Arx Expression Suppresses Ventralization of the Developing Dorsal Forebrain

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Early brain development requires a tight orchestration between neural tube patterning and growth. How pattern formation and brain growth are coordinated is incompletely understood. Previously we showed that aristaless-related homeobox (ARX), a paired-like transcription factor, regulates cortical progenitor pool expansion by repressing an inhibitor of cell cycle progression. Here we show that ARX participates in establishing dorsoventral identity in the mouse forebrain. In Arx mutant mice, ventral genes, including Olig2, are ectopically expressed dorsally. Furthermore, Gli1 is upregulated, suggesting an ectopic activation of SHH signaling. We show that the ectopic Olig2 expression can be repressed by blocking SHH signaling, implicating a role for SHH signaling in Olig2 induction. We further demonstrate that the ectopic Olig2 accounts for the reduced Pax6 and Tbr2 expression, both dorsal specific genes essential for cortical progenitor cell proliferation. These data suggest a link between the control of dorsoventral identity of progenitor cells and the control of their proliferation. In summary, our data demonstrate that ARX functions in a gene regulatory network integrating normal forebrain patterning and growth, providing important insight into how mutations in ARX can disrupt multiple aspects of brain development and thus generate a wide spectrum of neurodevelopmental phenotypes observed in human patients.

The establishment of dorsoventral (DV) identity in the developing neural tube enables the formation of separable progenitor zones and ultimately the generation of distinct neural subtypes. For example, dorsal forebrain progenitors produce excitatory (glutamatergic) projection neurons that make up approximately 80% of the neurons in the mature cerebral cortex. In contrast, inhibitory interneurons, which use γ-aminobutyric acid (GABA) as a neurotransmitter, originate from the ganglionic eminences (GE) in the ventral forebrain and migrate dorsally to the cerebral cortex, making up approximately 20% of cortical neurons. In addition to establishing the DV axis, the neural tube also undergoes substantial expansion of progenitor populations, a function that ultimately contributes to forebrain size. Interestingly, multiple genes involved in early DV patterning also play important roles in the control of brain size.

ARX is a vertebrate homologue of Drosophila aristaless (Al), a paired-like homeodomain transcription factor (TF). Mutations in al result in pattern disruptions in a subset of appendages of the adult fly. The affected appendages show reduced size, which led to the speculation that al may also be a ‘region specific growth control gene’. In fact, it has been shown that al is required for the growth and differentiation of the tip of the developing leg. In developing mice, ARX is expressed in the progenitor cells located both in the ventricular zone (VZ) of the embryonic cortex (dorsal forebrain) and in the subventricular zone (SVZ) of the GE (ventral forebrain). In the GE, its expression is maintained even after the cells undergo migration and differentiation, while its dorsal expression is restricted to progenitor cells. Patients with mutations in ARX present with intellectual disability and epilepsy, with or without structural defects in the brain such as lissencephaly (smooth brain), microcephaly (small brain), and agenesis of the corpus callosum, as well as abnormal genitalia. These human phenotypes have largely been recapitulated in genetic mouse models, supporting a direct role of ARX mutations in the pathogenesis of this wide spectrum of phenotypes.
Using a dorsal forebrain specific Arx mutant male mice (Arxflox/y; Emx1cre) (ARX/Arx is on the X-chromosome), we have previously shown that ARX modulates cortical progenitor proliferation and neurogenesis by directly repressing the expression of Cdkn1c (Kip2), a cell-cycle inhibitor gene. Progenitor cells deficient for Arx prematurely exit the cell cycle, resulting in depletion of the proliferating progenitor cell pool and a reduction in upper layer neurons. This has been postulated as the mechanism for the reduced brain size (microcephaly) reported in mice as well as in patients.

In the present study, we show that the loss of Arx from the dorsal forebrain results in DV gene expression defects. A subset of predominantly ventral genes, including Olig2, are aberrantly overexpressed in the dorsal forebrain. Olig2 is known as a ‘multifaceted TF’ that promotes neuronal and oligodendrocyte fates, and directs both differentiation and proliferation based on spatial and temporal dependent expression. Our data reveal that the aberrant induction of Olig2 leads to a reduction in Pax6 and Tbr2, both dorsally restricted TFs crucial for proliferation and/or differentiation of the cortical progenitor cells. Our findings further indicate that ARX can regulate the specification of cortical progenitors by suppressing ventral identity while promoting dorsal identity.

Taken together, we propose that ARX coordinates telencephalic patterning and forebrain size by regulating DV gene expression, including the suppression of dorsal Olig2, which modulates the expression of genes including Pax6 and Tbr2, ultimately influencing forebrain patterning and growth.

Results

Olig2 is ectopically expressed in ARX-deficient dorsal forebrain progenitors. We previously identified 83 differentially expressed genes in the Arx−/− cerebral cortex by microarray analysis (embryonic day 14.5, E14.5) and validated a subset by reverse transcription-quantitative real time PCR (RT-qPCR). Among the validated genes, Olig2 showed the highest upregulation. To confirm this finding, we compared Olig2 immunostaining from wild type (WT) (Arx+/+; Emx1fl/fl, also referred to as Arx+/y) and Arx cKO (Arxflox/y; Emx1cre, also referred to as ArxKO/y) embryonic brain sections. In WT mice, Olig2 expression was strongly detected in the ventral forebrain (GE) at E11.5-16.5 (Fig. 1 and Supplementary Fig. S1a). Given that CRE-mediated recombination only occurs in the dorsal but not in the ventral forebrain, at the Arx locus in Arxflox/y; Emx1cre mice, our results indicate that the loss of Arx in the dorsal forebrain leads to a dramatic increase in Olig2 positive cells in the dorsal forebrain. Interestingly, this abnormal Olig2 expression exhibits a strong anterior-high to posterior-low gradient (Fig. 1), and variable levels in different cells (compare three arrows in Fig. 1 inset).
We next sought to establish if the OLIG2 positive cells result from ectopic expression of Olig2 in the dorsal forebrain, or from increased migration of ventrally derived OLIG2 positive cells. To distinguish these possibilities, we electroporated a GFP expression construct in utero to the cortical ventricular zone (VZ) of the Arx cKO/y brains to mark dorsally positioned progenitor cells. We found that OLIG2 positive cells were also labeled with GFP, ensuring that these cells originated from the dorsal forebrain and not from the ventral GE (Supplementary Fig. S1b). Together these data indicate that the cortical progenitor cells in the dorsal forebrain of the Arx cKO mice abnormally overexpress OLIG2, which is normally repressed by ARX.

**ARX represses Olig2 expression.** To determine if the abnormal OLIG2 expression is a result of cell autonomous or non-autonomous function of ARX, we used Arx Het female (ArxHet) double immunolabeled with Olig2 and ARX antibodies (a’-a”; magnified images of the boxed area in left panel). ARX+ cells are Olig2−, and Olig2+ cells are Arx−. Dotted lines mark the ventricular surface. Longer brackets indicate example areas with high number of ARX+ cells, while shorter brackets denote areas with high number of Olig2+ cells. (b) Representative images of the immunofluorescent labeling (GFP and Olig2) of the ArxKO/y cortex electroporated with either pCIG (encoding GFP only) or pCIG-Arx (encoding ARX-IRES-GFP) (EP at E13.5 and harvested at E14.5). GFP antibody was used to label electroporated (EPed) cells (green, cytoplasmic staining pattern). Right most images are magnified images of the boxed areas. Long arrows indicate examples of GFP electroporated cells expressing Olig2 and short arrows indicate examples of ARX electroporated cells not expressing Olig2. (c) Quantification of results in B. The ratio of Olig2+ GFP+ cells over GFP+ cells was plotted for both GFP (pCIG) and Arx (pCIG-Arx) electroporated samples. Error bars: mean ± s.d (n = 3 for GFP EP and n = 4 for Arx EP; ***P = 0.0008; unpaired t-test).

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expression. Interestingly, we noted this repression was not observed in a subset of cells outside of the VZ (see marked area with * in Fig. 2b). One explanation for this observation is that ARX suppresses OLIG2 only in VZ progenitor cells but not cells that have exited the VZ.

Arx cKO dorsal forebrain is partially ventralized. Given that Olig2 normally shows strong expression in the developing ventral neural tube, we asked if the Arx cKO dorsal forebrain is abnormally ventralized. We closely re-examined the changes of the ventral genes in our previous Arx−/− cortex microarray data (E14.5) and noticed a subset of upregulated ventral genes in the cortex (Supplementary Fig. S2a). Using RT-qPCR, we confirmed an up-regulation of a subset of the ventral genes (Dlx2, Dlx5, Ascl1(Mash1), Pbx3 and Otx2) in the Arx cKO cortex at E12.5 (Fig. 3a). Unlike Olig2 that remained elevated (although at lower levels), these other genes no longer showed significant changes by E14.5 when compared to WT (Fig. 3b), suggesting the role for ARX in suppressing ventral gene expression is temporally restricted. We also found that Dbx1, whose expression is normally
restricted to the pallial-subpallial boundary (PSB)\textsuperscript{27}, is also ectopically expressed in the dorsal forebrain (Fig. 3d), further supporting an abnormal ventralization of the dorsal forebrain. Curiously, DLX2 protein, a ventral marker requiring high SHH activity for its induction\textsuperscript{28}, was not detected despite its mRNA elevation (data not shown). Finally, we found small decreases in the level of expression for a subset of dorsal genes in the cortical progenitors including Pax6, Tbr2, Lhx2, Emx1/2, NeuroG2, and Dmrta1, when we re-examined our previous Arx\textsuperscript{−/−} cortex microarray data (E14.5)\textsuperscript{17} (Supplementary Fig. S2b). Reduction in PAX6 and TBR2 expression in protein level have been confirmed in our previous study\textsuperscript{17}. Together, these data suggest dorsal progenitor cells are partially ventralized with a mixed identity.

Activation of the SHH signaling pathway participates in the ectopic induction of Olig2. During embryonic development, SHH is known to induce ventral gene expression in the ventral neural tube\textsuperscript{29}. Olig2 is one of the genes induced at a low concentration of SHH\textsuperscript{30}. Given the up-regulation of ventral genes including Olig2, we examined if SHH signaling was activated in the dorsal forebrain of Arx\textsuperscript{cKO} mice. No Shh transcript was detected in the cortex of either WT or Arx\textsuperscript{cKO} mice (E13.5), while its transcript was detected ventrally in both mice (Supplementary Fig. S2c), consistent with our previous microarray data\textsuperscript{17}. Interestingly, Gli1, a SHH downstream target, was upregulated in the cortex of Arx\textsuperscript{cKO} mice (Fig. 3c–i and Supplementary Fig. S3). Furthermore, both RT-qPCR and microarray assays demonstrate upregulation of PtcH1 as well as Gli1 in Arx mutant cortices, both SHH targets (Supplementary Fig. S3). Together these results suggest a possible upregulation of SHH signaling pathway, without changes in Shh transcript level itself, in Arx mutant cortices.

To determine if SHH signaling is required for the abnormal Olig2 induction in the Arx\textsuperscript{cKO} cortex, we electroporated Gli3R (a Gli3 repressor construct to block SHH signaling)\textsuperscript{31} and analyzed OLIG2 expression. When Gli3R was electroporated to the Arx\textsuperscript{cKO} cortex in utero, the ectopic OLIG2 overexpression was reduced (Fig. 4a,b), whereas a GFP expression construct did not change OLIG2 expression (Fig. 4a,b). These results support a role for the SHH pathway in the abnormal induction of Olig2 and that blocking SHH signal is critical for normal cortical development.

OLIG2 represses Pax6 and Tbr2 expression. We previously showed reduction in PAX6 as well as TBR2 expressing cells in cortical progenitors of Arx\textsuperscript{cKO} mice\textsuperscript{17}. A loss of PAX6 or TBR2 has been associated with a precocious cell cycle exit of intermediate progenitor cells (IPCs) and a disproportionate reduction in later-born,
upper layer neurons, likely contributing to the small brains observed in mice mutant for these two genes\(^{32,33}\). These same phenotypes are observed in Arx\(^{cKO}\) mice\(^{17}\). Interestingly, a transgenic mouse line with cortical Olig2 overexpression also shares many findings with these mice\(^{34}\). These data suggest a common pathway involving Arx, Olig2, Pax6 and Tbr2 that results in proliferation/neurogenesis defects and small brains. During spinal cord development, it has been inferred that Olig2 represses Pax6 expression, although this was not directly tested\(^{35}\). Moreover, Pax6 is known to directly regulate Tbr2 expression in the developing cortical IPCs\(^{32}\). We thus hypothesized that ectopically induced Olig2 suppresses Pax6 transcription and consequently Tbr2, and this suppression likely accounts for the reduction in Pax6 positive as well as Tbr2 positive staining in Arx\(^{cKO}\) mice\(^{17}\).

To test this hypothesis, we took several approaches. First, we used Arx Het (Arx\(^{cKO/+}\)) mice where WT and mutant cell columns, by virtue of random X-chromosome inactivation, are clonally distributed in the cortical VZ\(^{25,26}\). Pax6 expression levels were assayed in Olig2 positive vs Olig2 negative cells, taking advantage of the fact that Olig2 positive cell columns (ARX negative) are clonally distributed adjacent to Olig2 negative cell columns (ARX positive) in the VZ of the dorsal forebrain (see Fig. 5a). Lower levels of Pax6 were detected in Olig2 positive cells (29.90 ± 1.301, n = 153) when compared to the adjacent Olig2 negative cells (49.51 ± 1.945, n = 59) (Fig. 5a), supporting a repressive role of Olig2 in Pax6 expression.

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**Figure 5.** Olig2 expressing cells have lower level of Pax6 and Tbr2. (a) Representative images of Pax6 and Olig2 double immunolabeling of Arx Het (Arx\(^{cKO/+}\)) forebrain (E14.5). Longer brackets indicate areas with low Pax6 and high ARX, while shorter brackets mark areas with high Pax6 and no ARX. The quantification of Pax6 intensity in Olig2+ or Olig2- cells is shown in the graph. Error bars: mean ± s.e.m (n = 59 cells for WT; n = 153 cells for Arx\(^{cKO/+}\); ****P < 0.0001; unpaired t-test). (b) Representative images of Pax6 and Olig2 double immunolabeling, or Tbr2 immunolabeling of control or R26SmoM2; Emx1\(^{Cre}\) embryonic cortex (E14.5).
Second, we examined PAX6 and TBR2 expression in control and R26SmoM2; Emx1cKO mice (Fig. 5b) which have abnormal OLIG2 induction in the dorsal forebrain due to constitutively activated SHH signaling46. OLIG2 positive cells in the VZ of the dorsal forebrain show little or no PAX6 expression and PAX6 positive cells are located where OLIG2 is not present (Fig. 5b), also suggesting repression of PAX6 expression by OLIG2. We also observed almost complete loss of TBR2 expression in the dorsal VZ of R26SmoM2; Emx1cKO mice (Fig. 5b) when compared to controls (Fig. 5b).

Third, we tested if forced expression of Olig2 can repress PAX6 and TBR2. Electroporation of a GFP-tagged empty vector (pCIG) or GFP-tagged Olig2 expression construct (pCIG-Olig2) into the cortex of WT mice (E13.5) showed that both PAX6 (Fig. 6b) and TBR2 (Fig. 6a) expression were reduced in Olig2 electroporated cortices (PAX6: 0.68 ± 0.039, n = 5)(TBR2: 0.11 ± 0.009, n = 3)(harvested at E14.5) compared to control electroporated cortices (PAX6: 1.00 ± 0.000, n = 3)(TBR2: 0.83 ± 0.028, n = 3), although the reduction of TBR2 was greater than that observed with PAX6 (Fig. 6c,d). Given that PAX6 has been shown to positively regulate Tbr2 expression32, we postulate that the TBR2 reduction could be explained by reduced PAX6 expression.

Finally, we investigated if OLIG2 can directly regulate PAX6 expression at the level of transcription. For this, we first performed a ChiP-seq assay using an OLIG2 antibody and E14.5 embryonic forebrain. Our OLIG2-ChiP-seq data identified putative OLIG2 binding sites in the upstream PAX6 genomic sequence (Fig. 7a), which are consistent with previously identified sequences in the spinal cord and neural progenitor cells from embryonic mouse stem cells (ref.33; publicly available data, see methods). Next, to validate these binding sites, we performed two independent experiments; ChiP-qPCR and reporter gene assays. For ChiP-qPCR, ChiP was conducted with an OLIG2 antibody as well as IgG, followed by qPCR with the primer sets, OligPax6, for the PAX6 genomic sequence identified in OLIG2 ChiP-seq (chr2:105515511-105515700 on mm9) as well as nCPax6, for the PAX6 genomic sequence distal to the identified sequence as a negative control (Fig. 7b). A dramatic enrichment of the putative OLIG2 binding sites were detected when compared to negative control sequences, when OLIG2-ChiPed DNAs were used (3,323 ± 198, n = 3 vs 15.96 ± 0.504, n = 3). These data validate our ChiP-seq identified sequences (Fig. 7b). Next, a Pax6-Luc reporter construct was generated, which contains PAX6 genomic sequence (Pax6ncPax6 including ChiP-seq-identified PAX6 sequence) as an upstream promoter driving luciferase expression, in addition to the herpes virus thymidine kinase minimal promoter (Fig. 7a). Reporter gene assays were conducted using Pax6-Luc construct as well as Shox2-Luc construct, negative control, which contains Shox2a promoter sequence instead (Shox2a is an ARX target; ref.38) (Fig. 7c). Upon co-transfection, an OLIG2 expression construct (pCIG-Olig2) significantly down-regulated Pax6-Luc reporter activity when compared to a control construct (pCIG) (45,160 ± 19,007, n = 4 vs 276,615 ± 34,939, n = 4), demonstrating that the ChiP-identified PAX6 genomic sequences act as a transcription regulatory element responsive to OLIG2. In contrast, an OLIG2 expression construct did not change Shox2-Luc reporter activity (49,815 ± 14,009, n = 4 vs 46,490 ± 10,874, n = 4) (Fig. 7c), supporting OLIG2-mediated repression being specific to the PAX6 promoter. These results strongly suggest that OLIG2 can repress PAX6 expression at the level of transcription. In contrast, we did not find Tbr2 regulatory sequences in our OLIG2-ChiP-seq data, implying that Tbr2 transcription may not be directly regulated by OLIG2.

Collectively, our data presented here provide evidence to support our postulate that the ectopic induction of Olig2 in Arx cKO forebrain represses PAX6, which leads to a reduction in Tbr2 expression, likely contributing to the microcephaly phenotype observed in these mice and patients with ARX mutations21–24. These findings support a model wherein OLIG2 expression is actively repressed by ARX during normal development in the dorsal forebrain, permitting PAX6 and TBR2 expression. Therefore, our data implicate a role for ARX in modulating brain size through regulating Olig2-Pax6-Tbr2 pathway. Previously we have shown another mechanism whereby ARX participates in brain size control; ARX regulates Cdkn1c transcription and the upregulation of Cdkn1c in Arx cKO mice, which results in premature cell cycle exit of progenitors, is likely cause of decrease in IPC population, eventually small brain. Thus, we tested whether OLIG2 overexpression would influence Cdkn1c expression. Our data demonstrate that upon Olig2 overexpression in WT mice, Cdkn1c expression does not change (Supplementary Fig. S4). These data suggest that these two pathways do not converge but act in parallel. Taken together, our data indicate that ARX participates in cortical size control through at least two different mechanisms: by regulating Olig2-Pax6-Tbr2 pathway and/or by repressing Cdkn1c transcription.

Discussion

Disorders of brain development commonly include multiple and complex phenotypes that cannot be explained by perturbation in a single process (e.g. cell proliferation). Brain abnormalities associated with ARX mutations are an excellent example; patients with ARX mutations show brain size and structure anomalies, which suggest disruptions in more than one process. Our data provide insight into how mutations in one gene, ARX, can disrupt both brain growth and patterning. In mice where Arx has been conditionally abrogated from the cerebral cortex, we found ventral genes, such as Olig2, are abnormally expressed dorsally. Furthermore, the ectopically expressed OLIG2 represses dorsal specific PAX6 and TBR2 expression, both important for cortical progenitor proliferation32,33. Thus, our current findings together with our previous work37 identify ARX as a critical transcription factor impacting DV specification as well as proliferation of cortical progenitor cells.

OLIG2 is a basic helix-loop-helix (bHLH) transcription factor that is known to have essential roles in cell fate specification and cell proliferation31–34. During early spinal cord development, OLIG2 first specifies motor neuron precursors and then promotes their cell cycle exit and neuronal differentiation39,40. Later in spinal cord development, OLIG2 directs the formation of oligodendrocyte precursors and mature oligodendrocytes41,42. In the developing forebrain, OLIG2 is expressed in progenitor cells of the GE (with the highest levels of expression in the MGE domain) that give rise to subtypes of cortical interneurons as well as oligodendrocytes43,44. We found no detectable changes in the number of oligodendrocytes as well as no detectable changes in cell death in the Arx cKO brain (data not shown). These data demonstrate that the enhanced OLIG2 expression does not contribute
Figure 6. Forced expression of Olig2 represses Pax6 and Tbr2 expression. (a,b) Representative images of the immunofluorescent labeling of the WT embryonic cortex electroporated with a control GFP (pCIG) or Olig2 expression construct (pCIG-Olig2) (E13.5 → E14.5). Antibodies against GFP (green) and TBR2 (red) (a) or PAX6 (red) (b) were used. The number of PAX6+ and TBR2+ cells are reduced in the Olig2 electroporated cortices compared to the control. (c,d) Quantification of results in a (c) and b (d). The ratio of TBR2+ GFP+ or PAX6+ GFP+ cells over GFP+ cells was plotted for GFP or Olig2 electroporated brains. Error bars: mean ± s.d (For Tbr2, n = 3 for each sample; ***P = 0.0007; For Pax6, n = 3 for GFP; n = 5 for Olig2; **P = 0.0012; unpaired t-test).
to the generation of additional cells of the oligodendrocyte lineage in the mature brain. A recent study in an Olig2 transgenic mouse showed OLIG2 overexpression inhibits cortical progenitor proliferation and neurogenesis, leading to a severe reduction in brain size and a disruption in cortical lamination. These brain phenotypes are similar to what we observe in Arx cKO mice which also overexpresses Olig2. Given that OLIG2 maps to the Down’s syndrome critical regions on chromosome 21, these data support a provocative model for a potential role of OLIG2 in the developmental brain defects associated with Down syndrome such as intellectual disability. In this syndrome OLIG2 is triplicated and over-expressed, suggesting a common pathway resulting in intellectual disabilities as well as microcephaly in patients with Trisomy 21 and ARX mutations.

During development Olig2 is known to be induced by low levels of SHH signaling in the ventral spinal cord and brain. Our Gli1 RNA in situ hybridization data support that SHH signaling activity might be present, although weak, during normal cortical development. Another support for this comes from our observation of a weak OLIG2 expression in dorsal forebrain progenitor cells, prior to the arrival of migrating OLIG2 positive cells from the GE, which has not been previously reported (Fig. 1). With the loss of ARX, both Gli1 and Olig2 expressions become strongly activated, suggesting an elevated SHH signaling activity. Although the role of SHH in ventral specification of the forebrain is well established, the role of SHH signaling in cerebral cortical development is less clear, as the source of SHH protein is uncertain. There are at least two potential SHH sources for cortical development; the cerebrospinal fluid (CSF) and the developing cortex itself. SHH levels peak in the CSF at E10.5 and fall to low levels by E14.5. Our RNA in situ hybridization data suggest that Shh transcript is not present or in very low levels (undetectable) in the developing cortex (E14.5) (Supplementary Fig. S2).
Moreover, our data provide indirect support for CSF being a SHH source that influences cortical development, as the overexpression of ventral markers in Arx cKO coincides with this timing of SHH exposure; their upregulation was observed at E12.5 but not E14.5, except for Olig2 which was still detected at E14.5 but at lower levels, consistent with the known inducibility of Olig2 even at low levels of SHH signaling56. One possible role for ARX in the dorsal forebrain is to suppress Olig2 induction either by directly repressing Olig2 transcription or by indirectly repressing SHH signaling that can induce Olig2, or a combination of the two. Since we did not find Olig2 regulatory sequences in previously published ARX ChIP-on-ChIP data51, ARX appears not to regulate Olig2 transcription directly. However, we cannot rule out the possibility that ARX could bind to the Olig2 enhancer region and regulate its transcription from a distant location, since this study only interrogated the promoter region. Further studies are required to elucidate the specific mechanism by which ARX suppresses Olig2 induction during normal cortical development.

The enhanced expression of Olig2 at an early stage of neurogenesis in the Arx cKO likely has a significant impact on neurogenesis. In addition to the Cdkn1c overexpression as we previously reported57, reduced Pax6 and TBR2 expression likely accounts for some of the neurogenesis defects reported in the Arx mutant mice and the associated microcephaly57. Given that Cdkn1c expression does not change upon Olig2 electroporation, it appears that Cdkn1c upregulation in Arx cKO is independent from Olig2 overexpression. Pax6 is essential for controlling the balance between neural stem cell self-renewal and neurogenesis58,59. Altering the levels of Pax6, either up or down, leads to a small brain through different mechanisms: increasing Pax6 levels drives the system towards neurogenesis, while removing Pax6 reduces cortical stem cell self-renewal37. TBR2 is expressed in the IPCs and can direct conversion of RGs into IPCs53. Its loss in the developing forebrain results in the loss of IPCs53. Its expression is dependent on Olig23, and R26 SmoM2 (Stock No. 008831) mice from The Jackson Laboratory. The floxed Arx cKO (subsequently referred to as Arx cKO) mice were generated by mating Arx flox/y males to Emx1cre mice (Stock No. 005628) and R26 SmoM2 (Stock No. 008831) mice from The Jackson Laboratory. The floxed Arx (Arx flox/male) cKO mutant mice and TBR2 expression likely accounts for some of the neurogenesis defects reported in the Arx cKO mice.

In summary, the misregulation of Olig2 in the Arx cKO cortex and its relationship with Pax6 and TBR2 repression, link the control of cellular identity to the regulation of the cortical size. Our data also indicate ARX plays a critical role in coordinating dorsal progenitor cell proliferation and specification in the mammalian forebrain. Furthermore, our data implicate early patterning defects as components in the pathogenesis of the developmental anomalies and neurological phenotypes associated with ARX mutations in human patients.

Materials and Methods

Animals. All animal experiments were performed in accordance with the relevant guidelines and regulations approved by the Harvard Medical School Institutional Animal Care and Use Committee (protocol no. 04946), Brigham and Brown’s Hospital Institutional Animal Care and Use Committee (protocol no. 2016N000244). CD1 mice (Stock No. 022) were purchased from Charles River Laboratories, and the Emx1cre (Stock No. 005628) and R26 SmoM2 (Stock No. 008831) mice from The Jackson Laboratory. The floxed Arx (Arx flox/male) cKO mice were generated by mating Arx flox/y or Arx flox/female females to Emx1cre males, and R26SmoM2: Emx1cre mice were generated by mating R26 SmoM2 (homozygote) females to Emx1cre males. As Arx is an X-chromosome gene, we used only male conditional knock out (Arx flox/ male) and male wild type (Arx+/+ males) mice for consistent comparison unless noted otherwise.

In utero electroporation (IUEP). In utero electroporation (IUEP) was performed at embryonic day 12.5 (E12.5) or E13.5 as described previously54. One or two days after EP dams were sacrificed, and the brains were removed for analysis. For quantification, GFP+ electroporated cells were manually marked and counted as indicated.

DNA constructs. The pCAG-IREs-Gfp (pCIG) was used as a control for IUEP. The pCAG-Arx-IREs-Gfp (pCIG-Arx) was described previously57. To generate pCIG-Olig2, full length cDNAs encoding Olig2 (NM_016967) was derived by PCR from mouse cDNAs generated from the total RNA extracted from E12.5 brain lysates (see Table 1 for primer sequences). These PCR products were cloned into pCIG vector (EcoRI and MluI sites of pCIG-Arx) and replaced Arx cDNA sequences, using GeneArt Seamless Cloning and Assembly kit (Life Technologies). pCAG-Gli3R-ires-dsRed was generated by inserting PCR product containing 1–645 amino acid residues of GLI3 to EcoRI and MluI sites of pCAG-IREs-Gfp and by replacing GFP sequence with dsRed coding sequence. To generate the Pax6-Luc reporter construct, the 925 bp Pax6 genomic sequence (chr2:105515511-105516246) containing a putative Olig2 binding region identified in Olig2 ChIP-seq (chr2:105515511-105515700 on mm9), was PCR amplified from genomic DNA and cloned into BamHI and HindIII sites of the empty TK-Luc construct (MCS-TK-Luc) that carries the herpes virus thymidine kinase minimal promoter (−105/+51) and luciferase coding sequence. The Shox2-Luc reporter gene construct has been described previously58.

Immunohistochemistry (IHC), immunofluorescent (IF) labeling and quantification. Embryonic mouse brains were fixed overnight in 4% paraformaldehyde and processed for cyrosections (15 μm) as previously
described55. Control and experimental sections were collected on the same slide. IHC and IF labeling were performed using previously described protocols 55. Primary antibodies used in this study included rabbit monoclonal anti-OLIG2 (1:100, Abcam, ab109186), mouse monoclonal anti-OLIG2 (1:100, Millipore Sigma, MABN50), chicken polyclonal anti-GFP (1:500, Invitrogen, A10262), rabbit polyclonal anti-RFP (1:500, MBL International, PM005), rabbit polyclonal anti-ARX (1:100, Dr Kitamura), rabbit polyclonal anti-PAX6 (1:1000, Covance, PRB-278P), and rabbit polyclonal anti-TBR2 (1:200, Abcam AB15894 or a gift from Dr. Robert Hevner, Seattle Children’s Hospital). Appropriate secondary antibodies either biotinylated (1:500, Vector Lab) or conjugated with a fluorescent dye (Alexa-Fluor 488 or Alexa-Fluor 594; 1:200; Invitrogen) were used for IHC or IF, respectively. Tyramide amplification (Invitrogen) was used for mouse anti-OLIG2. AB reagents (Vector Laboratories) and 3,3′-Diaminobenzidine (DAB) (Vector Laboratories) were used as recommended by manufacturer to detect the signals in IHC. IF nuclear labeling was with 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes). Light microscopy images were captured on an Olympus BX43 microscope equipped with an Olympus DP26 camera using Cell Sens software, or Nikon eclipse E400 microscope with Leica DFC 420 camera using LAS AF Lite software (version 2.6.3).

Immunofluorescent images were captured on Zeiss Observer Z1 inverted microscope equipped with a Hamamatsu ORCA-Flash4.0 camera using Zeiss Zen Pro software. For some fluorescent images, multiple tiled images were taken at 20x and stitched using Zeiss Zen Pro software. When necessary, entire image level and brightness were adjusted with Adobe Photoshop CS5, or Image J (version 2.0.0). For PAX6 intensity quantification, DAPI staining was used to mark each cell as individual ROI, and the mean intensity of OLIG2 and PAX6 in each ROI was measured in Image J. OLIG2+ cells were called if the mean intensity was higher than background.

**mRNA in situ hybridization and quantification.** Embryonic mouse brains were fixed overnight in 4% paraformaldehdy and processed for cryosections (15 μm) as previously described55. Control and experimental sections were collected on the same slide. To detect mouse *Dbx1*, *Gli1*, and *Shh* mRNAs, RNAscope® 2.5 HD detection kit (brown) (ACDBio) was used following manufacturer’s recommendation. For quantification, four serial sections from each genotype were imaged (Nikon eclipse E400 microscope with Leica DFC 420 camera using LAS AF Lite software (version 2.6.3)) and *Gli1* positive granules within 200 μm wide area spanning the entire neural tube (from ventricular surface to pial surface) taken from the middle part of the section (see

| Subcloning | Forward primer (F’S to 3’) | Reverse primer (R’S to 3’) |
|------------|---------------------------|---------------------------|
| Gli3R      | F-CTACATTTTGGCAAAAGCAGCCATTGGAGGCCGCCAGGGCCCGACAGCTTACG | R-AGAAAGCCCTTGGCGAAGAGCTACTCCAGCGGCATGTGAAAC |
| Olig2      | F-TCCAGACCTCAAGCCTGACATTGGAGGCGATTGCCGAAAG | R-CAGATGAGGGCAGCGCTGAAAGG |
| **RT-qPCR**| Forward primer (F’S to 3’) | Reverse primer (R’S to 3’) |
| Olig2      | F-CAAATCTAATTCATTGCCGAAAGGTTG | R-AGGATTCAGCAGTCAGTCAG |
| Dlx2       | F-GTGCTCTACCTCGGCCCAAGACG | R-GGATTTTCAGGCAAGGTCCGG |
| Dlx5       | F-CAACTCTGAGAGGACATGCCGAC | R-GGCTTGCCCTGCAAGGGAGG |
| Lhx6       | F-CGTTTGAGGAGAAGGTCCTTGTCG | R-GCTTGGGCTGACTGTCCTTGTC |
| Ascl1      | F-CGGAACTAGTGCGCTGCAAAAGG | R-GGCAGAAACCAAGAGTGAGG |
| Nrt2f1     | F-CCAACAGGGAAGCTGCTGAGGTA | R-CGCTTGTAGTGTAGTATAG |
| Pbx3       | F-CGCGAGCCAAATGTGGCAGCACA | R-TTGGCGTCCTGCCAGCCTG |
| Otx2       | F-TGAGGGAAGAGGTGGCACG | R-GCCTCAGCTTTGCTGAGCCTG |
| Pch1       | F-GGATGCCCCATCCAGGCTG | R-CTGCGCTGACATGAGAGG |
| Gli1       | F-CTCAAAATCGCCAGTACACTA | R-TGGGGCTGACATGAGAGG |
| Gapdh      | F-ATCTCTTCTGTCAGTGACCCGACGGCTGCTGCCG | R-AGTGGAGGCTCAGAAAGGGGCTCTGATTTG |
| Cdkn1c     | F-AGCCTGAGGACGCGCTCTTC | R-AAGCTTGGTCAGCCCTGTT |
| **Chip-qPCR** | Forward primer (F’S to 3’) | Reverse primer (R’S to 3’) |
| ncPax6     | F-GAATTTGAGAATCCCAAGGGCAG | R-CGCTTGTAGTGTAGTATAG |
| ogPax6     | F-CCGCCAGACGCGAAAAAGAGG | R-TCGCTCTAGCAGGAAAAACACTTTCCT |

**Table 1.** Primer sequences used in this study.
Supplementary Fig. S3a–f for examples) were automatically counted using Image J (Images underwent thresholding using Otsu method, cut off value 243). Once quantification was completed, the slides were stained with diluted Hematoxylin for weak counterstaining and imaged again.

**Reverse transcription quantitative real time PCR (RT-qPCR).** Total RNA was isolated using the RNaseasy plus kit (Qiagen, CA, USA) from E12.5 or E14.5 neocortices. For Cdkn1c RT-qPCR, total RNA was isolated from E14.5 neocortices that were in utero electroporated with pCIG-Olig2 or pCIG at E13.5. cDNA was synthesized by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, using random hexamers) according to the manufacturer's instructions. RT-qPCR was performed in StepOne Plus Real Time PCR System (AB applied biosystems) using SYBR green PCR master mix (AB applied biosystems) in 20 μl reaction volume in triplicate. The reaction was done as following: 95°C for 10 min, 95°C for 15 sec, and 60°C for 1 min, 40 cycles. Primers used are listed in Table 1. Cycle threshold (CT) values were normalized by glyceraldehyde-3-phosphate dehydrogenase (Gapdh).

**Chromatin immunoprecipitation-sequencing (ChIP-seq) procedure.** Embryonic dorsal forebrains (E14.5) were triturated in Hank's Balanced Salt Solution (Thermo Fisher Scientific) and fixed in 1% fresh paraformaldehyde in PBS for 5 min at room temperature. Fixation was quenched with 125 mM Glycine at room temperature for 5 min and washed with cold PBS twice. Chromatin immunoprecipitation was performed using MAgNiFY Chromatin immunoprecipitation system (Thermo Fisher Scientific) with minor modifications as follows. Chromatin was fragmented into 100–300 bp by setting the Bioruptor UCD-200 (Diagenode) to high power and sonicated for 3 rounds of 10 cycles (30 sec ON/30 sec OFF). For immunoprecipitation of Olig2-bound chromatin, 2 μg of anti-Olig2 antibody [EPR2673] (Abcam, ab109186) was incubated with cleared chromatin lysate and 2 μg of whole rabbit IgG was used as a control. Input and ChIPed DNA libraries were prepared using an Illumina Next Seq (single-end reads of 75 bp).

**ChIP-seq data analysis.** FASTQ sequences were aligned to the mouse mm9 genome sequence using HISAT2 and converted to SAM and then BAM files. Then, ChIP-seq peaks were called using MACS2, input DNA without ChIP as reference, and with the default settings. External data (GSE103324 and GSE74646) were analyzed by the same settings previously used. The HISAT2-aligned peaks and MACS-determined peak positions were visualized using WASHU Epi Genome Browser.

**Chromatin immunoprecipitation-quantitative real time PCR (ChIP-qPCR).** Control IgG- and OLIG2-Chiped DNA libraries were prepared as described above in ChIP-seq procedure and used for qPCR. StepOne Plus Real Time PCR System (AB applied biosystems) using SYBR green PCR master mix (AB applied biosystems) in 20 μl reaction volume in triplicate was used for qPCR. Following the same procedure described above in the RT-qPCR methods, qPCR was performed with a negative control primer pair (cPax6F1 and cPax6R1) whose sequences are from the Pax6 genomic region (chr2:105512527-105512827) not associated with OLIG2 binding, and an experimental pair (oPax6F1 and oPax6R1) whose sequences are from the Pax6 genomic region (chr2:10551551-105515700 on mm9) identified in our OLIG2-Chip-seq as well as previously published or publicly available OLIG2-Chip-seq data (GSE103324 and GSE74646). All reactions were performed in triplicate. Cycle threshold (CT) values were normalized to IgG control.

**Reporter gene assay.** Pax6-Luc or Shox2-Luc was co-transfected into HEK293T cells with pCIG or pCIG-Olig2. Twenty-four hours later luciferase activity was measured with a POLARStar Omega microplate reader (BMG LABTECH, Ortenberg, Germany) as previously described.

**Experimental Design and Statistical Analysis.** All IUEP experiments were repeated at least three times with three different litters. Each image for IUEP analysis was taken from representative images of the sections from at least three brains from two or three different litters (average 10 sections per brain). All IUEP were performed with appropriate controls such as empty vector expressing GFP, or compared with un-electroporated control side. All immunostaining or mRNA in situ hybridization on embryonic brain sections were also performed with appropriate controls such as wild type brain (only male mice were used for comparison between Arx cKO and WT) or Cre negative brain sections. All statistical analyses were done in Prism software using 2-tailed unpaired Student's t-test (with Welch's correction). All graphs are plotted as mean ± the standard deviation (s.d) or mean ± the standard error of the mean (s.e.m).

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**Author Contributions**

J.A.G. designed and supervised the project, analyzed the data, and revised the manuscript; G.C. designed and supervised the project, revised the manuscript, performed and analyzed *in situ* hybridization, immunostainings, and reporter gene assays, and analyzed microarray and ChIP-seq data: Y.L. wrote the manuscript, prepared the figures, and performed and analyzed *in utero* electroporation and cell counting (*in situ* hybridization); I.-T.C. performed and analyzed RT-qPCR and ChIP-qPCR; X.S. maintained mouse lines and prepared animal samples; J.B.G. supervised PLP and TUNEL staining and analysis; All authors reviewed the manuscript.

**Additional Information**

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