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Dynamic *FoxG1* expression coordinates the integration of multipolar pyramidal neuron precursors into the cortical plate

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Abstract

Pyramidal cells of the cerebral cortex are born in the ventricular zone and migrate radially through the intermediate zone to enter into the cortical plate. In the intermediate zone, these migrating precursors are able to move tangentially and initiate the extension of their axons by transiently adopting a characteristic multipolar morphology. We observe that expression of the forkhead transcription factor *FoxG1* is dynamically regulated during this transitional period. By utilizing conditional genetic strategies, we show that the down-regulation of *FoxG1* at the beginning of the multipolar cell phase induces *Unc5D* expression, the timing of which ultimately determines the laminar identity of pyramidal neurons. In addition, we demonstrate that the re-expression of *FoxG1* is required for cells to transit out of the multipolar cell phase and to enter into the cortical plate. Thus, the dynamic expression of *FoxG1* during migration within the intermediate zone is essential for the proper assembly of the cerebral cortex.

Keywords

multipolar cell phase; migration; intermediate zone; gene dosage; Unc5D; DCC; NeuroD1

Introduction

The mammalian cerebral cortex is composed of a sophisticated neuronal network that processes higher order information such as sensory perception, consciousness and memory. Construction of this network is dependent on the emergence of two major classes of cortical neurons, glutamatergic pyramidal neurons and GABAergic interneurons, both of which need to be produced and precisely assembled during the course of development (Barnes et al., 2008; Bystron et al., 2008; Kriegstein and Noctor, 2004; Marin and Rubenstein, 2003; Molyneaux et al., 2007; Nguyen et al., 2006). It is becoming increasingly clear that the coordination of tangential and radial migration is critical for the integration of both interneurons (Kriegstein and Noctor, 2004; Lodato et al., 2011; Marin and Rubenstein, 2003; Miyoshi and Fishell, 2011) and pyramidal cells into cortical circuits (Britanova et al., 2006; O'Rourke et al., 1992; Rakic, 2009; Tan and Breen, 1993; Tarabykin et al., 2001;

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Extended experimental procedures are included in the Supplemental Information

Torii et al., 2009). Until recently, pyramidal neurons, which are generated locally within the cortical germinal zones (Gotz and Huttner, 2005), were thought to achieve their appropriate laminar positions exclusively through vertical migration along radial glial fibers. However, it is now recognized that pyramidal neuron precursors, like interneurons, tangentially disperse during their integration into the developing cortex (O'Rourke et al., 1992). During this phase, pyramidal neuron precursors within the intermediate zone transiently assume a characteristic 'multipolar' morphology, detach from the radial glial scaffold and initiate axonal outgrowth (Barnes et al., 2007) prior to entering the cortical plate (Noctor et al., 2004; Tabata and Nakajima, 2003). However, the importance of this multipolar migratory phase for assembling a mature cortical network and the precise genetic control of this stage are not well understood (LoTurco and Bai, 2006). Intriguingly, we have observed that the forkhead box transcription factor *FoxG1*, previously identified as a critical regulator of early telencephalic development (Xuan et al., 1995), is expressed in a dynamic manner as pyramidal neurons transit through these migratory phases. Here, through the use of conditional genetic strategies, we demonstrate that the dynamic regulation of FoxG1 expression that normally occurs during the pyramidal cell multipolar stage is essential for the proper assembly of cerebral cortex.

FoxG1 is known to play a central role in cortical development in that it regulates progenitor proliferation (Hanashima et al., 2002; Martynoga et al., 2005), specification and telencephalic patterning (Danesin et al., 2009; Hanashima et al., 2004; Manuel et al., 2010; Muzio and Mallamaci, 2005; Roth et al., 2010; Shen et al., 2006b). However, studying *FoxG1* gene function in postmitotic cells has proven challenging, as the constitutive loss of this gene results in gross developmental abnormalities, including the complete absence of subpallial structures (Xuan et al., 1995). Hence, the function of *FoxG1* during later stages of pyramidal neuron maturation has remained completely unexplored.

The central importance of *FoxG1* as an essential transcriptional regulator is underscored by the observation that even subtle alterations in *FoxG1* expression levels can have profound effects on brain development. Mice with heterozygous mutations in the *FoxG1* gene have impaired pallial development suggesting that the cortex is highly sensitive to *FoxG1* gene dosage (Eagleson et al., 2007; Shen et al., 2006a; Siegenthaler et al., 2008). Similarly, in humans, cases of Rett syndrome have been attributed to haploinsufficiency of *FoxG1* (Ariani et al., 2008; Le Guen et al., 2010). Moreover, duplication of the *FoxG1* locus has been found in patients with epilepsy, mental retardation and speech impairment (Brunetti-Pierri et al., 2010). These observations strongly suggest that the precise regulation of *FoxG1* expression is critical for proper brain development.

We hypothesized that the dynamic expression of *FoxG1* in pyramidal neuron precursors is critical for proper cortical development, and to test this, we utilized both genetic gain- and loss-of-function approaches. Remarkably, we find that the observed dynamic variation in *FoxG1* expression during pyramidal cell migration is crucial for the development of the cerebral cortex. Specifically, we find that a failure to down-regulate *FoxG1* at the beginning of the multipolar phase transiently stalls pyramidal neuron precursors within the lower intermediate zone, as a result of the failure to express Unc5D. Cells perturbed in this fashion were ultimately displaced to more superficial layers, and their laminar identity was re-specified accordingly. While the down-regulation of FoxG1 was essential for pyramidal cell migration, the re-initiation of FoxG1 expression following Unc5D expression was also critical for cells to leave the multipolar cell phase and to enter into the cortical plate. Taken together, our findings demonstrate that the dynamic expression of *FoxG1* during the postmitotic multipolar cell phase critically regulates the assembly and integration of pyramidal neuron precursors into the cortical network.

Results

FoxG1 expression is dynamically regulated during the postmitotic multipolar cell phase

In the developing cerebral cortex, we found that FoxG1 expression is transiently down-regulated in nascent pyramidal neuron precursors located at the lower portion of the intermediate zone (E14.5 Figures 1A and 1B, see other embryonic stages for Supplemental Figures S1A to S1C). By comparing the expression of FoxG1 to other transcription factors expressed within the ventricular (VZ) and intermediate (IZ) zones (Hevner et al., 2006), such as *Neurogenin2* (*Neurog2*) (Hand et al., 2005; Miyata et al., 2004; Nguyen et al., 2006), *Tbr2* (Arnold et al., 2008; Sessa et al., 2008) (Figure 1C) and *NeuroD1* (Mattar et al., 2008) (Figure 1D), we found that NeuroD1 expression, which is restricted to postmitotic cells (Mattar et al., 2008), is complementary to FoxG1 (Figure 1B and 1B'). In addition, we observed that the expression of *Unc5D* (Figure 1E), which has been shown to be exclusively expressed during the multipolar phase of pyramidal neuron development (Sasaki et al., 2008), is initiated at the point at which FoxG1 expression is down-regulated (Figure 1E, asterisk).

By taking advantage of an inducible *Cre* (CreER) driver under the control of proneural gene *Neurog2* (Zirlinger et al., 2002), which is transiently expressed at the time progenitors become postmitotic (Bertrand et al., 2002; Miyata et al., 2004), we were able to sparsely label the multipolar cell population (Figure 1F and 1F', see details of this method in Supplemental Figures S1D to S1G). We found two distinct levels of FoxG1 expression within these genetically labeled multipolar cells (Figure 1G), suggesting that FoxG1 expression is dynamically regulated specifically during this phase. We confirmed that the majority of multipolar cells are postmitotic as they were not labeled by an acute pulse of EdU (DNA analog) (0%, n=81) (Figure 1H) and did not express high levels of the Ki67 antigen (Miyata et al., 2004) (Figure 1H). We observed that these multipolar cells located near the ventricular zone express NeuroD1 (Figure 1I) and low levels of Tbr2, and, not surprisingly, most of them express Unc5D (Figure 1J) (Sasaki et al., 2008). We have further utilized *in utero* electroporation and found that FoxG1 down-regulation occurs precisely at the beginning of the multipolar cell phase, at a time coincident with when NeuroD1 expression is initiated (see detailed analysis in Supplemental Figures S1H and S1I). We refer to this NeuroD1 expressing stage as the 'early phase' (Figure 1A). These cells subsequently up-regulate FoxG1 levels at a period we designate as the 'late phase' of multipolar cell migration where NeuroD1 (but not Unc5D) has been down-regulated (Figure 1A). Based on these observations, we hypothesized that the dynamic regulation of FoxG1 activity during these multipolar cell transition phases is critical for the migration of cells through the intermediate zone and their integration into appropriate cortical layers.

Failure to down-regulate *FoxG1* at the beginning of the multipolar cell phase delays migration within the intermediate zone and redirects laminar identity

We next carried out *FoxG1* gain-of-function experiments to test the importance of *FoxG1* down-regulation at the beginning of the multipolar cell phase. Using *in utero* electroporation, we transduced the E13.5 cortical ventricular zone with a control (*pCAG-IRESEGFP*; Figure 2A) or a *FoxG1* expression vector (*pCAG-FoxG1-IRESEGFP*; Figure 2B), both of which resulted in EGFP cell labeling from the ubiquitously expressed *CAG* promoter (Niwa et al., 1991) (see methods). Three days after this manipulation, the majority of *FoxG1* gain-of-function cells remained within the lower intermediate zone and possessed multipolar morphologies (Figure 2B, compare to control in Figure 2A). However, three days later at P0, *FoxG1* gain-of-function cells are found inside the cortical plate (Figures 2C and 2D, bracket indicates cortical plate), suggesting that the failure in *FoxG1* down-regulation caused delay in migration but did not permanently stall cells at the multipolar phase. We

compared the location of these E13.5 electroporated cells to later born cells by E14.5 EdU birthdating and found that in contrast to control cells (Figure 2C, compare the colored asterisks), *FoxG1* gain-of-function cells within the cortical plate were intermingled with the population born at E14.5 (Figure 2D), suggesting that they are either still migrating or had become ectopically positioned.

We further fate mapped control and *FoxG1* gain-of-function cells and at P3 found that while control cells were positioned below those born at E14.5, *FoxG1* gain-of-function cells were located more superficially (compare Supplemental figures S2A and S2B, colored asterisks). We next analyzed the molecular expression profiles at P7, a stage at which neuronal migration is largely complete (Figures 2E – 2J). Consistent with previous findings (Takemoto et al., 2011), we found that the majority of control cells electroporated at E13.5 were located in layer IV (Figures 2E – 2G) and expressed molecular markers characteristic of that layer (RORβ-on, Brn2-low, Cux1-on Figures 2E –2G, insets) (Molyneaux et al., 2007). In contrast, the majority of *FoxG1* gain-of-function cells were located in layers II/III (Figures 2H –2J) and showed molecular features consistent with their ectopic laminar location (Figures 2H –2J, insets, RORβ-off, Brn2-high and Cux1-on). We conclude that failure to down-regulate *FoxG1* at the beginning of the multipolar cell phase delays cells from entering the cortical plate and results in a superficial shift in their location and marker profiles indicating a shift in their laminar identity.

We confirmed that this change in laminar identity did not result from postmitotic cells re-entering the cell cycle after *FoxG1* gain-of-function (Supplemental figures S2C and S2D). We also ruled out the possibility that *FoxG1* over-expression within the progenitor pool was responsible for the switch in laminar position by repeating the *FoxG1* gain-of-function specifically in postmitotic multipolar cells using a *NeuroD1* promoter expression vector (Supplemental Figures S1H and S1I). This manipulation resulted in a similar delay in migration after two or three days of *in utero* electroporation (Supplemental Figures S3A to S3D) and changes in laminar identity at postnatal stages (Supplemental Figures S3E to S3H) as was observed with the broader *FoxG1* gain-of-function experiments shown in Figure 2.

FoxG1 down-regulation is necessary for *Unc5D* receptor expression and transition from the early to late multipolar phase of migration

We next tried to understand why failure to down-regulate *FoxG1* at the beginning of the multipolar cell phase leads to delayed migration in the intermediate zone. Consistent with their multipolar morphology, these cells had already extinguished Tbr2 (Figure 3A) but maintained NeuroD1 expression (Figure 3B, asterisk indicates the domain normally expressing NeuroD1), suggesting that they had failed to transit from the early to late multipolar cell phase (Figure 1A). Consistent with this idea, *FoxG1* gain-of-function cells failed to express Unc5D (compare Figure 3C, 3C' versus 3D, 3D'), a protein whose expression is normally initiated shortly after the onset of NeuroD1 and maintained into the late multipolar phase (Figure 1A, also see Supplemental Figures S3A to S3D for the similar absence of *Unc5D* expression in cells with postmitotic *FoxG1* gain-of-function). Since *NeuroD1* mis-expression by itself does not affect cell migration (Mattar et al., 2008), we hypothesized that the loss of *Unc5D* may be responsible for the delayed migration. We repeated the *FoxG1* gain-of-function and rescued Unc5D expression specifically at the postmitotic multipolar phase (Figure 3F) by using a *NeuroD1* promoter construct (Supplemental Figures S1H and S1I). Remarkably, restoration of Unc5D expression in NeuroD1-positive cells partially rescued the migration phenotype in that there was a dramatic increase in cells that entered the cortical plate after three days (Figure 3F) compared to the *FoxG1* gain-of-function cells (Figure 3E). We further examined whether *Unc5D* restoration in *FoxG1* gain-of-function cells could also correct their altered laminar identity (Figure 2 and Supplemental Figures S3E to S3H). When *Unc5D* expression was

restored in *FoxG1* gain-of-function cells at the multipolar phase, we observed that indeed, by P7, a substantial number of them were now appropriately located in layer IV (Figure 3G) and possessed the correct molecular profile for this layer (Figures 3G', 3H and 3I, Cux1-on, Brn2-low, RORβ-on, See also Figure 3J).

How could *Unc5D* play such a critical role in regulating the early to late transitions within the multipolar cell phase? It has been shown that Unc5D is a receptor involved in Netrin-signaling in the postnatal cortex (Takemoto et al., 2011) and, in the context of axonal guidance, alters the response of Dcc (Deleted in colorectal carcinoma) to Netrins in growth cone turning assays (Hong et al., 1999). We found that both *Dcc* and *Unc5D* are expressed within the intermediate zone (Supplemental Figures S4A and S4B) and, in fact, are the only known Netrin receptor molecules expressed in this region (*Unc5A, 5B, 5C, Neogenin* and *Dscam* are not expressed within the intermediate zone, see Supplemental Figures S4C to S4H). However, unlike the down-regulation of Unc5D we have observed in *FoxG1* gain-of-function cells (Figure 3D), we found that Dcc expression was not affected (data not shown). This suggests that, similar to what has been demonstrated in the context of axon guidance, disruption of the Unc5D/Dcc balance by loss of Unc5D might be responsible for the delay in migration. We directly tested this idea and found that *Dcc* over-expression delays cell migration at the intermediate zone (Figure 3K), in a manner similar to *FoxG1* gain-of-function, and this can be rescued by simultaneously increasing the levels of Unc5D (Figure 3L). In summary, transient down-regulation of FoxG1 at the beginning of the multipolar cell phase is crucial for the maintenance of an appropriate Unc5D/Dcc balance through Unc5D induction, and this event is required for cells to transit rapidly from the early to late multipolar phase and to acquire their proper laminar position and gene expression.

Up-regulation of *FoxG1* is required for pyramidal neuron precursors to exit the multipolar phase and to enter the cortical plate

As noted above, a prominent feature of the dynamic regulation of *FoxG1* is its up-regulation in cells in the late multipolar phase prior to their migration into the cortical plate (Figure 1A). To explore the significance of this up-regulation, we have generated a *Cre*-dependent conditional loss-of-function allele of *FoxG1* (Supplemental Figure S5) in order to allow us to remove *FoxG1* expression at specific stages of pyramidal cell migration. In constructing this conditional allele, the *Flpe* recombinase was inserted into the *FoxG1* locus such that its expression is initiated upon removal of the loxP flanked *FoxG1* gene (Figure 4A scheme, Supplemental Figure S5). Prior to Cre-mediated recombination, the expression of *Flpe* is attenuated by the *FoxG1* coding and 3'UTR domains, which act as a transcriptional stop cassette (Dymecki and Kim, 2007; Joyner and Zervas, 2006; Luo et al., 2008; Miyoshi and Fishell, 2006). By combining this conditional allele with a Flpe-dependent reporter line (*R26R-CAG-FRTstop-EGFP*; Figure 4A bottom) (Miyoshi et al., 2010; Sousa et al., 2009), recombined cells can be selectively and permanently labeled with EGFP. To mediate the selective removal of *FoxG1* (and the initiation of Flpe expression) in postmitotic multipolar cells, we used a *Neurog2-CreER* driver line (Figure 4A, top, also see Figures 1F to 1J).

Experimentally, we compared the migration behavior of the recombined *FoxG1-C:Flpe/+* cells (heterozygous controls) with *FoxG1-C:Flpe/−* cells (*FoxG1* loss-of-function mutants). One day after tamoxifen administration at E13.5, many of the control cells were found in both the intermediate zone (Figures 4B and 4C) and the cortical plate (Figures 4B and 4C, brackets). By contrast, although the mutant cells had successfully down-regulated NeuroD1 and Unc5D (Figures 4D and 4E), they maintained a multipolar morphology and were restricted to a position below the cortical plate (Figures 4D and 4E, asterisks). Moreover, while three days after tamoxifen administration at E13.5 the majority of control cells had entered into the cortical plate (Figures 4F and 4G), all of the *FoxG1* loss-of-function cells were still positioned within the intermediate zone and maintained a multipolar morphology

(Figures 4H and 4I). Interestingly, at this stage many of the mutant cells expressed NeuroD1 (Figure 4H) and Unc5D (Figure 4I), strongly suggesting that they had regressed back to the early multipolar phase (Figure 1A). In addition, mutant cells had begun to form aggregates within the intermediate zone (Figures 4H and 4I). To ascertain if these results can be generalized to other stages of cortical development, we carried out similar experiments at different embryonic stages (E11.5 and E15.5) and obtained results comparable to those we observed after a E13.5 manipulation (Figures 4J to 4M). Furthermore, even 7 days after *FoxG1* removal, EGFP-labeled cells were restricted to positions below the cortical plate (E11.5 to E18.5 survivals, Supplemental Figures S6A and S6B, and from E15.5 to P3 in *FoxG1* conditional homozygous background, data not shown). These data suggest that when multipolar cells fail to re-express *FoxG1* they permanently lose their ability to enter into the cortical plate. In addition, we observed this mutant phenotype across all neocortical areas examined. These data further support the idea that all pyramidal neurons transit through the multipolar cell phase during development and that up-regulation of *FoxG1* at the end of this phase is universally required.

FoxG1 re-expression facilitates the integration of pyramidal neuron precursors into the cortical plate by regulating the late multipolar cell phase

When cells fail to up-regulate *FoxG1* during the late multipolar phase, in addition to failing to enter the cortical plate, they revert/regress to the early multipolar phase by re-expressing genes associated with this phase (NeuroD1 and Unc5D) and form aggregates (Figure 4). One possibility is that cells re-enter the multipolar phase simply as a consequence of their failure to migrate properly into the cortical plate. Alternatively, this phenotype may be due to a direct requirement of *FoxG1* for exiting from the multipolar phase. In order to distinguish these possibilities, we took advantage of our inducible genetic mosaic loss-of-function strategy (Figure 4A, scheme) and compared gene expression profiles in control versus *FoxG1* conditional mutant cells using microarray analysis. Two days after administrating tamoxifen to E11.5 pregnant dams, we dissected out the cortices from control and mutant embryos (E13.5) and isolated EGFP-expressing cells using fluorescently-activated cell sorting. We then extracted total RNAs from these samples and carried out microarray gene expression analyses (n=3 each) using Affymetrix MOE 430A.2 arrays.

Before the analysis of the results, we first tested whether *FoxG1* acts as a transcriptional activator or repressor during this transition period. We found that, both a wild type or a constitutive repressor form of *FoxG1* allow mutant cells to enter the cortical plate, suggesting that either can largely rescue the postmitotic loss-of-function phenotype (Supplemental Figures S7A and S7B). By contrast, mis-expression of an activator form of *FoxG1* in the wild type cortex prevented EGFP-labeled cells from entering the cortical plate, suggesting that this construct acts in a dominant negative fashion (See detailed analysis in Supplemental Figures S7D). We conclude that *FoxG1* functions as a transcriptional repressor (Yao et al., 2001) in the postmitotic multipolar cells.

Having ascertained this, we reasoned that one explanation for the observed phenotype is that genes associated with radial migration are up-regulated in mutant cells. To our surprise, comparison in control versus mutant populations revealed no change in the expression of genes known to regulate the migration of pyramidal neuron precursors (Table1A), including *Doublecortin* (Gleeson et al., 1999; Ramos et al., 2006), *Filamin A* (Nagano et al., 2004), *Pafah1b1* (*Lis1*) (Tsai et al., 2007), *Ndel1* (Hippenmeyer et al., 2010; Shu et al., 2004), *Rnd2* (Alfano et al., 2011; Heng et al., 2008; Nakamura et al., 2006), *Rnd3* (Pacary et al., 2011) and *Tubb2b* (Jaglin et al., 2009), suggesting that none of these genes are directly regulated by *FoxG1*. One exception to this overall trend was an observed 10-fold reduction in *Dab1*, which encodes an adaptor protein that mediates Reelin-signaling (Table 1B) (Franco et al., 2011; Morimura and Ogawa, 2009; Olson and Walsh, 2008; Sanada et al.,

2004). However, studies of *Dab1* indicate that it is required in early (layers V/VI) but not late (layers II/III/IV) born pyramidal neuron precursors to enter into the cortical plate (Franco et al., 2011). Since we found *FoxG1* to be required for the development of all pyramidal neurons (Figure 4), *Dab1* is an unlikely down-stream mediator of *FoxG1* loss-of-function. Consistent with this prediction, restoration of *Dab1* alone or even together with *Csk*, a kinase that stimulates Dab1 activity (Bock and Herz, 2003), did not allow *FoxG1* mutant cells to leave the multipolar phase and enter into the cortical plate (see detailed analysis in Supplemental Figures S7E and S7F). These data suggest that neither changes in the cell's migration apparatus nor changes in Reelin signaling could account for the failure of *FoxG1* mutant cells to enter the cortical plate.

Having ruled out that FoxG1 acts by regulating radial migration, we examined the alternative hypothesis that it is required for cells to exit from the multipolar phase. In concordance with this idea, we observed a marked up-regulation of genes normally restricted to pyramidal neuron precursors within the intermediate zone (Table 2 and Supplemental Figures S8). In addition to *NeuroD1, Unc5D* (Figure 4) and *Reelin* (Table 1B) (Kubo et al., 2010; Uchida et al., 2009), we observed up-regulation of *Cdh10, Nhlh1* and *Slc17a6* (*vGlut2*). We thus conclude that the most parcimonious explanation of our findings is that *FoxG1* up-regulation during the late multipolar phase is directly controlling the exit from this cellular state.

Postmigratory pyramidal neuron precursors do not require FoxG1 function to prevent reentry into the multipolar phase

While we have shown that *FoxG1* up-regulation is specifically required during the late multipolar cell phase, *FoxG1* expression levels are further increased within the postmigratory cells inside the cortical plate (Figures 1A and 1B, Supplemental Figures S1A to S1C). This raised the possibility that *FoxG1* up-regulation is required not only at the multipolar cell phase but also during later stages of maturation. In order to test this hypothesis, we conditionally removed *FoxG1* from postmigratory pyramidal neurons located within the cortical plate (see details of this method in the legend of Supplemental Figures S6C and S6D). At E19.5, compared to the control cells (heterozygous in *FoxG1-C:Flpe/+* background) (Figures 5A and 5A'), cells three days after the postmigratory removal of *FoxG1* generally maintained pyramidal cell morphologies and did not resemble multipolar cells, although their dendritic branching pattern was somewhat diminished (Figures 5B and 5B') (Also, see Supplemental Figures S6C and S6D for detailed comparison of wild type, heterozygous and null mutant cells for *FoxG1* in two genetic backgrounds). Moreover, *FoxG1* removal in postmigratory cells did not result in the re-expression of the multipolar cell markers NeuroD1 and Unc5D (data not shown). These data strongly suggest that *FoxG1* has a specialized function during the transition from the late multipolar cell phase into the cortical plate and does not play similar roles in the postmigratory populations.

In summary, dynamic *FoxG1* expression during the multipolar cell phase specifically coordinates pyramidal cell integration into the cortical plate (Figure 6A). This process appears to be mediated by two equivalently important steps: 1) a down-regulation of *FoxG1,* allowing pyramidal neuron precursors to promptly transit through the multipolar phase by inducing *Unc5D* and 2) subsequent up-regulation of *FoxG1* to leave the multipolar cell phase and enter into the cortical plate.

Discussion

In the present study, we have examined the role of *FoxG1* in regulating the migration and maturation of postmitotic pyramidal neuron precursors (Figures 6A). Specifically, we have observed that FoxG1 protein levels are dynamically regulated as pyramidal neurons migrate

from the ventricular zone to the cortical plate. We demonstrate that the transient down-regulation of *FoxG1* at the beginning of the multipolar phase enables cells to initiate *Unc5D* expression, which facilitates their transition from the early to late multipolar phase and is thus critical for their migration through the intermediate zone. Failure to down-regulate *FoxG1* during this period delays the entrance into the cortical plate, resulting in a superficial shift in both the laminar position and marker expression of pyramidal neurons. Subsequently, the up-regulation of *FoxG1* is specifically required for cells to transit out of the multipolar state and enter into the cortical plate. Taken together, we conclude that the dynamic regulation of *FoxG1* is a crucial mechanism for controlling the incorporation of pyramidal neuron precursors into the cerebral cortex (Figures 6A and 6B). These findings may have relevance to the etiology of specific classes of mental disorders observed in human patients, including congenital variants of Rett syndrome (Ariani et al., 2008; Brunetti-Pierri et al., 2010; Le Guen et al., 2010).

The importance of the multipolar cell phase in cortical circuit assembly

Only relatively recently has it been recognized that pyramidal neuron precursors transiently adopt a characteristic multipolar morphology while they are migrating within the intermediate zone (Tabata and Nakajima, 2003; Noctor et al., 2004; this study). However, the significance of this phase for the establishment of mature cortical networks remains unclear (LoTurco and Bai, 2006). Recent work suggests that the lateral dispersion of migrating pyramidal neuron precursors is mediated by *EphrinA - EphA* signaling, most likely during the multipolar phase, and, as a consequence, the width of radial columns is established (Torii et al., 2009). Given recent evidence that radially aligned cells arising from a common progenitor have a high probability of interconnecting (Yu et al., 2009), the tangential dispersion at the multipolar cell phase may also be critical for establishing intercolumnar cortical connectivity (Costa and Hedin-Pereira, 2010). Our work adds to these findings by demonstrating that the timing and duration of the multipolar phase is precisely regulated by *FoxG1* activity.

The laminar fate of pyramidal neurons remains labile at the early multipolar cell phase

Pulse-chase studies have shown that cell birth date within the cortex predicts laminar position (Angevine and Sidman, 1961; Rakic, 1974). A now classic transplantation study found that cell fate could be altered depending on whether pyramidal neurons underwent their last neuronal division in an isochronic or heterochronic host environment (McConnell and Kaznowski, 1991). Our study adds to this finding by demonstrating that the laminar position and postnatal marker expression of pyramidal neurons remains labile at least up to the early multipolar phase. In this regard, both the laminar (Kwan et al., 2008; Lai et al., 2008) and areal (Joshi et al., 2008) identity of pyramidal neurons require the persistent expression of the transcription factors (*Sox5* and *BhlhB5*), which are exclusively restricted to postmitotic cells. It will take further analysis to establish whether the mispositioning of pyramidal neurons upon *FoxG1* gain-of-function results in changes in their hodological identity. Nonetheless, it is becoming evident that rather than being irreversibly fixed, pyramidal neurons require active maintenance in their identity, demonstrating that the line between developmental programs and adult plasticity is less absolute than previously recognized.

The role of *Unc5D*-mediated signaling in cell migration

In addition to its roles in axon outgrowth and growth cone turning in commissural projection neurons (Serafini et al., 1994), Netrin-signaling has been shown to mediate both attraction and repulsion during cell migration (Ackerman et al., 1997; Alcantara et al., 2000; Hu and Rutishauser, 1996; Stanco et al., 2009; Xu et al., 2010). It has also been suggested that Netrin-signaling controls axon outgrowth and cell migration through distinct downstream

mechanisms (Causeret et al., 2004). Here, we show that, in the case of pyramidal neuron precursor migration, *Unc5D* and *Dcc* function in concert during the multipolar cell phase. In this context, *FoxG1* appears to regulate the expression of *Unc5D* but not *Dcc*. Interestingly, in *Drosophila* motorneurons akin to the present context, *Unc5* is positively regulated by the transcription factor *Even-skipped,* while *Frazzled* (the fly homolog of *Dcc*) is not (Labrador et al., 2005).

Netrins and, more recently, Flrts (Fibronectin type III domain and leucine-rich repeats transmembrane protein) have been demonstrated to interact with Unc5 receptors (Karaulanov et al., 2009; Yamagishi et al., 2011). However, we find that Unc5D-expressing multipolar cells do not undergo obvious changes in their behavior upon over-expression of either *Netrin4* or *Flrt2* in the ventricular zone (Data not shown due to space limitation). This suggests that Unc5D/Dcc signaling is binary rather than graded, which is consistent with it playing a role in multipolar to radial phase transition but not chemotropic guidance. An area of future interest will be to investigate whether different ligands initiate distinct down-stream signaling cascades upon Unc5D-activation.

Iterative roles of *FoxG1* throughout telencephalic development

It is striking to compare the early role of *FoxG1* demonstrated for suppressing the production of Cajal-Retzius cells (Hanashima et al., 2007; Hanashima et al., 2004; Shen et al., 2006b) with our present finding that *FoxG1* can suppress the late multipolar cell phase of postmitotic pyramidal neuron precursors (Figure 6B). Although quite distinct lineages, Cajal-Retzius cells and pyramidal neuron precursors in the multipolar migratory phase have in common their expression of Reelin (Uchida et al., 2009; Yoshida et al., 2006) and their propensity for tangential migration. Interestingly, we observe a similar dynamic regulation of *FoxG1* in telencephalic GABAergic interneuron precursors, where this gene is selectively down-regulated during the tangential phase of their migration and reinitiated when they have invaded the cortical plate (GM, unpublished observation and Supplemental Figures S1A to S1C). Furthermore, *FoxG1* is also essential for the integration of interneuron precursors into the cortical plate (GM, unpublished observation). Taken together, there may be a universal requirement for *FoxG1* down-regulation during the tangential phases of neuronal migration within the telencephalon. These findings lead us to conjecture that *FoxG1* function has been evolutionarily adapted in mammals as a means to regulate radial versus tangential modes of neuronal migration and is therefore vital to the assembly of the laminar and columnar organization that is the hallmark of the cerebral cortex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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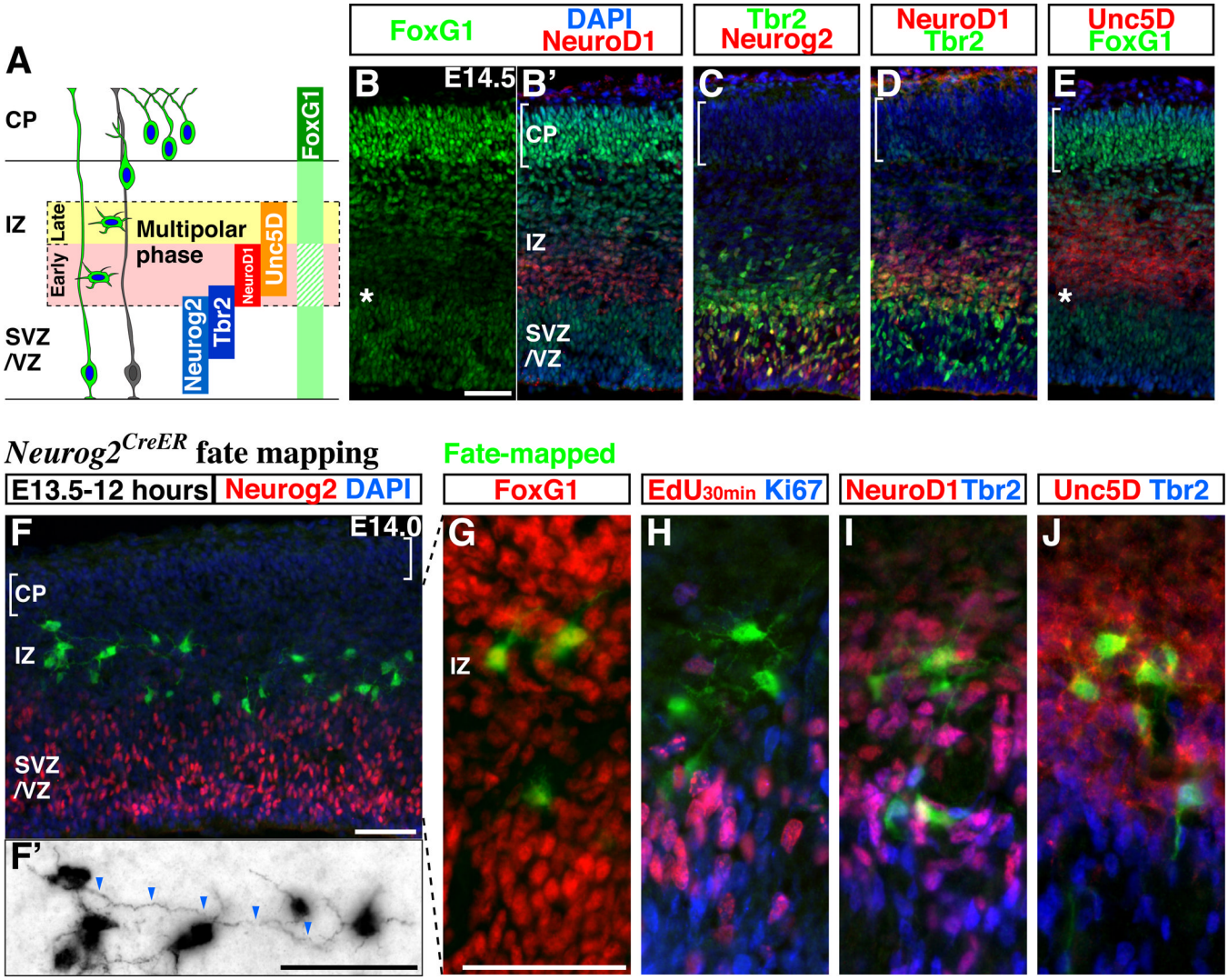

Figure 1. *FoxG1* expression is dynamically regulated during the multipolar phase of pyramidal neuron development

(A) A schematic drawing comparing the expression of genes in pyramidal neuron precursors as they proceed from the proliferative zone to the cortical plate. The area surrounded by dotted lines indicates the region in which pyramidal neuron precursors adopt a multipolar identity. CP: cortical plate, IZ: intermediate zone, SVZ/VZ: subventricular and ventricular zones (B, B') FoxG1 protein is expressed at different levels in E14.5 cortical cells at distinct stages of their maturation (See also Supplemental Figures S1A to S1C). FoxG1 expression is down-regulated (asterisk) in cells concomitant with their initiation of NeuroD1 expression. We observe similar results from the use of two different polyclonal antibodies for FoxG1. (C) A section adjacent to (B) indicating the region where the expression of Neurog2 and Tbr2 overlap. (D) Comparison of Tbr2 and NeuroD1 expression. (E) Down-regulation of FoxG1 (indicated by asterisk) occurs in the lower IZ (early multipolar phase) slightly below (i.e., before) initiation of Unc5D expression. Notably, in the uppermost IZ (late multipolar phase) Unc5D-expressing cells re-express FoxG1 below (i.e., prior) to where Unc5D-expression is extinguished. (F–J) Multipolar cells are labeled with EGFP by an acute (12 hour) fate mapping of the *Neurog2*-expressing population. This is achieved by combining the *Neurog2-CreER* driver and the *R26R-CAG-loxPstop-EGFP* reporter lines (See also

Supplemental Figures S1D to S1G) and initiated through tamoxifen administration at E13.5. Note that EGFP-expressing cells have already shut off Neurog2 by this time. The majority of EGFP-labeled cells are found within the intermediate zone and possess multipolar morphology. (F') In order to better visualize the cell morphology during the multipolar phase, a picture with EGFP expression is shown with reverse contrast to highlight their morphology. Tangentially oriented process resembling axon (arrowheads) are found in the multipolar cells. (G) Dependent on the position within the intermediate zone, multipolar cells express distinct levels of FoxG1 protein. (H) Multipolar cells are neither labeled by acute pulse-chase analysis using EdU (DNA analog) nor by antibodies against the Ki67 antigen suggesting that they are postmitotic. (I, J) Many *Neurog2* fate-mapped cells with multipolar morphology expressed NeuroD1 and Unc5D but only low levels of Tbr2. Scale bars: 50μm

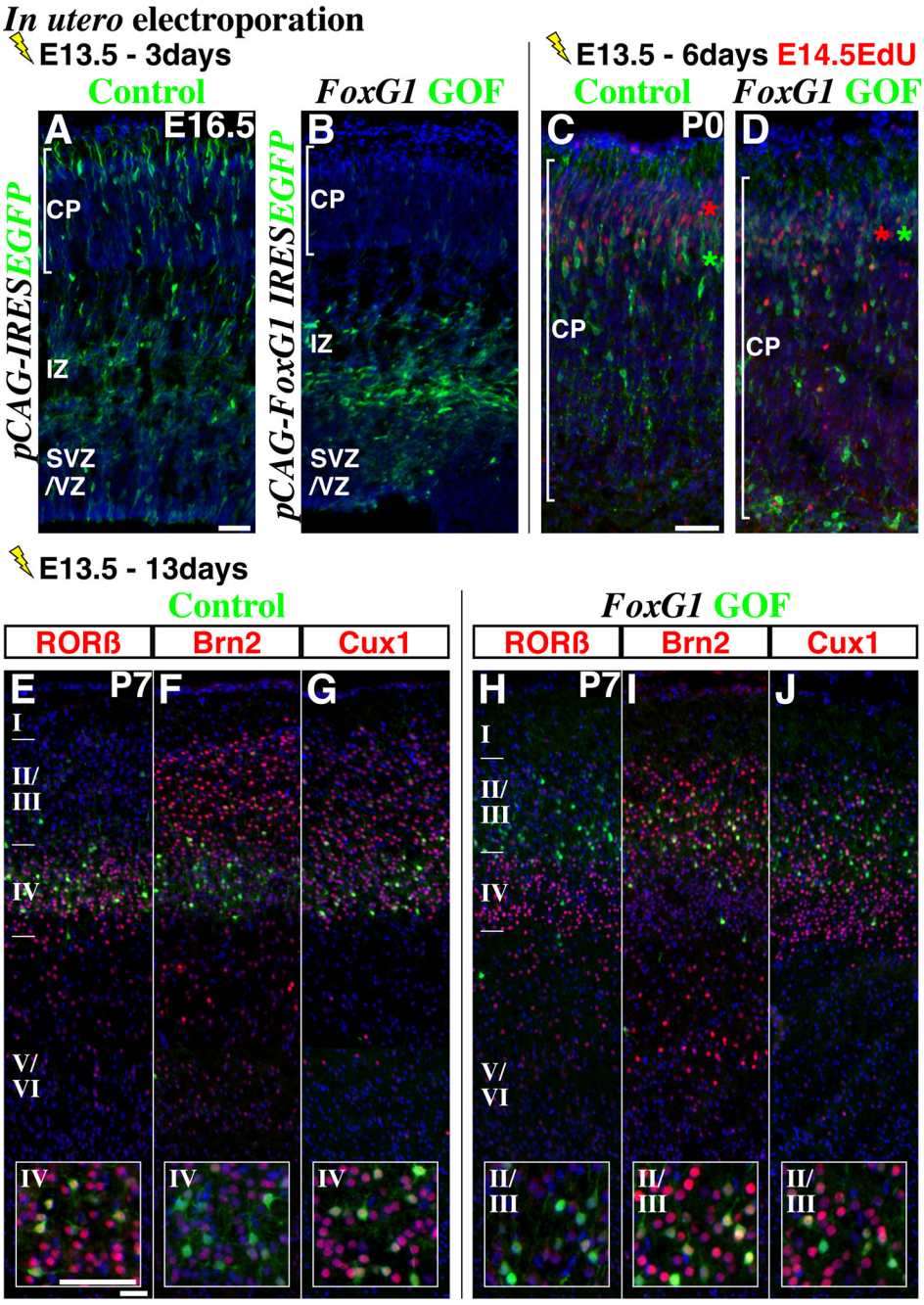

Figure 2. Failure to down-regulate *FoxG1* delays the migration of pyramidal neuron precursors at the multipolar phase and alters their laminar fate

In utero electroporation of either (A) control (*pCAG-IRES EGFP)* or (B) *FoxG1* gain-of-function (GOF) (*pCAG-FoxG1-IRES EGFP)* vectors under the regulation of a ubiquitous *CAG* promoter were carried out at E13.5 and brains were analyzed at E16.5. Three days after manipulation, while most control cells have entered the cortical plate (A), the majority of *FoxG1* gain-of-function cells (B) were found in the lower part of the intermediate zone and displayed multipolar morphologies. (C, D) 6 days later, many *FoxG1* gain-of-function cells delayed in migration by this manipulation now enter the cortical plate (bracket indicates cortical plate). Control cells (C) were generally found in lower positions compared

to cells born one day after the electroporation (single pulse of EdU at E14.5), while *FoxG1* gain-of-function cells (D) were intermingled with them. (E–G) At P7, the majority of EGFP-labeled control cells were found in layer IV and expressed molecular profiles consistent with cells within this layer (i.e., RORβ-on, Brn2-low, Cux1-on, insets). (H–J) The majority of *FoxG1* gain-of-function cells were located in layers II/III and possessed molecular features consistent with this location (insets, RORβ-off, Brn2-high and Cux1-on). Note that in case of *FoxG1* gain-of-function, a few cells were also found within the white matter and lacked expression of the layer specific markers we have examined. See also Supplemental Figures S2 and S3.
Scale bars: 50μm

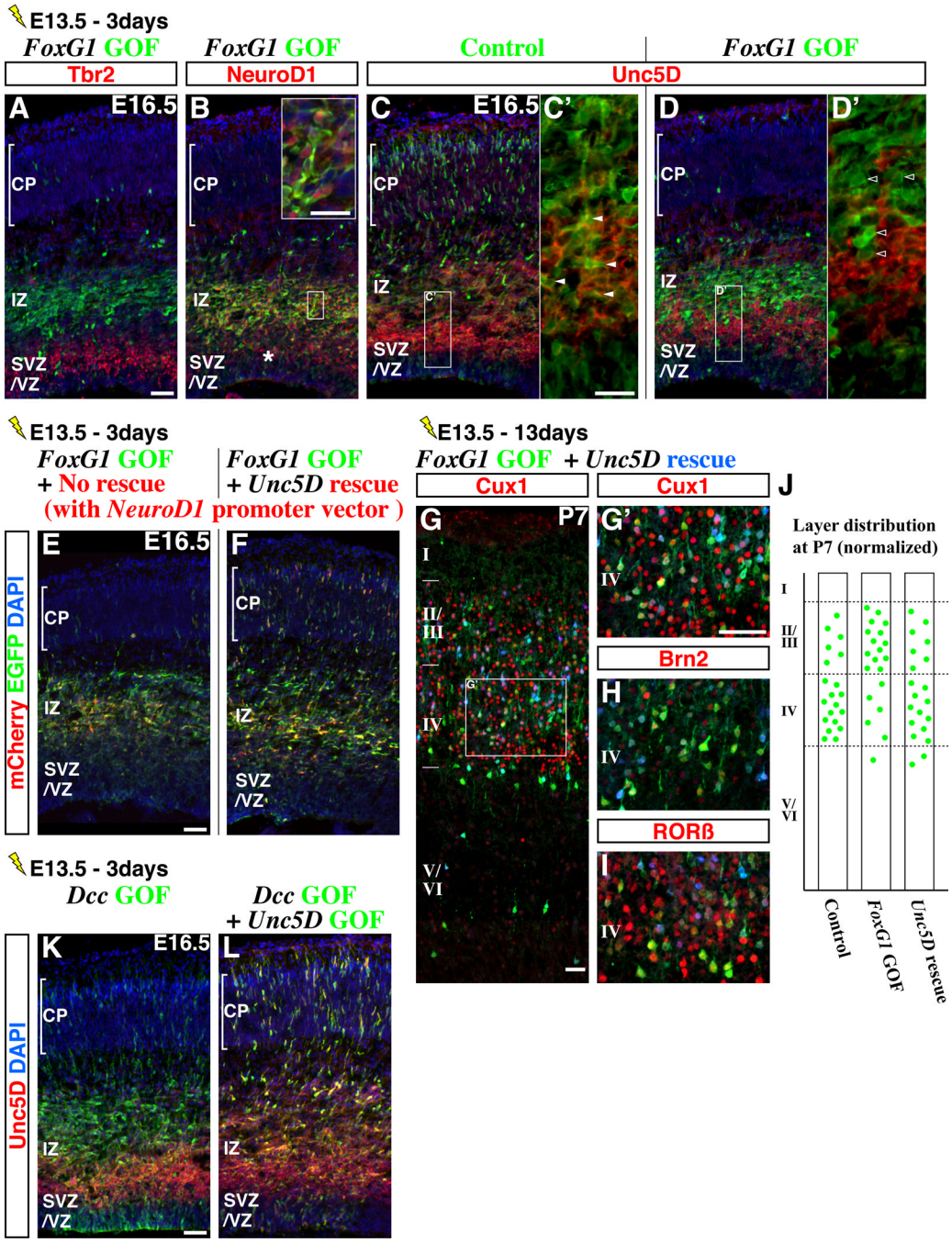

Figure 3. *FoxG1* down-regulation promotes an early to late multipolar phase transition through induction of *Unc5D*

Migrating pyramidal neuron precursors that failed to down-regulate *FoxG1* were delayed within the lower part of the intermediate zone (Figure 2). These cells shut off Tbr2 (A) but ectopically maintained NeuroD1 expression (B) (in a region above the normal expression domain, asterisk), suggesting that they become arrested at the early multipolar phase. (C, C') Control cells within the intermediate zone expressed Unc5D (arrowheads in C'). (D, D') *FoxG1* gain-of-function cells, although they possess multipolar morphology, failed to express Unc5D protein (open arrowheads). (E) As we have previously shown, three days after electroporation at E13.5, *FoxG1* gain-of-function cells (with *pCAG-FoxG1-IRES*

EGFP) remained within the lower part of the intermediate zone. Note that mCherry was expressed in NeuroD1-expressing cells by co-introducing a *pNeuroD1-IRES mCherry* control vector. (F) When *Unc5D* expression was restored in NeuroD1-expressing *FoxG1* gain-of-function cells (by using a *pNeuroD1-Unc5D-IRES mCherry* vector), a subset of these pyramidal neuron precursors migrated normally into the cortical plate after three days (green and red cells) and after 13 days at P7 (G, G', H, I), we observed cells located in both layers II/III and layer IV (see summary in J). The partial restoration in the laminar location of this population at P7 was consistent with the degree of rescue in migration we observed after three days (F, compare with Figure 2A). In *FoxG1* gain-of-function cells, both rescued (in layer IV) and non-rescued (in layers II/III) populations express molecular signatures appropriate to their laminar locations. Specifically, *Unc5D*-rescued cells in layer IV showed molecular expression profiles consistent with them being layer IV cells, i.e., Cux1-on (G'), Brn2-low (H) and RORβ-on (I). (J) Schematized layer distribution of Control (*pCAG-IRES EGFP*) (Figures 2E to 2G), *FoxG1* gain-of-function (*pCAG-FoxG1-IRES EGFP*) (Figures 2H to 2J) and *Unc5D*-rescued *FoxG1* gain-of-function (*pCAG-FoxG1-IRES EGFP* + *pNeuroD1-Unc5D-IRES mCherry*) experiments. Note that the numbers of EGFP-labeled cells (including the low-expressors) within the cortical plate is normalized (20 cells) and represented in this scheme. (K) Similar to *FoxG1* gain-of-function, *Dcc* over-expression (*pCAG-Dcc-IRES EGFP*) delays cell migration at the intermediate zone. (L) *Unc5D* over-expression (*pCAG-Unc5D-IRES EGFP*) rescues the impaired migration phenotype observed by *Dcc* over-expression. Thus, a precise balance between *Dcc* versus *Unc5D* expression is important for cells to migrate through the intermediate zone (See also Supplemental Figures S4). This balance appears to be critically controlled by transient *FoxG1* down-regulation as *FoxG1* gain-of-function affects Unc5D (D, D') but not Dcc expression (data not shown). Scale bars: 50μm, except for C' and D': 20μm

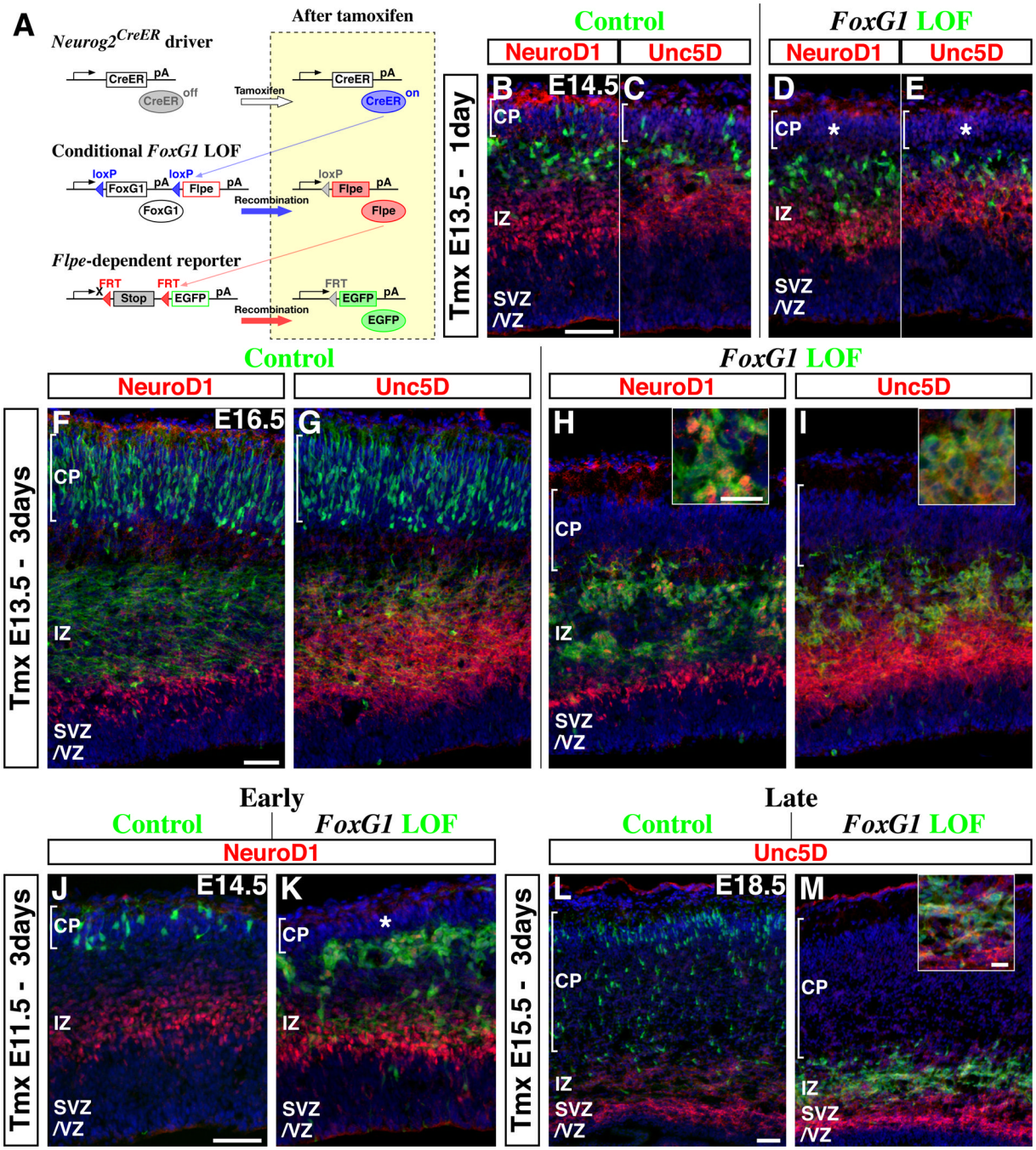

Figure 4. Up-regulation of *FoxG1* is required for pyramidal neuron precursors to transit out of the multipolar cell phase and to enter into the cortical plate

(A) Schematic drawings of our genetic strategy to mosiacally remove *FoxG1* at the multipolar cell phase and to selectively follow the fate of recombined cells. (Top) *Neurog2-CreER* driver was used to target multipolar cells at specific time points. (Middle) A conditional *FoxG1* loss-of-function (LOF) allele that expresses *Flpe* after *Cre*-mediated recombination (Supplemental Figure S5). (Bottom) A *Flpe*-dependent *EGFP* reporter line was utilized to visualize the cells in which the *FoxG1* conditional loss-of-function allele has been recombined. (Right) Subsequent to tamoxifen administration, the *CreER* expressed in *Neurog2*-positive cells becomes active (top) and recombines the *FoxG1* conditional allele

resulting in the removal of *FoxG1* coding region and initiation of *Flpe* expression from the *FoxG1* locus (middle), *Flpe* in turn removes a stop cassette in the reporter, allowing us to permanently trace the fate of manipulated cells with EGFP (Bottom). (B–I) Comparison of control (*FoxG1* heterozygous) versus *FoxG1* loss-of-function cells at various time points after tamoxifen administration at E13.5. (B, C) One day after tamoxifen administration, some control EGFP-labeled cells are already found inside the cortical plate (See also Supplemental Figure S1E). (D, E) By contrast, *FoxG1*-null cells remain excluded from the cortical plate (asterisk) and generally maintained multipolar morphologies. Note that neither control nor *FoxG1*-null cells located right below the cortical plate express NeuroD1 (B, D) or Unc5D (C, E). (F, G) Three days after tamoxifen administration, the majority of control cells are found within the cortical plate. Note that in these cortices, EGFP-labeled axonal fibers derived from the cortical plate cells are readily evident within the intermediate zone. (H, I) *FoxG1*-null cells in littermate embryos were only found below the cortical plate and maintained multipolar morphologies. Note also that the majority of *FoxG1*-null cells now express NeuroD1 (H, inset) and Unc5D (I, inset) suggesting that these cells have reverted back to the early multipolar phase. In addition, we observed aggregation of *FoxG1*-null cells at this time point (H, I). (J–M) Similar *FoxG1* loss-of-function experiments were carried out at earlier (E11.5: J, K) or later (E15.5: L, M) time points. At both early and late time points, *FoxG1*-null cells maintained multipolar morphologies, did not enter the cortical plate (asterisk in K), re-initiated genes specifically expressed during the early multipolar phase (NeuroD1 in K and Unc5D in M, inset) and formed aggregates. Hence, *FoxG1* up-regulation during the late multipolar cell phase seems universally required for pyramidal cells throughout development. See also Supplemental Figures S6A and S6B for an analysis after 7 days of manipulation.
Scale bars: 50μm, except for insets in H, I and M: 20μm

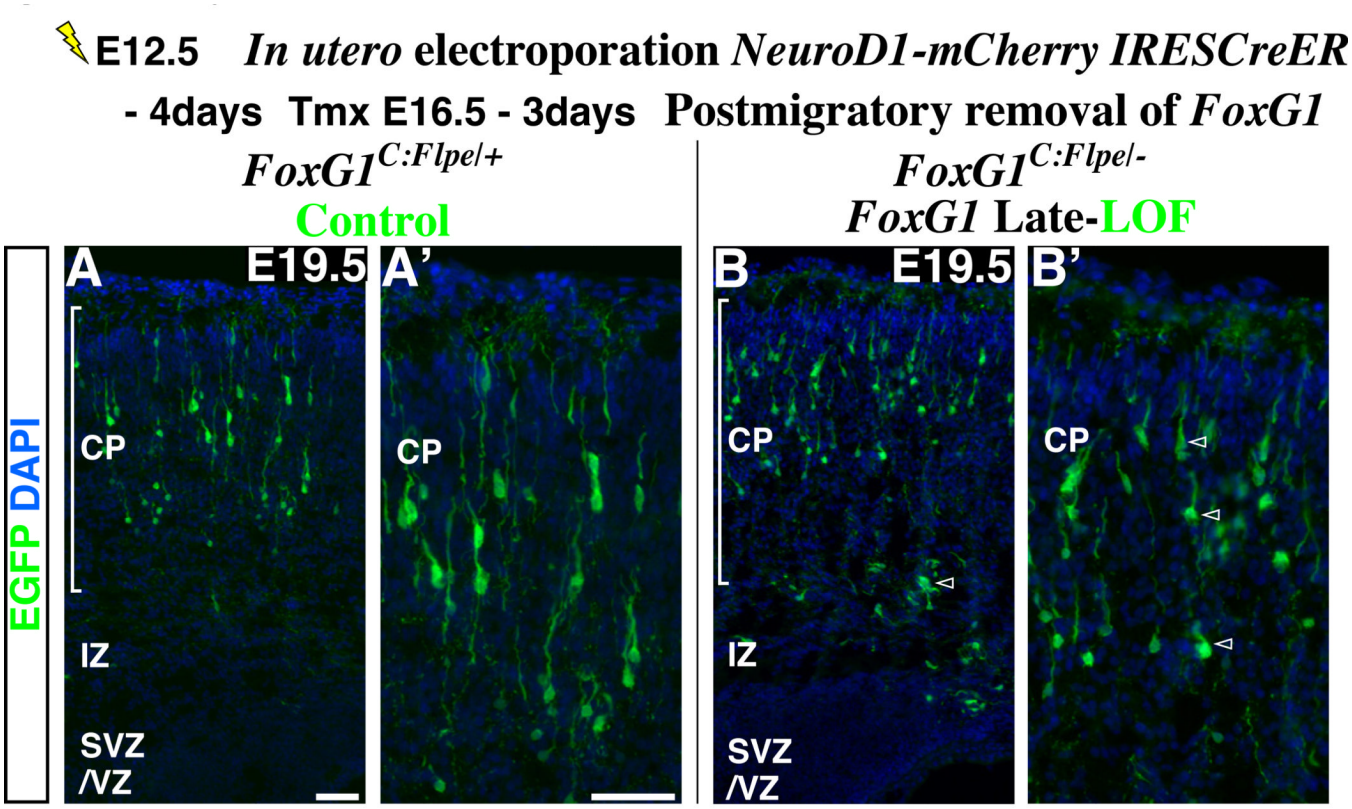

Figure 5. The role of *FoxG1* in the multipolar cells is distinct from its function in postmigratory neurons within the cortical plate

In order to remove *FoxG1* in postmigratory pyramidal neuron precursors inside the cortical plate, we have introduced a *pNeuroD1-mCherry-IRES CreER* vector into the ventricular zone by *in utero* electroporation at E12.5. This manipulation allows us to simultaneously visualize the morphologies of postmitotic cells through their expression of mCherry, as well as enabling us to recombine the conditional *FoxG1* loss-of-function allele at a desired time point. As the *FoxG1* loss-of-function allele expresses *Flpe* after gene removal (A scheme in Figure 4A), *FoxG1* mutant cells can be visualized with EGFP directed from the *Flpe*-activated reporter line (*R26R-CAG-FRTstop-EGFP*) (Miyoshi et al., 2010). At E16.5, a time by which the majority of cells electroporated at E12.5 have completed migration and settled inside the cortical plate (data not shown), tamoxifen administration was carried out to remove *FoxG1* in these cells. These embryos were analyzed at E19.5. (A, high magnification in A') The control experiment was carried out in the *FoxG1-C:Flpe/+* background. Pyramidal neurons labeled by EGFP are heterozygous for *FoxG1*. (B, B') The loss-of-function experiment was carried out in the *FoxG1-C:Flpe/−* background. Note that control and *FoxG1*-null cells have pyramidal morphologies and occupy similar positions within the cortex. In addition, removal of *FoxG1* gene function in postmigratory cells did not result in up-regulation of NeuroD1 and Unc5D (data not shown). Although the dendritic branching of mutant pyramidal neurons appears somewhat retarded compared to the control population (A, A'), they are not different from the internal control cells (Supplemental Figure S6D', red without green cells, arrowheads). Most importantly, these cells do not morphologically or molecularly resemble the multipolar population. Note that for the purpose of presentation, cells labeled red by mCherry are not visualized in these panels. For the mCherry expression and also for the detailed comparison of wild type, heterozygous and null cells for *FoxG1* in both *FoxG1C:Flpe/+* and *FoxG1C:Flpe/−* backgrounds, see Supplemental Figures S6C and S6D.

Scale bars: 50μm

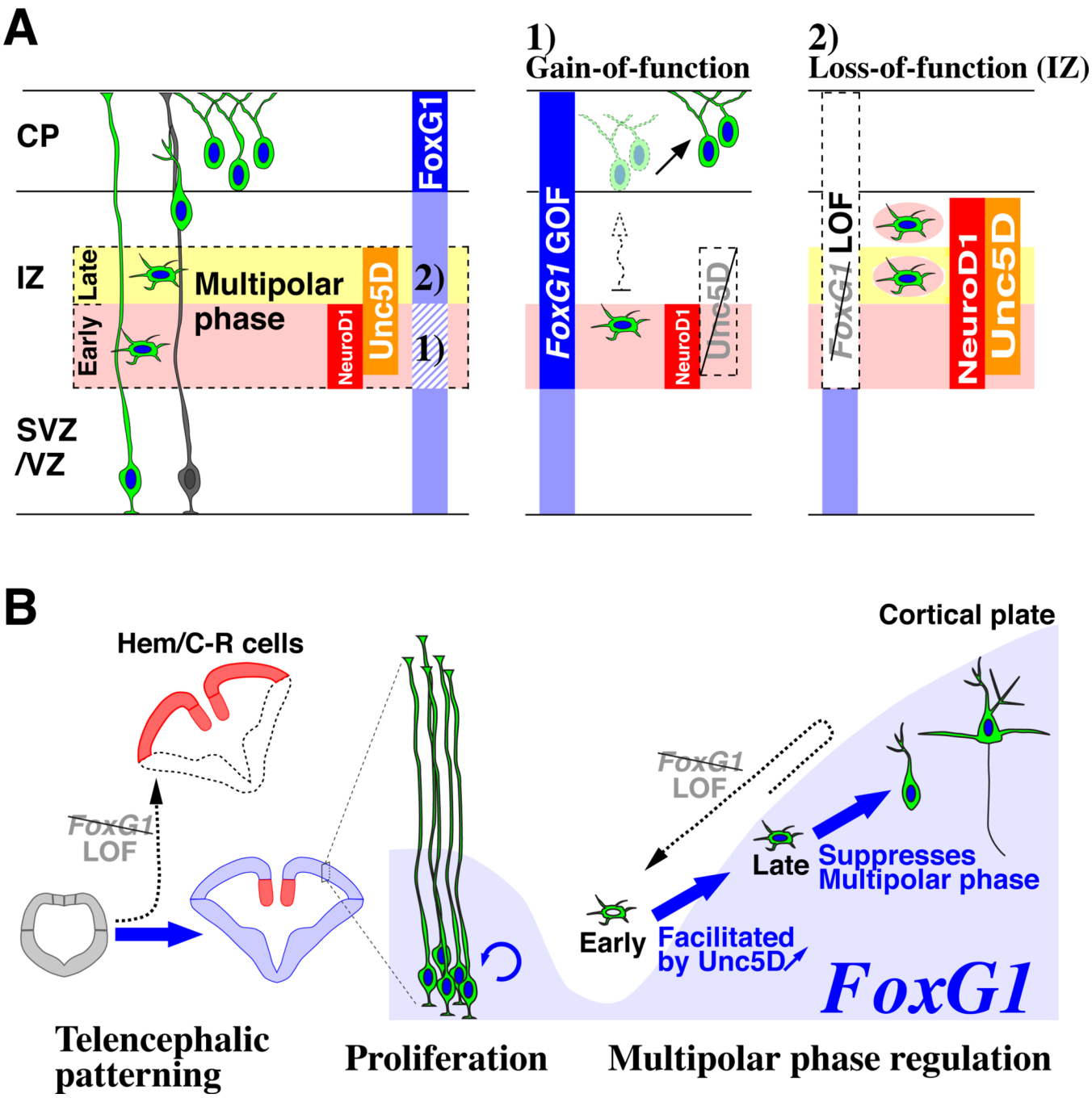

Figure 6. Dynamic *FoxG1* expression during the postmitotic multipolar cell phase coordinates pyramidal neuron development

(A, left panel) FoxG1 expression (blue vertical bar) is dynamically regulated throughout pyramidal neuron development. Especially within the intermediate zone (IZ), FoxG1 is transiently down-regulated at the beginning of the multipolar cell phase (Pink) and subsequently re-initiated during the late multipolar phase (Yellow). (A, middle panel) *FoxG1* down-regulation is necessary for multipolar cells to initiate *Unc5D* expression and to rapidly proceed from the early to late multipolar phase. Mis-regulation of *FoxG1* at this early phase delays entry into the cortical plate and redirects the laminar fate of postmitotic multipolar cells. (A, right panel) Multipolar cells need to re-initiate *FoxG1* expression

during the late phase in order to enter the cortical plate; otherwise, they remain in the intermediate zone and regress to the early multipolar phase. (B) Model diagram summarizing the roles of *FoxG1* during pyramidal neuron development. FoxG1 expression and function (arrows) are indicated in blue. At the early stage when the telencephalon is emerging from the anterior regions of the neural tube, *FoxG1* is required for the patterning by suppressing cortical hem (Hem) / Cajal-Retzius (C-R) cell fate (Hanashima et al., 2004; Muzio and Mallamaci, 2005; Shen et al., 2006b). After this period, *FoxG1* is further required for the proliferation of neocortical progenitors (Hanashima et al., 2002; Martynoga et al., 2005). In this study, we have demonstrated the requirement for *FoxG1* during the postmitotic period, when pyramidal neurons transit through their multipolar phase of development. At the beginning of the multipolar phase (Early), transient down-regulation of *FoxG1* allows cells to initiate Unc5D expression, which is crucial for the rapid transition from the early to late phase. Failure to undergo this transition delays entry of pyramidal neuron precursors into the cortical plate, resulting in a switch in their laminar identity (See middle panel of A). Subsequent to this, *FoxG1* expression is re-initiated during the late multipolar phase (Late) and is required for pyramidal neuron precursors to enter into the cortical plate. As indicated by the dashed arrow, failure to re-express *FoxG1* at this late phase results in a regression of pyramidal neuron precursors into the early multipolar phase and permanently prevents them from entering the cortical plate (See right panel of A). Hence, as illustrated here, *FoxG1* has iterative roles during pyramidal neuron development in patterning, proliferation and postmitotic regulation of the multipolar cell phase.

Table 1

A Cell migration and polarity related genes			
Gene Symbol	**Fold Change (Mut/Ctrl)**	**Gene Title**	**References**
Cdk5	0.958, 0.944	cyclin-dependent kinase 5	(Chae et al., 1997) (Howell et al., 1997)
Dclk1	0.959,0.934,0.871, 0.800, 0.756, 0.738	doublecortin-like kinase 1	(Deuelet al.,2006) (Koizumi et al., 2006)
Dcx	0.985, 1.191,0.673, 0.868	doublecortin	(Gleeson et al., 1999) (Ramos et al., 2006)
Flna	1.166	filamin, alpha	(Nagano et al., 2004)
Mapk8 (Jnk1)	1.180, 1.126,0.948	mitogen-activated protein kinase 8	(Westerlund et al., 2011)
Ndel1	1.097, 1.044	nuclear distribution gene E-like homolog 1	(Shu et al., 2004) (Hippenmeyer et al., 2010)
Pafah1b1 (Lis1)	1.239, 1.072, 1.028, 0.853	platelet-activating factor acetylhydrolase, isoform 1b, beta1 subunit	(Hirotsune et al., 1998) (Tsai et al., 2007) (Hippenmeyer et al., 2010)
Rac1	1.082,0.973,0.943	RAS-related C3 botulinum substrate 1	(Kawauchi et al., 2003)
Rhoa1	0.954	ras homolog gene family, member A	(Govek et al., 2011) (Pacary et al., 2011)
Rnd2	1.985	Rho family GTPase 2	(Heng et al., 2008)
Rnd3	0.940, 0.850	Rho family GTPase 3	(Pacary et al., 2011)
Stmn2 (SCG10)	1.110,0.969	stathmin-like 2	(Westerlund et al., 2011)
Tubb2b	0.985	tubulin, beta 2b	(Jaglin et al., 2009)

B Reelin signaling pathway related genes			
Gene Symbol	**Fold Change (Mut/Ctrl)**	**Gene Title**	**References**
Dab1	0.138, 0.138, 0.109, 0.106, 0.105	disabled homolog 1	(Morimura and Ogawa, 2009) (Franco et al., 2011)
Fyn	0.901, 0.900	Fyn proto-oncogene	(Howell et al., 1997)
Lrp8 (ApoER2)	0.925	low density lipoprotein receptor-related protein 8, apolipoproteine receptor	(Trommsdorff et al., 1999)
Reln	17.249	reelin	(Uchida et al., 2009) (Kubo et al., 2010)
Src	1.156, 1.080	Rous sarcoma oncogene	(Howell et al., 1997)
Vldlr	0.977,0.614, 0.593, 0.551	very low density lipoprotein receptor	(Trommsdorff et al., 1999)

No significant changes were observed in these genes. Fold change indicates intensity of the signals of Mutant / Control raw values observed in our microarray expression analysis. Note that multiple values are shown when several probes were assigned for a particular gene.

Table 2

Intermediate zone expressed genes

Gene Symbol	Fold Change (Mut/Ctrl)	Gene Title	Gene Paint ID
1190002N15Rik	47.62, 41.66	RIKEN cDNA 1190002N15 gene /// hypothetical protein LOC100044725	EH4432
Cdh8	0.4937	cadherin 8	DA115
Cdh10	3.219	cadherin 10	DA84
NeuroD1	4.142, 4.104	neurogenic differentiation 1	DA125
Nhlh1	3.909	nescient helix loop helix 1	EN679
Pcp4	7.795	Purkinje cell protein 4	EB2090
St8sia4	5.160, 4.640	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase4	EG45
Slc17a6 (vGlut2)	10.70, 8.819	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6	EB854

We have cross-correlated genes showing over 2-fold changes in our microarray expression analysis to the E14.5 expression database at Genepaint (www.genepaint.org). From this analysis, we have identified several genes showing specific expression within the intermediate zone (Listed in this table, also see Supplemental Figures S8) consistent with our hypothesis that *FoxG1* regulates the exit from the late multipolar phase.