008). Expression of Cited2 in the SVZ peaks during generation of superficial layers, and loss of Cited2 function disrupts generation of superficial layers specifically. CUX2 similarly is expressed by IPCs only during late neurogenesis, and its loss perturbs superficial layer neuron production specifically (Cubelos et al., 2008), further highlighting the increased molecular regulation, in which Cited2 has a substantial role, controlling the production of this set of neuronal populations.

The reduction in Cited2; Emx1-Cre cKO neocortical superficial layers arises from a significant reduction of TBR2+ IPCs at E15.5, the peak of superficial layer neuron birth. This reduction in TBR2+ IPCs likely results from both an increase in basal progenitor death and a decrease in IPC proliferation/cell cycle re-entry. We identified an ~2-fold increase in cell death in the Cited2 cKO neocortex, including both progenitors and postmitotic cells. Increased apoptosis is also evident in the midbrain of Cited2−/− embryos during neural tube closure (Bamforth et al., 2001; Barbera et al., 2002), and the somatosensory (extending into motor) neocortex at P6, and contralateral callosal projections were analyzed at 6 weeks of age. In contrast to the precise homotopic projections observed in Cited2 WT1 (G), callosal projections in the Cited2 cKO somatosensory neocortex are diffuse (J). Relative GFP fluorescence intensity was measured in matched sagittal sections of the left injected neocortical hemisphere and in the same region in the contralateral projection hemisphere, demonstrating consistent caudal spread of callosal projections in the Cited2 cKO neocortex. N = 3 WT, 3 cKO. ANOVA analysis finds no change in the anterior tail (−1400 to −800 μm) or center (−700 to 700 μm) regions, but the posterior tail (800–1400 μm) of the cKO distribution is significantly different from that of the WT (p < 0.0001). Error bars denote SEM. *p < 0.05, (Student’s t test) in C "p < 0.0011 (Bonferroni post-test) in K. Scale bars: A–B’, 1.5 mm; C, D, F, G, I, J, 1 mm; G’, J’, 500 μm.

Figure 8. Interhemispheric CPN axonal connectivity is disrupted in the adult Cited2 cKO neocortex. A, B, HARDI tractography analysis of interhemispheric connections demonstrates a significant reduction in the size of the CC and in the number of callosal fibers in the juvenile/youth adult (9 weeks old) Cited2; Emx1 cre cKO neocortex, compared with littermate controls, particularly within the mid-CC (corresponding to the somatosensory cortex). Reconstructed pathways are superimposed on the mean diffusion-weighted MRI of the brain. Pathways running between right and left are red; dorsal and ventral are green; and anterior and posterior are blue. The images do not include anterior commissure or olfactory bulb fibers; these were removed a priori to focus diffusion-weighted MRI of the brain. Pathways running between right and left are red; dorsal and ventral are green; and anterior and posterior are blue. The images do not include anterior commissure or olfactory bulb fibers; these were removed a priori to focus on the CC. Images in A’ and B’ similarly exclude hippocampal commissure fibers. N = 2 WT, 2 cKO. C, D, Staining for MBP in sagittal sections of Cited2 WT and cKO brains (C) followed by measurement of midsagittal CC area (D) identifies a reduction in CC area in Cited2 cKO brains, but demonstrates the structural integrity of the CC throughout all areas. N = 3 WT, 4 cKO. E–K, To investigate precision of callosal projections in the absence of Cited2 function, a focal injection of the anterograde tracer AAV-GFP was made in
CITED2 regulates death of cortical neurons in vitro after induced DNA damage (Gonzalez et al., 2008).

In addition to increased apoptosis, there is a significant reduction in the number of proliferating IPCs at E15.5, and cell-autonomous Cited2 loss of function results in reduced cell cycle re-entry between E14.5 and E16.5, suggesting that Cited2 function is required to maintain and expand these transit-amplifying progenitors. In line with this interpretation, Cited2 is highly upregulated by actively proliferating transit-amplifying progenitors during induced regeneration in the olfactory epithelium (Shetty et al., 2005). Further, CITED2 controls proliferation in fibroblasts (Kranz et al., 2003), hematopoietic stem cells (Du and Yang, 2013), and nonsmall-cell lung cancer cells (Chou et al., 2012). Together, the reduction in TBR2+ IPCs in E15.5 Cited2 cKO SVZ is likely due, at least in large part, to reduced proliferation within this progenitor population, in addition to the identified apoptosis increase.

**Cited2 functions in concert with distinct transcriptional regulators**

Transcription factors that play roles in both areal and laminar identity have been identified. For example, TBR1 functions in postmitotic neurons to regulate the appropriate differentiation of layer VI broadly, as well as the establishment of frontal cortex identity (Hevner et al., 2001; Bedogni et al., 2010), and PAX6 functions in progenitor cells to both regulate neurogenesis and promote rostral identity (Bishop et al., 2002; Schuurmans et al., 2004). The dual functions of Cited2 in CPN development appear to be quite different from these previously described mechanisms, however. Cited2 functions broadly in SVZ progenitors to regulate the generation of (primarily) layer II/III neurons; Cited2 also has a progenitor function leading to the acquisition of appropriate areal identity of a subpopulation of superficial layer CPN. Unlike areal identity genes, such as Pax6, Bhlhb5, and Tbr1 (Joshi et al., 2008; Bedogni et al., 2010), loss of Cited2 function does not disrupt establishment of areal identity, per se. This is demonstrated by the apparently normal development of the barrel field, the hallmark of the primary somatosensory cortex, in the context of an areal disruption in somatosensory layer II/III. These results strongly suggest that CITED2 functions as part of a complex network of transcriptional coregulators that interact, compete, and compensate to regulate and refine appropriate acquisition of areal identity within a particular projection neuron subpopulation, layer II/III CPN of the somatosensory cortex.

We hypothesized that the transcriptional coregulator LMO4 might function as part of this network, regulating the acquisition of areal identity of layer II/III CPN of the motor cortex. We found that additional removal of Lmo4 from the Cited2 cKO neocortex results in a balanced reduction of molecularly defined motor and somatosensory areas within layer II/III, compared with the specific reduction in layer II/III somatosensory area with loss of Cited2 alone, and an increase in layer II/III somatosensory area with loss of Lmo4 alone. Loss of Cited2 and Lmo4 function, alone and in combination, does not result in complete loss of areal identity; rather, areal boundaries shift, highlighting that these transcriptional coregulators provide a mutually dependent and partially antagonistic level of precise regulation of neuronal subtype-specific areal identity acquisition.

**Precise connectivity of somatosensory CPN**

Laminar composition and circuit organization is distinct within the primary somatosensory neocortex compared with other neocortical areas (Polleux et al., 2001; Rash and Grove, 2006; Dehay and Kennedy, 2007; O’Leary et al., 2007). The unique misalignment of the layer II/III somatosensory neocortex relative to the layer IV barrel cortex in the Cited2 cKO neocortex likely profoundly perturbs this precise circuitry, as evidenced, in part, by the disrupted dendritic complexity and precise axonal connectivity of Cited2 cKO CPN. The dendritic complexity of layer II/III CPN is specific to each neocortical area in mice, with dendritic arbors becoming progressively more complex from caudal to rostral areas (Benavides-Piccione et al., 2006). CPN of the Cited2-null somatosensory cortex display increased dendritic complexity that highly resembles that normally found in CPN of the motor cortex. This is despite the fact that CPN from the motor/somatosensory border regions, where molecular boundaries shift during development, were excluded from this analysis. Layer II/III CPN within the molecularly defined somatosensory cortex (i.e., BHLHB5+/LMO4−) of Cited2 cKO mice develop dendritic morphology of CPN of a normal motor cortex, indicating critical roles for Cited2 in somatosensory CPN afferent connectivity.

Within the young adult Cited2 cKO neocortex, CPN projections from the disrupted motor/somatosensory region are also quite imprecise, demonstrating a bimodal distribution of projections rostrocaudally within the somatosensory cortex. Further, HARDI identifies reduced callosal connectivity, particularly in the midcallosum, correlating with the somatosensory cortex. Normally, ectopic CPN projections are eliminated through activity-dependent mechanisms over the first postnatal weeks (Innocenti and Price, 2005; Luo and O’Leary, 2005; Mizuno et al., 2007; Wang et al., 2007; Zhou et al., 2013). The atypical projections identified in the adult Cited2 cKO neocortex might be aberrantly maintained, and might indicate earlier disrupted neuronal activity of Cited2-null CPN during somatosensory cortex development. It has recently been shown that balanced thalamic input regulates targeting of callosal projections in the somatosensory cortex (Suárez et al., 2014). Even though postmitotic loss of Cited2 does not disrupt the metrics assessed here in somatosensory CPN, it would be of interest in future studies to assess whether Cited2 might be induced or maintained in postmitotic somatosensory CPN in response to such balanced activity, leading to specific acquisition of other somatosensory CPN features.

In humans, even subtle disruptions in callosal connectivity are associated with defects in abstract reasoning, problem solving, and generalization (Paul et al., 2007), as well as with multiple neurodevelopmental disorders, including autism spectrum disorders (ASDs; Egaas et al., 1995; Piven et al., 1997; Herbert and Kenet, 2007; Frazier and Hardan, 2009; Hardan et al., 2009), attention deficit hyperactivity disorder (Hynd et al., 1991; Roessner et al., 2004; Seidman et al., 2005), Tourette’s syndrome (Plessen et al., 2006), and schizophrenia (Swayze et al., 1990; Tibbo et al., 1998; Innocenti et al., 2003; Wolf et al., 2008). Further, perturbed dendritic complexity of layer II/III CPN is observed in multiple neurodevelopmental disorders, including Rett syndrome (Armstrong et al., 1995; Kishi and Macklis, 2004), ASD (Mukaetova-Ladinska et al., 2004; Srivastava et al., 2012), and schizophrenia (Broadbelt et al., 2002). Greater understanding of molecular regulation of precise temporal and area-specific development of diverse CPN subpopulations might elucidate perturbations underlying such complex neurodevelopmental disorders.

**Conclusions**

Together, our results demonstrate that Cited2 functions differently from previously described mechanisms to regulate two
stages of precise CPN development, acting in neocortical progenitors to both broadly regulate generation of superficial layer CPN throughout the neocortex, and in an areally restricted manner to refine the distinct identity and precise connectivity of somatotopic CPN. This novel biology of Cited2 adds yet another layer of complexity to the multistage control and regulation of neocortical development.

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