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DEVELOPMENT AND DISEASE

Loss of *Emx2* function leads to ectopic expression of *Wnt1* in the developing telencephalon and cortical dysplasia

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SUMMARY

Leptomeningeal glioneuronal heterotopias are a focal type of cortical dysplasia in which neural cells migrate aberrantly into superficial layers of the cerebral cortex and meninges. These heterotopias are frequently observed as microscopic abnormalities in the brains of individuals with central nervous system (CNS) malformations and epilepsy. Previous work has demonstrated that the function of *Emx2*, which encodes a homeodomain transcription factor, is essential for development of the cortical preplate, which gives rise to the marginal zone and subplate. However, transcriptional targets of EMX2 during CNS development are unknown. We report that leptomeningeal glioneuronal heterotopias form in *Emx2*$^{-/-}$ mice that are equivalent to human lesions. Additionally, we observed ectopic expression of *Wnt1* in the embryonic roofplate organizer region and dorsal telencephalon. To determine the phenotypic consequences of such *Wnt1* misexpression, we deleted a putative EMX2 DNA-binding site from the *Wnt1* enhancer and used this to misexpress *Wnt1* in the developing murine CNS. Heterotopias were detected in transgenic mice as early as 13.5 days postcoitum, consistent with a defect of preplate development during early phases of radial neuronal migration. Furthermore, we observed diffuse abnormalities of reelin- and calretinin-positive cell populations in the marginal zone and subplate similar to those observed in *Emx2*-null animals. Taken together, these findings indicate that EMX2 is a direct repressor of *Wnt1* expression in the developing mammalian telencephalon. They further suggest that EMX2-*Wnt1* interactions are essential for normal development of preplate derivatives in the mammalian cerebral cortex.

Key words: *Emx2*, *Wnt1*, Dysplasia, Cerebral, Cortex, Telencephalon, Marginal zone, Subplate, Preplate, Cortical hem, Organizer, Transgenic, Leptomeningeal, Glioneuronal, Heterotopia

INTRODUCTION

During development of the elegantly structured mammalian cerebral cortex, generation and subsequent migration of specialized neurons and glia within the brain follows a characteristic sequence. The earliest differentiated neurons emigrate from the telencephalic ventricular zone to form a superficial primordial layer called the preplate (Allendoerfer and Shatz, 1994). In mice, the marginal zone (MZ, layer I) and subplate (SP) derive from splitting of the preplate at ~11-12.5 days postcoitum (dpc) as successive waves of neurons from the underlying ventricular zone undergo radial migration into the cortical plate proper (layers II-VI). The MZ and SP comprise a heterogeneous population of cells with neuronal properties, including reelin-expressing Cajal-Retzius (CR) cells, subpial granule cells (glutamate-positive) and subpallial-derived interneurons (GABA-positive) (Fairen et al., 2002; Meyer et al., 1998; Parnavelas, 2000; Zecevic et al., 1999). Important roles for MZ and SP cells during tangential and radial migration of cortical neurons have been previously described (Parnavelas, 2000; Xie et al., 2002). For example, proper formation of the MZ and SP has been shown to be necessary for tangential emigration of GABA-expressing interneurons from ventral forebrain regions (e.g. the ganglionic eminence) into the cortical plate (Lavdas et al., 1999). Second, cells in the MZ provide repulsive or stop cues to radially migrating cortical

neurons (Rice and Curran, 1999). Indeed, the MZ includes the foot processes of radial glia that form the subpial glia limitans, a structure that ultimately limits the radial migration of neural cells and defines the external boundary of the central nervous system (CNS) (Super et al., 2000).

The term cortical dysplasia is used to describe a heterogeneous group of CNS malformations in which the organization of the cortical plate is disrupted, typically owing to abnormal cellular migration (Friede, 1989; Gleeson and Walsh, 2000). Marginal zone heterotopias (MZH) or leptomeningeal glioneuronal heterotopias (LGH) are one form of dysplasia in which ectopic nests of glial and neuronal cells are observed in the cortical MZ or overlying leptomeninges, respectively (Greenfield et al., 2002). These lesions are frequently seen in association with other features of dysplasia as well as other CNS malformations and congenital syndromes such as Trisomy 13 or type II lissencephalies (Walker-Warburg syndrome) (Friede, 1989; Greenfield et al., 2002; Hirano et al., 1992). They are among the most common neuropathological malformations found in human brains with cortical dysplasia (Mischel et al., 1995). Although their clinical significance is not fully understood, they are presumed foci of seizure activity because of their cortical location, neuronal composition and high incidence in individuals with epilepsy (Mischel et al., 1995).

Insights into the genetic and molecular causes of cortical dysplasia are beginning to emerge from general investigation of cortical development in mice and humans (Gleeson and Walsh, 2000). In particular, certain transcription factors have been described with important roles in MZ and SP development. Mutation of *Lmx1a*, a LIM-homeodomain factor that is expressed in the dorsomedial telencephalon, results in diffuse and focal abnormalities of the MZ (Costa et al., 2001). Second, function of the homeodomain protein EMX2 is required for several aspects of cortical development including regulation of neural precursor cell proliferation (Galli et al., 2002; Heins et al., 2001; Tole et al., 2000) and lamination of the cortical plate (Galli et al., 2002; Heins et al., 2001; Mallamaci et al., 2000a; Tole et al., 2000), as well as formation of preplate derivatives. Loss-of *Emx2* function or *Emx1/2* function is associated with a decrease or absence, respectively, of Cajal-Retzius and SP cells within regions of the cortex (Bishop et al., 2003; Mallamaci et al., 2000a; Shinozaki et al., 2002). Additionally, defects in tangential neuronal migration and axon pathfinding from the ventral forebrain to the cortex of *Emx*-null animals have been described (Bishop et al., 2003; Shinozaki et al., 2002).

Despite its pivotal roles during brain development, downstream transcriptional targets of Emx2 have yet to be identified. *Emx2* is expressed in the cortical ventricular zone and cortical hem, which is located adjacent to the dorsal midline of the embryonic telencephalon. The cortical hem is generally considered to be an organizing center and source of secreted molecules with important roles in the specification and proliferation of forebrain cell types (Furuta et al., 1997; Grove et al., 1998). Numerous Wnt genes are expressed in overlapping patterns within the cortical hem (Grove et al., 1998), and *Wnt3a* has been shown to be essential for proliferation and expansion of hippocampal precursor cells in this region (Lee et al., 2000). Although expression of *Wnt1* is observed throughout most of the dorsal midline of the CNS it is excluded from the telencephalon and cortical hem. We have previously proposed that this aspect of *Wnt1* regulation relies on transcriptional repression, because deletion of certain *Wnt1* cis-acting regulatory sequences results in ectopic reporter gene expression in the telencephalon of transgenic mice (Rowitch et al., 1997). Mutation of a single homeodomain core-binding site (HBS1) within the 5.5 kb *Wnt1* enhancer results in ectopic dorsomedial telencephalic reporter gene expression (Iler et al., 1995; Rowitch et al., 1998) in a pattern reminiscent of *Emx2* (Boncinelli et al., 1993). EMX2 as well other homeodomain proteins have been shown to bind HBS1 in vitro (Iler et al., 1995), and in a previous analysis of *Emx2*-null embryos, weak ectopic expression of *Wnt1* in the 10.5 dpc telencephalon was observed (Yoshida et al., 1997). Taken together, these experiments are consistent with a model in which EMX2 functions to repress *Wnt1* expression in the developing telencephalic organizer regions (Rowitch et al., 1997). However, further experiments to demonstrate functional consequences of ectopic *Wnt1* expression in the cortex and functional or phenotypic correlation between this and *Emx2*-null animals are required to assess this putative interaction and establish its possible significance for normal development.

We report the presence of marginal zone and leptomeningeal heterotopias in *Emx2*$^{-/-}$ mice. Our data suggest that this lesion and other abnormalities of the superficial cortical plate (layer I) in *Emx2*$^{-/-}$ mice result from a failure to repress *Wnt1*. First, we observed striking ectopic expression of *Wnt1* in the cortical hem and medial cortical ventricular zone of *Emx2*$^{-/-}$ mice but not heterozygous littermates. Second, *Wnt1* gain-of-function analysis produced heterotopias identical to those in *Emx2*$^{-/-}$ mice. Finally, we observed diffuse abnormalities of the early-born Calretinin- and reelin-positive cells and also the plexiform network of cellular processes derived from these cells within the marginal zone and subplate, similar to those described for *Emx*-null animals. These findings indicate that EMX2 acts to repress *Wnt1* in the developing telencephalon. Furthermore, they suggest that ectopic activation of *Wnt1* signaling is sufficient to cause anomalous formation of the marginal zone and subplate, as well as defects in neural migration and heterotopias in *Emx2*$^{-/-}$ mice.

MATERIALS AND METHODS

DNA constructs

Construction of the *lacZ* reporter (*Wnt-lacZ*-ΔHBS) or *Wnt1* misexpression transgenes (*Wnt1*-mutHBS1 and *Wnt1*ΔHBS) was carried out as follows. Full-length *lacZ* or *Wnt1* genomic sequences, including 1.25 kb of upstream DNA (promoter) and all *Wnt1* exons, introns and poly A sequence (Danielian and McMahon, 1996), were cloned upstream of the 1.1 kb *Wnt1* regulatory elements that were modified to have mutation of HBS1 (Iler et al., 1995), or deletion of HBS1 and 300 bp of surrounding sequence (construct #16), (Rowitch et al., 1998). Confirmation of plasmid identity was by restriction endonuclease digestion and DNA sequencing. Prior to pronuclear injection, transgenes were purified from the plasmid backbone after digestion with the restriction endonuclease, *Sal*I (*Wnt-lacZ*-ΔHBS) or *Aat*II (*Wnt1*-mutHBS1 and *Wnt1*ΔHBS).

Generation of transgenic mice, animal husbandry and genotyping

Four independent founder lines of transgenic mice that expressed the

Wnt-lacZ-ΔHBS reporter construct were generated by pronuclear injection of one-cell BL6CBAF1/J embryos as described (Rowitch et al., 1998). Genotyping was by Southern blot with a probe directed against *Wnt1* (details supplied upon request). Of 12 independent *Wnt1*ΔHBS transgenic founders generated, one was analyzed at 13.5 dpc, and three at 18.5 dpc-PN1. One stable *Wnt1*ΔHBS transgenic line was followed and could be maintained in a hemizygous state. Homozygous offspring (n=4) were generated at 12.5 dpc for analysis. This line was subsequently lost because of infection with MHV. Extent *Emx2* mutant mice (generously provided by Prof. Peter Gruss, Goettingen, Germany) on a mixed C129Sv/J-C57Bl6 genetic background were generated and genotyped as previously described (Pellegrini et al., 1996). Matings of transgenic mice were timed and embryos harvested such that noon the day of vaginal plug discovery was considered to be 0.5 dpc.

Human heterotopias

Surgical biopsy and autopsy material containing leptomeningeal glioneuronal heterotopias was identified by review of the case records and archival material in the Neuropathology Division of Children's Hospital, Boston. Four appropriate cases were identified and analyzed by Hematoxylin and Eosin analysis in addition to immunohistochemistry for glial (GFAP) and neuronal markers (NeuN, synaptophysin).

Histopathology

Mouse embryos and brains were fixed in freshly made 4% paraformaldehyde/PBS overnight at 4°C. Tissue for frozen sections was then impregnated in 50% Sucrose/PBS overnight at 4°C, frozen in OCT, and then sectioned at 16 μm. Tissue for paraffin wax-embedded sections was dehydrated, embedded and sectioned at 5 μm according to standard protocols. Human neuropathological specimens were fixed in 10% buffered formalin, according to standard clinical protocols, and then embedded in paraffin wax for sectioning. Sections were stained with Hematoxylin and Eosin and photomicrographs were taken digitally using a Zeiss Axioskop and Axiocam imaging system.

Immunohistochemistry and whole-mount β-galactosidase staining

Paraffin wax embedded and frozen sections were subjected to heat antigen retrieval at 99°C in 10 mM sodium citrate buffer for 20 minutes for all antibodies. Staining was performed using the HRP/DAB based Envision+ staining system (DAKO) according to the manufacturers specifications with the only modification being increased incubation time for primary antibodies (overnight, 4°C) and secondary antibodies (1 hour, room temperature). Primary antibodies were Calretinin (Zymed #18-0211), GFAP (DAKO #Z0334), Nestin (BD #611658), NeuN (Chemicon #MAB377) and laminin (DAKO). Staining for β-galactosidase activity in transgenic embryos was performed as previously described (Whiting et al., 1991).

RNA in situ hybridization

Frozen and paraffin wax-embedded section RNA in situ hybridization was performed as previously described (Lu et al., 2001). Protocol modifications specific to use with paraffin wax-embedded sections included pretreatment of sections with 0.2 N HCl for 10 minutes at room temperature followed by incubation in 2× SSC for 10 minutes at 70°C. Sections were then treated with Proteinase K (50 μg/ml) for 10 minutes at 37°C. After hybridization, slides were treated with RNaseA (20 μg/ml) for 15 minutes at 37°C, then washed and developed according to standard protocols. The probes used were for the genes *Wnt1* (Parr et al., 1993), *Emx2* and *Wnt2b* (Tole et al., 2000), *Bmp4* (Lee et al., 2000), reelin (Alcantara et al., 1998) and *Gfap* (modified construct from A. Ruiz i Altaba).

RESULTS

Superficial cortical heterotopias in *Emx2*$^{-/-}$ mice resemble human leptomeningeal glioneuronal heterotopias

Loss of *Emx2* function in mice has been shown to result in generalized cortical dysplasia with defects in cortical lamination and hippocampal growth, as well as abnormal formation of the marginal zone (MZ) and subplate (SP) (Boncinelli et al., 1993; Mallamaci et al., 2000a; Mallamaci et al., 2000b; Shinozaki et al., 2002; Yoshida et al., 1997). We performed further neuropathological analysis of the cortex of *Emx2*$^{-/-}$ mice at 18.5 dpc, which revealed abnormal features of the marginal zone that had not previously been described (Fig. 1). The MZ of *Emx2*$^{-/-}$ mice exhibited diffusely increased cellularity and disorganization with an indistinct boundary between the marginal zone and the developing cortical plate proper (layers II-VI) (Fig. 1D-F). By contrast, wild-type and *Emx2* heterozygous mice showed no such defects (Fig. 1A-C). Focal lesions in the *Emx2*$^{-/-}$ MZ consisted of heterotopic eruptions of cells from the cortical plate into the overlying marginal zone, the subpial space or even into the overlying leptomeninges (Fig. 1D-F). Serial sectioning of the lesions showed them to consistently have a polypoid shape with a stalk and oftentimes they were associated with a central penetrating blood vessel. In the area of the lesions the disorganization of the migrating neural cells was noted to extend through the full thickness of the cortical plate and into the subplate (Fig. 1E).

The phenotype was highly penetrant, as heterotopias were observed in all *Emx2*$^{-/-}$ mice examined (n=6) whereas no lesions were detected in heterozygous littermates (n=3) or wild-type controls. Between two and six lesions were detected in each *Emx2*$^{-/-}$ brain on serial sectioning at 18 μm intervals. The lesions ranged from ~30-200 μm in size. The lesions were preferentially located in the dorsal frontoparietal neocortex. Heterotopias were not found in ventral or medial cortex or within the diencephalon of wild-type or mutant mice. The distribution of the lesions overlapped that of the normal expression domain of *Emx2* to a significant degree (Boncinelli et al., 1993).

The distribution and morphological appearance of the marginal zone heterotopias were strikingly similar to human heterotopias we identified in surgical and autopsy tissue from individuals with cortical dysplasia (Fig. 1G-I). The lesions most closely resembled those referred to in humans as leptomeningeal glioneuronal heterotopias (nodular heterotopias or 'brain warts') (Friede, 1989; Greenfield et al., 2002). Comparison of the histological features of representative lesions from a 36-week-old infant and lesions from an 18.5 dpc *Emx2*$^{-/-}$ mouse, demonstrated the shared findings of frontoparietal regional restriction, extension of cells into the overlying meninges, frequent association with a central penetrating blood vessel and a polypoid shape (Fig. 1F,G) (Ellison, 1998; Friede, 1989; Greenfield et al., 2002; Hirano et al., 1992; Iida et al., 1994). Serial sectioning of the lesions confirmed that nearly all the lesions involved the subarachnoid space or leptomeninges. The more differentiated appearance of the human lesions was probably due to the older developmental age of the individual relative to the mouse. Both human and mouse lesions contained NeuN-positive neurons as well as GFAP-expressing glia by immunohistochemical and in situ

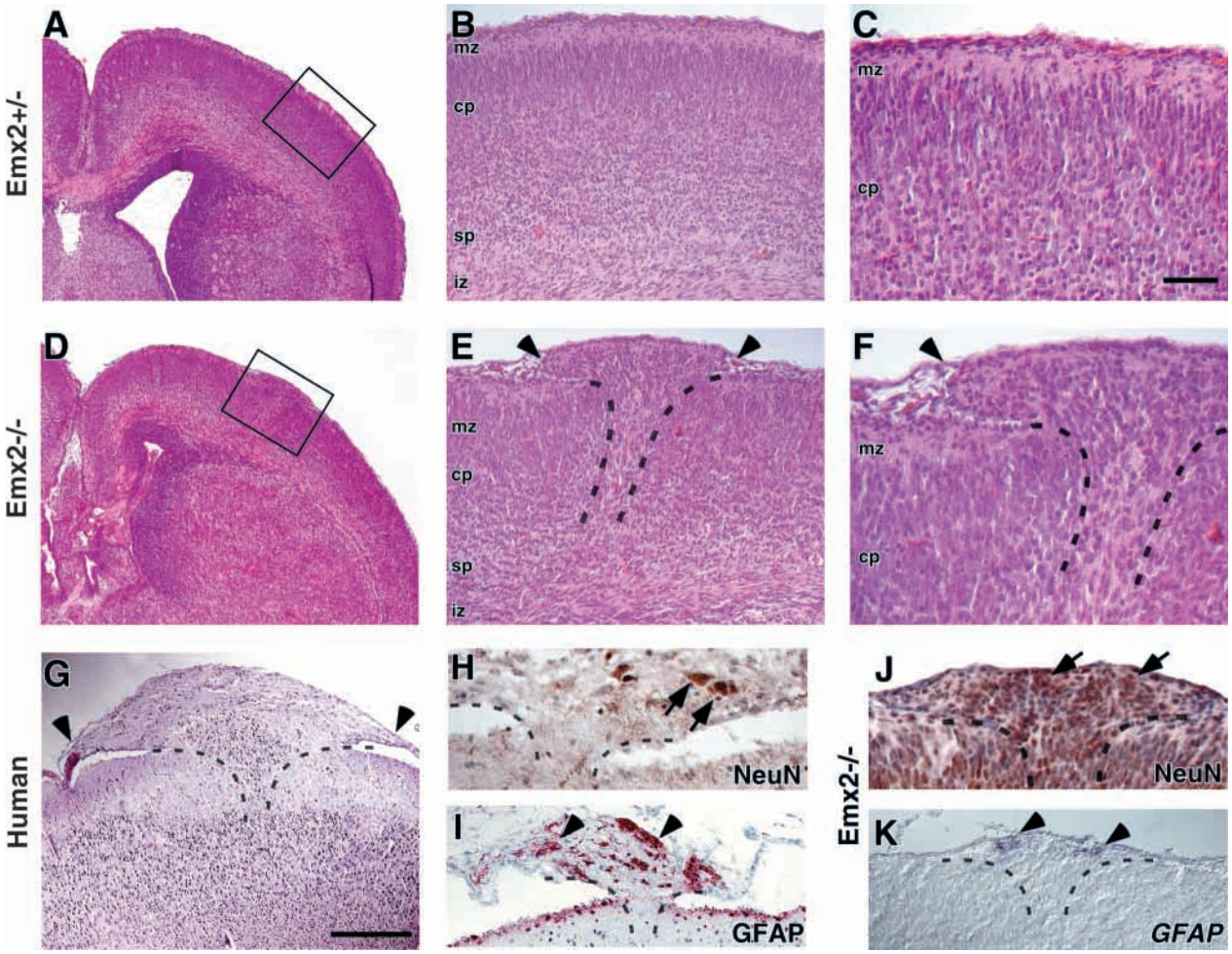

Fig. 1. Marginal zone heterotopias in *Emx2*$^{-/-}$ mice are similar to human heterotopias in individuals with cortical dysplasia. Histological analysis of Hematoxylin and Eosin stained sections from *Emx2*$^{-/-}$ mouse brains demonstrated diffuse changes in the marginal zone with increased cellularity and poor distinction of the marginal zone from the underlying cortical plate proper (D-F). Nodular protrusions of cells into the marginal zone and overlying leptomeninges (arrowheads) were also detected and were frequently associated with focal disorganization of the subplate and full thickness abnormalities of the cortical plate in the same regions (broken line). No such abnormalities were detected in *Emx2*$^{+/-}$ littermates (A-C). Hematoxylin and Eosin stained human leptomeningeal glioneuronal heterotopia from the cortex of a 36-week-old human infant had a similar morphology (G). Immunohistochemical analysis of human and *Emx2*$^{-/-}$ lesions demonstrated the presence of NeuN-positive neurons within both lesions (H,J arrows). Immunohistochemistry in the human (I) and in situ hybridization in the mouse (K) demonstrated GFAP-positive astrocytes within the lesions (arrowheads). mz, marginal zone; cp, cortical plate; sp,subplate; iz, intermediate zone. Scale bar: in G, 200 μm for G; in C, 50 μm for C,F,J,K.

hybridization analysis, confirming shared features within the heterotopic tissue (Fig. 1H-K). We conclude that formation of marginal zone and leptomeningeal glioneuronal heterotopias comprises a novel neuropathological feature in *Emx2*-null mice, and that such lesions are comparable to human LGH.

Loss of *Emx2* function results in strong ectopic expression of *Wnt1* in the dorsomedial telencephalon

The findings above and previous observations indicate critical roles for EMX2 during cortical development and highlight its possible relevance in the understanding of human LGH. In order to investigate mechanisms underlying *Emx2* function, we focused on possible transcriptional targets of EMX2 during brain development. As we have previously proposed, one candidate locus for repression by EMX2 is *Wnt1* (Iler et al., 1995; Rowitch et al., 1997; Rowitch et al., 1998). However, it was unclear whether ectopic expression of *Wnt1* occurred in *Emx2*$^{-/-}$ embryos at times associated with formation of preplate derivatives (~11-12.5 dpc) (Monuki and Walsh, 2001). To investigate this, we performed whole-mount in situ hybridization on wild-type and *Emx2*-null embryos at 12.5 dpc. *Wnt1* is normally expressed along the dorsal midline of the CNS with a rostral limit in the rostral diencephalon (Fig. 2A). In contrast to the wild-type pattern, *Emx2*$^{-/-}$ embryos also showed strong ectopic expression of *Wnt1* in the dorsomedial telencephalon and the rostral diencephalon that peaked at 12.5 dpc (Fig. 2B). Ectopic expression was barely detectable at 14.5 dpc and was absent at 18.5 dpc (data not shown).

A dissected coronal view of a stained *Emx2*-null telencephalon indicated that ectopic *Wnt1* expression was localized both to the cortical hem ventricular zone, which is

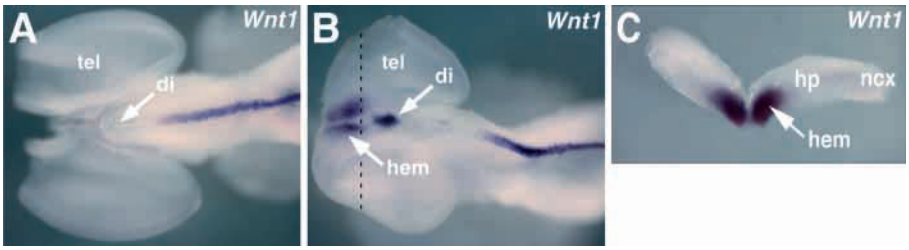

Fig. 2. Ectopic *Wnt1* expression in the cortical hem and cortical ventricular zone of *Emx2*-null mutant mice. Whole-mount in situ hybridization analysis of *Wnt1* expression in 12.5 dpc wild-type embryos showed normal expression of *Wnt1* within the roof plate of the neural tube and extending to the rostral diencephalon (A). In addition to the wild-type pattern, $Emx2^{-/-}$ embryos (B) exhibited strong ectopic expression of *Wnt1* within the dorsomedial telencephalon, cortical hem organizer region and in the diencephalon (arrows). A dissected coronal view (C) of same embryo at the level of the broken line in B showed ectopic *Wnt1* within the cortical hem, hippocampal primordium and cortical ventricular zone (arrow). tel, telencephalon; di, diencephalon; hem, cortical hem; hp, hippocampal primordium; ncx, neocortex.

abnormally small in *Emx2*-null mice (Muzio et al., 2002), as well as the adjacent hippocampal and neocortical VZ (Fig. 2C). These findings confirmed that robust ectopic expression of *Wnt1* occurs in neuroepithelial precursor cells of the cortical hem and medial cortical plate of $Emx2^{-/-}$ mice in a pattern reminiscent of endogenous *Emx2*.

A homeodomain DNA-binding site in the *Wnt1* enhancer regulates Wnt1 expression within the *Emx2* domain

The findings above suggested that EMX2 activity was necessary for repression of *Wnt1* expression in the telencephalon during the time of formation of the preplate and its derivatives. Indeed, previous work has suggested that EMX2 and other homeodomain proteins (e.g. DLX2, MSX1) can directly bind the *Wnt1* enhancer via a core-binding site (HBS1) (Iler et al., 1995) that is conserved at the pufferfish *wnt1* locus (Rowitch et al., 1998). Additionally, mutation or deletion of HBS1 resulted in ectopic expression of a reporter transgene in the dorsomedial telencephalon (Iler et al., 1995; Rowitch et al., 1998). To test whether ectopic expression of *Wnt1* was sufficient to recapitulate aspects of the *Emx2* cortical phenotype, we first over expressed *Wnt1* using a strong 1.1 kb enhancer fragment that carried a mutation of HBS1 (Fig. 3A) (Iler et al., 1995). However, preliminary analysis indicated that this resulted in defects of neural tube closure in the midbrain region (data not shown), most probably because of the proliferative effects of *Wnt1* and excessive neural overgrowth, as previously observed (Danielian and McMahon, 1996; Dickinson et al., 1994). To address this problem, we characterized the expression of additional reporter transgene constructs with various deletions within the 1.1 kb *Wnt1* enhancer (Rowitch et al., 1998). An enhancer fragment with deletion of HBS1 and 300 bp of surrounding sequence (Fig. 3B, referred to hereafter as ΔHBS) drove strong ectopic expression of the *lacZ* reporter gene in the telencephalon (Fig. 4A-D). Yet, in comparison to previous results with the full-length 1.1 kb enhancer (Iler et al., 1995; Rowitch et al., 1998), β-galactosidase activity was relatively weak in regions of endogenous *Wnt1* expression, such as the roofplate and midbrain-hindbrain region (Fig. 4B). Ectopic telencephalic expression of the ΔHBS reporter transgene was first detectable at ~10-somites (8.75-9 dpc, Fig. 4A) and was maintained at least until 16.5 dpc (Fig. 4C). These studies suggested that ΔHBS DNA regulatory sequences were suitable to drive transgene expression within the dorsomedial telencephalon while avoiding adverse consequences of *Wnt1* overexpression in its endogenous domain (e.g. neural tube defects).

As shown (Fig. 3C), the *Wnt1* locus was cloned upstream of the ΔHBS enhancer, and independent founder transgenic mice were generated for analysis at embryonic (13.5 dpc, n=1), fetal (18.5 dpc, n=2) and neonatal (P1, n=1) stages (hereafter referred to as *Wnt1*-Tg). Although 10-40% of litters genotyped antenatally carried the transgene, less than 10% of postnatal animals were transgenic. Only one stable line proved viable past one month of age and this was used primarily to generate embryos for analysis at 12.5 dpc (n=4). Although these data indicate that expression of *Wnt1*-ΔHBS-transgene caused lethality after birth, no transgenic animals showed neural tube defects.

Analysis of *Wnt1* mRNA transcripts in situ showed that the expression pattern of the *Wnt1*-ΔHBS-transgene in the dorsomedial telencephalon of animals derived from the stable line closely resembled that of *Emx2* (compare Fig. 4E with Fig. 4G,H). At 12.5 dpc, the transgene was most strongly expressed in the ventricular zone neuroepithelium of the cortical hem region adjacent to the choroid plexus primordium (cpp, Fig. 4G). Moreover, we observed an apparent gradient of transgene expression within the adjacent cortical ventricular zone that was stronger in dorsomedial regions than dorsolateral regions of the telencephalon (Fig. 4G,H) similar to the expression gradient of endogenous *Emx2* (Fig. 4E). Ectopic expression was reduced but maintained within the medial most cortical regions within the ventricular zone and subplate at 18.5 dpc (data not shown). Even so, *Wnt1* expression was not seen within the marginal zone at early or late stages in the transgenic mice.

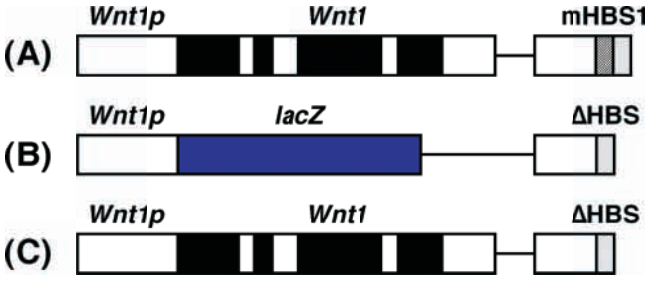

Fig. 3. *Wnt1* reporter and misexpression transgene constructs. (A) Schematic of *Wnt1* promoter (*Wnt1p*) and gene under control of the strong 1.1 kb enhancer that contains a point mutation in HBS1 (mHBS1) (Iler et al., 1995). (B) The *lacZ* reporter transgene driven by ΔHBS1 regulatory sequences. (C) The *Wnt1*-Tg misexpression transgene driven by ΔHBS1 regulatory sequences. For descriptions, see text.

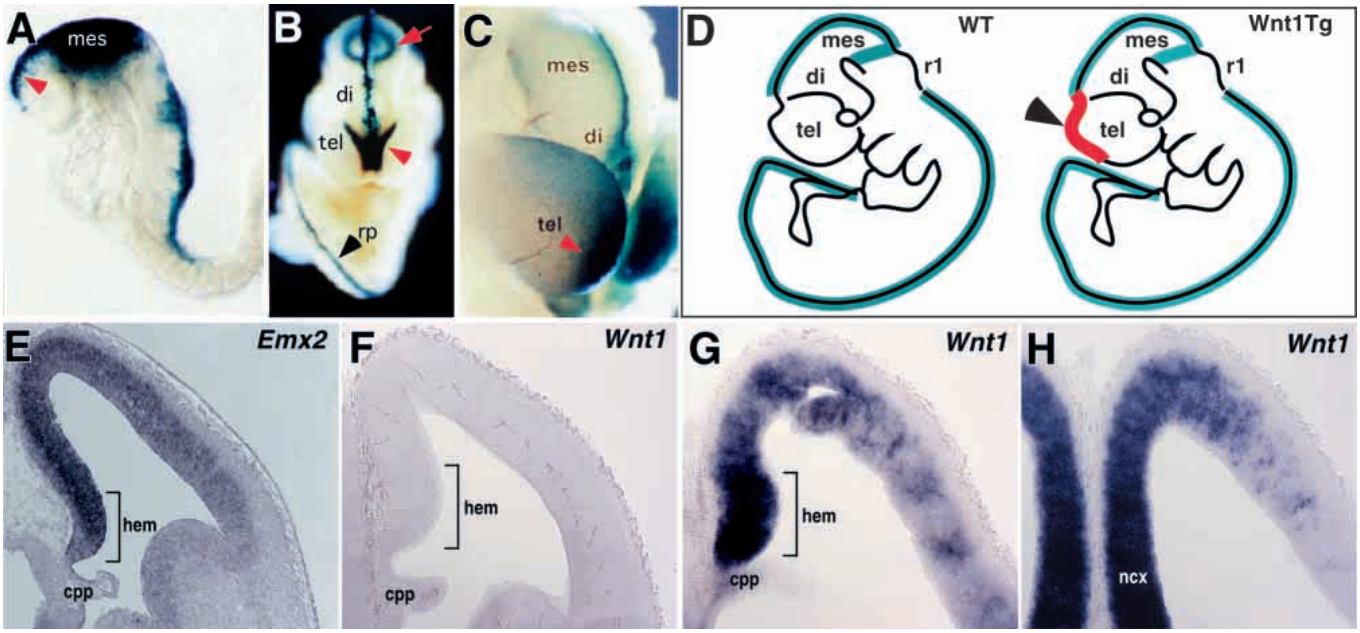

Fig. 4. Deletion of the HBS1 site in the 1.1 kb 3′ Wnt1 enhancer element results in ectopic expression of transgenes within the telencephalon. (A-C) Whole-mount histochemical analysis of β-galactosidase expression in *Wnt1-lacZ*-ΔHBS1 transgenic mice at 8.75 (A), 10.5 (B) and 16.5 dpc (C). Ectopic expression was highest in the dorsomedial telencephalon (A-C, red arrowheads) with a gradient of expression diminishing ventrally (C). Ectopic expression was also noted in the rostral diencephalon (B,C). Expression of the transgene was relatively weak in the endogenous *Wnt1* expression domain, including the roof plate and mid-hindbrain junction compared with previous analyses of the full-length *Wnt1* 1.1 kb enhancer (Iler et al., 1995; Rowitch et al., 1998) (B, red arrow). (D) Summary of the wild-type endogenous *Wnt1* expression pattern (blue) and ectopic telencephalic expression (red), as observed in *Wnt1*-Tg and *Emx2*$^{-/-}$ transgenic mice. (E-H) Representative results of in situ hybridization analysis of coronal sections from 12.5 dpc control (n=4) (E,F) and *Wnt1*-Tg (n=4) (G,H) telencephalon demonstrated a striking gradient of ectopic *Wnt1* expression in the cortical hem and cortical ventricular zone of the transgenic (G,H) mice in a pattern recapitulating that of endogenous *Emx2* (E). mes, mesencephalon; di, diencephalon; tel, telencephalon; rp, roof plate of neural tube; r1, rhombomere 1; hem, cortical hem; cpp, choroid plexus primordium; ncx, neocortical ventricular zone.

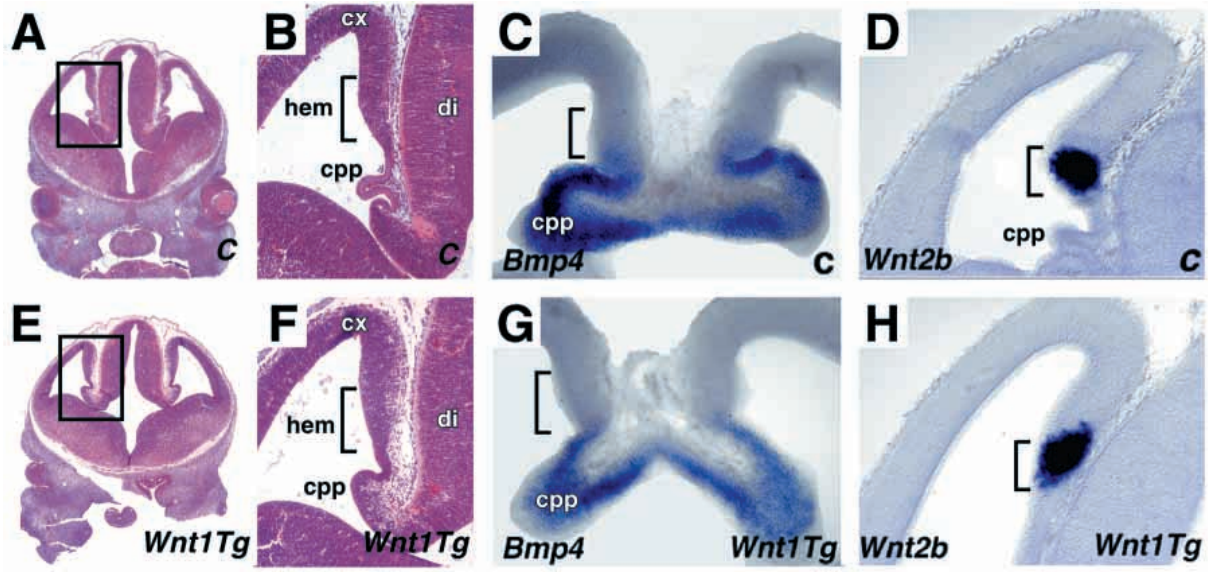

Fig. 5. Absence of cortical hem patterning defects in *Wnt1*-Tg mice. Hematoxylin and Eosin staining of control littermates (A,B) and *Wnt1*-Tg mice (E,F) at 12.5 dpc showed only mild disorganization of the cortical ventricular zone and cortical hem. Poor definition of the choroid plexus primordium from the surrounding thalamic eminence was noted. Expression of the cortical hem marker *Wnt2b* (D,H) and the roofplate organizer marker *Bmp4* (C,G) were normal in *Wnt1*-Tg mice by in situ hybridization at 12.5 dpc. cpp, choroid plexus primordium; hem, cortical hem; cp, cortical ventricular zone; di, diencephalon. The bracket delineates the cortical hem region. All sections are coronal.

Ectopic expression of *Wnt1* in the dorsomedial telencephalon does not alter cortical patterning

During forebrain development, organizing centers are important determinants of regional specialization. One such organizing center, termed the cortical hem, is markedly reduced in size in *Emx2*-null mice (Muzio et al., 2002). The finding of ectopic expression of *Wnt1* in the cortical hem of *Wnt1*-Tg mice initially suggested the possibility of early patterning abnormalities. However, histopathological analysis of *Wnt1*-Tg and littermate controls at 12.5 dpc showed only subtle differences in the delineation of the choroid plexus primordium from the adjacent thalamic eminence and cortical hem in transgenic animals, and no reduction in size of the cortical hem (Fig. 5E,F). The most medial part of the cortical primordium (hippocampal anlagen) was also examined just before birth with a range of hippocampal field-specific gene expression markers as previously described (Tole et al., 2000). Although this region was sometimes significantly larger than normal in *Wnt1*-Tg mice, hippocampal field specification was comparable with that in littermate controls (data not shown).

Analysis of cortical hem markers at 12.5 dpc revealed normal *Emx2* expression within the cortical hem and adjacent cortical ventricular zone in *Wnt1*-Tg animals, including preservation of the characteristic medial to lateral gradient of cortical expression (data not shown). Indeed, expression of numerous markers of the roofplate organizer and cortical hem showed no significant alterations in pattern (e.g. Fig. 5C,D,G,H and data not shown). We conclude that patterning of the telencephalic roofplate organizer and dorsomedial telencephalon is normal despite ectopic expression of *Wnt1*.

Ectopic expression of *Wnt1* results in diffuse abnormalities in the marginal zone and subplate

To investigate phenotypic similarities between *Emx2* null and *Wnt1*-Tg mice further, we focused on development of the marginal zone and subplate, which show a marked reduction in the number of reelin-positive CR cells and Calretinin-positive cells within the MZ and SP of *Emx2*-null mice (Fig. 6A,B) (Mallamaci et al., 2000a; Shinozaki et al., 2002; Yoshida et al., 1997). As shown (Fig. 6C), analysis of the anterolateral cortex of *Wnt1*-Tg mice at 18.5 dpc revealed patchy areas containing markedly reduced numbers of reelin-positive CR cells. Scattered ectopic reelin-positive cells were seen in the cortical plate, marginal zone and subplate of both *Emx2*$^{-/-}$ and *Wnt1*-Tg mice. These cells lacked normal Cajal-Retzius morphology and were extremely small, possibly representing reelin-positive cortical interneurons or abnormally differentiated Cajal-Retzius-like cells (Fig. 6B,C, arrowheads).

Calretinin is a Ca^{2+}-binding protein that is strongly expressed in the cell bodies and processes of Cajal-Retzius cells and other neurons within the preplate, marginal zone and subplate. As shown (Fig. 6E,F), immunohistochemical analysis revealed a marked reduction in the number and complexity of Calretinin-positive CR cells and neuronal processes within the marginal zone and subplate of the *Emx2*$^{-/-}$ and *Wnt1*-Tg mice at 18.5 dpc (Fig. 6D-F). Although abnormal reelin and calretinin expression was observed diffusely throughout the

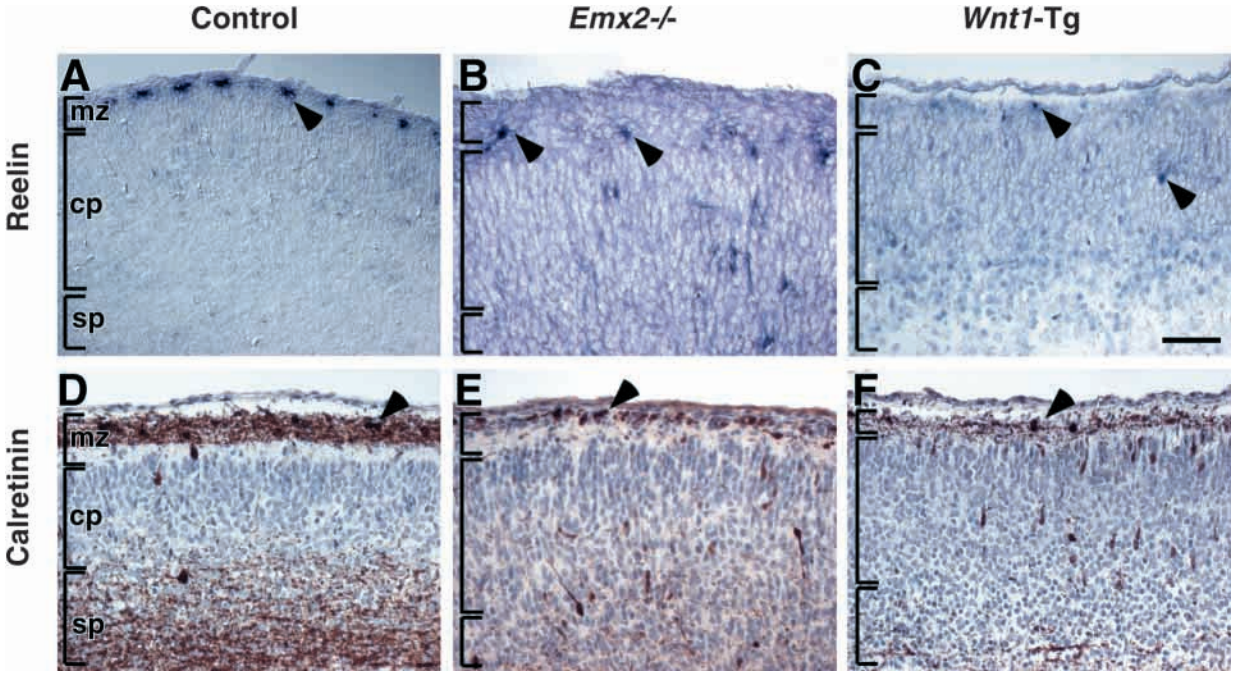

Fig. 6. *Emx2*$^{-/-}$ and *Wnt1*-Tg mice exhibit diffuse abnormalities of the marginal zone and subplate at 18.5 dpc. (A-C) In situ hybridization analysis shows a reduction in the number of reelin-positive Cajal-Retzius (CR) cells in the anterior regions of the marginal zone and subplate of *Emx2*$^{-/-}$ (B) and a patchy reduction in *Wnt1*-Tg (C) mice when compared with control wild-type littermates (A) at 18.5 dpc. Note, that scattered reelin-positive cells (arrowheads) with small cell morphology persisted at 18.5 dpc but few cells with clear CR type morphology were observed in anterior dorsolateral cortex. (D-F) Immunohistochemistry demonstrating a marked reduction in the number and complexity of calretinin-positive cells and CR cells (arrowheads), and their processes within the marginal zone and subplate of *Emx2*$^{-/-}$ and *Wnt1*-Tg mice. cp, cortical plate proper (layers II-VI); mz, marginal zone (layer I); sp, subplate. Scale bar: 50 μm.

anterior to mid-cortex of both *Emx2*$^{-/-}$ and *Wnt1*-Tg mice, the changes appeared more severe and widespread in *Emx2*$^{-/-}$ mice than in *Wnt1*-Tg mice at 18.5 dpc. Medial and posterior regions were relatively less affected in both mice. Together, these findings suggest that ectopic expression of *Wnt1* in the telencephalon under control of the ΔHBS1 regulatory element produces abnormalities in preplate derived structures (MZ and SP) that are remarkably similar to those seen in *Emx2*$^{-/-}$ mice (Mallamaci et al., 2000a; Shinozaki et al., 2002).

Wnt1-Tg mice develop leptomeningeal glioneuronal heterotopias

Having observed abnormal formation of preplate derivatives, we next examined *Wnt1*-Tg mice for other features of cortical dysplasia. Histological analysis at 18.5 dpc revealed that *Wnt1*-Tg founder mice had well-formed LGH (Fig. 7A-C). Approximately two to four lesions/brain were observed in two out of three independent founder animals examined at 18.5dpc-PN1. Moreover, the lesions occurred in an anterior and dorsal distribution similar to that seen in *Emx2*$^{-/-}$ mice. Immunohistochemistry confirmed the presence of numerous NeuN-positive neurons (Fig. 7D), as well as calretinin-positive neurons and processes (Fig. 7E,F) within the lesions from both mice.

The presence of cells within the leptomeninges suggested that the mechanism of heterotopia formation might involve radial migration of cells through a focal defect of the glia limitans and along misplaced radial glial foot processes. Analysis of *Emx2*$^{-/-}$ and *Wnt1*-Tg mice at 18.5 dpc showed that the lesions in both cases were associated with well-defined focal gap defects in the nestin positive radial glial foot processes forming the glia limitans (Fig. 7G-I). In addition, Nestin positive radial glial processes were noted to extend through the SP and MZ and into the substance of the heterotopias in an abnormal fashion and without formation of organized foot processes at the surface of the lesions (Fig. 7H,I, white arrowheads). The glia limitans in regions away from the lesions in both *Wnt1*-Tg and *Emx2*$^{-/-}$ mice had a normal appearance (Fig. 7, red arrowheads). This novel finding suggests that radial glia may provide the substrate for abnormal continued radial migration of cells through outer layers of the marginal zone and into the leptomeninges.

Ectopic expression of *Wnt1* causes heterotopias and abnormalities of preplate derivatives early in cortical development

Ectopic *Wnt1* expression in the telencephalon of *Emx2*$^{-/-}$ mice showed a peak at 12.5 dpc, and was undetectable by 18.5 dpc (Fig. 2 and data not shown). This suggested that heterotopias might form contemporaneously with splitting of the preplate

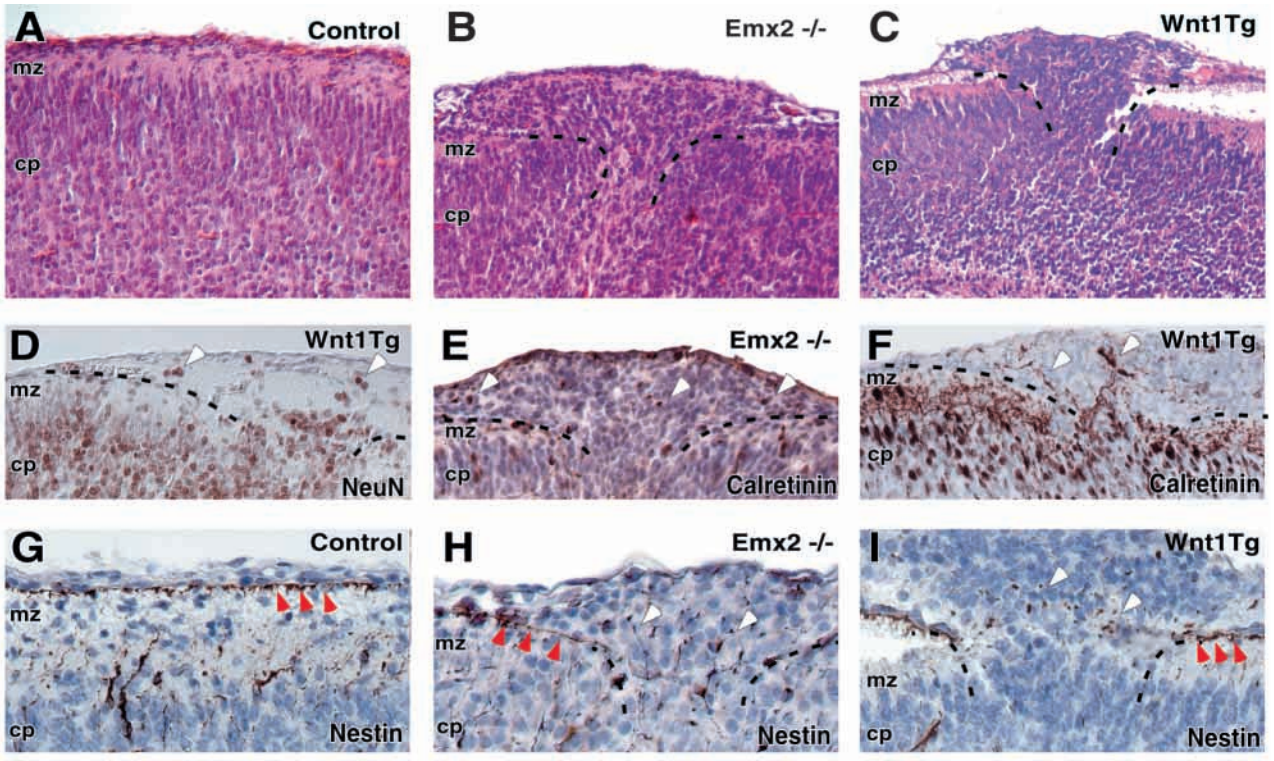

Fig. 7. Ectopic *Wnt1* expression in the telencephalon produced heterotopias identical to those seen in *Emx2*$^{-/-}$ mice. (A-C) Representative histological analysis (Hematoxylin and Eosin staining) of the 18.5 dpc cortex of *Emx2*$^{-/-}$ (*n*=6) (B) and *Wnt1*-Tg (*n*=2) (C) mice demonstrated heterotopias that were identical in morphological appearance. A further *Wnt1*-Tg founder so analyzed at P1 also showed heterotopias. Lesions in the *Wnt1*-Tg mice contained NeuN-positive neurons (D, arrowheads) as well as calretinin-positive neuronal cell bodies and processes (F, arrowheads) similar to those seen in *Emx2*$^{-/-}$ mice (E, arrowheads). Immunohistochemistry for Nestin demonstrated focal defects in the subpial glia limitans in direct association with heterotopias of *Emx2*$^{-/-}$ (H) and *Wnt1*-Tg (I) mice, which were not seen in wild-type littermates (G). Staining was normal away from the lesions (red arrowheads). Radial glial processes (white arrowheads) were seen to extend directly into the lesions from the underlying cortical plate but did not form a glia limitans (H,I). mz, marginal zone; cp, cortical plate.

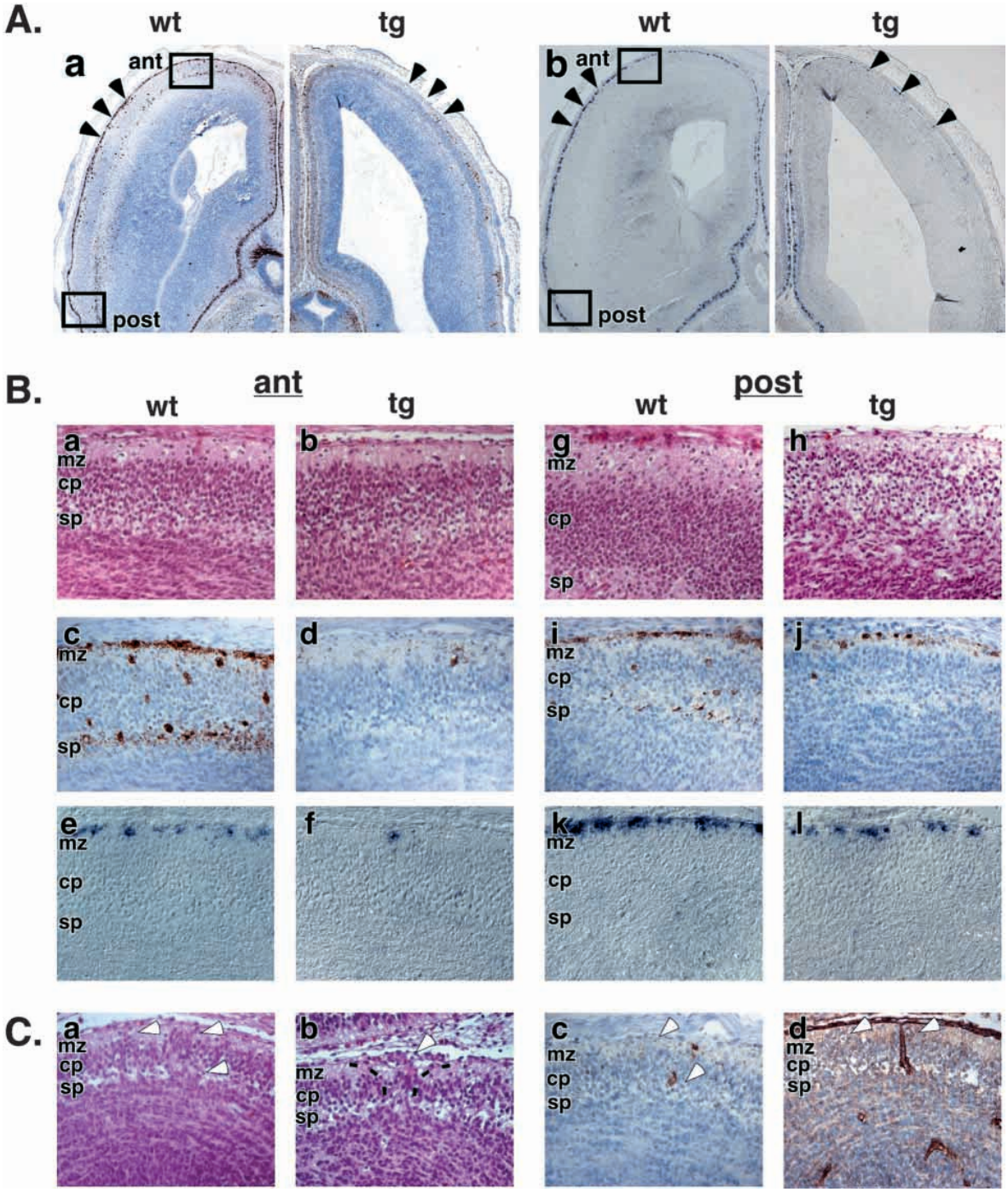

Fig. 8. Early heterotopias and defects of preplate derivatives in *Wnt1*-Tg mice. (A, part a) Immunohistochemical staining in wild-type and *Wnt1*-Tg mice at 13.5 dpc showed a marked reduction in the number of Calretinin-positive cells and processes (arrowheads) within the marginal zone and subplate of *Wnt1*-Tg transgenic mice in anterior and lateral neocortex. (A, part b) In situ hybridization for reelin mRNA transcripts demonstrated a similar reduction of Cajal-Retzius cells in the same distribution (arrowheads). Note that the cortex of *Wnt1*-Tg mice also exhibited a marked increase in size. (All sections cut in the horizontal plane; boxed regions correspond to sections photographed in B, parts a-l). (B, parts a-f) Analysis of anterior regions of the neocortex of *Wnt1* transgenic animals showed a loss of calretinin-positive processes and cells within the marginal zone and subplate as well as loss of reelin-positive Cajal-Retzius cells within the marginal zone at 13.5 dpc. (B, parts g-l) Reductions in cell number and processes were not as evident in more posterior regions of neocortex. No significant abnormalities of cortical plate architecture were detected in *Wnt1*-Tg animals by Hematoxylin and Eosin morphological evaluation (a,b,g,h). (C, parts a,b) Heterotopias were detected within the marginal zone and leptomeninges at 13.5 dpc in *Wnt1*-Tg mice (b) by Hematoxylin and Eosin staining (arrowheads, broken lines) and were associated with defects in the subplate morphology. (C, part c) Marginal zone heterotopias were not consistently associated with an absence of calretinin-positive cells as some positive cells were detected in close proximity to an overlying heterotopia (arrowheads). (C, part d) Defects in the laminin-positive pial basement membrane were not detected in association with or distant from *Wnt1*-Tg heterotopias (white arrowheads), or at any other location by immunohistochemistry at 13.5 dpc. mz, marginal zone; cp, cortical plate; sp,subplate.

and the onset of radial neuronal migration. To assess timing further, we analyzed effects of ectopic *Wnt1* expression in an additional transgenic founder at 13.5 dpc. Similar to findings at 18.5 dpc, we observed a severe reduction in the number of calretinin- and reelin-positive cells and processes within the anterior to mid-cortical fields in the marginal zone and subplate of 13.5 dpc *Wnt1*-Tg animals (Fig. 8A,B). Posterior and medial cortical regions showed a relatively normal number and distribution of calretinin and reelin-positive cells within the marginal zone although the processes of cells within the subplate were variably reduced within these areas (Fig. 8B, parts i-l).

In addition, small MZH (Fig. 8C, part a, arrowheads) and LGH (Fig. 8C, part b, arrowhead) were readily discernable on Hematoxylin and Eosin staining in a distribution that correlated well with the abnormal areas of calretinin and reelin staining (Fig. 8C, parts a,b) in the most anterolateral portions of the telencephalon. The morphology at this early stage was strikingly similar to that seen in 18.5 dpc *Emx2*$^{-/-}$ and *Wnt1*-Tg mice (Fig. 8C, parts a,b). Marginal zone heterotopias were not exclusively associated with an absence of calretinin-positive cells as several such cells (Fig. 8C, part c, lower arrowheads) were detected by immunohistochemistry immediately underlying a MZH (Fig. 8C, part c, upper arrowhead). In addition, no abnormalities were detected within the laminin-containing pial basement membrane (Fig. 8C, part d) by IHC or Nestin-positive processes of radial glia (data not shown) either in association with or distant from marginal zone heterotopias. These observations suggest that ectopic *Wnt1* expression adversely affects development of preplate-derived calretinin-positive cell types at ~12.5-13.5 dpc at the onset of cortical plate formation and are further confirmation of phenotypic similarities between *Emx2*$^{-/-}$ and *Wnt1*-Tg mice.

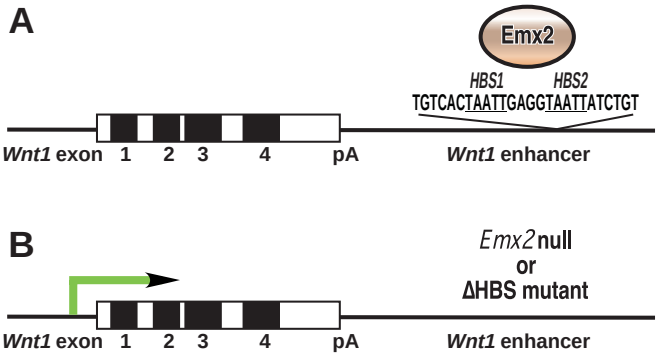

Fig. 9. EMX2 is a direct repressor of *Wnt1* in the developing telencephalon. (A) Model for normal regulation of *Wnt1* in the embryonic forebrain. Previous work indicates that EMX2 directly binds HBS sequences located in the 3′ enhancer region (Danielian and McMahon, 1996; Iler et al., 1995). We speculate that EMX2 represses *Wnt1* expression via local effects on activating transcription factors that directly bind *Wnt1* cis-acting regulatory sequences or via long-range effects. (B) In the absence of *Emx2* function or intact HBS DNA-binding sequences, *Wnt1* is ectopically expressed in the developing telencephalon (green arrow), causing abnormalities of marginal zone and subplate development in the superficial cerebral cortex. Our data do not rule out that interactions of other homeodomain factors (e.g. MSX1) with EMX2 at the HBS sequences may also be necessary for repression of *Wnt1* expression in the forebrain.

DISCUSSION

Emx2$^{-/-}$ mice comprise a novel mouse model of human heterotopias

Loss of *Emx2* function results in cortical dysplasia including defects in cortical lamination and tangential cell migration in the mouse (Mallamaci et al., 2000a; Shinozaki et al., 2002; Tole et al., 2000). We have found that an additional feature of cortical dysplasia in *Emx2*$^{-/-}$ mice is the frequent occurrence of MZH and LGH. We detected MZH/LGH in six out of six *Emx2*$^{-/-}$ mice analyzed, but not *Emx2*$^{+/-}$ littermates or wild-type controls. These heterotopias are a focal type of dysplasia of cortical layer I and are probably a more severe manifestation of the previously described marginal zone abnormalities in *Emx2*$^{-/-}$ mice (Mallamaci et al., 2000a; Shinozaki et al., 2002). The finding of extension of the heterotopias into the leptomeninges might have gone unnoticed in previous analyses of *Emx2*-null mice because only two to six of these relatively subtle neuropathological lesions are detectable per brain. Moreover, varied penetrance of the phenotype might occur on different genetic backgrounds.

Because the heterotopias in *Emx2*$^{-/-}$ mice at late stages are histopathologically identical to MZH and LGH found in humans with cortical dysplasia, we propose that *Emx2*$^{-/-}$ mice may be useful as a model to better understand this disorder. Our analysis indicates that both human and mouse lesions have a morphology suggestive of overmigration of radially migrating cells into the marginal zone and leptomeninges. In addition, the cortical plate below the lesion is commonly disrupted into deeper layers, sometimes through to the subplate or subcortical regions. Although we were not able to obtain early human lesions to confirm a similar time of onset to the mouse (i.e. prior to 13.5 dpc), mature large heterotopias have been histologically identified in human fetal autopsy specimens as early as 18 and 20 weeks gestation, implying that their onset could coincide with that of the mouse upon evaluation of earlier stage human embryos (Iida et al., 1994).

As MZH and LGH are seen in association with a wide range of CNS disorders and functional mutations in mice and humans, the genetic basis of these lesions is likely to be complex (Costa et al., 2001; Halfter et al., 2002; Moore et al., 2002). Although *EMX2* and *WNT1* might play a direct role in human heterotopia formation or subplate abnormalities, no such role has been described previously in the literature. Alterations of Wnt pathway signaling have been described within human cortical resections containing focal intra-cortical dysplasias, but the presence of heterotopias was not addressed in this study (Cotter et al., 1999). Loss-of-function mutations in *EMX2* have been described in individuals with the radiological diagnosis of schizencephaly, a rare cortical dysplasia syndrome (Brunelli et al., 1996; Faiella et al., 1997; Granata et al., 1997). However, further work will be required to determine whether *EMX2* loss in these individuals is associated with heterotopias or MZ/SP abnormalities as the neuroimaging techniques used to identify the individuals lacked the resolution to detect such microscopic lesions and the neuropathological findings in these cases have not been described.

Emx2 function is required for repression of *Wnt1* within the dorsomedial telencephalon

Several lines of evidence support the proposal that EMX2

is a direct repressor of *Wnt1* expression during normal development of the telencephalon. We have previously shown that EMX2 binds the highly conserved HBS1-binding site in the *Wnt1* enhancer, and that mutation or deletion of HBS1 results in ectopic reporter transgene expression in the dorsomedial telencephalon (Iler et al., 1995; Rowitch et al., 1998). Second, Yoshida et al. (Yoshida et al., 1997) have previously noted weak ectopic telencephalic expression of *Wnt1* in *Emx2*$^{-/-}$ mice at 10.5 dpc. By contrast, our results establish robust expression of *Wnt1* in the cortical hem organizer region and dorsal cortical ventricular zone at 12.5 dpc, extending the observations of Yoshida et al. (Yoshida et al., 1997) and raising the possibility of phenotypic consequences of ectopic *Wnt1* expression at a critical stage in telencephalic development. Indeed, we confirmed by histological and expression analysis that the cortex of *Wnt1*-Tg mice was a partial phenocopy of *Emx2*$^{-/-}$ cortical dysplasia, sharing profound abnormalities of the preplate and their derivative cell types. Finally, we identified LGH in the cortex of *Emx2*$^{-/-}$ mice and verified that precise ectopic expression of *Wnt1* within the *Emx2* domain was sufficient for LGH formation. Together, these findings strongly support a model in which EMX2 directly binds the *Wnt1* enhancer and represses expression of *Wnt1* in the developing telencephalon (Fig. 8).

Previous work has shown that EMX2 regulates several distinct aspects of cortical development. First, EMX2 activity regulates proliferation of neural precursors of the developing telencephalon and neural stem cells (Galli et al., 2002; Heins et al., 2001), which may account in part for the small size of the cortex and cortical hem in the *Emx2*-null mice. Additionally, *Emx2* function is necessary for proper development, but not specification, of the hippocampus (Tole et al., 2000). A similar phenotype is observed in *Wnt3a* mutants (Lee et al., 2000). Thus, one possibility is that EMX2 serves as a 'competence factor' that is necessary for neural precursors to respond to a growth signal, while Wnt3a and other Wnt proteins may act principally as mitogens within the developing telencephalon. We noted that the hippocampus and cortical hem are normally formed in the majority in *Wnt1*-Tg animals, indicating that hypoplasia of these structures in *Emx2*-null mice is unrelated to ectopic expression of *Wnt1*. Indeed, the overall size of *Wnt1*-Tg brain was typically larger than normal (megalencephalic), consistent with the known mitogenic effects of *Wnt1* and β-catenin signaling on a wild-type genetic background (Chenn and Walsh, 2002; Danielian and McMahon, 1996; Dickinson et al., 1994; Megason and McMahon, 2002). Ectopic expression of *Wnt1* alone also did not reproduce the diffuse architectural abnormalities of cortical plate lamination and radial glial morphology present in the cortex of *Emx2*$^{-/-}$ mice, most probably because of variability of gene dosage and/or non-uniform (variegated) expression commonly observed in the analysis of founder transgenic mice. Nevertheless, we observed LGH and abnormal formation of subplate and marginal zone populations in a total of 3/4 (75%) independent transgenic founders. In summary, our results suggest that repression of *Wnt1* by EMX2 is important in its capacity as a determinant of cortical layer I and SP structure and function, but not for its other roles in the regulation of neural precursor proliferation, cortical lamination in layers II-VI or development of the hippocampus.

Factors underlying cortical dysplasia and heterotopia formation in *Emx2*$^{-/-}$ mice

The preplate (PP) forms as the initial wave of neural precursors leaves the ventricular zone and migrates radially to the margin of the developing telencephalon. The PP, MZ and SP then serve as a framework for the subsequent influx of neurons from the ventricular zone, cortical hem and ventral forebrain, potentially along tangential and radial processes of cells that reside in these layers (Parnavelas, 2000). Because ectopic *Wnt1* expression was never detected in the PP or MZ of *Emx2*-null or *Wnt1*-Tg mice, it is likely that it acts principally on the early ventricular zone progenitors of the dorsomedial cortex. It is now known that cells from this region, especially the cortical hem, appear much more migratory than originally assumed. Several reports suggest that neurons from the hem migrate extensively into the adjacent cortical primordium (Meyer et al., 2002; Monuki et al., 2001). As a result, ectopic *Wnt1* is expressed early on in the same progenitor cells that will later migrate into and throughout the neocortex thereby creating the potential for very long-range functional effects.

Although ΔHBS *Wnt1* regulatory sequences drove ectopic expression in the dorsomedial telencephalon commencing at ~10-somites (8.75 dpc), we first observed heterotopias in *Wnt1*-Tg mice at 13.5 dpc, contemporaneous with PP maturation and the earliest formation of the MZ and SP. This finding is consistent with a model in which heterotopias result from defects in the developing MZ and SP or pre-existing defects in the PP. We observed striking and diffuse deficiencies of reelin-expressing CR cells and calretinin-expressing neurons as early as 13.5 dpc in the telencephalon of *Wnt1*-Tg mice. Evidently, Wnt1 signaling inhibits development or initial migration of preplate cells and their processes.

Another feature common to heterotopias of *Emx2*$^{-/-}$ and *Wnt1*-Tg mice is the novel finding of abnormally located processes of radial glia outside the glia limitans and limiting basement membrane of the leptomeninges. Such ectopically placed processes imply the existence of focal defects in the glia limitans and laminin 1-containing subpial basement membrane during the period of radial migration of cells. In fact, mice with an unstable basement membrane, owing to a mutation in laminin-g-1, develop defects in the basement membrane and glia limitans at early stages (10-12 dpc) associated with numerous LGH (Halfter et al., 2002). Although we found defects in the glia limitans/basement membrane complex associated with heterotopias in *Emx2*$^{-/-}$ and *Wnt1*-Tg mice using Nestin immunostaining at late stages (18.5 dpc), we were unable to detect primary defects in the limiting plate in regions overlying MZ heterotopias of *Wnt1*-Tg mice at early stages of radial migration (13.5 dpc) by laminin 1, Nestin or TuJ1 immunostaining (Nestin and TuJ1, data not shown). Together, these data suggest that positioning of the radial glial foot process and formation of the pial basement membrane may be linked to early events in formation of the marginal zone and subsequent leptomeningeal heterotopia formation, but that such defects are not required for the formation of heterotopias within the marginal zone.

EMX2-*Wnt* interactions during forebrain development

Regulatory interactions between Wnt signaling and *Emx2* expression in the telencephalon have recently been described

(Theil et al., 2002). However, we did not observe abnormal expression of *Emx2* in the telencephalon of *Wnt1*-Tg mice (K.L., E.G. and D.R., unpublished), nor would one expect regulation of *Emx2* directed by Wnt1 given their non-overlapping patterns of expression during normal development. Because *Emx2* expression is unaltered in the ΔHBS *Wnt1* transgenic mice in precisely the same regions where we see ectopic *Wnt1* expression, it is evident that EMX2 cannot repress expression from the ΔHBS *Wnt1* enhancer construct. Similar observations apply to analysis of *lacZ* reporter constructs with mutated or deleted HBS sequences (Iler et al., 1995; Rowitch et al., 1998). Although we do not have evidence for direct EMX2 interaction/repression of *Wnt1* at the HBS sequences in vivo, previous work suggests this to be the case. Within the entire 5.5 kb *Wnt1* enhancer, which has been shown to be necessary and sufficient for regulation of *Wnt1* in vivo (Danielian et al., 1997; Echelard et al., 1994), homeodomain proteins have been found to bind only the HBS1 and HBS2 sites located ~5 kb downstream of the *Wnt1* polyA (Iler et al., 1995; Shang et al., 1994). Together, these observations suggest that regulation of *Wnt1* by EMX2 is via direct binding to HBS *cis*-acting regulatory sequences.

Because multiple Wnt genes are expressed in the developing forebrain (e.g. *Wnt2b*, *Wnt3a*, *Wnt5a*, *Wnt 7a*, *Wnt7b*, *Wnt 8a* and *Wnt8b*), it is not obvious why there should be a mechanism for repression of *Wnt1*. It might be important to regulate the total dosage of Wnt protein during development; alternatively, *Wnt1* could have specific signaling effects that do not overlap with other Wnt proteins. In this regard it is interesting to note that ectopic expression of *Wnt1* driven by ΔHBS1 regulatory sequences overlaps that of *Wnt7a* in the cortical ventricular zone (Lee et al., 2000). *Wnt* family members can be divided into functional classes based on downstream signaling via β-catenin: *Wnt1* and *Wnt3a* signal via β-catenin, in contrast to *Wnt5a* and *Wnt7a* (see http://www.stanford.edu/~rnusse/wntwindow.html). Thus, it is possible that ectopic activation of β-catenin signaling per se affects the radial migration of neurons emigrating from the ventricular zone and/or positioning of radial glia. Shinozaki et al. have recently shown requirements for *Emx1/2* function during tangential migration of MGE precursors into the neocortex (Shinozaki et al., 2002). In particular, wild-type MGE precursors failed to migrate into the Emx-null cortex, indicating a non-cell-autonomous defect. Bishop et al. have suggested that Emx activity is critical for afferent and efferent axonal projections in the neocortex (Bishop et al., 2003). Further work will be required to establish whether aberrant expression of β-catenin or WNT1 protein is directly related to abnormal patterns of neural migration, axon pathfinding and preplate development in Emx mutant mice.

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REFERENCES

Alcantara, S., Ruiz, M., D'Arcangelo, G., Ezan, F., de Lecea, L., Curran, T., Sotelo, C. and Soriano, E. (1998). Regional and cellular patterns of reelin mRNA expression in the forebrain of the developing and adult mouse. *J. Neurosci.* **18**, 7779-7799.

Allendoerfer, K. L. and Shatz, C. J. (1994). The subplate, a transient neocortical structure: its role in the development of connections between thalamus and cortex. *Annu. Rev. Neurosci.* **17**, 185-218.

Bishop, K. M., Garel, S., Nakagawa, Y., Rubenstein, J. L. and O'Leary, D. D. (2003). Emx1 and Emx2 cooperate to regulate cortical size, lamination, neuronal differentiation, development of cortical efferents, and thalamocortical pathfinding. *J. Comp. Neurol.* **457**, 345-360.

Boncinelli, E., Gulisano, M. and Broccoli, V. (1993). Emx and Otx homeobox genes in the developing mouse brain. *J. Neurobiol.* **24**, 1356-1366.

Brunelli, S., Faiella, A., Capra, V., Nigro, V., Simeone, A., Cama, A. and Boncinelli, E. (1996). Germline mutations in the homeobox gene EMX2 in patients with severe schizencephaly. *Nat. Genet.* **12**, 94-96.

Chenn, A. and Walsh, C. A. (2002). Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. *Science* **297**, 365-369.

Costa, C., Harding, B. and Copp, A. J. (2001). Neuronal migration defects in the Dreher (Lmx1a) mutant mouse: role of disorders of the glial limiting membrane. *Cereb. Cortex* **11**, 498-505.

Cotter, D., Honavar, M., Lovestone, S., Raymond, L., Kerwin, R., Anderton, B. and Everall, I. (1999). Disturbance of Notch-1 and Wnt signalling proteins in neuroglial balloon cells and abnormal large neurons in focal cortical dysplasia in human cortex. *Acta Neuropathol. (Berl)* **98**, 465-472.

Danielian, P. S. and McMahon, A. P. (1996). Engrailed-1 as a target of the Wnt-1 signalling pathway in vertebrate midbrain development. *Nature* **383**, 332-334.

Danielian, P. S., Echelard, Y., Vassileva, G. and McMahon, A. P. (1997). A 5.5-kb enhancer is both necessary and sufficient for regulation of Wnt-1 transcription in vivo. *Dev. Biol.* **192**, 300-309.

Dickinson, M. E., Krumlauf, R. and McMahon, A. P. (1994). Evidence for a mitogenic effect of Wnt-1 in the developing mammalian central nervous system. *Development* **120**, 1453-1471.

Echelard, Y., Vassileva, G. and McMahon, A. P. (1994). Cis-acting regulatory sequences governing Wnt-1 expression in the developing mouse CNS. *Development* **120**, 2213-2224.

Ellison, D. (1998). *Neuropathology: A Reference Text of CNS Pathology*. London, Chicago, IL: Mosby.

Faiella, A., Brunelli, S., Granata, T., D'Incerti, L., Cardini, R., Lenti, C., Battaglia, G. and Boncinelli, E. (1997). A number of schizencephaly patients including 2 brothers are heterozygous for germline mutations in the homeobox gene EMX2. *Eur. J. Hum. Genet.* **5**, 186-190.

Fairen, A., Morante-Oria, J. and Frassoni, C. (2002). The surface of the developing cerebral cortex: still special cells one century later. *Prog. Brain Res.* **136**, 281-291.

Friede, R. L. (1989). *Developmental Neuropathology*. Berlin, New York: Springer-Verlag.

Furuta, Y., Piston, D. W. and Hogan, B. L. (1997). Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. *Development* **124**, 2203-2212.

Galli, R., Fiocco, R., de Filippis, L., Muzio, L., Gritti, A., Mercurio, S., Broccoli, V., Pellegrini, M., Mallamaci, A. and Vescovi, A. L. (2002). Emx2 regulates the proliferation of stem cells of the adult mammalian central nervous system. *Development* **129**, 1633-1644.

Gleeson, J. G. and Walsh, C. A. (2000). Neuronal migration disorders: from genetic diseases to developmental mechanisms. *Trends Neurosci.* **23**, 352-359.

Granata, T., Farina, L., Faiella, A., Cardini, R., D'Incerti, L., Boncinelli, E. and Battaglia, G. (1997). Familial schizencephaly associated with EMX2 mutation. *Neurology* **48**, 1403-1406.

Greenfield, J. G., Graham, D. I. and Lantos, P. L. (2002). *Greenfield's Neuropathology*. London, New York: Arnold.

Grove, E. A., Tole, S., Limon, J., Yip, L. and Ragsdale, C. W. (1998). The hem of the embryonic cerebral cortex is defined by the expression of multiple Wnt genes and is compromised in Gli3-deficient mice. *Development* **125**, 2315-2325.

Halfter, W., Dong, S., Yip, Y. P., Willem, M. and Mayer, U. (2002). A critical function of the pial basement membrane in cortical histogenesis. *J. Neurosci.* **22**, 6029-6040.

Heins, N., Cremisi, F., Malatesta, P., Gangemi, R. M., Corte, G., Price, J., Goudreau, G., Gruss, P. and Gotz, M. (2001). Emx2 promotes symmetric cell divisions and a multipotential fate in precursors from the cerebral cortex. *Mol. Cell. Neurosci.* **18**, 485-502.

Hirano, S., Houdou, S., Hasegawa, M., Kamei, A. and Takashima, S. (1992). Clinicopathologic studies on leptomeningeal glioneuronal heterotopia in congenital anomalies. *Pediatr. Neurol.* **8**, 441-444.

Iida, K., Hirano, S., Takashima, S. and Miyahara, S. (1994). Developmental study of leptomeningeal glioneuronal heterotopia. *Pediatr. Neurol.* **10**, 295-298.

Iler, N., Rowitch, D. H., Echelard, Y., McMahon, A. P. and Abate-Shen, C. (1995). A single homeodomain binding site restricts spatial expression of Wnt-1 in the developing brain. *Mech. Dev.* **53**, 87-96.

Lavdas, A. A., Grigoriou, M., Pachnis, V. and Parnavelas, J. G. (1999). The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. *J. Neurosci.* **19**, 7881-7888.

Lee, S. M., Tole, S., Grove, E. and McMahon, A. P. (2000). A local Wnt-3a signal is required for development of the mammalian hippocampus. *Development* **127**, 457-467.

Lu, Q. R., Park, J. K., Noll, E., Chan, J. A., Alberta, J., Yuk, D., Alzamora, M. G., Louis, D. N., Stiles, C. D., Rowitch, D. H. et al. (2001). Oligodendrocyte lineage genes (OLIG) as molecular markers for human glial brain tumors. *Proc. Natl. Acad. Sci. USA* **98**, 10851-10856.

Mallamaci, A., Mercurio, S., Muzio, L., Cecchi, C., Pardini, C. L., Gruss, P. and Boncinelli, E. (2000a). The lack of Emx2 causes impairment of Reelin signaling and defects of neuronal migration in the developing cerebral cortex. *J. Neurosci.* **20**, 1109-1118.

Mallamaci, A., Muzio, L., Chan, C. H., Parnavelas, J. and Boncinelli, E. (2000b). Area identity shifts in the early cerebral cortex of Emx2$^{-/-}$ mutant mice. *Nat. Neurosci.* **3**, 679-686.

Megason, S. G. and McMahon, A. P. (2002). A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development* **129**, 2087-2098.

Meyer, G., Soria, J. M., Martinez-Galan, J. R., Martin-Clemente, B. and Fairen, A. (1998). Different origins and developmental histories of transient neurons in the marginal zone of the fetal and neonatal rat cortex. *J. Comp. Neurol.* **397**, 493-518.

Meyer, G., Perez-Garcia, C. G., Abraham, H. and Caput, D. (2002). Expression of p73 and Reelin in the developing human cortex. *J. Neurosci.* **22**, 4973-4986.

Mischel, P. S., Nguyen, L. P. and Vinters, H. V. (1995). Cerebral cortical dysplasia associated with pediatric epilepsy. Review of neuropathologic features and proposal for a grading system. *J. Neuropathol. Exp. Neurol.* **54**, 137-153.

Monuki, E. S., Porter, F. D. and Walsh, C. A. (2001). Patterning of the dorsal telencephalon and cerebral cortex by a roof plate-Lhx2 pathway. *Neuron* **32**, 591-604.

Monuki, E. S. and Walsh, C. A. (2001). Mechanisms of cerebral cortical patterning in mice and humans. *Nat. Neurosci. Suppl.* **4**, 1199-1206.

Moore, S. A., Saito, F., Chen, J., Michele, D. E., Henry, M. D., Messing, A., Cohn, R. D., Ross-Barta, S. E., Westra, S., Williamson, R. A. et al. (2002). Deletion of brain dystroglycan recapitulates aspects of congenital muscular dystrophy. *Nature* **418**, 422-425.

Muzio, L., DiBenedetto, B., Stoykova, A., Boncinelli, E., Gruss, P. and Mallamaci, A. (2002). Emx2 and Pax6 control regionalization of the pre-neuronogenic cortical primordium. *Cereb. Cortex* **12**, 129-139.

Parnavelas, J. G. (2000). The origin and migration of cortical neurones: new vistas. *Trends Neurosci.* **23**, 126-131.

Parr, B. A., Shea, M. J., Vassileva, G. and McMahon, A. P. (1993). Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. *Development* **119**, 247-261.

Pellegrini, M., Mansouri, A., Simeone, A., Boncinelli, E. and Gruss, P. (1996). Dentate gyrus formation requires Emx2. *Development* **122**, 3893-3898.

Rice, D. S. and Curran, T. (1999). Mutant mice with scrambled brains: understanding the signaling pathways that control cell positioning in the CNS. *Genes Dev.* **13**, 2758-2773.

Rowitch, D. H., Danielian, P. S., Lee, S. M., Echelard, Y. and McMahon, A. P. (1997). Cell interactions in patterning the mammalian midbrain. *Cold Spring Harb. Symp. Quant. Biol.* **62**, 535-544.

Rowitch, D. H., Echelard, Y., Danielian, P. S., Gellner, K., Brenner, S. and McMahon, A. P. (1998). Identification of an evolutionarily conserved 110 base-pair cis-acting regulatory sequence that governs Wnt-1 expression in the murine neural plate. *Development* **125**, 2735-2746.

Shang, Z., Ebright, Y. W., Iler, N., Pendergrast, P. S., Echelard, Y., McMahon, A. P., Ebright, R. H. and Abate, C. (1994). DNA affinity cleaving analysis of homeodomain-DNA interaction: identification of homeodomain consensus sites in genomic DNA. *Proc. Natl. Acad. Sci. USA* **91**, 118-122.

Shinozaki, K., Miyagi, T., Yoshida, M., Miyata, T., Ogawa, M., Aizawa, S. and Suda, Y. (2002). Absence of Cajal-Retzius cells and subplate neurons associated with defects of tangential cell migration from ganglionic eminence in Emx1/2 double mutant cerebral cortex. *Development* **129**, 3479-3492.

Super, H., del Rio, J. A., Martinez, A., Perez-Sust, P. and Soriano, E. (2000). Disruption of neuronal migration and radial glia in the developing cerebral cortex following ablation of Cajal-Retzius cells. *Cereb. Cortex* **10**, 602-613.

Theil, T., Aydin, S., Koch, S., Grotewold, L. and Ruther, U. (2002). Wnt and Bmp signalling cooperatively regulate graded Emx2 expression in the dorsal telencephalon. *Development* **129**, 3045-3054.

Tole, S., Goudreau, G., Assimacopoulos, S. and Grove, E. A. (2000). Emx2 is required for growth of the hippocampus but not for hippocampal field specification. *J. Neurosci.* **20**, 2618-2625.

Whiting, J., Marshall, H., Cook, M., Krumlauf, R., Rigby, P. W., Stott, D. and Allemann, R. K. (1991). Multiple spatially specific enhancers are required to reconstruct the pattern of Hox-2.6 gene expression. *Genes Dev.* **5**, 2048-2059.

Xie, Y., Skinner, E., Landry, C., Handley, V., Schonmann, V., Jacobs, E., Fisher, R. and Campagnoni, A. (2002). Influence of the embryonic preplate on the organization of the cerebral cortex: a targeted ablation model. *J. Neurosci.* **22**, 8981-8991.

Yoshida, M., Suda, Y., Matsuo, I., Miyamoto, N., Takeda, N., Kuratani, S. and Aizawa, S. (1997). Emx1 and Emx2 functions in development of dorsal telencephalon. *Development* **124**, 101-111.

Zecevic, N., Milosevic, A., Rakic, S. and Marin-Padilla, M. (1999). Early development and composition of the human primordial plexiform layer: an immunohistochemical study. *J. Comp. Neurol.* **412**, 241-254.