Foxg1: a pleiotropic regulator of telencephalic development

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This thesis is dedicated to my father, Andy Martynoga, 1948-1995

Disclaimer

I (Benjamin Martynoga) performed all of the experiments presented in this thesis unless otherwise clearly stated in the text. No part of this work has been, or is being, submitted for any other degree or qualification

Signed:

Date:

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ABBREVIATIONS

Term	Definition
A/P	Anterior to Posterior
Bmp	Bone Morphogenetic Protein
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CNS	Central Nervous System
D/V	Dorsal to Ventral
ddH_2O	Double distilled, column purified water
Di	Diencephalon
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotides
Dtel	Dorsal Telencephalon
E	Embryonic age (days)
Fgf	Fibroblast Growth Factor
Hh	Hedgehog
IddU	Iododeoxyuridine
LacZ	Beta-Galactosidase
mRNA	Messenger RNA
NEB	New England Biolabs
PCR	Polymerase Chain Reaction
QRT-PCR	Quantified RTPCR
RA	Retinoic Acid
RNA	Ribonucleic Acid
rpm	Rotations per minute
RT	Room Temperature (18-20 °C)
RT-PCR	Reverse Transcriptase PCR
SDS-PAGE	SDS Polyacrylamide Gel Electrophoresis
Shh	Sonic Hedgehog
Tel	Telencephalon
Vtel	Ventral Telencephalon
Wt	Wild-type

Abstract

During embryonic development the telencephalon develops from a simple neuroepithelium to give rise to some of the most complex and multi-functional regions of the adult brain. The molecular mechanisms regulating this dramatic metamorphosis are gradually being elucidated. This thesis focuses on the role of the forkhead box factor Foxg1 in the development of the vertebrate telencephalon. Mice mutant for *Foxg1* exhibit multiple telencephalic defects demonstrative of an important pleiotropic role for Foxg1 in telencephalic organogenesis. The results presented here provide a range of novel insights into the function of Foxg1 at the molecular level and make an important contribution to the understanding of the mechanisms regulating telencephalic development in general.

Severe hypoplasia of the null mutant telencephalon demonstrates that Foxg1 is required for the regulation of telencephalic growth. Experiments described in this thesis provide the most systematic study to date of the mechanisms by which Foxg1 regulates this process. Broadly, the results demonstrate that Foxg1 is required to maintain progenitor cell fate, at the expense of differentiated cell fate, and to maintain the proliferation rate of progenitors. This is consistent with previously published reports. The earliest requirement for Foxg1 in regulating proliferation is shown to be in the rostro-medial telencephalon at embryonic day 10.5 (E10.5). Decreased proliferation in this region is spatially and temporally correlated with a decrease in expression of the putative mitogen Fgf8. This molecular change may also underlie a decrease in the number of apoptotic cells observed in the same region. Following these early, localised, changes in progenitor behaviour, proliferative defects become apparent more globally in the *Foxg1*$^{-/-}$ mutant and unusually high levels of neuronal differentiation are observed. A few days later, at E15.5, numerous cells with astroglial properties are observed in ectopic positions in the mutant telencephalon. Therefore, in addition to regulating the rate of telencephalic proliferation and differentiation, Foxg1 also contributes to the highly regulated process of neural cell type specification.

In addition to telencephalic growth defects, it has been documented that ventral telencephalic cell fates are lost in *Foxg1*$^{-/-}$ embryos. This dramatic phenotype has received remarkably little attention. This thesis demonstrates that all ventral telencephalic lineages, which give rise to the basal ganglia and neuronal and glial constituents of the cerebral cortex, are completely absent in *Foxg1*$^{-/-}$ embryos. By generating chimeric embryos, consisting of a mixture of *Foxg1*$^{-/-}$ and wt cells, new insights are gained into the mode of action of Foxg1 in directing the development of the ventral telencephalon. Most striking of these is the demonstration that *Foxg1* is required cell-autonomously for the specification of ventral telencephalic fate. Even when provided with appropriate developmental signals produced by wild type cells in the chimeric embryo, *Foxg1*$^{-/-}$ cells fail to express ventral markers and default to an identity with molecular characteristics of dorsal telencephalic lineages.

The requirement for Foxg1 to repress ectopic dorsal genes and to activate ventral genes is consistent with a role in regulating the response to ventralising signals. The hypothesis that Foxg1 is required for telencephalic cells to respond appropriately to the ventralising morphogen Shh is advanced. A range of experimental approaches is then taken to test this hypothesis. The hedgehog signalling pathway can be activated in *Foxg1*$^{-/-}$ cells, but this activation cannot be translated into ventral identity. Evidence is provided for a role of Foxg1 in the regulation of the expression and activity of Gli3, the major antagonist of the hedgehog signalling pathway. Genetic removal of *Gli3* on a *Foxg1*$^{-/-}$ background provides an *in vivo* test of the significance of this finding. In *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ telencephalon, some aspects of ventral telencephalic fate are recovered. This demonstrates that Foxg1 is required for ventral fate specification, in part through the antagonism of Gli3 action, consistent with the original hypothesis. However, markers of ventro-medial telencephalic fate are not recovered and ectopic expression of dorsal markers persists in double mutants. From these findings it is argued that *Foxg1* has hedgehog-independent roles in dorso-ventral patterning of the telencephalon. The nature of these functions is still opaque, but elucidating them will provide exciting challenges for future investigators.

CHAPTER 1: INTRODUCTION

Introduction to the telencephalon and its derivatives

The embryonic telencephalon gives rise to the most complex and multi-functional regions of the vertebrate brain. These include the cerebral cortex, the hippocampus and the basal ganglia. It is principally within these brain structures that conscious thoughts are generated, memories are stored, voluntary movements are controlled and emotions are engendered. It appears that the elaboration of these mental faculties is part of what defines our unique existences as human beings. However, it seems clear that none of these brain regions is unique to the primate lineage. Indeed, the basic organisation of the central nervous system (CNS) is remarkably conserved across all living vertebrates. For example, the nervous system of the primitive cephalochordate Amphioxus features an anterior neural structure with some of the molecular and morphological characteristics of the mammalian telencephalon (Toresson et al., 1998). Rather than synthesising *de novo* brain structures, capable of new brain functions, it appears that over evolutionary time, the human lineage has co-opted existing structures. These have then been driven to a dramatic and disproportionate expansion in both size and complexity through the process of natural selection to provide the computing power required for conscious thought and other high level cognitive abilities. The deep conservation of mechanisms involved in CNS development validates the use of a wide range of model organisms from frogs and fish to chickens and mice. Consequently our understanding of the fundamental principles

of the development of the nervous system, including the development of the telencephalon itself, is growing apace.

In humans a huge range of developmental, congenital and neuro-degenerative disorders affecting patterning and neurogenesis in the telencephalon and its derivatives have been identified. Many of these have devastating consequences for the individuals involved. However, in most cases the molecular mechanisms underlying the conditions remain opaque. A thorough understanding of the mechanisms regulating patterning and neuronal generation in the normal brain of model organisms and humans is a pre-requisite if we hope to develop effective, targeted strategies for the prevention and treatment of these neurological disorders. It is also hoped that an understanding of the process involved in controlling brain development will help to improve our comprehension of how the immensely complicated neural networks of the adult brain are connected and how they become functional.

The development of the telencephalon

The telencephalon is a major subdivision of the forebrain or prosencephalon. The forebrain is specified from anterior neural ectoderm during the process of gastrulation (Rallu et al., 2002a; Wilson and Houart, 2004). Early in its development, the forebrain becomes patterned in the anterior-posterior (A/P) axis into caudal/diencephalic (principally giving rise to the thalamus, pre-thalamus and hypothalamus) and rostral/telencephalic territories, with the neural component of the eyes developing from a region at the juncture of these major forebrain divisions. Each transverse forebrain region

is then patterned along the dorsoventral (D/V) axis. Groups of cells in specific D/V and A/P positions then become the progenitors of distinct brain regions and spawn the neural and glial cells that will ultimately populate the subdivisions of the adult brain. Fate mapping and gene expression studies have shown that the fundamental patterning of the forebrain into A/P and D/V domains is fairly well defined at neural plate stages, when the forebrain constitutes a flat sheet of simple neuroepithelium. After neural plate stages, the forebrain undergoes a series of dramatic morphogenetic changes. The lateral edges of the neural plate swell, fold upwards and medially, and eventually meet and fuse at the dorsal midline to form the neural tube. Soon after neural tube closure, rapid proliferation causes the anterior neural tube to swell to form the three primary brain vesicles, the anterior-most of which is the telencephalic vesicle. Extensive growth of the telencephalic vesicle, coupled with dorsal midline invagination then leads to the division of the primary telencephalic vesicle into paired, fluid-filled, cerebral vesicles. The organisation of the telencephalon at this stage of development is essentially maintained through to adulthood, giving rise to the characteristic bilaterally symmetrical cerebral hemispheres of the vertebrate brain. During these extensive morphological re-organizations the A/P and D/V subdivisions of the forebrain are maintained and further refined to ensure the correct cell types are produced in the right place, at the right time.

The signalling centre model of telencephalic development

The progression from a relatively homogeneous neuroepithelium at neural plate stages, to a functional and hugely complex, multi-laminar brain, populated by multiple cells types is a remarkable feat of transformation. Similar to the development of other embryonic

structures, the imposition of early positional identity in the telencephalic anlage appears to be driven by the emergence of discrete groups of cells, which have become known as signalling centres (although they have also been named organizing centres and secondary organizers by various authors), at the margins of the telencephalic territory (reviewed in (Hebert, 2005). Cells contributing to signalling centres are able to influence the fate and behaviour of surrounding cells by the production of extracellular signalling molecules (reviewed in (Echevarria et al., 2003)). Although signals from extra-telencephalic sources almost certainly impact on telencephalic development, experimental embryology and genetic manipulations have demonstrated the presence of just three major signalling centres within the telencephalic territory during early stages of development. As in other developing structures, the range of different signalling factors produced by these signalling centres is relatively small. It therefore appears that there is no simple mapping of a single signalling molecule, from a single source, to the development of a distinct lineage. Rather, cells in the receptive fields of signalling centres receive a range of inputs, which are then spatiotemporally integrated to achieve a distinct readout in terms of cell fate and behaviour. A major mechanism by which signalling factors control the behaviour of receiving cells in a lasting fashion is by altering their gene expression profile. This appears to be achieved by controlling the expression and activity of developmentally regulated transcription factors, which control the expression of specific downstream genes to effect morphogenetic control, by regulating regional patterning, and tissue proliferation, apoptosis and cellular differentiation. The variety of signalling inputs a cell receives will therefore be reflected in its profile of developmental transcription factors expressed, which then co-ordinate gene expression patterns more globally, across the genome. It must be noted that the expression of transcription factors downstream of

signalling factors may alter the response of a cell to other extrinsic inputs and may also lead to the expression of further factors that go on to affect neighbouring cells. Consequently, layer on layer of feedforward, feedback and combinatorial regulatory loops add great complexity to the linear progression from signalling molecule to transcription factor expression to transcriptional change to altered behaviour, and thus facilitate the emergence of hugely complex cellular behaviours from a relatively small number of early signals. Nevertheless, the idea that a combinatorial code of transcription factor expression leads to the unique development of distinct D/V and A/P partitions of nervous system is a powerful one, and one that opens the door to experimental testing of such a model.

Experiments conducted over the last one to two hundred years focussed on the physical manipulation of developing embryos have been crucial in our understanding of developmental systems, in particular the lineages and inductive interactions within and between tissues (reviewed in (Schoenwolf, 2001)). However, it is the marriage of this knowledge with our ever-expanding grasp of the genetic factors controlling embryonic development that has, in recent decades, fuelled our accelerating understanding of the principles of developmental biology at the molecular level. Genetic manipulations which remove, replace or over-represent factors controlling embryonic development provide fascinating insights into the role of these factors in the normal embryo. Returning to telencephalic development and the predominant signalling centre model, one approach to further our understanding is to genetically ablate transcription factors that are expressed in a regulated pattern in the developing telencephalon and, by studying the resultant developmental defects, determine the normal role of that genetic factor (reviewed in

(Zaki et al., 2003)). It is this approach that is taken in this study to improve our understanding of the role of a particular transcription factor, *Foxg1*, in the development of the telencephalon.

Foxg1 is a member of the Forkhead box family of transcriptional regulators

The *Foxg1* gene encodes a protein belonging to a conserved family of proteins typified by the presence of a distinctive 110 amino acid DNA binding domain called a forkhead box, which is highly conserved from *Drosophila* to mammals (reviewed in (Carlsson and Mahlapuu, 2002; Kaufmann and Knochel, 1996)). Interestingly, there is a correlation between anatomical complexity and Forkhead box (Fox) gene number. The genomes of *Saccharomyces* and *Schizosaccharomyces* yeast contain 4 fox genes, *Caenorhabditis* worms have 15, *Drosophila* 17 and *Homo sapiens* 39 (Carlsson and Mahlapuu, 2002; Lee and Frasch, 2004). Due to their ability to bind specific DNA sequences, Forkhead box-containing proteins are thought to function, at least in part, as transcriptional regulators. Experimental and genetic manipulations have demonstrated that Fox genes are required for a multiplicity of functions in both developing and adult organisms across a wide range of phyla. During development many Fox genes have highly specific and developmentally regulated expression patterns and are required for the genesis of a wide range of tissues (Carlsson and Mahlapuu, 2002). For example, Foxh1 is required early in mouse development, during gastrulation, for proper initiation of the A/P axis and for formation of the critical signalling centre known as the node (Hoodless et al., 2001; Yamamoto et al., 2001). By contrast, Foxp2 appears to be required rather later in

development and has been linked to a human disorder called specific language impairment (MacDermot et al., 2005) and is required for proper ultrasonic vocalization in mice (Shu et al., 2005).

Foxg1 was first identified by its homology to the prototypical vertebrate Forkhead box genes of the FoxA subfamily (formerly known as hepatocyte nuclear factors (HNF)) and was originally named *Brain Factor-1* (*BF-1*) (Tao and Lai, 1992). The strong expression of Foxg1 in the telencephalon of mid-gestation mouse embryos gave the first clue that Foxg1 may be important in telencephalic development. Subsequent detailed analyses of the expression of *Foxg1* in mice and other organisms have further clarified this suggestion and are summarised in the following paragraphs.

The expression of Foxg1 during development is evolutionarily conserved

The first appearance of Foxg1 expression in the mouse embryo is at the 1-3 somite stage in the anterior non-neural ectoderm adjacent to the prosencephalic neural plate, cells known as the anterior neural ridge (ANR) (Dou et al., 1999). As somitogenesis proceeds, by the 5-8 somite stage, Foxg1 expression appears in the anterior neural plate and then rapidly spreads throughout the entire rostral portion of the neural plate, the region that will give rise to the telencephalon and its derivatives, and to the anterior neural components of the eye (Dou et al., 1999; Shimamura et al., 1995). During the 9^{th} and 10^{th} embryonic days (E8-E9.5), the anterior neural plate closes to form the telencephalic vesicle, which expresses Foxg1 throughout. Soon after this stage, Foxg1 is lost from cells

in the dorsomedial telencephalic roofplate (Dou et al., 1999; Shimamura et al., 1995). Apart from the roofplate region which gives rise to the cortical hem and choroid plexus epithelium (Currle et al., 2005) and a population of early born Cajal-Retzius neurons which appear between E11 and E12 (Hanashima et al., 2004), Foxg1 appears to be expressed throughout the telencephalon in every cell type during mouse embryonic development. During adulthood, Foxg1 is expressed intensely in components of the hippocampus and in scattered cells of both glial and neuronal morphology throughout the telencephalon-derived structures (Dou et al., 1999).

The primary site of Foxg1 expression in the anterior-most CNS structures is strikingly conserved through vertebrate evolution. Human *Foxg1* transcripts are expressed in adult and foetal telencephalic cells (Murphy et al., 1994). The chicken homolog of *Foxg1* (formerly known as *qin*) is also expressed throughout the early telencephalon (Ahlgren et al., 2003; Chang et al., 1995; Li and Vogt, 1993) and in zebrafish a similar pattern is seen (Toresson et al., 1998). In *Xenopus laevis* (Bourguignon et al., 1998) *Foxg1* (originally *XBF-1*) is expressed in an anterior transverse stripe on the neural plate, which is the forebearer of the telencephalon and the olfactory placode, structures which continue to express Foxg1 later in development. Even *Amphioxus*, which are cephalochordates, thought to represent the closest living group of animals to a common ancestor of the chordates, contain a conserved Foxg1 gene whose expression is localised to cells in the anterior cerebral vesicle, an evolutionary fore-runner of the chordate telencephalon (Toresson et al., 1998). Interestingly, at an even more evolutionary distant level, the closest *Drosophila* orthologues of *Foxg1*, the *sloppy paired* genes (*Slp1/2*), which have extensive homology in both the forkhead box domain and an N-terminal conserved

region (Bourguignon et al., 1998; Lee and Frasch, 2004), are also expressed in anterior structures, including anterior neuroblasts of the Drosophila embryo and are required for development of the head and for aspects of cephalic neurogenesis (Andrioli et al., 2004; Bhat et al., 2000; Urbach and Technau, 2003). In summary, the impressive conservation of *Foxg1* protein structure (Bourguignon et al., 1998; Toresson et al., 1998) and gene expression patterns provide a strong indication that *Foxg1* is likely to play important roles in the formation of anterior structures including the telencephalon. Moreover, these factors suggest that regulatory networks that Foxg1 contributes to may be equally conserved and that helpful insights into Foxg1 function can be contributed by the study of a wide range of experimental organisms from insects to mammals.

Due to its highly specific and conserved expression pattern throughout the telencephalon from early stages of its development, Foxg1 has a unique position as a marker of telencephalic identity. Two basic and very important questions stem from this discovery. Firstly, it is very interesting to consider which signals, from which signalling centres, direct Foxg1 expression to the telencephalon and whether the appearance of Foxg1 expressions co-incides with the appearance of telencephalon character? Secondly it is important to determine whether Foxg1 is required for either the initial specification of telencephalic fate or, if not, whether Foxg1 regulates later aspects of telencephalic development?

The first identification of signals regulating Foxg1 expression came from Shimamura and Rubenstein in 1997 (Shimamura and Rubenstein, 1997). Using anterior explants of early neural plate stage mouse embryos, they demonstrated that surgical removal of the ANR (now considered an important signalling centre for forebrain development (Wilson and Houart, 2004)) prior to the onset of Foxg1 expression in neural ectoderm led to the failure of Foxg1 induction. This suggests that an inductive signal from the ANR directs early Foxg1 expression in the anterior neural plate. Furthermore they showed that ectoderm from more lateral and caudal regions of the embryo were not capable of rescuing Foxg1 expression in ANR excised explants. Thus the inductive signal is specific to the ANR. The authors examined the expression patterns of a range of candidate signalling molecules of the fibroblast growth factor (Fgf) and bone morphogenetic protein (BMP) families. Having fixed upon Fgf8 as the molecule that is expressed specifically in the ANR just prior to Foxg1 expression in the neural plate (Crossley and Martin, 1995), Shimamura and Rubenstein demonstrated that Fgf8-soaked beads were sufficient to induce Foxg1 in ANR-excised explants. Interestingly they went on to show that the ability of Fgf8 to induce Foxg1 was limited to the anterior neural plate; when Fgf8 beads were introduced to more caudal regions of the neural explants, the midbrain marker En2 was induced. Thus, the early rostral neural plate appears to be pre-patterned in some way to be competent to respond to Fgf8 by inducing Foxg1. It therefore follows that the induction of Foxg1 itself may not be the first step in telencephalic induction and other factors already expressed there or active there are pre-requisite for Foxg1 induction. Indeed, a recent paper showed that co-expression of Six3 and constitutively

active Fgf receptors in caudal brain regions facilitated the induction of *Foxg1* in the chick hindbrain (Kobayashi et al., 2002). This suggests that Six3 expression may contribute to the telencephalic pre-pattern that allows Foxg1 induction. Accordingly, the telencephalon and other anterior structures are lost in mouse *Six3*$^{-/-}$ mutants (Lagutin et al., 2003).

In zebrafish the anterior neural border (ANB, the name for the zebrafish ANR) also appears to be required for the induction of a range of telencephalic markers, demonstrating that ANB cells are necessary for telencephalic induction (Houart et al., 1998). However, unlike Fgf8 beads in the mouse, zebrafish ANB cells were able to induce Foxg1 and other telencephalic markers when transplanted to more caudal regions of the neural plate, suggesting that the ANB is sufficient for Foxg1 expression and telencephalic induction in a non cell-autonomous fashion (Houart et al., 2002). This suggests that there may be additional factors in the ANR/ANB required for telencephalic induction, at least in zebrafish embryos. The authors identify a secreted Wnt signalling antagonist, Tlc, as a crucial molecule in this process. Ectopic Tlc can induce Fgf8 and telencephalic markers including Foxg1, whilst knock-down of Tlc function severely impairs telencephalic development (Houart et al., 2002). Assuming this mechanism is conserved, Wnt antagonist such as Tlc may be genetically upstream of Fgf8 in Foxg1 induction and telencephalic induction in general.

The identification of further exogenous signalling molecules that are able to regulate Foxg1 expression came from the realisation that the first telencephalic cells to lose Foxg1 expression (between E9.5 and E10.5 in mouse), in the dorsal midline express various Bmp family members in a complementary pattern to that of Foxg1 (Furuta et al.,

1997). They followed this up by demonstrating that Bmp4 and Bmp2, but not Bmp6 or Bmp7, repress Foxg1 in telencephalic explants that normally maintain high-level Foxg1 expression. It has since been demonstrated in chicks (Ohkubo et al., 2002) and in mice (Panchision and McKay, 2002) that high levels of exogenous Bmp signalling can repress Foxg1 expression. Although it has not been tested directly, it is possible that Bmp ligands expressed laterally and caudally to the anterior neural plate limit Foxg1 expression to the forebrain anlage.

Although the regulation may not be direct, it also appears that the signalling molecule sonic hedgehog (Shh) may also be required for the maintenance, if not the induction of Foxg1 expression. In support of this, Foxg1 expression is initiated in *Shh*$^{-/-}$ mouse mutants, but is lost by E9.5 (Ohkubo et al., 2002).

It therefore appears that Foxg1 expression is, directly or indirectly, under the control of at least three different families of signalling molecule: Fgfs, Bmps and Hhs. Furthermore, these signals emanate from the three distinct signalling centres mentioned above that are thought to exert the greatest influence on telencephalic development. These are the rostrally-positioned ANR, the dorso-medially positioned cortical hem (which also expresses multiple Wnt ligands (Grove et al., 1998) and is at the lateral edge of the neural plate prior to neural tube closure), and ventrally positioned Shh-secreting cells of the prechordal mesendoderm (Shimamura and Rubenstein, 1997). Later in telencephalic development, the activities of these three signalling centres are all found within the telencephalic neuro-epithelium itself. They appear to be inter-dependent and to some extent mutually cross-repressive (Aoto et al., 2002; Kuschel et al., 2003; Ohkubo et al.,

2002; Shimogori et al., 2004) and they continue to influence the development of telencephalic cells in terms of both patterning and growth (recently insightfully reviewed (Hebert, 2005)). The signalling centre model therefore provides an excellent framework from which to study the development of the telencephalon and to help interpret the effects of experimental and genetic manipulations, including the effects of null mutation of developmentally regulated transcription factors such as Foxg1, as is considered in this work.

Foxg1 is not required for telencephalic specification

The construction of a mouse mutant with a targeted null mutation of the *Foxg1* locus (Xuan et al., 1995) provided the direct test for whether Foxg1's very early expression in the telencephalon reflected a requirement for Foxg1 in telencephalic induction. It is readily apparent that the telencephalic vesicle does form in *Foxg1*$^{-/-}$ embryos and at a superficial morphological level cannot be distinguished from wild-type (wt) control embryos during the first half of gestation (Xuan et al., 1995). Thus, Foxg1 is not required for the specification of telencephalic identity as a whole from anterior neural ectoderm. Analysis of the telencephalon of *Foxg1*$^{-/-}$ embryos during the second half of embryonic development has revealed a range of defects in telencephalic development demonstrating that Foxg1 is required for normal development of the telencephalon. Early analyses of the *Foxg1* mutant provide a strong basis for the experiments carried described in this thesis. I will therefore summarise these reports with special regard to the unanswered questions relating to the role of Foxg1, some of which are addressed in this work.

Foxg1$^{-/-}$ *embryos have a hypoplastic telencephalon*

The most striking defect in the *Foxg1*$^{-/-}$ telencephalon towards the end of embryogenesis is its hugely reduced size. *Foxg1*$^{-/-}$ embryos die around the time of birth with cerebral hemispheres 95% smaller than littermate controls (Xuan et al., 1995). Reduced telencephalon size is morphologically evident at E10.5, during a period when the wt telencephalon undergoes dramatic growth. From E10.5 onwards, the *Foxg1*$^{-/-}$ telencephalon cannot match the exuberant growth rate of the wt telencephalon and becomes increasingly hypoplastic.

In order to address the requirement for Foxg1 in regulating telencephalic growth, the authors used pulse-labelling with bromodeoxyuridine (BrdU) as a first step in the examination of precursor proliferation rates and they carried out immunohistochemical labelling to identify the proportion of precursors leaving the cell cycle to terminally differentiate as neurons. Reduced BrdU labelling and increased numbers of cells expressing neuronal markers are consistent with a reduction in proliferation rate and an increase in the rate of terminal differentiation. Thus it was proposed that Foxg1 regulates telencephalic growth by maintaining precursors in an undifferentiated, proliferative state and by maintaining the timely progression through the cell cycle (Xuan et al., 1995); a process that is required to produce the large number of cells required to populate the mature telencephalon (Caviness et al., 1995).

Consistent with the suggestion that murine Foxg1 regulates precursor proliferation and differentiation, Foxg1 is capable of similar activities in non-mammalian vertebrates.

Indeed, the chicken Foxg1 ortholog was first identified as the avian cellular homolog of v-qin, the oncogenic determinant of the avian sarcoma virus 31 (Chang et al., 1995). Both viral and cellular versions of Foxg1 are able to induce oncogenic transformation in chick embryonic fibroblasts and live chickens (Li et al., 1997), consistent with an ability to maintain cells in a rapidly proliferating, de-differentiated state. Some of the effects of ectopic expression of Foxg1 in *Xenopus* embryos are also consistent with a role in promoting proliferation and suppressing neuronal differentiation. In this model high levels of Foxg1 can inhibit endogenous neuronal differentiation and promote neural progenitor fates in both neural and non-neural ectodermal regions (Bourguignon et al., 1998; Hardcastle and Papalopulu, 2000).

There is, therefore, good evidence that Foxg1 may function in an evolutionarily conserved fashion to maintain progenitor identity and promote progenitor proliferation to maintain the growth of the telencephalon. There were, however, several unanswered questions, the answers to which may help to understand, at a more mechanistic level, how Foxg1 promotes telencephalic growth. Some of these questions are as follows:

i) Do *Foxg1*$^{-/-}$ telencephalic cells actually progress through the cell cycle at a reduced rate? Reduced BrdU labelling following a short pulse need not reflect reduced proliferation rate, as discussed in detail in Chapter 3. Although the group that made the *Foxg1* knock-out published evidence in 2002 (after this study had commenced) that *Foxg1*$^{-/-}$ cells do indeed proliferate at a reduced rate (Hanashima et al., 2002), their estimates did not provide kinetic data on

the extent of lengthening of cell cycle time or when reduced proliferation rates are first apparent.

ii) If reduced proliferation rates are confirmed, are they homogeneous, or confined to specific regions? This could give useful clues as to whether reduced proliferation results from failure of exogenous signals from discrete signalling centres, or a direct requirement for Foxg1 in all telencephalic cells to proliferate at the appropriate rate.

iii) When is accelerated neuronal differentiation first apparent and where? Xuan and colleagues showed the presence of an increased number of neurons at E12.5, but they did not look at earlier ages (Xuan et al., 1995). A more thorough analysis of the timing and locality of changes in differentiation rate is also important in determining the causes of these changes.

iv) Is the rate of apoptosis altered in *Foxg1*$^{-/-}$ telencephalon? Cell death must be correctly regulated to ensure proper growth rate of a tissue. This had not been thoroughly analysed in *Foxg1*$^{-/-}$ mouse embryos and a report published during the study suggested that ectopic Foxg1 reduced the rate of apoptosis in chick embryos to fuel increased growth (Ahlgren et al., 2003).

v) Are there any molecular correlates to changes in growth rate in terms of changes in signals produced from telencephalic signalling centres? It had already been determined that some members of the Bmp family are over-expressed in *Foxg1*$^{-/-}$ telencephalon (Dou et al., 1999; Hanashima et al., 2002) and that Shh is not induced in the mutant telencephalon (Huh et al., 1999). The expression of signals from the ANR had not been examined.

These five questions are addressed by the experiments described in Chapter 3. As well as dealing with the answers generated, the discussion section of Chapter 3 summarises our current understanding of the some of the molecular mechanisms through which Foxg1 may regulate telencephalic growth and neurogenesis.

Foxg1$^{-/-}$ embryos lack ventral telencephalon

In addition to the severe growth impairment, the most obvious phenotypic defect of the murine *Foxg1$^{-/-}$* telencephalon is the complete absence of ventral telencephalic structures, the ganglionic eminences (which later give rise to the basal ganglia). Despite two early and rather inconclusive attempts to define why Foxg1 is required for ventral telencephalic development (Huh et al., 1999; Xuan et al., 1995), this glaring phenotype has received remarkably little attention in the decade or so since study of the *Foxg1$^{-/-}$* mice was first published. Work in other species has also focused on Foxg1's role in regulating forebrain neurogenesis, rather than its role in controlling the development of sub-divisions of the telencephalon, such as the ventral telencephalic lineages. Consequently, progress in this area has been extremely limited.

In wt mouse embryos by E11.5, the ganglionic eminences are apparent as distinct dilations in the ventral telencephalic neuroepithelium. These eminences do not form in the *Foxg1$^{-/-}$* telencephalon (Xuan et al., 1995). At a molecular level it was also shown that the expression of transcription factors usually restricted to the ventral telencephalon were absent from the mutant telencephalon from E10.5 onwards and, in their absence, dorsal telencephalic markers are expressed in an expanded domain (Xuan et al., 1995).

The authors of this original study claim that the telencephalon is specified normally and then, due to decreased proliferation, the ventral regions fail to grow at an adequate rate and are lost. However, since they did not look at the telencephalon earlier in development, when ventral telencephalic markers are in place in the wt, their claim that the telencephalon is 'normally specified' was not substantiated. Their explanation also fails to explain why ventral markers are not present (even in a small number of cells) at E10.5 when they describe the onset of proliferative defects. Even at E10.5 ventral *Foxg1*$^{-/-}$ telencephalic cells are still proliferating, as evidenced by their continued uptake of BrdU, albeit at a reduced level. It remained equally possible, and perhaps more likely, that ventral telencephalon was never specified in the *Foxg1*$^{-/-}$ telencephalon. This question is explored in some detail in Chapter 4 of this thesis by the analysis of *Foxg1*$^{-/-}$ embryos and *Foxg1*$^{-/-}$ cells in *Foxg1*$^{-/-}$↔wt chimeras (see below). The issue of ventral specification gained extra pertinence following the failure to replicate the ventral-specific proliferative defects documented by Xuan and colleagues (1995), as discussed in Chapter 4. Consequently, I suggest that Foxg1 is required for the specification of the ventral telencephalon.

This hypothesis was explored further by the generation of *Foxg1*$^{-/-}$↔wt chimeras. The use of chimeric embryos can provide a range of interesting and subtle insights into the analysis of complex gene requirements as follows (this area has also been reviewed recently (Tam and Rossant, 2003)).

1) *The lineage specific requirements for a gene can be assessed by examining which lineages contain mutant cells and whether mutant cells are represented in appropriate numbers.* This data can be difficult to obtain from mutant embryos due to early loss of entire lineages and compensatory effects. A good example of this type of analysis comes from Voiculescu and colleagues, who examined the role of Krox20 in hindbrain lineage assignment (Voiculescu et al., 2001). In the context of Foxg1, consideration of which telencephalic structures *Foxg1*$^{-/-}$ cells contribute to allows assessment of which lineages Foxg1 is required for.
2) *Determination of cell-autonomy and non-cell-autonomy of gene function.* A gene can function directly, only in cells expressing that gene (cell-autonomously) or it can also exert an action on other cells that do or do not express the gene (non-cell-autonomously). A cell autonomous role for a gene means that mutant cells maintain their mutant phenotype in any cellular context. In development this often suggests that mutant cells cannot respond to extrinsic developmental cues. Non-cell-autonomy is inferred when mutant cells are either phenotypically rescued by surrounding wt cells or, conversely, they impose a mutant phenotype on their genotypically wildtype neighbours. This issue is particularly pertinent to the study of a transcription factor such as Foxg1. The factor may be required cell autonomously, for example in the transcriptional readout of incoming developmental cues, or non-cell autonomously if the factor is required to drive or repress the transcription of a

molecule designed to influence the fate of neighbouring cells. Since mutant embryos lack gene function in all cells, it is impossible to determine whether gene functions are cell autonomous by straightforward mutant analysis. This type of analysis is used in Chapter 4 to determine whether Foxg1 is required cell autonomously for ventral telencephalic fate specification.

3) *Distinguishing between primary and secondary defects.* A mutant phenotype represents the culmination of defects in the primary target cells (the primary effect) and other effects elicited in other cells and tissues by changes in function of the primary target cells (the secondary effects). An example of such secondary defects compounding analysis of a mutant phenotype comes from analysis of Retinoblastoma (Rb) mutants. Severe defects in neural and erythrocyte lineages were recently shown to be, at least in part, secondary to a primary requirement for Rb in extra-embryonic tissues. This was resolved by analysis of chimeric embryos (de Bruin et al., 2003; Wu et al., 2003). In chimeras the rescue of primary target cell function by wt cells can help distinguish between primary and secondary defects. With regards to Foxg1 it is possible that the primary target for Foxg1 is in ventral telencephalic cells and the defects in the dorsal telencephalon are secondary to the loss of the ventral telencephalon in *Foxg1*$^{-/-}$ mutants. Provision of wt ventral telencephalic cells allows this idea to be tested.

4) *Analysis of later phenotypes.* Frequently if a gene has a primary function in a given lineage, that lineage will be completely absent in a null mutant embryo. This precludes analysis of the gene in later stages of development of that lineage. In chimeric embryos, the presence of wt cells may allow the lineage

to persist and to incorporate mutant cells and it may, therefore, be possible to assess later roles of the gene of interest in a way that is simply impossible in the mutant embryo. In the context of Foxg1, the ventral telencephalon is absent (results in Chapter 4 and (Martynoga et al., 2005; Xuan et al., 1995)). Thus chimeras theoretically provide an opportunity to determine whether Foxg1 gene function is required in the ventral telencephalon, when this structure is rescued by the presence of large numbers of wt cells.

Foxg1 is required cell-autonomously for ventral telencephalic specification

A few years after the publication of the Foxg1 knock-out, the same group showed that Shh expression is absent from the *Foxg1*$^{-/-}$ telencephalon at E10.5 (Huh et al., 1999). Since Shh is required for proper development of ventral cell types at all levels of the nervous system (Ericson et al., 1995a; Ingham and McMahon, 2001; Patten and Placzek, 2000), including the telencephalon (Zaki et al., 2005), this result was used to suggest that loss of telencephalic Shh expression was the primary (and non-cell-autonomous) molecular defect in ventral telencephalic development in *Foxg1*$^{-/-}$ embryos (Huh et al., 1999). However, as demonstrated in Chapter 4, the defect in ventral specification in *Foxg1*$^{-/-}$ cells is cell autonomous (it cannot be rescued by the expression of pro-ventral cues from wt cells *in vivo*) and consequently a failure of Shh expression is unlikely to be the primary defect in ventral telencephalic development. It is then proposed in Chapter 5 that a more likely primary defect in ventral telencephalic development is that Foxg1 is required by telencephalic cells to respond appropriately to signals that regulate D/V

patterning. Chapter 5 focuses on the possibility that the failure of ventral specification reflects a failure to respond appropriately to Shh signals.

Chapter 2: Materials and Methods

ANIMALS

All mice used in this study were maintained on a mixed CBA x C57/Bl6 background. Mice were housed and cared for according to Home Office regulations, in a dedicated facility. Two lines of mice carrying null alleles of *Foxg1* were used in this thesis: $Foxg1^{cre}$ and $Foxg1^{lacZ}$. In both of these alleles all but the first 13 amino acids of the *Foxg1* coding sequence is replaced by *cre* recombinase (Hebert and McConnell, 2000) or *lacZ* reporter (Xuan et al., 1995) coding sequences. *Foxg1* heterozygous mice are viable and fertile and were maintained throughout the project by breeding pairs consisting of one wt and one $Foxg1^{+/-}$ parent. In addition mice heterozygous for both $Foxg1^{lacZ}$ and $Gli3^{Xt}$ (the mutant allele Xt^{J} is a functionally null allele of *Gli3* ((Hui and Joyner, 1993)) were generated by mating $Foxg1^{+/lacZ}$ and $Gli3^{Xt/+}$ mice.

PRODUCTION OF TRANSGENIC EMBRYOS

Foxg1-null embryos were obtained from timed matings of *Foxg1* heterozygous mice. $Foxg1^{cre/cre}$, $Foxg1^{lacz/lacz}$ and $Foxg1^{cre/lacz}$ embryos were all observed to have the same phenotypic abnormalities, in agreement with previous authors (Hebert and McConnell, 2000); (Pratt et al., 2002), and are henceforth denoted $Foxg1^{-/-}$. In all analyses *Foxg1* heterozygous embryos were found to be phenotypically identical to wild-type as

previously described (Pratt et al., 2002; Xuan et al., 1995) and in some cases were included in the control group. Inter-crossing compound heterozygous mice facilitated the generation of double knock-out $Foxg1^{lacZ/lacZ}$,$Gli3^{Xt/Xt}$ mice. The day of the vaginal plug following mating was designated E0.5. On the required embryonic day, pregnant females were killed by cervical dislocation and embryos dissected from uteri and either fixed or dissected further for analysis of primary cell cultures, as described below.

INJECTION OF S-PHASE TRACERS

For pulse labelling with bromodeoxyuridine (BrdU) pregnant females were injected intra-peritoneally with 200µl of 100µg/ml (in 0.9% NaCl) (Sigma) and embryos were collected after 30mins. For double labelling experiments females were injected with iododeoxyuridine (IddU) (200µl of 100µg/ml (in 0.9% NaCl) (Sigma)) and then 1.5hr later with BrdU and sacrificed after 30 minutes. For 8 hr BrdU cumulative labelling four BrdU injections were given at 0, 2, 4 and 6 hours before sacrifice at 8 hours.

GENOTYPING OF MICE

DNA was prepared from embryonic tissues and adult adult earclips and used to genotype mice by polymerase chain reaction (PCR) as described Appendix A.

FIXATION AND HISTOLOGICAL STAINING OF EMBRYONIC TISSUES

Embryonic tissues were fixed by submersion in a solution of 4% paraformaldehyde in PBS for 2-16 hours at 4°C with agitation. Following fixation they were washed and dehydrated through increasing concentrations of ethanol prior to embedding in paraffin wax. This processing was carried out with the aid of an automated tissue processor (Tissue-Tek VIP, Sakura). 7-10μm sections were cut on a microtome (Reichert-Jung 2050) and the sections mounted on poly-l-lysine (Sigma) coated or superfrost plus (BDH) slides. Unless specifically stated in the figure legend, all sections illustrated in this thesis are in the coronal plane.

IMMUNOHISTOCHEMISTRY

Chromogenic endpoint

Sections were dewaxed in xylene then rehydrated through graded ethanol solutions to PBS. To denature DNA and reveal antigens the slides were boiled in 10mM sodium citrate (pH 6) in a microwave oven for four bursts of 5 minutes each at full power (900W). Slides were allowed to cool for at least thirty minutes and were then washed in PBS. After blocking in 10% normal goat serum (NGS) in PBS with 0.1% TritonX-100 (Sigma) (PBT) sections were incubated overnight at 4°C with the primary antibodies. All primary antibodies used in this thesis and their optimal concentrations for immunohistochemistry are listed in Table2.1. Following incubation in primary antibody, slides were washed at least twice for 10 minutes in PBT and then incubated with species-

specific biotin-conjugated secondary antibodies (goat anti-mouse IgG and goat anti-rabbit IgG, 1:200, Dako) for 1-2hr at room temperature. After washing twice for 10 minutes in PBT, Vectastain ABC-elite peroxidase reagent (Vectorlabs) was added to the slides for 45-60 minutes. Slides were again washed twice in PBS and then antibody staining was revealed with 3,3'-diaminobenzidine (DAB)/hydrogen peroxide chromogenic substrate (Vectorlabs), which produces a dark brown precipitate. For some reactions, nickel chloride (Vectorlabs) was added to the DAB reagent to produce a black/grey stain. Following DAB reaction some slides were counterstained with Harris-haematoxylin (Thermo Shandon). Once staining was complete slides were dehydrated through increasing concentrations of ethanol, then cleared in two changes of xylene prior to mounting under coverslips in DPX mountant (BDH).

Fluorescent endpoint

For immunofluorescence of brain sections slides were processed in an identical fashion to slides reacted for chromogenic immunohistochemistry until after incubation with primary antibodies (see above). Following binding of primary antibodies, staining was either revealed directly or amplified to enhance sensitivity. For the more direct approach slides were incubated with species-specific secondary antibodies conjugated to fluorescent molecules (Goat anti-mouse, goat anti-rabbit or goat-anti-rat, all conjugated to either Alexfluor-488 or Alexafluor-568. Diluted to 1:200 and all purchased from Molecular Probes). For staining with amplification, sections were incubated with species-specific biotin-conjugated secondary antibodies (goat anti-mouse IgG and goat anti-rabbit IgG, 1:200, Dako) for 1-2hr at room temperature prior to addition of streptavidin-conjugated fluorescent probes (streptavidin-Alexafluor-488 or Alexafluo-

568, diluted 1:200, Molecular Probes). Following fluorescent detection of primary antibody binding nuclei were counterstained with the DNA dye TO-PRO-3 iodide (diluted 1:100 in water, Molecular Probes) and mounted under coverslips in Vectashield hardset (Vectorlabs) to preserve fluorescence.

For simultaneous immunofluorescent detection of two primary antibodies, antibodies from two different species were selected (two avoid cross-reactivity of secondary antibodies) and incubated together. Staining was then either revealed directly by incubation with species-specific fluorescently conjugated secondary antibodies, or one target was amplified via the biotin/streptavidin system described above and the other revealed directly. Appropriate controls were performed to ensure that secondary and tertiary reagents did not cross react. For IddU/BrdU double labelling primary antibodies used were: mouse anti-BrdU/IddU (which recognises both BrdU and IddU) (clone B44, 1:100 in blocking solution; Becton Dickinson) and rat anti-BrdU (clone BU1/75, 1:100; Abcam). These primary antibodies were visualised with highly cross-absorbed goat anti-mouse IgG conjugated to Alexa488 (Molecular Probes A11029, 1:200) and goat anti-rat Alexa568 (Molecular Probes A11077, 1:200), respectively (NB. Non-highly cross-absorbed anti-mouse secondaries exhibit some cross reactivity with rat-derived primary antibodies. Appropriate controls were performed to ensure that primary antibodies were indeed specific for the halogenated pyrimidines and that there was no cross reactivity of secondary antibodies.

	Species	Clone name	Monoclonal/ polyclonal	Optimal dilution	Source	Reference
β-III-Tubulin	Mouse	Tuj1	monoclonal	1 : 800	Sigma	-
PCNA	Mouse	PC10	monoclonal	1 : 400	Dako	-
BrdU	Rat	BU1/75	monoclonal	1 : 100	Abcam	-
BrdU/IddU	Mouse	B44	monoclonal	1 : 100	BD Bioscience	
GFAP	Rabbit	-	polyclonal	1 : 400	Sigma	-
Reelin	Mouse	G10	monoclonal	1 : 400	Chemicon	-
Calretinin	Rabbit	-	polyclonal	1 : 1000	Sigma	-
Calbindin	Mouse	CB-955	monoclonal	1 : 100	Sigma	-
Calbindin	Rabbit	-	polyclonal	1 : 1000	Sigma	-
Nestin	Mouse	RAT401	monoclonal	1 : 50	DSHB	S. Hockfield
Nkx2.1	Mouse	8G7G3/1	monoclonal	1 : 100	Abcam	-
Nkx2.1	Rabbit	-	polyclonal	1 : 800	Biopat	-
Pax6	Mouse	PAX6	monoclonal	1 : 200	DSHB	A. Kawakami
Olig2	Rabbit	-	polyclonal	1 :10000	D.Rowitch	(Ligon et al., 2004)
Gsh2	Rabbit	-	polyclonal	1 : 1500	K. Campbell	(Toresson et al., 2000)
pan-Dlx	Rabbit	-	polyclonal	1 : 100	R. Hevner	(Hevner et al., 2004)
Mash1	Mouse	24B7.2D11	monoclonal	1 : 100	BD Pharmingen	
Islet1	Mouse	40.2D6	monoclonal	1 : 100	DSHB	T.M. Jessell
Pax3	Mouse	Pax3	monoclonal	1 : 400	DSHB	C.P. Ordahl
Ngn2	Mouse	7G4	monoclonal	1 : 10	D. Anderson	(Lo et al., 2002)
Tbr1	Rabbit	-	polyclonal	1 : 500	R. Hevner	(Englund et al. 2005)
Tbr2	Rabbit	-	polyclonal	1 : 500	R. Hevner	(Englund et al. 2005)
Lhx2/9	Rabbit	-	polyclonal	1 : 1000	T. Jessell	(Liem et al., 1997)
Foxg1	Rabbit	-	polyclonal	1 : 500	Y. Sasai	(Watanabe et al., 2005)
Lhx6	Rabbit	-	polyclonal	1 : 500	V. Pachnis	(Grigoriou et al., 1998)
PSmad158	Rabbit	-	polyclonal	1 : 100	Cell Signaling Technology	
$P21^{cip1}$	Rabbit	-	polyclonal	1 : 100	BD Pharmingen	

DSHB denotes Developmental Studies Hybridoma Bank. Several monoclonals were obtained from the DSHB developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

Table 1. Primary antibodies used in this thesis.

LacZ HISTOCHEMISTRY

E12.5 *Foxg1*$^{lacZ/+}$ and *Foxg1*$^{lacZ/lacZ}$ embryonic heads were dissected and fixed for 1 hour at 4°C in 4% paraformaldehyde, 0.02% NP40, 0.01% sodium deoxycholate, 5 mM EGTA, 2mM $MgCl_2$ in PBS. Heads were equilibrated in 30% sucrose/PBS and sectioned (15 μm) on a cryostat. Sections were rinsed several times in wash buffer (2 mM $MgCl_2$, 0.02% NP40, 0.01% sodium deoxycholate in PBS), transferred to staining solution (wash buffer supplemented with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml X-gal), and stained overnight at 37°C in darkness. Staining was stopped with 20 mM EDTA in PBS and sections were counterstained with Nuclear Fast Red.

DETECTION OF APOPTOSIS BY TUNEL REACTION

TUNEL on embryonic brain sections

Terminal deoxynucleotidyl nick end labelling (TUNEL) was carried out with an Apoptag plus Fluorescein in situ apoptosis detection kit (Chemicon international) according to the manufacturer's protocol. Following staining, nuclei were counterstained with TO-PRO-3.

Whole mount TUNEL

Whole mount terminal deoxynucleotidyl nick end labelling (TUNEL) was performed according to a published protocol (Smith and Cartwright, 1997) using the ApopTag *in situ* apoptosis detection kit (Chemicon International). TUNEL labelled embryos were

either photographed or visualised by optical projection tomography (OPT) by Harrison Morrison according to a published technique (Sharpe et al., 2002).

LIGHT MICROSCOPY

Slides were examined and photographed with a Leica DMLB upright compound microscope connected to a Leica DSC480 digital camera driven by Leica IM50 image management software.

Whole embryos were examined and photographed on a Wild M5A dissecting microscope connected to a Nikon Coolpix995 digital camera.

CONFOCAL MICROSCOPY

Fluorescently stained images were obtained using a Leica TCS NT confocal system and associated software with a Leica DMRE compound microscope in a confocal facility run by Linda Wilson. Alexafluor-488 staining was collected in the FITC (green) channel, Alexafluor-568 in the TRITC (red) channel and TO-PRO-3 in the Cy3 (far-red, pseudo-coloured blue) channel.

ENUMERATION OF BrdU AND IddU-LABELLED NUCLEI FOR CELL CYCLE KINETIC ANALYSIS

Evenly spaced sections throughout both the antero-posterior and dorso-ventral dimensions of the embryonic telencephalon were immunofluorescently stained and imaged with the confocal microscope. For each section analysed images were acquired in three channels (appearing red, green and blue in all figures) as separate files. These images were then stacked with Photoshop software (Adobe) and used for the enumeration of labelled cells. Sections were divided into evenly spaced counting bins as described in the legend to Figure1.4 and then counted as described in Figure1.2.

CALCULATION OF BRDU LABELLING INDEX IN VENTRAL TELENCEPHALON AT E10.5

For the calculation of BrdU labelling index in the ventral-most telencephalon, the number of BrdU-labelled cells in two 100μm sampling bins spaced 100μm apart in the ventral-most region of the telencephalon were counted and expressed as a percentage of all VZ cells. A total of 14 counts were made in each of three control and *Foxg1*$^{-/-}$ embryos in sections spaced evenly through the rostro-caudal axis of the telencephalon.

IMAGE ANALYSIS

Images were analysed and cropped for publication with Photoshop 7 software (Adobe). Occasionally the brightness and contrast of pictures were adjusted to improve clarity of images. In all images that were adjusted, changes were linear and applied across the entire image.

DATA ANALYSIS AND GRAPH PLOTTING

Data were analysed and graphs plotted with the following computer applications: Excel (Microsoft), Sigmaplot (Systat Software Inc.), SPSS (SPSS software), and Prism 4 (GraphPad).

WHOLE MOUNT IN SITU HYBRIDISATION

i) Preparation of digoxigenin-labelled RNA probes.

Plasmids encoding sequences to be used as probes for in situ hybridisation were cloned, purified and linearised according to standard techniques of molecular biology as summarised in Appendix A. 1.5μg of linearised plasmid was then used as a template to synthesise digoxygenin-labelled probe using T7 or T3 RNA polymerase as appropriate with a pre-made digoxygenin-labelled nucleotide mix (Roche). For each anti-sense probe, the corresponding sense probe was synthesised from the opposite strand of the DNA template as a negative control for non-specific probe binding. Labelled probes were then precipitated with ammonium acetate and ethanol and re-suspended in 50μl of sterile RNase-free water ready for use. An aliquot of each probe was run on an agarose gel for approximate quantitation against DNA standards and the known concentration of DNA template, still present in the reaction mix. The following probes were used in this thesis: *Fgf8* (Crossley and Martin, 1995), *Wnt8b* (Richardson et al., 1999), *Nkx2.1* (Shimamura et al., 1995) and *Patched1* (Goodrich et al., 1996). Control hybridisation with sense probe confirmed specificity of hybridisation.

ii) Preparation and Hybridisation of embryos

Embryos for in situ were fixed overnight in 4% PFA in PBS overnight at 4°C and then gradually dehydrated to 100% MeOh. Prior to hybridisation, embryos were rehydrated back into PBT prior to partial digestion with proteinase K (10mg/ml, Sigma) for 2-10 minutes depending on the size of the embryo. Embryos were then post-fixed for 20 minutes in 4% PFA, 0.1% glutaraldehyde in PBT. After washing, embryos were placed in hybridisation mix (50% formamide, 5xSSC, 0.1% Triton X-100, 50mg/ml heparin, 0.1mg/ml yeast tRNA, 5mM EDTA) and pre-hybridised at hybridisation temperature (in each case this was 65°C) for 12-16 hours. After pre-hybridisation, fresh pre-warmed hybridisation mix containing approximately 0.2mg/ml of labelled probe was added and embryos hybridised at 65°C for 16-24 hours.

iii) Post-hybridisation washes

Embryos were washed twice in hybridisation mix then in decreasing concentrations of formamide prior to two washes in maleic acid buffer (MABT: 100mM maleic acid, 150mM NaCl, 0.1% Triton X-100). Embryos were then blocked in a solution of MABT containing 20% sheep serum and 2% blocking reagent (Roche) for 2-4 hours at room temperature. An alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (1:2000 in blocking solution) was then added and embryos incubated for 16 hours at 4°C with agitation. After at least 6 hour long washes in MABT at room temperature and an overnight wash at 4°C, embryos were washed twice in NTMT solution (100mM NaCl, 100mM Tris-HCl, pH9.5, 50mM $MgCl_2$, 0.5% Tween-20, 0.2mg/ml levamisole). Staining was then revealed by incubation of embryos in NTMT containing 0.34 mg/ml p-

nitrotetrazolium blue chloride and 0.18 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate in (1:50 from stock, Roche). When the colour had developed to the desired extent embryos were fixed in 4% PFA in PBS and then cleared through increasing concentrations of glycerol.

DISSOCIATION AND CULTURE OF PRIMARY TELENCEPHALIC CELLS

Telencephalic vesicles were isolated and dissected in ice-cold, oxygenated Earle's buffered salts solution (EBSS, see Appendix B for constituents). Control telencephalon was divided into dorsal and ventral portions and the whole *Foxg1*$^{-/-}$ telencephalon was used. Telencephalic tissues were dissociated by incubation for 30-45 minutes at 37°C in EBSS containing papain (20 units/ml) and DNase1 (Papain Dissociation System, Worthington Biochemical). Following trituration with a pipette, cells were pelleted by centrifugation at 300g for 5 minutes and gently resuspended in EBSS containing 10% ovomuciod inhibitor and DNase1. Cell suspensions were then layered over 100% ovomucoid inhibitor solution and cells centrifuged at 70g for 6 minutes to remove cellular debris and inactivate the papain. Cells were gently resuspended in serum-free defined culture medium (see Appendix B for constituents), counted with the aid of a haemocytometer, adjusted to the corrected concentration and plated at 4000 cells/mm^2. Viability was routinely tested with the trypan blue exclusion assay prior to plating was always found to be very high. For immunocytochemical analysis, cells were plated in Lab-Tek II 8-well chamber-slides (Nunc-154534) and fixed after 24 hours. For Shh response experiments, cells were plated in 24 well plates (Nunc). 5 hours after plating recombinant mouse amino terminal Shh peptide (Shh-N, R&D Systems) was added to

experimental wells to a final concentration of 50nM. After one hour, medium was removed and RNA extracted for RT-PCR and qRT-PCR as described below. RNA lysis buffer was added directly to the cells, which were homogenised by vortexing and repeated syringing though a 25-gauge needle prior to RNA extraction with RNeasy mini kit (Qiagen) according to the manufacturers instructions.

TELENCEPHALIC EXPLANT CULTURES

Embryos at E10.5 were dissected in cold EBSS. At this age *Foxg1*$^{-/-}$ embryos can be distinguished from controls by the distinctive defects in telencephalic and eye morphology (Martynoga et al., 2005). Explants of dorsal telencephalon were manually dissected from *Foxg1*$^{-/-}$ and control telencephalon and cultured on tissue culture inserts (Falcon inserts 0.4μm pore size, Becton Dickinson) in wells containing 2ml of defined culture medium (see Appendix B for constituents) containing 10% foetal bovine serum (Sigma). 1hr after placement on insert, 3-6 Affi-gel blue beads (100-200 mesh, Bio-rad) containing either 0.5μg/μl Shh-N or 0.1% BSA were placed on the explants, which were then cultured for 24-36 hours. Explants were fixed in 4% PFA and processed for immunofluorescence or placed in RNAlater reagent (Qiagen) for RNA extraction (see below).

IMMUNOCYTOCHEMICAL STAINING OF CULTURED DISSOCIATED CELLS

Dissociated cells were plated on poly-L-lysine coated wells (Lab-Tek-IITM; Nunc Life Technologies) at a density of 4000 cells/mm^2 in serum-free medium for 16-24 hours. Cells were then fixed in 4% paraformaldehye for 15 minutes and washed in PBS. Cells were permeabilized in blocking buffer (20% goat serum, 0.1% Triton X-100, PBS) for one hour at room temperature. Primary antibodies were then diluted in blocking buffer and applied for one hour at room temperature or overnight at 4°C. Mouse primary antibodies were detected with goat anti-mouse or goat anti-rabbit antibodies conjugated to Alexa 488 or 568 secondary antibodies at 1:200 (Molecular Probes). Cells were counterstained with TO-PRO-3 iodide (Molecular Probes) in PBS and mounted in Vectashield Hardset (Vectorlabs).

IMMUNOCYTOCHEMISTRY OF CULTURED TELENCEPHALIC EXPLANTS

Following fixation, explants were dehydrated through increasing concentrations of Methanol/PBTw (PBS, 0.5% Tween-20) (25%, 50%, 75%, 100% MeOh, 5-10 minutes each) to permeabilise the cell membranes, then rehdrated back to PBSTw throught the same series. Explants were then blocked in PBSTw with 20% normal goat serum for 1hr at room temperature. Primary antibodies were added at the appropriate concentration (Mash1, 1:100; pan-Dlx, 1:80, Islet-1, 1:100) and explants incubated for 16 hours at 4°C with agitation. Following two 10 minute washes in PBTw, fluorescently conjugated secondary antibodies (goat anti-mouse-Alexafluor 488 and goat anti-rabbit-Alexafluor 568, both 1:100, Molecular Probes) were added for 1-2hr at room temperature in the

dark. Explants were then transferred to microscope slides and mounted under coverslips in Vectorshield hardest (Vectorlabs) for confocal microscopy.

PRODUCTION OF CHIMERIC ANIMALS

Generation and aggregation of pre-implantation embryos

Chimeras were produced in a similar manner to those previously described (Quinn et al., 1996). Four to eight-cell embryos at the morula stage were obtained from the parental cross *Foxg1*$^{lacZ/+}$, *Gpi1*$^{b/b}$ female × *Foxg1*$^{+/Cre}$, *Gpi1*$^{b/b}$, *Tg/Tg* male[1], where Tg denotes the presence of the reiterated β-globin transgene TgN(Hbb-b1)83Clo (Keighren and West, 1993). To increase the number of embryos recovered, females were super-ovulated by the sequential injection of pregnant mares serum gonadotrophin (Folligon, Intervet. 5 i.u) and human chorionic gonadotrophin (Chorulon, Intervet. 5 i.u.). Embryos of the following four genotypes were obtained from this parental cross: *Foxg1*$^{+/+}$, *Foxg1*$^{lacZ/+}$, *Foxg1*$^{+/Cre}$ and *Foxg1*$^{lacZ/Cre}$(*Foxg1*$^{-/-}$), all of which were *Gpi1*$^{b/b}$ and contained a single copy of the β-globin transgene (Tg+). Wt donor embryos for aggregation were obtained from (BALB/b x A/J) F_1 and F_2 intercrosses which produced embryos which were *Foxg1*$^{+/+}$, *Gpi1*$^{a/a}$ and negative for the β-globin transgene (Tg-). Embryos were collected from the oviducts and uteri of superovulated females at 2.5 days post coitum and aggregated according to West and Flockhart (West and Flockhart, 1994). In brief, the

[1] As described in the Results section of this chapter, unfortunately some males must have been heterozygous for the β-globin transgene, since some of the chimeric embryos did not carry the transgene.

zona pelucida was removed by immersion in acid tyrodes solution, then the embryo was washed through several drops of handling medium (see Appendix B for constituents). Drops of KSOM culture medium (see Appendix B for constituents) under silicon oil were prepared on the base of a tissue culture dish and a small dimple created with a purpose-made aggregation needle (BLS, Hungary). Two embryos, one donor and one experimental, were placed in each dimple with the culture drop and apposed. Aggregated embryos were cultured overnight (37°C, 5% CO_2, humidified) to the blastocyst stage and transferred to the uteri of recipient pseudopregnant (CBA x C57/Bl6) F_1 females (*Foxg1*$^{+/+}$, *Gpi1*$^{c/c}$, Tg-) and recovered at E12.5.

Recovery and analysis of chimeric embryos

Embryos were dissected into cold PBS and staged according to forelimb development (Palmer and Burgoyne, 1991; Wanek et al., 1989). Heads were fixed overnight in 4% PFA in PBS, then processed for histology as described above. Tail, forelimb, hindlimb and liver samples were taken for analysis of glucose phosphate isomerase 1 (GPI1) isotype contribution to give a proportion of global chimerism for each embryo, as described previously (West and Flockhart, 1994). In all embryos, cells derived from the eight cell wild-type embryo were Gpi1$^{a/a}$, and all cells derived from the *Foxg1*$^{lacZ/+}$ × *Foxg1*$^{+/Cre}$ cross were Gpi1$^{b/b}$. The mean percentage GPI1B was taken as an estimate of the proportion of cells in the chimera derived from the *Foxg1*$^{-/-}$ or *Foxg1*$^{+/-}$ 8-cell embryo. The genotype of each chimera was determined by PCR analysis of genomic DNA as described in previously (Martynoga et al 2005). The use of two null mutant *Foxg1* alleles allowed distinction between chimeras containing *Foxg1*$^{lacZ/Cre}$, compound

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heterozygous (*Foxg1*$^{-/-}$), and those containing heterozygous cells (*Foxg1*$^{lacZ/+}$ or *Foxg1*$^{+/Cre}$) or wild type (*Foxg1*$^{+/+}$) cells.

DNA:DNA in situ hybridisation for the β-globin transgene

Histological visualisation of cells derived from the *Foxg1*$^{lacZ/+}$ x *Foxg1*$^{+/Cre}$ embryos was achieved by DNA-DNA in situ hybridisation using a digoxigenin labelled probe to the reiterated β-globin transgene as previously described (Keighren and West, 1993). Probe synthesis was carried out as described above for RNA in situ hybridisations. Paraffin embedded tissue sections on microscope slides were dewaxed in xylene, rehydrated through graded ethanol series then microwaved for five five minute bursts at full power (900 watts). Following pre-hybridisation (60°C for 15 minutes in 5x Denhardts solution, 45% formamide, 6x SSC and 500mg/ml salmon sperm DNA), slides were hybridised with approximately 20ng of labelled probe for 16 hours at 60°C in hybridisation mix (5x SSC, 10% dextran sulphate, 500mg/ml salmon sperm DNA). Detection was with peroxidase-labelled antibody to digoxigenin visualised by diaminobenzidine staining (Keighren and West, 1993). Slides were counterstained with haematoxylin, dehydrated and mounted in DPX.

WESTERN BLOTTING

Pax6 Western Blotting

Telencephalons were dissected in ice-cold PBS and then frozen on dry ice. Protein extracts were made by homogenization in buffer H (20mM Tris-HCl (pH7.4) containing a cocktail of protease inhibitors (Sigma)) and then sonication after the addition of Laemmli SDS sample buffer (25mM Tris-HCl (pH6.8), 2% SDS). Extracts were quantified with the BSA reagent kit (Pierce) according to the manufacturer's instructions. Absorbance at 562nm was measured on a spectrophotometer. A standard curve was drawn, linear regression performed and the resulting equation used to calculate the concentration of total protein of samples. Samples were boiled for at least 10 minutes at 100°C and then loaded into a lane filled with 1X SDS Running Buffer. The gels were set up in the Xcell SureLock Mini-Cell (Invitrogen) and run for 1 hour and 40 minutes at 150V. For Pax6 western blotting 10μg of protein were diluted with Tris glycine running buffer (25mM Tris Base, 192mM Glycine, 0.1% SDS, pH8.3) and run on a Novex 12% Tris glycine gel (Invitrogen) prior to transfer to nitrocellulose (Biorad) for 1-2hr at 150V <400mA at 4°C (Transfer buffer: 12mM Tris Base, 96mM Glycine, pH8.3). Successful transfer was confirmed by staining the membrane with Ponceau S (Sigma). Pax6 protein was immunodetected with a mouse monoclonal to Pax6 (diluted 1:200, DSHB) and then ECL+ reagents (Amersham Bioscience) were used for exposure to X-ray film. Following detection of Pax6, a rabbit polyclonal to β-actin (Sigma) was used to detect β-actin levels to confirm even loading and allow sample normalisation. Band intensity was determined by scanning densitometry (Biorad GS710 calibrated imaging densitometer with Image One software).

Gli3 Western Blotting

Protein extracts were prepared and quantified as for Pax6 (see above). 10μg of protein were run on a Nupage 3-8% Tris acetate gel (Invitrogen) (1hr 45minutes, 150V, Nupage TA running buffer (Invitrogen)) prior to transfer to nitrocellulose (1-2hr 150V, Nupage

TA transfer buffer, Invitrogen). Gli3 protein was immunodetected with a rabbit polyclonal to Gli3 (1:100, Santa-Cruz) and then ECL+ reagents (Amersham Bioscience) were used for exposure to X-ray film. Band intensity was determined by scanning densitometry (Biorad).

RT-PCR

RNA was extracted from embryonic tissues or cultured cells with Qiagen RNEasy mini or micro kits (Qiagen), according to amount of starting material. cDNA was then synthesised from RNA template with M-MLV (Promega) or Sensiscript (for small amounts of material, Qiagen) RT polymerase with random hexanucleotide primers (See Appendix A). PCR (see Appendix A) was then performed on the cDNA template with gene specific primers (Table 2) to determine the presence of transcripts. 'No RT' and 'no cDNA' controls were included where appropriate. When possible primers were designed to span introns to control for contamination from genomic DNA (although RNA samples were also treated with DNase prior to cDNA synthesis).

QUANTITATIVE RT-PCR

To enable the repeatable and efficient quantification of levels of transcript between telencephalic tissues of wt and *Foxg1*$^{-/-}$ mice, quantitative RT-PCR (qRT-PCR) was used (Bustin, 2000). In this method the incorporation of a fluorescent dye (in this case, Sybr green) into dsDNA PCR product is measured over the course of a PCR reaction. Quantitect Sybr Green PCR reagents (Qiagen) were used on an Opticon single

wavelength PCR machine (MJ Research) driven by Opticon Monitor software. All primers were designed to span introns and to have an annealing temperature ≈58°C, such that PCR reactions could be carried out with a standard annealing temperature. Each reaction was monitored over 36 PCR cycles and then a melting curve analysis performed to ensure target specificity. Amplicons were all designed to be <250bp to ensure linearity of Sybr Green incorporation.

For each gene analysed (i.e. for each PCR) a series of dilutions of control cDNA (usually a wt sample known to contain target cDNA) were performed, from 1μl to 0.1μl of cDNA. This allows a plot of concentration of cDNA against fluorescence observed after a given number of cycles (the cycle threshold, where all PCR reactions are in the linear phase). In each case, the plots were linear, confirming that amplification efficiencies were identical over the concentration range. The concentration series of control cDNA also generates a standard curve of arbitrary units at a given cycle threshold. Fluorescence levels of control and experimental reaction products at this cycle threshold can then be interpolated from this graph, to yield the relative amounts of gene-specific template present in the cDNA.

For each experiment, qRT-PCR was performed to a control or house-keeping gene β-actin. Transcript levels of this gene did not appear to fluctuate between samples from each experimental group and this was confirmed by qRT-PCR to another commonly used control gene, GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), confirming that β-actin was a suitable control. The inclusion of the β-actin gene allowed the level of the gene of interest to be normalised to the level of β-actin, and therefore control for

variations in the initial concentrations of cDNA in the reactions. Following normalisation to β-actin, expression levels for each gene were then expressed as a ratio to normalised levels of a control group (wt telencephalon for expression analyses or untreated samples for Shh-N induction analyses). For every gene analysed samples were run in triplicate on cDNA pooled from two synthesis reactions to control for variation in amplification efficiency between PCR reactions and for variations in cDNA synthesis.

	Forward Primer	**Reverse Primer**
Beta-actin	CCT GTC AGC AAT GCC TGG GT	CCA GCC TTC CTT CTT GGG TA
GAPDH	GGG TGT GAA CCA CGA GAA AT	CCT TCC ACA ATG CCA AAG TT
Ptc1	CTG GGA GGA AAT GCT GAA TA	GTT TTC CAG TGG CAT TCT TG
Smo	GTT CGT GGT CCT CAC CTA TG	TCA CGG AGT CTC CAT CTA CC
Gli1	GTT ATG GAG CAG CCA GAG AG	GAG TTG ATG AAA GCC ACC AG
Gli3	ACA CAG CCC TCC TCT CAT C	CAT CAG GCT TGA TCT TGG AC
CyclinD1	CGC CCT CCG TAT CTT ACT TC	CTC TTC TTC AAG GGC TCC A
Nkx2.1	GAA AGA CAG CAT CAG CTT CC	CCA TGC CCA CTT TCT TGT AG
Shh	AGG AAC TCA CCC CCA ATT AC	ACT GCT CGA CCC TCA TAG TG

Table 2. RT-PCR primer sequences used in this thesis

Chapter 3: Foxg1 regulates proliferation, differentiation and apoptosis in the dorsal telencephalon

INTRODUCTION

Foxg1 null mutant mice die around the time of birth with extremely hypoplastic telencephalon. The cerebral hemispheres of newborn *Foxg1*$^{-/-}$ mice are 95% reduced in mass relative to control littermates (Xuan et al., 1995), demonstrating that Foxg1 has a central role in the regulation of telencephalic growth. Telencephalic development during embryogenesis is governed by the proper regulation of three distinct developmental phases. Soon after its specification at the rostral-most end of the neural plate, the telencephalic neuroepithelium undergoes a period of rapid expansion whereby both the progeny of each mitotic division re-enter the cell cycle to bolster the progenitor pool (Bhide, 1996; Caviness et al., 1995). Following this period of explosive progenitor expansion, at around E10.5 in the mouse (Bhide, 1996; Caviness et al., 1995; McConnell, 1995), telencephalic neurogenesis is initiated with the birth of the first post-mitotic neurons. During neurogenesis there is a gradual slowing of the telencephalic growth rate as the average duration of the progenitor cell cycle lengthens and an increasing proportion of the progeny leave the cell cycle and terminally differentiate to become neurons, thus depleting the progenitor pool. The telencephalic neurogenetic interval continues until around E17.5 (Caviness et al., 1995). During this period the vast majority of neurons that will populate the adult telencephalon are born. As the neurogenetic period wanes, gliogenesis is initiated, first with astrocytes being produced in the dorsal

telencephalon during late embryogenesis. The other major glial cells, oligodendrocytes, are principally generated during early post-natal development.

During the three temporally distinct, but overlapping periods of progenitor expansion, neurogenesis and gliogenesis, four crucial factors must be exactly regulated to ensure that the telencephalon grows at the correct rate such that it can produce the appropriate number of cells in the right place at the right time. These factors, which are the same for any growing tissue, are as follows:

i) Proliferation rate

ii) Rate of cell cycle exit (which in the CNS is tightly coupled with differentiation)

iii) Rate of cell death

iv) Rate of migration in and out of the tissue

Any failure to correctly regulate these crucial processes will have a profound effect on the number of cells produced and the structure of the brain region concerned.

The severe size reduction of the *Foxg1*$^{-/-}$ telencephalon demonstrates that the gene must regulate at least one of these fundamental processes. Previous reports have utilised bromodeoxyuridine (BrdU) labelling to infer that progenitor proliferation rate is reduced, partly through a proposed lengthening of the cell cycle (Hanashima et al., 2002; Xuan et al., 1995). Immunohistochemical labelling of markers of post-mitotic neurons has also been used to show a precocious production of post-mitotic neurons (Hanashima et al.,

2002; Xuan et al., 1995). To date, no description of defects in the regulation of apoptosis of cells in the telencephalon have been described in *Foxg1*$^{-/-}$ mutants. There is also no evidence of unusual patterns of cell migration into or out of the telencephalon and this issue was not pursued further in the present work. Thus it is accepted that Foxg1 is involved in the regulation of at least two dimensions of the growth matrix: progenitor proliferation rate and differentiation rate.

In this chapter the role of Foxg1 in regulating the growth of the mouse telencephalon is investigated systematically by studying the processes set out above. A powerful technique for accurately assessing cell cycle kinetics *in vivo* is developed and applied to measure, for the first time, cell cycle parameters in groups of *Foxg1*$^{-/-}$ and control progenitor cells in discrete regions of the telencephalon during each phase of telencephalic development. During the pre-neurogenetic progenitor expansion period, cell cycle kinetics are the same in *Foxg1*$^{-/-}$ and controls. As neurogenesis begins (~E10.5) rostrally positioned cells begin to proliferate more slowly in the absence of *Foxg1*. This slowing becomes more widespread and severe as development proceeds, coinciding with an increased rate of telencephalic neurogenesis from E11.5. Evidence that astrogliogenesis is prematurely initiated in the absence of Foxg1 is also presented. Concomitant with the initial slowing of the progenitor cell cycle at E10.5 a localised decrease in induction of apoptosis is observed. This chapter provides a detailed characterisation of telencephalic growth parameters in *Foxg1*$^{-/-}$ mutants, which support the idea that three fundamental roles for Foxg1 are to maintain telencephalic progenitor proliferation rate, to delay progenitor differentiation and to regulate the orderly specification of neuronal and glial cell types.

Many of the results presented in this chapter have already been published (Martynoga et al., 2005). This paper is bound into the back of this thesis and will not be cited again in this chapter.

RESULTS

Calculation of cell cycle kinetic parameters

From mid-embryogenesis the telencephalon of mice lacking *Foxg1* is severely hypoplastic (Xuan et al., 1995). To examine whether misregulation of cell cycle progression of telencephalic progenitors during early, mid and late telencephalic development contributes to the defective growth of the *Foxg1*$^{-/-}$ telencephalon, cell cycle kinetic parameters were analysed. To achieve accurate estimates of the average cell cycle time (T_c), a bromodeoxyuridine/iododeoxyuridine (BrdU/IddU) double labelling paradigm (Shibui et al., 1989) was adapted and optimised to measure cell cycle kinetic parameters in the developing telencephalon *in vivo*. This technique allows the estimation of T_c and the duration of S-phase (T_s) in a single specimen.

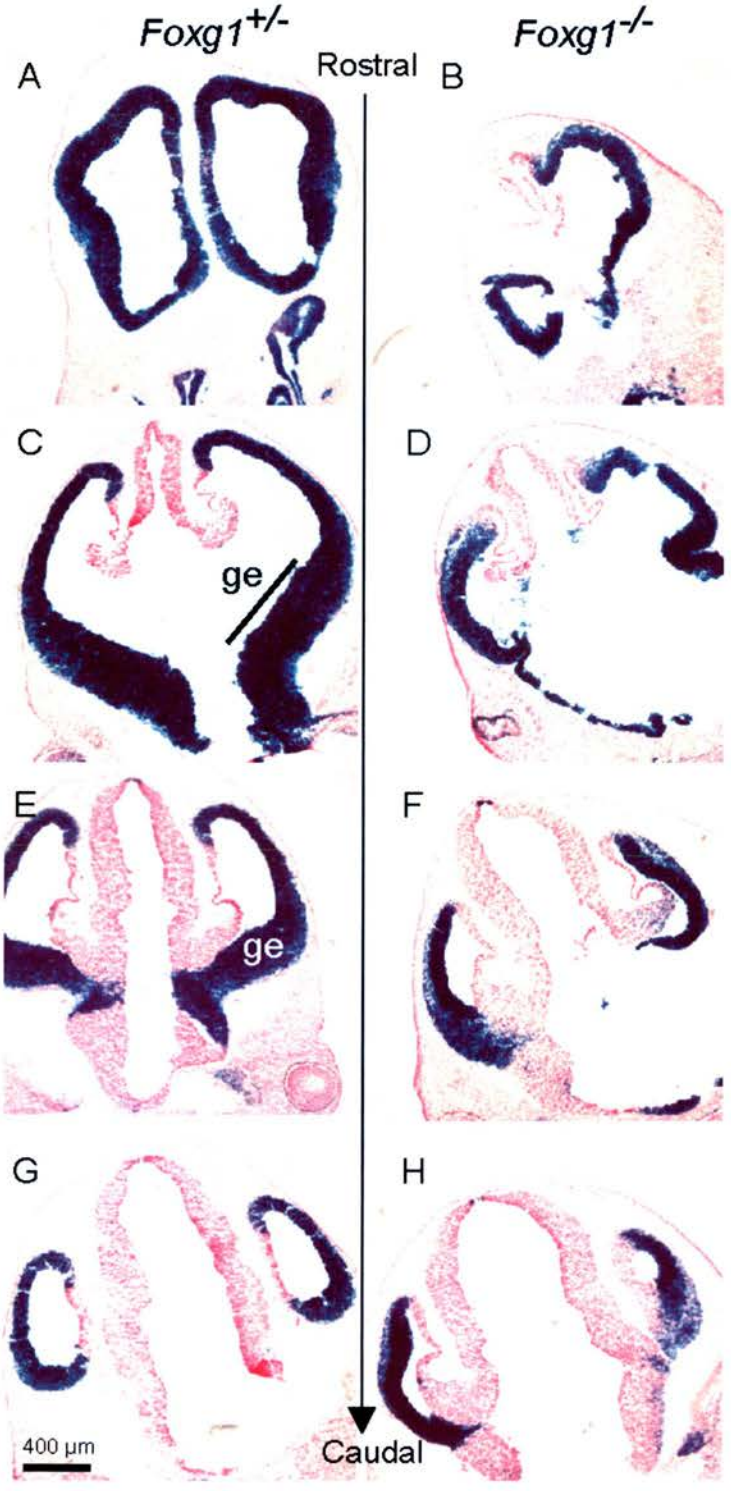


Fig.3.1. Morphological abnormalities of the *Foxg1*$^{-/-}$ telencephalon. At E12.5 the mutant telencephalon is markedly reduced in size relative to control and ventral ganglionic eminences are notably absent. LacZ reporter activity driven from the Foxg1 locus marks the telencephalic territory in *Foxg1*$^{+/-}$ and *Foxg1*$^{-/-}$ tissue sections in the coronal plane. The size reduction is more pronounced in rostral regions (compare A and C with B and D) than in caudal sections (compare E and G with F and H). ge, ganglionic eminences.

In the telencephalic neuroepithelium precursors progress through the cell cycle asynchronously (Takahashi et al., 1993). In such a population of proliferating cells, the fraction of cells in a given phase of the cell cycle is directly proportional to the length of that phase relative to the total length of the cell cycle (Nowakowski et al., 1989). BrdU and IddU are halogenated thymidine analogues that are incorporated into DNA synthesised during S-phase. Sequentially exposing proliferating cells to IddU and BrdU allows differentiation between defined populations of cells. Determining the relative sizes of these populations facilitates calculation of T_c and T_s of the proliferating pool. In this technique, telencephalic progenitors are exposed to IddU *in vivo* at T=0hr such that all cells in S-phase at the beginning of the experiment are labelled with IddU (Fig.3.2A). At T=1.5hr, cells are exposed to BrdU to label all cells in S-phase at the end of the experiment (S_{cells}) (Fig.3.2A). These cells will also be labelled with IddU, which is still present in the bloodstream. Animals are killed at T=2hr. It takes up to 30mins for injected IddU and BrdU to circulate and label the DNA of S-phase cells to detectable levels (Nowakowski et al., 1989), therefore, the interval during which cells can incorporate IddU but not BrdU (T_i) is 1.5hr (Fig.3.2A). Since telencephalic neural precursors are not synchronised, cells in the initial IddU-labelled S-phase cohort will leave S-phase at a constant rate during T_i. Consequently, this leaving fraction (L_{cells}) will be labelled with IddU but not BrdU, as summarised in Fig.3.2A. By staining tissue sections with monoclonal antibodies that allow distinction between cells labelled with just IddU from those which incorporated BrdU and IddU it is possible to count the L_{cells} and S_{cells} fractions, see Fig.3.2B,C. The ratio of the length of any one period of the cell cycle to that of another period is equal to the ratio of the number of cells in the first

period to the number in the second period (Nowakowski et al., 1989). Therefore, the ratio between the number of cells in the L_{cells} and the S_{cells} fractions is equal to the ratio between T_i (which equals 1.5hr) and T_s (Shibui et al., 1989):

$T_i/T_s = L_{cells}/S_{cells} \quad \therefore T_s = T_i/(L_{cells}/S_{cells})$

(where $L_{cells} = IddU^+/BrdU^-$ and $S_{cells} = IddU^+/BrdU^+$)

By exactly the same logic the calculated T_s can be used to estimate T_c:

$T_s/T_c = S_{cells}/P_{cells} \quad \therefore T_c = T_s/(S_{cells}/P_{cells})$

P_{cells} is the total number of proliferating cells in the sampling area. In this case P_{cells} is estimated by counting the total number of cells in the ventricular zone (VZ) in the sampling area. This equation, therefore, rests on the assumption that all cells in the ventricular zone are proliferating. Previous authors have shown that 98-100% of cells in the VZ of the wild-type telencephalon are indeed actively proliferating (Takahashi et al., 1993); (Takahashi et al., 1995a); (Caviness et al., 1995); (Estivill-Torrus et al., 2002). Here, 8hr cumulative BrdU labelling at E10.5 (Fig.3.3I,J) and proliferating cell nuclear antigen (PCNA) staining at each age (Fig.3.3A-H) in *Foxg1*$^{-/-}$ mutant and wild-type brains labelled all VZ cells, showing that they are all proliferating. Very few post-mitotic neurons, as assessed by immunofluoresence to β-III-Tubulin, were observed in the ventricular zone (Fig.3.3K,L). This technique also assumes that the precursor cells consist of a single proliferating population with the same cycling kinetics. Evidence suggests this is indeed the case during early telencephalic development. However, around

E13.5 in the dorsal telencephalon (Takahashi et al., 1995b; Takahashi et al., 1996) and E11.5 in the ventral telencephalon (Sheth and Bhide, 1997) a second proliferative population arises within the germinal zone. Since the cell cycle kinetics of these populations are very similar to those of the primary proliferative population (Sheth and Bhide, 1997; Takahashi et al., 1995b), this aspect of telencephalic development should not affect results generated by this technique. In the future, if antibodies are generated that specifically recognise sub-populations of proliferating cells, this third label could easily be incorporated into the labelling scheme to allow separate cell cycle kinetic analyses for each population.

The IddU/BrdU double labelling technique for measuring cell cycle kinetics is fast, reliable and can be used to estimate parameters in defined groups of cycling cells in individual embryos. Estimates of cell cycle parameters in wild-type embryos generated in this study are very similar to those obtained at a range of ages by various authors using the more traditional technique of BrdU cumulative labelling (Takahashi et al., 1995a); (Bhide, 1996); (Estivill-Torrus et al., 2002); (Yuasa et al., 2002) (Fig.3.2E). The correlation between the wild-type values of T_c obtained here and those previously published at comparable ages is statistically significant ($p=0.022$, Pearson correlation test, $n=9$).

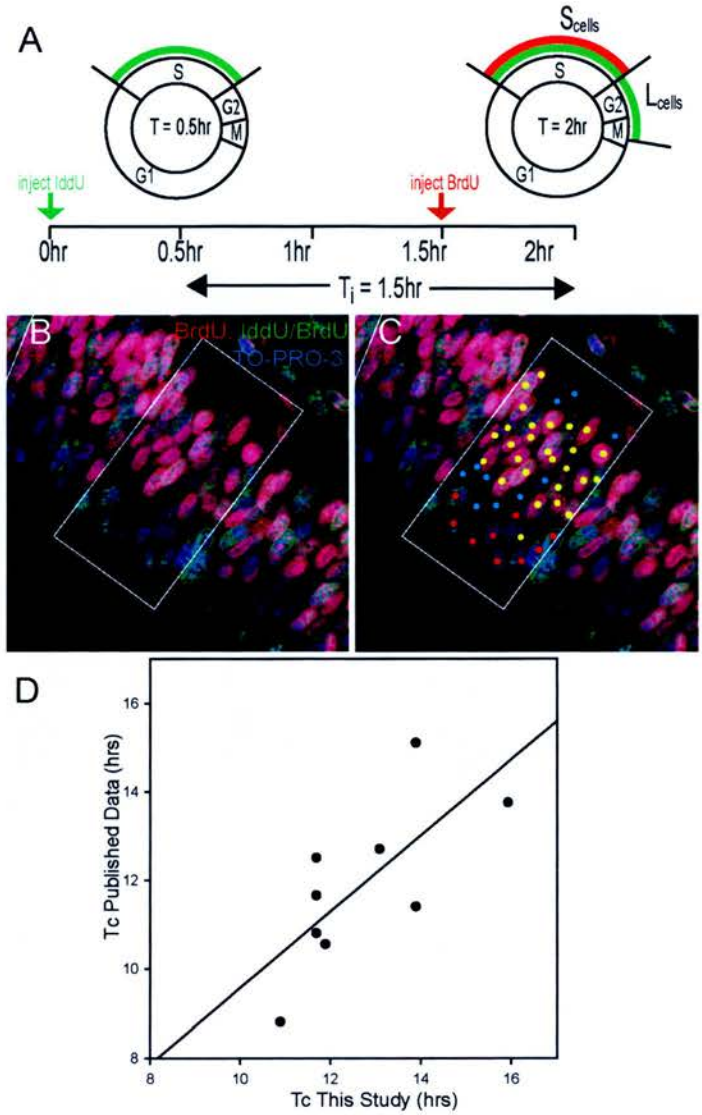


Fig.3.2. Calculation of cell cycle kinetics by IddU/BrdU double labelling. (A) To estimate cell cycle kinetic parameters, the pregnant mouse is injected with IddU at T=0hr to label all cells in S-phase at the beginning of the experiment. At T=1.5hr, an injection of BrdU is given and the embryos are fixed after a short survival period of 0.5hrs, sufficient to label the S-fraction (S_{cells}) at the end of the labelling period (Nowakowski et al., 1989). During the 1.5hr interval when cells are exposed to IddU but not BrdU (T_i), some of the initial S-phase cohort will leave S-phase and consequently will be labelled just with IddU; this is the leaving fraction (L_{cells}). (B,C) Monoclonal antibodies specific for both BrdU and IddU (green), and BrdU alone (red) can then be used to identify cells in the S-fraction (red and green double-labelled cells in B marked with yellow spots in C) and cells in the L-fraction (green only in B and C, marked with red spots in C); blue spots in C mark unlabelled cells. A nuclear counterstain can be used to count all proliferating VZ cells which represents the P_{cells} fraction (all stained nuclei in B and yellow, red and light blue marked nuclei in C, please refer to Fig.2. to assess the validity of this). The L_{cells}, S_{cells} and P_{cells} fractions can then be used to calculate T_c and T_s (see Materials and Methods). (D) When T_c estimates obtained in wild-types with this technique at a range of ages are plotted against those obtained using BrdU cumulative labelling for matched ages in the same tissues

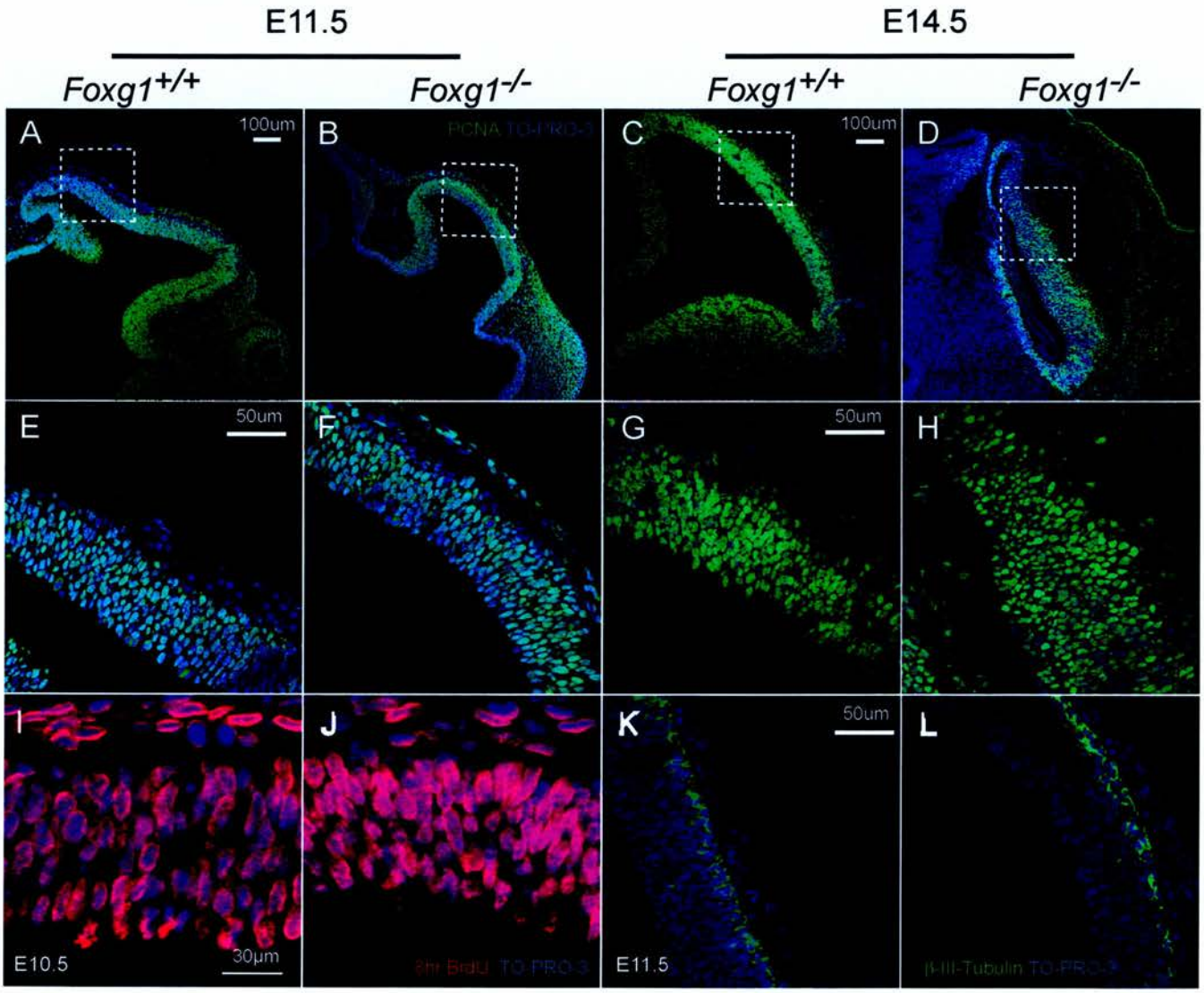


Fig.3.3. All Ventricular Zone cells are proliferative in wt and *Foxg1*$^{-/-}$ telencephalon. PCNA and β-III-Tubulin immunohistochemistry at E11.5 and E14.5 and cumulative BrdU labelling at E10.5. (A,D,C,D) Low power images show PCNA staining throughout the ventricular zone in all regions of wildtype and *Foxg1*$^{-/-}$ telencephalon. (E,F,G,H) High power views of regions highlighted in A,B,C,D show that the overwhelming majority of cells in the ventricular zone (VZ) of control and mutant dorsal telencephalon express PCNA and are therefore actively proliferating. (I,J) At the beginning of telencephalic neurogenesis (~E10.5) essentially all VZ cells are actively proliferating in both wild-type and *Foxg1*$^{-/-}$ telencephalon, since they all become labelled with BrdU following 8hrs cumulative labelling at E10.5. (K,L) β-III-Tubulin staining at E11.5 labels newly differentiated neurons. In both wildtype and *Foxg1*$^{-/-}$ telencephalon newborn neurons migrate rapidly to the pial surface and very few are witnessed within the VZ. Nuclei are counterstained with TO-PRO-3 (blue).

Cell cycle time increases precociously in the Foxg1$^{-/-}$ telencephalon

Cell cycle kinetics of telencephalic progenitor cells were estimated at several ages during early and mid neurogenesis in control (wild-type and *Foxg1*$^{+/-}$ heterozygotes, see Materials and Methods) and *Foxg1*$^{-/-}$ embryos (Fig.3.4). Numerous counts were made in sampling bins throughout the telencephalon at E9.5, E10.5 and E11.5 and in the dorsal telencephalon at E14.5, to give an average estimate of T_c for each embryo at each age. Patterns of IddU and BrdU labelling were very similar in control (Fig.3.4A) and mutant sections (Fig.3.4B). Cells in S-phase are BrdU labelled (red) and, as expected, were found in the outer half of the ventricular zone. Cells which had left S-phase during the labelling period (IddU-labelled only, green) had moved towards the ventricular surface. The presence of unlabelled mitotic figures (asterisks in Fig.3.4A and B) shows that T_i is shorter than the length of G2 and M-phases (T_{G2+M}) so the number of cells in the L_{cells} fraction is not artificially inflated by the counting of recently divided cells. These findings suggest that interkinetic nuclear migration and the general mode of telencephalic precursor proliferation is not severely affected by the loss of *Foxg1*. This is in agreement with the findings of Hanashima et al (2002). Fig.3.4C shows that at E9.5 mean T_c is approximately 7hr in control and *Foxg1*$^{-/-}$ telencephalon. Around E10.5, neurogenesis commences and the first post-mitotic neurons appear at the pial surface of the telencephalic neuroepithelium. As neurogenesis proceeds there is a gradual lengthening of T_c with age in both control and *Foxg1*$^{-/-}$ telencephalon (Fig.3.4C). This slowing of progenitor proliferation rate is more severe and occurs earlier in the *Foxg1*$^{-/-}$ telencephalon. At E14.5, the mid-point of wild-type dorsal telencephalic neurogenesis,

when cell output should be at its peak (Takahashi et al., 1996); (Caviness et al., 1995), the mean cell cycle time of *Foxg1*$^{-/-}$ dorsal telencephalic precursors is more than twice that of their wild-type counterparts (29hr and 14hr respectively). The difference in T_c between controls and mutants observed over time is statistically significant ($p=0.002$, univariate general linear model, $n=12$).

Interestingly, the length of S-phase is very similar in wild-type and mutant precursors, averaging between 4 and 5 hours (very similar to wild-type T_s measured by other authors (Takahashi et al., 1995a); (Bhide, 1996); (Estivill-Torrus et al., 2002)) at all ages except E14.5, where it is lengthened as the mutant cell cycle becomes more disregulated (Fig .3.4D).

These results suggest that while the mode of progenitor cell division is not grossly altered in the *Foxg1*$^{-/-}$ telencephalon, there is an early and significant reduction in proliferation rate from E10.5, co-incident with the start of neurogenesis. From this age onwards the mutant telencephalon is notably reduced in size (Xuan et al., 1995); and this study). This reduction in proliferation rate is very likely to contribute to the observed hypoplasia.

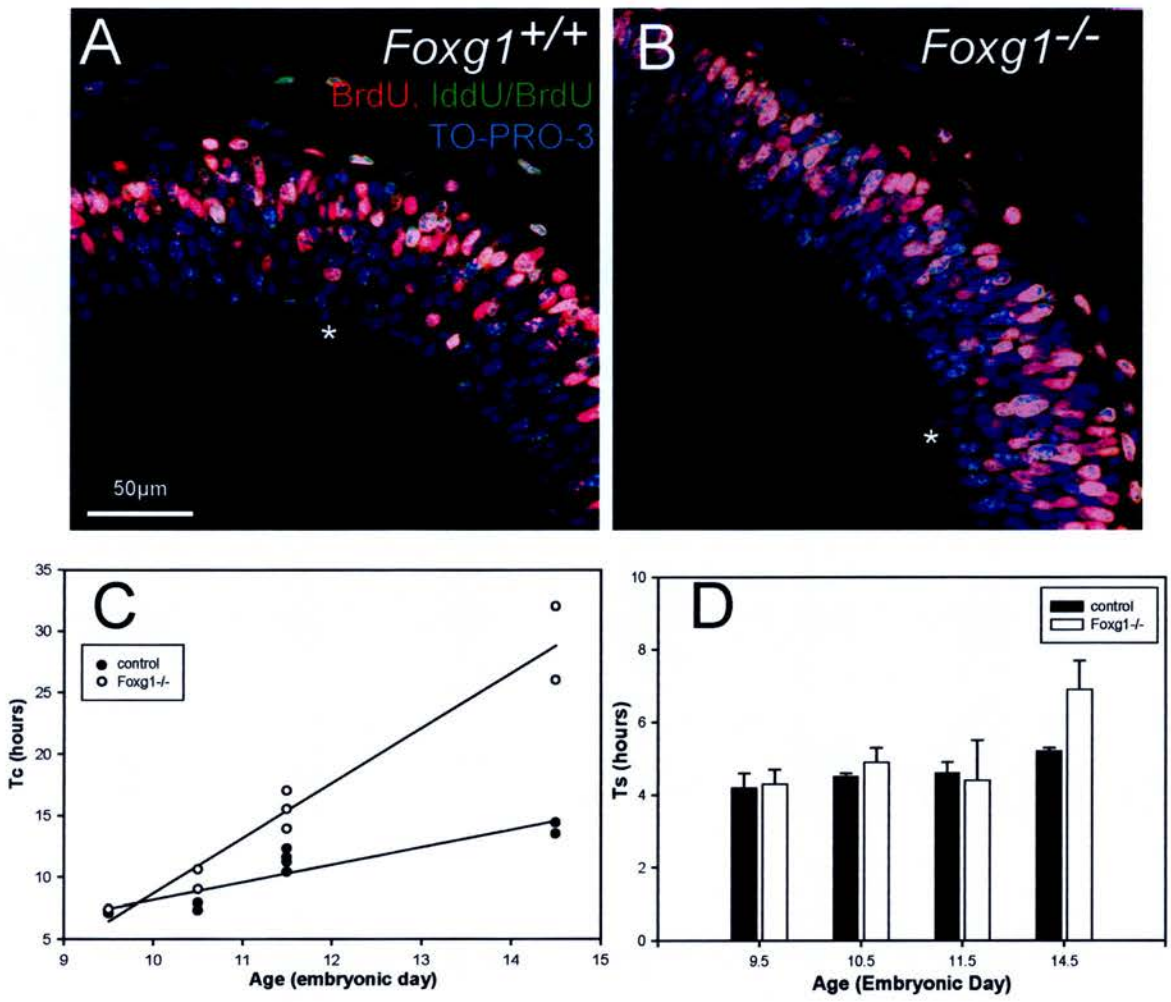


Fig.3.4. Cell cycle times increase precociously in the *Foxg1*$^{-/-}$ telencephalon. Patterns of IddU and BrdU labelling within the ventricular zone are very similar in (A) wild-type and (B) *Foxg1*$^{-/-}$ telencephalon at E10.5, suggesting that inter-kinetic nuclear migration and the general mechanics of precursor cell division are not severely perturbed in the mutant. The presence of non-IddU labelled mitotic figures (asterisks) shows that T_i is shorter than the length of G2 and M-phases (T_{G2+M}). These facts suggest that it is appropriate to apply the IddU/BrdU labelling technique to control and experimental embryos. (C) Mean T_C increases with developmental age in both control and *Foxg1*$^{-/-}$ telencephalon, but the trend is accelerated in mutant embryos, as shown by linear regressions. The difference in T_C between control and mutant telencephalon over developmental time is significant ($p=0.002$, analysis of variance by general linear model, $n=12$ embryos). (D) Histogram showing mean T_S ±SD. at a range of ages in *Foxg1*$^{-/-}$ embryos (open bars) and control embryos (filled bars). T_S remains relatively constant at 4-5hrs in both wild-type and mutant at all ages analysed, except at E14.5

Cell cycle time is altered in a region-specific fashion

At E9.5, the *Foxg1*$^{-/-}$ telencephalon is similar in size to wild-type controls. However, as development proceeds, the mutant telencephalon appears to grow more slowly than wild-type telencephalon. At E12.5, the telencephalon of *Foxg1*$^{-/-}$ embryos is clearly hypoplastic relative to that of controls and this is more apparent rostrally than caudally (Fig.3.1, compare A and C with B and D and E and G with F and H). Morphologically there is also a loss of ventral telencephalic structures. The ganglionic eminences, which are usually evident by ~E11 and are well established by E12.5 (Fig.3.1C,E) fail to form in the absence of *Foxg1* (Fig.3.1D,F). Previous studies have also failed to detect genetic markers of ventral telencephalic fate at E10.5 and E12.5 ((Dou et al., 1999; Xuan et al., 1995) and results presented in subsequent chapters).

IddU/Brdu labelling was used to determine whether the reductions in rostral and ventral telencephalic tissues are related to region-specific variations in the rate of cell cycle progression. To achieve this, the telencephalon was systematically divided into sampling bins across the full dorso-ventral and rostro-caudal axes (Fig.3.5A). T_c was determined in each bin in several control and *Foxg1*$^{-/-}$ embryos at E9.5, E10.5 and E11.5. The results were plotted relative to position and graphs were consistent between each genotype and at each age; representative examples are shown in Fig.3.5B-G. At E9.5, when there is no overall difference in mean T_c between the mutant and wild-type (Fig.3.4C), the T_c values obtained were fairly constant across the whole telencephalon in embryos of both genotypes (Fig.3.5B,C). At E10.5, when loss of *Foxg1* causes an increase in the mean T_c

(Fig.3.4C), the defect was not observed throughout the telencephalon, but was region-specific. Consistently, cells in rostral and dorsal territories had an inflated T_c, whilst cells in caudal and ventral regions continue to progress through the cell cycle at a rate comparable to control littermates (Fig.3.5D,E). This rostral defect is even more pronounced at E11.5 and at this stage, groups of more slowly proliferating cells also appeared in more caudal regions (Fig.3.5F,G).

At E14.5 T_c and T_s were estimated in three equally spaced domains along the rostro-caudal axis (Fig.3.5H) in a single 200μm wide sampling bin in the dorsal telencephalon of control (Fig.3.5J) and mutant (Fig.3.5K) embryos. T_c was significantly increased (Students t-test $p<0.05$, $n=3$) at all three rostro-caudal levels, with a particularly large increase rostrally, where mutant T_c is three times that of control, as shown in Fig.3.5I.

The finding presented here that cell cycle time was not increased in ventral regions of the *Foxg1*$^{-/-}$ telencephalon at E10.5 contrasts with the previous suggestion that proliferation rate is reduced in this territory in this mutant (Xuan et al., 1995). This finding was based on calculation of the proportion of cells that had incorporated BrdU following pulse labelling with the halogenated pyrimidine. In the present study no difference in BrdU labelling index (LI, the percentage of VZ cells labelled with BrdU) in the ventral-most region of *Foxg1*$^{-/-}$ and control embryo telencephalon was observed at E10.5 (see Fig.3.5L-O). LI is 56.7±8.3% in control and 54.6±3.1% in *Foxg1*$^{-/-}$ ventral telencephalon (n=3).

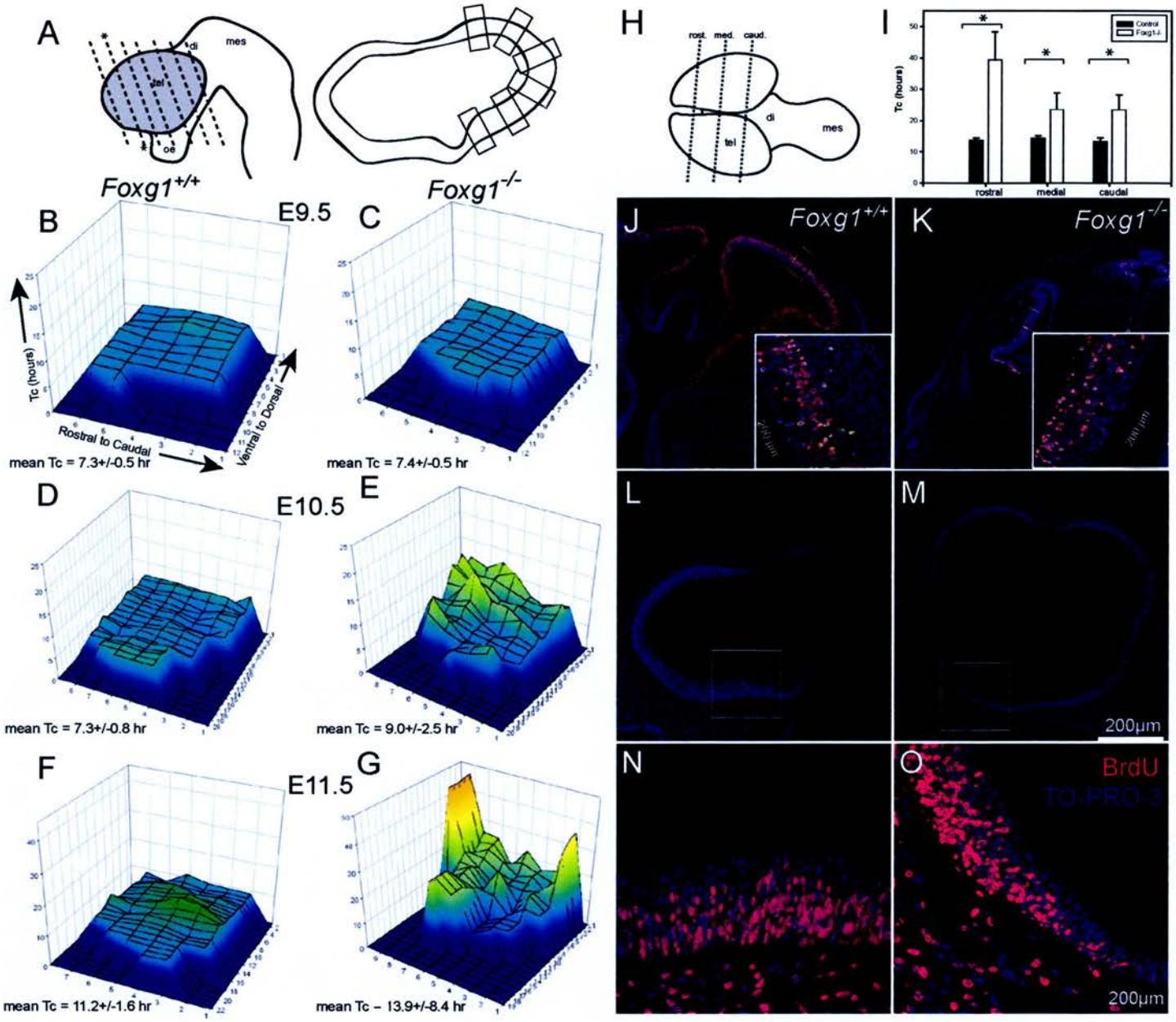


Fig.3.5. Cell cycle time is increased in a region-specific fashion in *Foxg1*$^{-/-}$ mutants. T_c and T_s were estimated in sampling bins covering the whole of the developing telencephalon at E9.5, E10.5 and E11.5 as schematised in A. Approximately every 6-10th section was imaged. The sections were divided into 100µm-wide sectors and cell counts were made through the depth of the telencephalic wall from the dorsal to ventral-most extent of the telencephalon in alternate sectors (the coronal section shown in A is taken from level marked by asterisks in the lateral view). This allowed the mapping of T_c values measured at evenly spaced interval across the surface of the telencephalon. (B-G) 3D plots from representative embryos at the three ages analysed. (B, C) At E9.5 T_c was constant across the telencephalon in control and mutant telencephalon. (D, E) By E10.5 populations of cells in rostral and dorsal portions of the *Foxg1*$^{-/-}$ had inflated T_c's relative to control. (F, G) At E11.5, lengthening of T_c was more severe and was observed through much of the mutant telencephalon. (H) At E14.5 groups of three non-adjacent sections in caudal, rostral and medial positions were analysed. (J,K) Cell cycle analysis was conducted in a 200µm counting bin placed in a dorso-lateral position of the neocortex in mutant and control sections. (I) At all three rostro-caudal levels T_c was significantly higher in the *Foxg1*$^{-/-}$ dorsal telencephalon (open bars) than in controls (filled bars) ($p<0.05$, Student's t-test, n=3). Histogram shows mean $T_c \pm$ SD. (L-O) BrdU labelling in

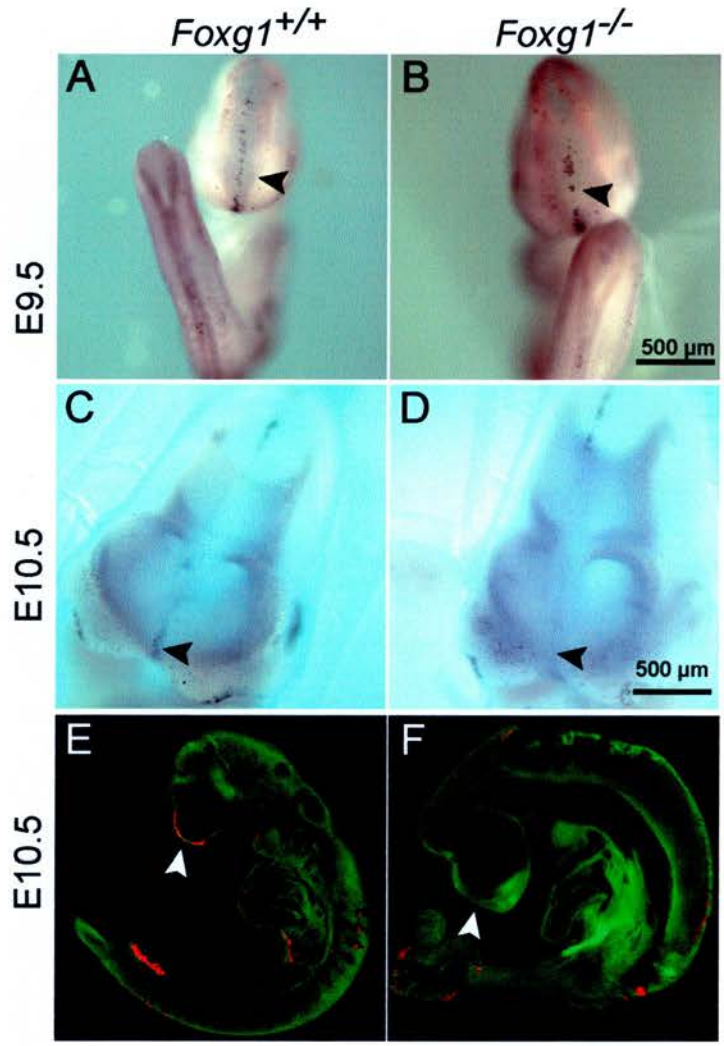


Fig.3.6. Apoptosis is reduced in the rostral telencephalic midline of *Foxg1*$^{-/-}$ mutants. (A, B) Whole mount TUNEL shows that apoptotic cells are present in the rostral midline of the wild-type (arrowhead in A) and *Foxg1*$^{-/-}$ telencephalon (arrowhead in B) at E9.5. At E10.5 TUNEL-labelled cells are still observed in the rostral telencephalon of controls (arrows in C, E) but almost completely absent in the *Foxg1*$^{-/-}$ mutant (arrows in D, F). Some TUNEL-labelled embryos were scanned with optical projection tomography (OPT) allowing for the digital reconstruction of the complete set of optical sections for each embryo, which provided verification that this rostral apoptotic population is in the neuroepithelium, rather than the overlying surface epithelium. Examples of pseudo-coloured optical sections are shown in E and F with regions of TUNEL-labelling in red.

Apoptosis is reduced in the rostral telencephalon of Foxg1$^{-/-}$ mutants

As well as the reduced precursor proliferation rate measured here, it is possible that an increase in the level of programmed cell death contributes to the morphological abnormalities and hypoplasia of the *Foxg1*$^{-/-}$ telencephalon. To examine levels of apoptosis in the developing telencephalon whole-mount TUNEL was performed on *Foxg1*$^{-/-}$ embryos and wild-type littermates. At E9.5, TUNEL labelled cells in the telencephalon are restricted to the dorso-rostral midline (Furuta et al., 1997);(Aoto et al., 2002); (Ohkubo et al., 2002)) (Fig.3.6A,B). At this age, although it appears that there may be slightly fewer labelled cells in *Foxg1*$^{-/-}$ telencephalon, this was not consistent (n=3 per genotype). A day later, at E10.5, there is a clear and consistent reduction in the number of TUNEL labelled cells in this rostral domain in the *Foxg1* mutant telencephalon at E10.5 in all embryos analysed (n=3) (compare Fig.3.6C and E with D and F). Scanning TUNEL-labelled embryos with optical projection tomography (OPT) (this was done by Harris Morission, according to a published technique ((Sharpe et al., 2002)) showed that apoptotic cells are in the telencephalic neuroepithelium, rather than the overlying ectoderm. Movies of OPT-scanned, TUNEL-labelled embryos can be seen online at the Developmental Biology website: http://www.elsevier.com/locate/inca/622816 (follow links to volume 283, issue 1, page 113). Few apoptotic cells were observed in ventral regions of *Foxg1* mutant or control embryos, and as such, increased cell death does not seem to contribute to the impaired development of the *Foxg1*$^{-/-}$ ventral telencephalon. Patterns of apoptosis in non-telencephalic parts of *Foxg1*$^{-/-}$ embryos are otherwise indistinguishable from those in

wild-types, with labelled cells in the midline of the diencephalon and mesencephalon, in the facial ectoderm, somites and limb buds (please refer to online movies), suggesting that the observed decrease in TUNEL labelled cells in the telencephalon results specifically from the loss of *Foxg1*.

Neurogenesis is accelerated in the Foxg1$^{-/-}$ telencephalon

In addition to the regulation of proliferation rate and the rate of cell death, the rate at which the progeny of neurogenic cell divisions withdraw from the cell cycle to differentiate must be tightly controlled to ensure neurons are produced in correct numbers at the correct time. Previous authors have observed an early accumulation of cells expressing markers of post-mitotic neurons in the mantle zone of the *Foxg1*$^{-/-}$ telencephalon from E12.5 onwards (Xuan et al., 1995); (Hanashima et al., 2002). Here, these observations are confirmed and extended to show that an excess of neurons is produced from E11.5 onwards.

At E11.5 the number of cells in sampling bins traversing the depth of the telencephalic wall was counted systematically in several rostral and caudal sections throughout the dorso-ventral extent of the telencephalon in *Foxg1*$^{-/-}$ embryos and in the control dorsal telencephalon (the neocortex). Sections used were taken from an embryo that had been labelled with a short pulse (30 minutes) of BrdU, which revealed the position of S-phase nuclei. These lie at the outside edge of the ventricular zone (Takahashi et al., 1992). Cell nuclei superficial to the S-phase zone were considered post-mitotic and counted as such. This was confirmed by double labelling with β-III-Tubulin, an early marker of

differentiating neurons, in control and *Foxg1*$^{-/-}$ telencephalons (Fig.3.7A,B). In both control and mutant embryos very few cells expressing this neuronal marker were observed within the ventricular zone (Fig.3.7A-D, 3.3K,L). The average number of cells in the sampling bin positioned superficial to the ventricular zone averaged across several rostral and caudal sections for 3-4 embryos per genotype are plotted in Fig.3.7E.

In both rostral and caudal telencephalon, the number of cells outside the ventricular zone is two to three times larger in *Foxg1*$^{-/-}$ embryos than in their normal littermates. These differences are statistically significant ($p<0.05$, Student's t-test, n=3-4). The early exit of precursor cells from the cell cycle must limit the proliferative potential of the mutant telencephalon and contribute to its serious growth defects. Indeed, by E14.5, the mantle zone in *Foxg1* mutants is much wider than in the control dorsal telencephalon and whole cerebral hemisphere is clearly smaller (Fig.3.7C,D). In contrast to the proliferative and apoptotic defects described earlier, we saw no significant difference in the number of post-mitotic neurons in rostral and caudal regions of the mutant telencephalon.

The mature cerebral cortex consists of several neuronal cell types organised in a stereotypical laminar array (McConnell, 1995). Cells of the six cortical layers are produced in an organised and temporally regulated sequence. Cortical layers are born in an inside out fashion, except for the most superficial Cajal-Retzius (CR) cells which are born first (McConnell, 1995). Recent reports have demonstrated that CR cells are over-produced in *Foxg1*$^{-/-}$ mutant telencephalon (Hanashima et al., 2004; Muzio and Mallamaci, 2005) and have suggested that Foxg1 is required to actively suppress CR fate. By immunohistochemical staining for Reelin, Calretinin and Tbr1, all expressed by

CR cells, it was shown in the present study that the majority of neurons in the *Foxg1*$^{-/-}$ telencephalon at E12.5 have characteristics of CR cells, in concurrence with the earlier reports (Fig.3.7F-K). Previous work also provides strong evidence that Foxg1 is required in telencephalic cells to resist the cytostatic action of Tgfβ signalling (Dou et al., 2000; Rodriguez et al., 2001; Seoane et al., 2004). Furthermore, at a molecular level, this has been related to Foxg1's ability to repress the transcription of Tgfβ-induced expression of the CDK inhibitor p21^{cip1} (Seoane et al., 2004). Immunohistochemistry for p21^{cip1} in this study recapitulates the findings of Seoane and colleagues (2004) (Fig.3.7L,M). As noted above, other reports have suggested that bone morphogenetic protein (BMP) signalling can also induce p21^{cip1} in telencephalic cells (Gomes and Kessler, 2001; Israsena and Kessler, 2002). Since several BMPs are overexpressed in Foxg1 mutants after E11.5 (Dou et al., 1999; Hanashima et al., 2002), this could also contribute to p21^{cip1} induction, cytostasis and neuronal differentiation.

In addition to excitatory glutamatergic neurons, which are produced locally by dorsal telencephalic progenitor cells, the mature cerebral cortex contains significant numbers of inhibitory GABAergic interneurons. The majority of cortical interneurons appear to be generated subcortically, in the ventral telencephalic progenitor zones, prior to their tangential migration to the developing cerebral cortex (Marin and Rubenstein, 2001). By E12.5, the wildtype telencephalon contains significant numbers of Calbindin+ and Lhx6+ cells (Fig.3.7N,P), both of these markers are expressed specifically in interneurons (Marin and Rubenstein, 2001). At this age and later in telencephalic development, *Foxg1*$^{-/-}$ telencephalon is completely devoid of Calbindin+ and Lhx6+ cells (Fig.3.7O,Q), consistent with a failure to generate telencephalic interneurons.

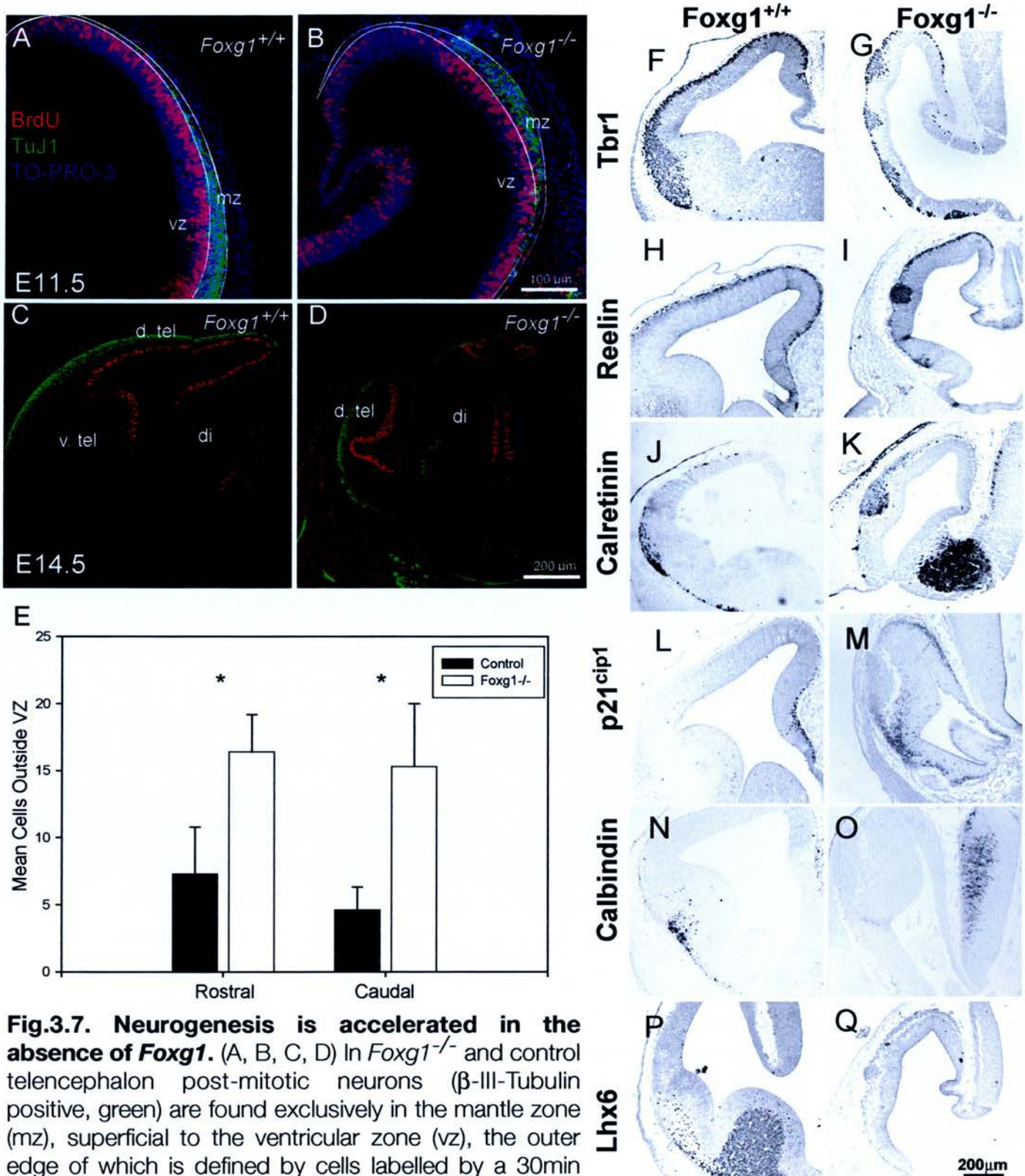


Fig.3.7. Neurogenesis is accelerated in the absence of *Foxg1*. (A, B, C, D) In *Foxg1*$^{-/-}$ and control telencephalon post-mitotic neurons (β-III-Tubulin positive, green) are found exclusively in the mantle zone (mz), superficial to the ventricular zone (vz), the outer edge of which is defined by cells labelled by a 30min pulse of BrdU (red). At both E11.5 (A, B) and E14.5 (C, D) the mutant mz appears thicker, relative to the vz. (E) Cells in the mantle zone (MZ) were counted to assess the average number of differentiated cells at E11.5. Counts were made in sampling bins in the dorsal telencephalon in groups of rostral and caudal sections. Mean number (±SD) of cells outside the VZ in control (filled bars) and *Foxg1*$^{-/-}$ (open bars) telencephalon are plotted. In both rostral and caudal regions the number of differentiated cells in the mutant dorsal telencephalon is significantly greater than

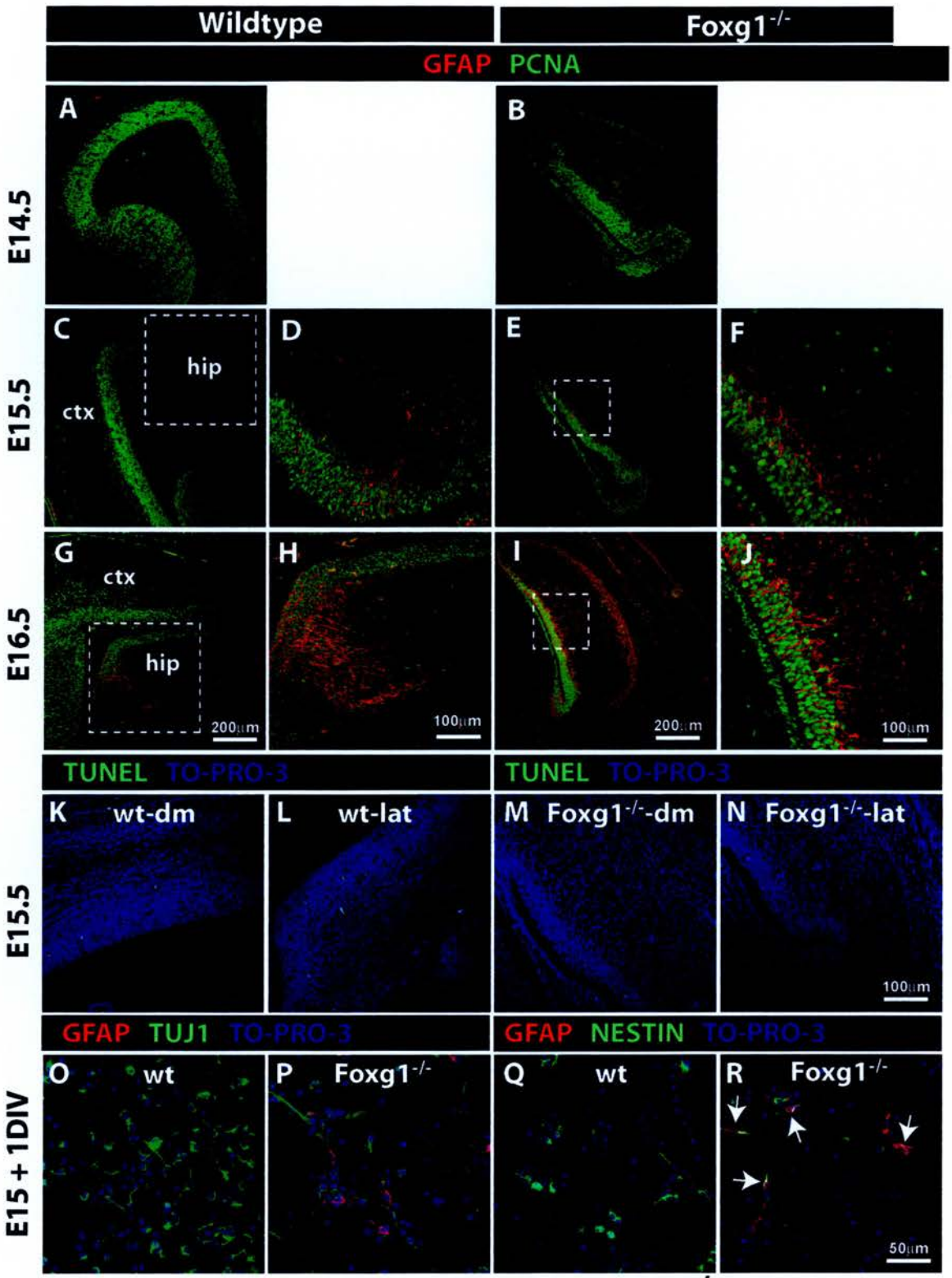


Fig.3.8. Premature onset of astrogliogenesis in *Foxg1*$^{-/-}$ telencephalon. (A-J) Immunofluorescence for GFAP (red) and PCNA (green) during mid to late neurogenesis. (A,B) At E14.5 no GFAP staining was observed wt and *Foxg1*$^{-/-}$ telencephalon. (C,D) By E15.5 a small group of GFAP+ cells are visible in the dorso-medial-most region of control telencephalon (D). (E,F) In *Foxg1*$^{-/-}$ telencephalon at E15.5 GFAP expression is evident throughout the telencephalon and is primarily associated with VZ cells (F). (G-J) At E16.5, GFAP+ cells are more numerous, but still confined to the wt cortical hem region (G,I), whereas GFAP staining is intense in the mutant. (J-M) TUNEL labelling of dying cells at E15.5 in wt lateral (K) and dorso-medial (L) telencephalon and

Extensive astrogliogenesis is initiated prematurely in the Foxg1$^{-/-}$ telencephalon

The production of astroglia is a relatively late event in telencephalic development, largely occurring after the period of neurogenesis in dorsal regions of the telencephalon (Sauvageot and Stiles, 2002). Since neurogenesis is initiated prematurely in the telencephalon of *Foxg1*$^{-/-}$ embryos, we were interested to determine whether astrogliogenesis was also accelerated in these mutants. Mature glia of the astrocyte lineage express glial fibrillary acidic protein (GFAP) and are first observed during late neurogenesis in defined midline populations where they have important roles in guiding commissural axons (Shu et al., 2003; Shu and Richards, 2001). In *Foxg1*$^{-/-}$ embryos extensive GFAP immunofluorescence is observed at E15.5, where it is associated with cells in or near the ventricular zone throughout the mutant telencephalon (Fig.3.8E,F). In wt littermates at this age, GFAP staining is confined to a small region of the medial telencephalon in the hippocampal complex (Fig.3.8C,D). A day later, at E16.5, a significant proportion of cells in the *Foxg1*$^{-/-}$ telencephalon are positive for GFAP (Fig.3.8I,J). At this age it appears that most VZ cells express GFAP and multiple GFAP+ processes are visible throughout the telencephalic wall. This marked upregulation of GFAP between E15.5 and E16.5 is not observed in wt controls. At E16.5, GFAP cells are somewhat more numerous, but still exclusively localised to the dorso-medial telencephalon (Fig.3.8G,H). To confirm that GFAP+ cells in the *Foxg1*$^{-/-}$ telencephalon were indeed astrocytes staining for S-100β protein, which is also expressed by astrocytes (Gomes et al., 2003), with two different antibodies was attempted. However, reliable staining was not achieved in control (including post-natal brain where S-100β+

astrocytes are known to be present (Gomes et al., 2003)) or mutant telencephalon and this issue was not pursued further.

One possibility for the sudden appearance of GFAP+ glia in the *Foxg1*$^{-/-}$ telencephalon is that it represents a non-specific gliosis type reaction, which can occur in degenerating or damaged brain regions (Horner and Palmer, 2003). To determine whether there was an increase in cell death at E15.5 (when GFAP upregulation occurs), TUNEL labelling of dying cells was performed. Qualitatively there appears to be no excess cell death in the telencephalon of *Foxg1*$^{-/-}$ mutants (Fig.3.8N,O) compared to wt (Fig.3.8K,L), suggesting that such a gliosis reaction is unlikely.

The appearance of GFAP immunofluorescence within the pseudostratified ventricular epithelium at the ventricular surface of the *Foxg1*$^{-/-}$ telencephalon suggested that radial glia in this zone may have been undergoing a terminal maturation to an astroglial phenotype, a transition that also happens in the wt telencephalon, but only much later in development (Hunter and Hatten, 1995; Schmechel and Rakic, 1979). To examine this issue, double immunofluorescence for GFAP and Nestin (an intermediate filament protein expressed by radial glia) was performed. Since both these proteins are expressed in cellular processes, it is difficult to determine accurately whether cells are truly co-labelled or whether the processes of separate cells are closely entwined. To facilitate such analysis, cells from E15.5 wt and *Foxg1*$^{-/-}$ telencephalon were dissociated and cultured overnight prior to immunofluorescence, to allow single cells to be analysed in isolation. In these conditions numerous Nestin+ cells co-expressed GFAP in *Foxg1*$^{-/-}$ cultures

(Fig.3.8S) but not in wt cultures (Fig.3.8R), consistent with an early transition from radial glial to astrocytic phenotype.

To ensure that the generation of GFAP+ glia was distinct from the generation of neurons in Foxg1 mutants, dual immunofluoresence for GFAP and β -III-Tubulin was performed, also on dissociated cells. Numerous GFAP+ cells were present in *Foxg1*$^{-/-}$ but not in control cultures and GFAP and β-III-Tubulin were never co-expressed (Fig.3.8P,Q).

Fibroblast growth factor 8 expression is greatly reduced in Foxg1$^{-/-}$ *rostral telencephalon.*

Members of the Fibroblast growth factor (Fgf) family have been shown to have numerous roles in the development of various structures in developing embryos, including the telencephalon. Fgf8 seems to be a particularly important morphogenetic cue for the development of the telencephalon. In the wild-type *Fgf8* is expressed in a discrete pattern at the rostral pole of the telencephalon from its initial specification (~E8) until at least E12.5 (Crossley and Martin, 1995), (Shimamura and Rubenstein, 1997); (Crossley et al., 2001). Gain and loss of function studies have demonstrated that *Fgf8* is important for controlling rostro-caudal patterning of the telencephalon (Fukuchi-Shimogori and Grove, 2001) and also for regulating cell proliferation (Lee et al., 1997); (Martinez et al., 1999)) and survival (Storm et al., 2003) of neural cells. Given its role as a potent controller of telencephalic morphogenesis, the pattern of *Fgf8* expression was examined in control and *Foxg1*$^{-/-}$ mutant embryos. At E9.5, before proliferative, apoptotic and differentiative defects are

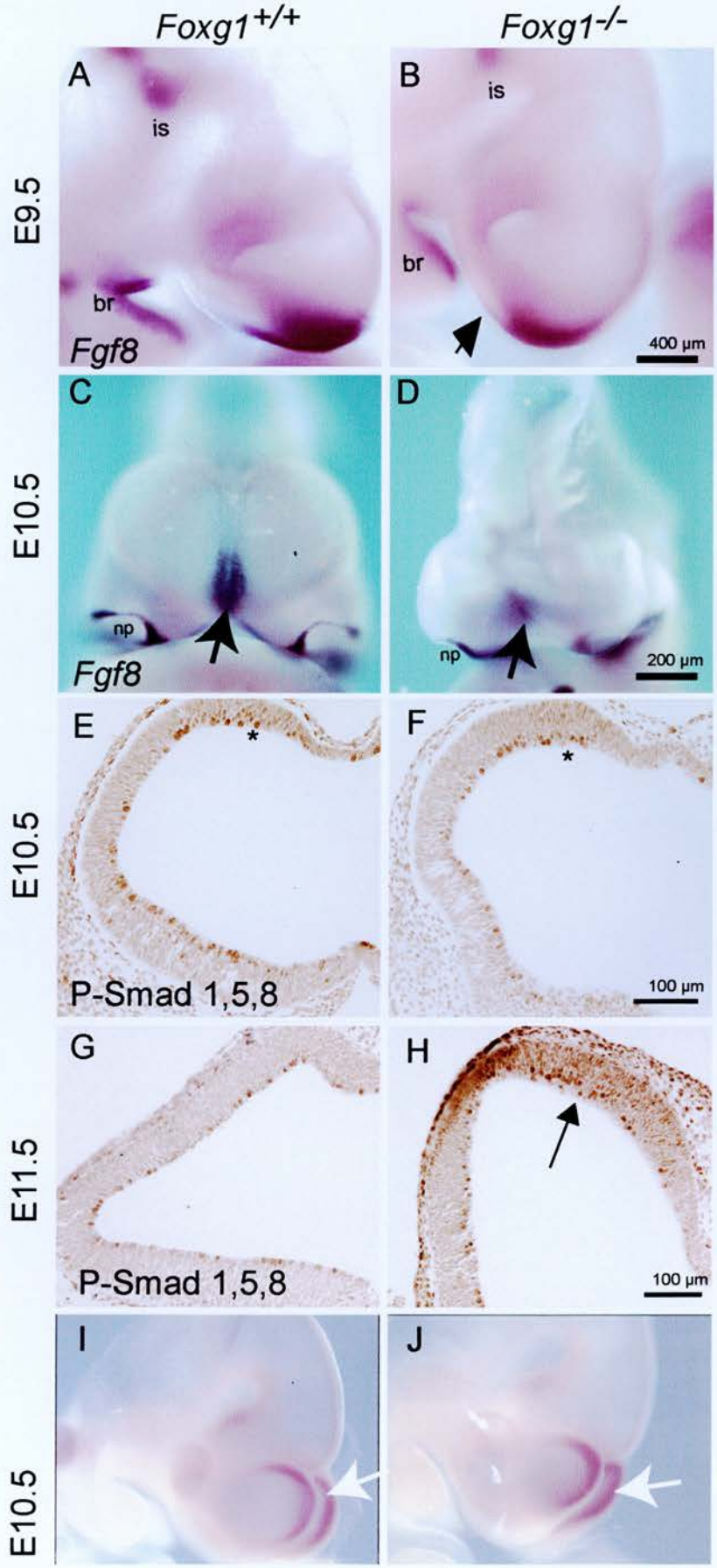


Fig.3.9. Fgf8, BMP and Wnt signalling pathways in control and *Foxg1*$^{-/-}$ telencephalon. (A, B) Whole mount in situ hybridisation at E9.5 for *Fgf8* shows this signalling molecule is expressed in the rostral telencephalon of both *Foxg1*$^{-/-}$ and wild-type embryos, although it may be slightly reduced and does not extend so far ventrally in the mutant (arrow in B). (C, D) At E10.5 there is dramatic reduction in the level of *Fgf8* expression in the same rostral telencephalic domain in *Foxg1*$^{-/-}$ embryos (white arrows). At both ages Fgf8 expression in other structures, notably the branchial arches (br), nasal pits (np) and isthmus (is), is not affected by the loss of Foxg1. (E,F,G,H) Immunohistochemistry on coronal sections for phosphorylated Smad-1,5,8 (P-smad), a readout of BMP signalling. At E10.5 there is no clear difference in P-smad staining between mutant and control, with positive cells around the ventricular surface and increased staining in the medial telencephalon (asterisks in E,F). By E11.5 there is increased P-smad staining in the null telencephalon, particularly in the medial telencephalon (arrow in H). (I,J) Whole mount in situ hybridisation for *Wnt8b* shows that this Wnt family member is expressed appropriately in a restricted dorsomedial band of control and mutant telencephalon (white arrows).

observed, *Fgf8* expression is detectable in the rostral telencephalon of the *Foxg1*$^{-/-}$ embryos and controls (n=3 per genotype), although expression levels may be slightly lower overall and the rostro-ventral limit of expression may be shifted in the mutant telencephalon (Fig.3.9A,B). At E10.5 there is a striking reduction in the level of *Fgf8* expression in the anterior telencephalon of *Foxg1* mutant embryos relative to controls (n=3 per genotype) (Fig.39C,D).

Since apoptosis is actually reduced in the same territory at this age, the reduction in *Fgf8* expression is not due to programmed cell death of expressing cells. The reduced proliferation rate described above, although it is first observed in the same rostro-dorsal domain that expresses *Fgf8*, also seems an unlikely cause since it co-incides with, rather than precedes, the reduction in expression. Previous researchers have shown that BMP4, 6 and 7, which are normally restricted to the dorsomedial wall of the telencephalon are expanded in the *Foxg1*$^{-/-}$ telencephalon after E11.5 (Dou et al., 1999; Hanashima et al., 2002). Furthermore, it has been shown that enhanced BMP signalling can repress Fgf8 expression in the chick telencephalon (Ohkubo et al., 2002). BMPs have also been shown to induce cell cycle exit, premature differentiation and apoptosis in telencephalic cells (Li et al., 1998; Panchision et al., 2001). To examine whether excess BMP signalling might contribute to the loss of Fgf8 expression and proliferative and differentiative defects described above, the expression of direct effectors of BMP signalling, tyrosine phosphorylated forms of Smad-1,5,8 (P-Smad), were examined. At E10.5 immunohistochemistry for P-smad revealed positive nuclei around the ventricular surface of the telencephalic neuroepithelium and a slightly increased level of staining in the roofplate region of the telencephalon, where BMP expression has been documented

(Furuta et al., 1997). There is no clear difference in P-smad staining in control and *Foxg1*$^{-/-}$ telencephalon at E10.5 (Fig.3.9E,F). A day later, however, at E11.5 when BMP expansions have been demonstrated (Dou et al., 1999; Hanashima et al., 2002), increased P-smad staining is observed in the *Foxg1*$^{-/-}$ telencephalon, especially in dorsomedial regions (Fig.3.9G,H), suggesting that the ectopic BMP expression is causing intracellular BMP signal transduction.

It is also possible that changes in other dorsally restricted signalling molecules, such as the Wnt proteins, may alter *Fgf8* expression and cause defects in telencephalic development in *Foxg1*$^{-/-}$ mutants. To address this, the expression of *Wnt8b* at E10.5 was assessed. In both control and mutant embryos, *Wnt8b* mRNA was restricted to the dorsomedial telencephalic wall (Fig.3.9 I,J).

These data suggest that BMP and Wnt8b signalling activity are present and correctly localised in the *Foxg1*$^{-/-}$ telencephalon at E10.5. However, since they are not expanded at the time that *Fgf8* expression is reduced (E10.5) it seems unlikely that increases in these dorsally derived signals cause this repression directly. It is possible that other Wnt family members are expanded prior to E11.5.

DISCUSSION

The following pages provide a detailed analysis of the various causes of telencephalic hypoplasia resulting from null mutation of the transcription factor Foxg1. Telencephalic

growth and development was approached by considering the three crucial factors that determine the size of a population during a defined developmental interval: proliferation rate, differentiation rate, and the rate of cell death.

IddU/BrdU Labelling for the analysis of cell cycle kinetics

During telencephalic development precursor cell cycle kinetics are very tightly regulated (Caviness et al., 1995; Takahashi et al., 1995a; Takahashi et al., 1995b). This is necessary to allow the orchestrated production of the correct type of neurons and glia, in the right place, at the right time. Even relatively small alterations in cell cycle time can have profound effects on the cellular output from the proliferative zones. Coletter Dehay and colleagues have described how region-specific regulation of cell kinetics during embryogenesis is used to generate the unique structure of different neocortical areas in the adult mammal (Dehay et al., 1993; Lukaszewicz et al., 2005; Polleux et al., 1997). For these reasons it is important that tools are available for the accurate assessment of cell cycle kinetics if we are to understand the development of the normal and experimentally perturbed developing telencephalon.

Halogenated pyrimidines such as BrdU and IddU are hugely powerful tools for developmental biologists studying cell proliferation. The proportion of cells in a population labelled during a short exposure, or pulse, of BrdU (the labelling index, LI) is frequently used a proxy measure of the rate of proliferation and/or number of proliferating cells. However, there is no necessary correlation between BrdU LI and proliferative pool size or proliferative rate. Differences in LI between two or more

populations can be caused by a range of factors, which each have different biological implications. The cause of the change in LI is usually impossible to determine without further analysis. To illustrate this point we must consider a hypothetical population of asynchronously cycling cells, all of which are proliferating. If the LI of this population is 0.25, Ts is one quarter the length of T_c. If we take a second, comparable population and find that this population also has a LI of 0.25, we cannot assume that T_s or T_c (and therefore the rate of proliferation) is the same. If the length of Tc was half that of the first population, but T_s was also half that of population 1, then LI would still be 0.25, but the second population would be proliferating twice as fast. Equally, if we observed a third population with an LI of 0.15, this population may still have the same T_c as the first population, but just a relatively shorter S-phase. Therefore, the BrdU LI can only reliably tell us the proportion of cells in S-phase at a given time. Although it has been reported that S-phase is relatively invariant through normal telencephalic development (Caviness et al., 1995), this need not be the case during experimental or genetic manipulations, especially those interventions likely to affect cell cycle progression and the rate of proliferation. Where people have measured T_s, there are good examples of variation in its length. For example in mice mutant for the transcription factor Emx2, T_c of cortical progenitors is increased, largely due to a significant increase in T_s (Muzio et al., 2005). Similarly, Rachel et al (2002) clearly show a rather dramatic lengthening of T_s in the retina during the second half of embryogenesis (Rachel et al., 2002). In both of these examples a BrdU pulse would give an increased LI (since a greater proportion of cells will be in S-phase) and researchers may be tempted to infer erroneously a greater rate of proliferation, when proliferation rate is actually reduced.

It is therefore clear that the investigator cannot rely solely on BrdU labelling indices if the aim is to determine the rate of proliferation. This requires cell cycle kinetic information, most importantly, the time taken for an average member of the population to traverse a cell cycle (T_c), an important determinant of how quickly a population can grow. Traditionally developmental neurobiologists have used cumulative BrdU labelling to measure T_c and T_s (this technique is described in detail in Nowakowski et al, 1989). Although many interesting data have been generated with this technique (Estivill-Torrus et al., 2002; Hodge et al., 2004; Muzio et al., 2005; Takahashi et al., 1995a; Yuasa et al., 2002), it is somewhat cumbersome, in that it involves multiple counts in several specimens and averaging across litters to generate estimates of kinetic parameters. For these reasons, alternative techniques for assessing cell cycle kinetics are desirable. The IddU/BrdU double labelling strategy which was first described by Shibui et al (1989) for the estimation of cell cycle parameters in tumour cell lines, has here been adapted and optimised for use in the developing embryo *in vivo*. It is fast, reliable, allows parameters to be estimated in single specimens and also requires few *ad hoc* assumptions about the proliferating population under study. This technique will hopefully be of great utility for a wide range of developmental biologists.

Cell cycle time is increased in a regionally restricted fashion in the Foxg1$^{-/-}$ telencephalon

Previous authors have demonstrated that patterns of BrdU labelling are altered in the telencephalon of *Foxg1*$^{-/-}$ embryos, especially in rostral and ventral regions (Xuan et al., 1995), and inferred that cell cycle time is increased after E11.5 (Hanashima et al., 2002).

Here IddU/BrdU labelling is used to provide cell cycle kinetic estimates in *Foxg1* mutants to show that cell cycle time is the same as controls during the preneuronogenetic progenitor expansion period, but becomes increased in rostral and dorsal portions of the telencephalon as neurogenesis is initiated at E10.5. The defective lengthening of T_c spread through the telencephalic neuroepithelium as neurogenesis proceeds. Reduced proliferation rate must be a primary causal factor for the reduced size and cell number in the *Foxg1*$^{-/-}$ telencephalon observed from mid-gestation until the perinatal death of these embryos (Xuan et al., 1995).

Interestingly, the earlier study of BrdU labelling indices in the *Foxg1* mutant found that fewer cells in the ventral region of the telencephalon took up BrdU than their wild-type counterparts at E10.5 (Xuan et al., 1995). This evidence was used to infer that proliferation rate was specifically reduced in this region and, by extension, was responsible for the loss of ventral telencephalic tissue, as assessed by genetic markers, observed at later ages (Xuan et al., 1995); and this study, please refer to the following chapters). I found no evidence for reduced proliferation rate in the ventral portion of the mutant telencephalon and consequently it seems very unlikely that abnormal proliferation causes the agenesis of this structure.

Possible molecular mechanisms for Foxg1's regulation of precursor proliferation rate

A very interesting insight into Foxg1's ability to regulate telencephalic progenitors stems from recent work by Hanashima and colleagues (Hanashima et al., 2002). Transgenic

expression of a mutated version of Foxg1 lacking direct DNA binding ability (Foxg1-NHAA) on a *Foxg1*$^{-/-}$ background appears to rescue the major proliferative defects of the Foxg1 null mutant. This striking finding shows that although Foxg1 is discussed as a 'transcription factor', it can have potent biological activity independent of its ability to directly bind its own DNA targets. Thus the primary requirement for the Foxg1 protein in maintaining progenitor proliferation rates (as defined by Hanashima and colleagues, 2002) appears to be mediated by its protein:protein interactions within the cell.

Over the past few years an increasing number of reports have offered insight into DNA binding independent actions of Foxg1, some of which are likely to impinge on Foxg1's role in regulating proliferation. Two groups independently identified Foxg1 as a powerful antagonist of the cytostatic action of Tgfβ (Dou et al., 2000; Rodriguez et al., 2001), a member of the transforming growth factor-β superfamily of extracellular signalling molecules (Miyazono, 2000). *Foxg1*$^{-/-}$ progenitors are significantly more sensitive than controls to cytostasis induced by TGFβ (Dou et al., 2000; Seoane et al., 2004) and ectopic Foxg1, or Foxg1-NHAA, expression can protect cells from Tgfβ-mediated growth inhibtion in a mink lung epithelial cell line (Dou 00, Rodriguez 01). On a molecular level TGFβ signalling has a negative impact on cellular proliferation by inducing, amongst other genes, the cyclin dependent kinase (CDK) inhibitors (CKIs) p15 and p21^{cip1} and by repressing the expression of cyclinA. Foxg1 is capable of antagonising all three of these transcriptional responses to TGFβ (Dou et al., 2000; Rodriguez et al., 2001; Seoane et al., 2004). Foxg1's role in blocking p21^{cip1} transcription has been particularly well characterised. TGFβ receptor binding results in phosphorylation of specific Sma-Mad homolog (Smad) transcription factors, which then enter the nucleus

and regulate the transcription of TGFβ target genes (reviewed in (Massague et al., 2005; Miyazono, 2000)). In the case of $p21^{cip1}$, specific Smads activated by TGFβ bind to forkhead box transcription factors of the FoxO family and the Smad/FoxO complex binds the $p21^{cip1}$ promoter to activate transcription (Seoane et al., 2004). Foxg1 has been shown to bind directly to FoxO1, FoxO3 and FoxO4 (Seoane et al., 2004) and also to Smad3, Smad4 and Smad1 (Rodriguez et al., 2001). The addition of Foxg1 to Smad/FoxO complexes appears to negate the ability of this complex to activate $p21^{cip1}$ expression (Seoane et al., 2004). Indeed, $p21^{cip1}$ is just one of many Smad targets downstream of TGFβ. Foxg1's ability to bind Smads and Smad partners such as FoxO may have an impact on a wide range of TGFβ targets in addition to $p21^{cip1}$. Further to this point, previous work has shown that other members of the TGFβ superfamily, BMPs can also induce $p21^{cip1}$ expression (Gomes and Kessler, 2001) and that most BMP transcriptional responses are mediated via Smad1. Given Foxg1's ability to interact with Smads and to inhibit Smad-dependent gene expression, it is also possible that Foxg1 is required to antagonise anti-proliferative BMP signalling in the developing telencephalon, independent of Foxg1's direct DNA-binding activity, and that excess $p21^{cip1}$ also stems from de-repressed BMP signalling.

The physiological significance of Foxg1's role in TGFβ antagonism is supported by the evidence that various TGFβ family members and their receptors are expressed in and around the telencephalic precursors (Flanders et al., 1991; Miller, 2003)and in the absence of Foxg1, $p21^{cip1}$ is expressed in numerous cells throughout the telencephalon (Seoane et al (2004) and repeated in this study). In the wt telencephalon, $p21^{cip1}$ protein expression is largely restricted to the cells of the Foxg1-negative dorso-medial

telencephalon, suggesting that Foxg1 might repress $p21^{cip1}$ expression throughout the rest of the telencephalon. $p21^{cip1}$ is known to inhibit the G1-S transition (Sherr and Roberts, 1999) and its ectopic expression in Foxg1 mutants may mediate aspects of the slowing of the cell cycle progression documented in this chapter. Furthermore, S-phase was found to be invariant between wt and *Foxg1*$^{-/-}$ during early neurogenesis, suggesting that the defect is not in S-phase progression, but may well be at the point of $p21^{cip1}$ activity (i.e. just prior to entry to S-phase).

As mentioned above, Foxg1 has been shown to bind FoxO transcription factors and inhibit their transcriptional activity (Aoki et al., 2004; Seoane et al., 2004). This is of great interest when considering additional ways that Foxg1 can maintain telencephalic precursor proliferation. FoxO factors are critical regulators of cell cycle progression and cell cycle progression and are negatively regulated targets of the PI3K (phosphatidyl inositol 3-kinase) mitogenic pathway (Czech, 2003; Tran et al., 2003; Vivanco and Sawyers, 2002). The PI3K pathway is activated by mitogens such as insulin-like growth factors (IGF) and platelet-derived growth factor (PDGF), both of which are present in the developing telencephalon (Hodge et al., 2004; Tekki-Kessaris et al., 2001). Furthermore, IGF1 has been shown to directly promote cell cycle progression in the telencephalon in vivo (Hodge et al., 2004). Foxg1 may, therefore, potentiate the activity of these mitogenic pathways by contributing to the antagonism of FoxO transcriptional activity.

A further clue to Foxg1's ability to promote the proliferation of telencephalic progenitors comes from the demonstration in this chapter that in *Foxg1*$^{-/-}$ mutants, telencephalic Fgf8 expression is dramatically reduced. Furthermore, the reduction in Fgf8 expression is

temporally and spatially correlated with the earliest increases in Tc, that is in the rostral midline of the telencephalon at E10.5. Members of the Fgf family are often mitogenic in activity. Indeed, several observations suggest that Fgf8 is capable of inducing cellular proliferation in a variety of tissues in the developing embryo. It is expressed in several major outgrowths of the embryo during their rapid expansion: the rostral telencephalon; the apical ectodermal ridge of the limb bud; and the developing tailbud (Crossley and Martin, 1995). More direct experimental evidence that Fgf8 is pro-proliferative in the developing central nervous system comes from experiments involving ectopic application of the protein to the midbrain and diencephalon (Martinez et al., 1999) and its transgenic misexpression in the mesencephalon (Lee et al., 1997). Both of these manipulations caused dramatic overgrowth of the midbrain and caudal forebrain. We know of no previous studies in vivo which have directly related telencephalic Fgf8 to proliferation rate. However, Gunhaga et al (2000) showed that exogenous Fgf8 increased proliferation and decreased differentiation of telencephalic explants in culture (Gunhaga et al., 2000). Also, the telencephalon of newborn mice expressing a single hypomorphic allele of Fgf8 is greatly reduced in size relative to control, although this has not yet been directly linked to reduced proliferation (Meyers et al., 1998). Correlative evidence presented here suggests that Fgf8 may have a pro-proliferative role in the most anterior brain structure and, morever, that this activity may be regulated by Foxg1. Reduced Fgf8 levels may contribute to the reduced precursor proliferation rate observed in the rostral telencephalon of the *Foxg1* mutant. This hypothesis is also testable in vitro or in vivo by exposing *Foxg1*$^{-/-}$ telencephalic cells to exogenous Fgf8 to attempt to improve their rate of proliferation. Such an experiment would also test another possibility: that Foxg1 is required cell autonomously for telencephalic cells to respond to Fgf signalling.

Loss of Fgf8 expression may be a direct or indirect consequence of Foxg1 mutation

It is very interesting to consider why *Fgf8* expression is lost from the *Foxg1*$^{-/-}$ telencephalon. One possibility is that it is a consequence, rather than a cause, of the reduced rostrodorsal proliferative defects described here. However, this seems an unlikely explanation for two reasons. Firstly, whilst the reduction in *Fgf8* is spatially correlated with reduced proliferation rate, it is also temporally coincident. Reduced proliferation would have to precede the *Fgf8* reduction to explain it. Secondly, the reduced cell death observed in the same area that expresses *Fgf8* would be predicted to cause an expansion, rather than a contraction of the number of cells expressing this factor. A more likely reason for the loss of *Fgf8* may be the absence of the potent ventrally derived signalling molecule Sonic Hedgehog (Shh) from the *Foxg1*$^{-/-}$ telencephalon at E10.5 (Huh et al 1999). In *Shh*$^{-/-}$ embryos, just like in the *Foxg1*$^{-/-}$ embryos, *Fgf8* is induced but not maintained, although in the former *Fgf8* expression is lost somewhat earlier, at E9 (Ohkubo et al 2002).

It is also possible that *Foxg1* may have a more direct effect on the *Fgf8* promoter itself and is perhaps required for the maintenance, if not the induction, of telencephalic *Fgf8* transcription after E9.5. However, if the hypothesis that Foxg1 promotes proliferation via promotion of Fgf8 signalling is correct, we would expect Fgf8 expression to be recovered in Foxg1-NHAA transgenic embryos which exhibit improved progenitor proliferation. If this was the case, it seems unlikely that Foxg1 directly regulates Fgf8

expression via its own DNA binding. However, given the ability for Foxg1 to affect transcription via its interactions with other transcription factors including Smad and FoxO factors, as discussed above, it seems entirely possible that Foxg1 could regulate Fgf8 expression in an indirect fashion. For these reasons it would be very interesting to determine whether Fgf8 expression is rescued in the Foxg1-NHAA transgenics, co-incident with the proliferative rescue.

Foxg1 is required for apoptosis in the telencephalic dorsal midline

In addition to a reduced proliferation rate in the rostral telencephalon at the onset of neurogenesis, the induction of apoptosis is misregulated in *Foxg1*$^{-/-}$ embryos. Contrary to the notion that increased apoptosis could be contributing to the hypo-cellularity of the mutant telencephalon, fewer apoptotic cells are observed in the rostral midline at E10.5. Interestingly, the reduction in apoptosis observed here is somewhat at odds with the results of Ahlgren and colleagues (Ahlgren et al 2003), which suggested that *Foxg1* acts primarily to suppress apoptosis. However, this study focussed on the chick neural tube, where the gene may have a rather different mode of action, and was based on retroviral over-expression of *Foxg1*. Despite these differences, the possibility that in particular contexts *Foxg1* may act to suppress neural apoptosis cannot be ruled out. No localised increase in apoptosis was observed in the ventral part of the *Foxg1*$^{-/-}$ telencephalon at each age examined. As such, increased cell death is unlikely to account for the loss of ventral telencephalon.

Foxg1's regulation of midline apoptosis may be mediated by mis-regulation of Fgf8 expression

While FGF8 level may have a direct relationship with proliferation rate, as discussed earlier, the relationship to cell survival does not seem to be so straightforward. High levels of apoptosis are seen in the same terminal outgrowths listed above, which express high levels of FGF8 and are proliferating rapidly, perhaps suggesting that very high exposure to FGF8 can promote programmed cell death. A recent paper suggested that such a paradoxical relationship is observed between FGF8 dosage and cell survival (Storm et al., 2003). Increasing or eliminating FGF8 caused increased apoptosis in telencephalic explants, whilst a hypomorphic allele that produces lower levels of FGF8 led to enhanced survival. The result presented here is directly comparable to this finding. At E10.5, when *Fgf8* expression is greatly reduced, but not totally eliminated in the *Foxg1* mutant, an enhancement of cell survival is seen.

In the absence of Foxg1 telencephalic cell cycle exit and neurogenesis are prematurely activated

In this chapter evidence is presented that Foxg1 regulates the timing of differentiation and the cell fate choice of progenitors as they leave the cell cycle. While neuron production appears normal at E10.5 (Hanashima et al, 2004), at E11.5 the number of post-mitotic neurons in the mantle zone of the mutant is significantly greater than that of controls. This finding suggests that either the neurogenic program is ectopically activated in precursors without cell division, or that there is an increased rate of neurogenic

divisions (either symmetrical or asymmetrical) in the *Foxg1*$^{-/-}$ telencephalon at this early stage of neurogenesis. This early depletion of the proliferative population combines with decreased proliferation rates of the remaining precursors to drastically limit the growth of the *Foxg1*$^{-/-}$ telencephalon. Like the proliferative and apoptotic defects described above, early differentiation was not specifically observed in ventral regions of the *Foxg1*$^{-/-}$ telencephalon and therefore cannot account for the loss of this structure.

Molecular mechanisms by which Foxg1 may regulate cell cycle exit and neurogenesis

The Foxg1-NHAA rescue mice described above provide a useful functional separation between Foxg1's roles in regulating proliferation and differentiation (Hanashima et al 2002). Whilst Foxg1 can promote progenitor proliferation independent of direct DNA binding, Foxg1-NHAA did not rescue the early and excessive neuronal differentiation associated with loss of Foxg1, suggesting that Foxg1 antagonises cell cycle exit and neurogenesis via binding its own target genes (Hanashima et al 2002). As a transcription factor, Foxg1 appears to function largely as a transcriptional repressor. This was first demonstrated for the avian homolog of Foxg1 (formerly known as qin) (Li et al., 1995). Chicken Foxg1 has been described as a proto-oncogene due to its ability to cause cellular transformation. This ability is retained by a chimeric protein with the DNA binding sequence of Foxg1 fused to the transcriptional repression domain of Engrailed (Li et al., 1997) and, furthermore, DNA binding defective versions of Foxg1 were incapable of inducing transformation (Ma et al., 2000). These findings suggest that Foxg1 can

antagonise cell cycle exit and promote an undifferentiated, progenitor-like state by directly repressing target genes.

Transcriptional repression by Foxg1 is greatly potentiated in cellular models by co-expression of Groucho/transducin-like enhancer of split homologues (henceforth TLE) (Marcal et al., 2005; Yao et al., 2001). TLE factors are strong transcriptional corepressors that lack intrinsic DNA binding activity. Their repressive activity is recruited to target genes via TLEs' ability to associate with DNA-binding transcription factors in a highly context dependent fashion (reviewed in (Chen and Courey, 2000; Gasperowicz and Otto, 2005). Among the best characterised binding partners of TLE factors in Drosophila and mammalian systems are the basic helix-loop-helix (bHLH) factors of the Hairy/Enhancer of split (Hes) family. The importance of TLE factors in regulating telencephalic neural differentiation has been clearly demonstrated in vitro and in vivo (Nuthall et al., 2004; Yao et al., 2001). TLE1 downregulation is required for telencephalic neural differentiation (Yao et al., 2001) and the anti-neurogenetic activity of TLE1 depends on its phosphorylation (Nuthall et al., 2004). TLE phosphorylation is, in turn, mediated by its transcription factor binding partners including Hes factors (Nuthall et al., 2004). Hes factors are also transcriptional repressors and are capable of repressing the acquisition of neural fate in the developing telencephalon as in other neural tissues and in species as diverse as Drosophila and zebrafish (Reviewed in (Ross et al., 2003)). Retroviral overexpression of Hes1 inhibits neuronal differentiation of telencephalic precursors (Ishibashi et al., 1994) and targeted mutation of Hes1 results in premature neurogenesis in the telencephalon and more caudal neural structures (Ishibashi et al., 1995).

Foxg1 has been shown to bind directly to TLE factors in mouse and chickens (Sonderegger and Vogt, 2003; Yao et al., 2001), leading to the formation of powerful co-repression complexes which include histone deacetylases (Yao, Lai et al. 2001). Furthermore Foxg1 causes phosphorylation of TLE1 in a manner analogous to Hes-mediated TLE phosphorylation (Sonderegger and Vogt 2003). There does, therefore, seem to be good evidence that Foxg1 can act as a transcriptional repressor and its ability to recruit TLE factors to DNA appears to be a major mode by which Foxg1 mediates transcriptional repression. Interestingly, this function of Foxg1 may be particularly highly conserved. The *Drosophila* orthologue of Foxg1, slp1, is also a groucho/TLE-dependent transcriptional repressor (Andrioli et al., 2004). Another recent report describes Foxg1's physical interaction with Plu1, another co-repressor (Tan et al., 2003). Foxg1 enhances Plu1-mediated transcriptional repression, perhaps through TLE recruitment (Tan et al., 2003). Foxg1 and Plu1 are temporally and spatially co-expressed in the developing telencephalon (Madsen et al., 2002). Since Plu1 was originally identified by its over-expression in breast cancer cells, a role for Plu1/Foxg1 complexes in promoting progenitor fate in the telencephalon may be a realistic possibility worthy of further research.

Despite these interesting insights into the mechanism by which Foxg1 can act as a transcriptional repressor, and the identification of an optimal DNA binding sequence for Foxg1 (TGTAAACAAA, (Li et al., 1997)), no direct targets of Foxg1 have been described. It is interesting to note, however, that the human promoter of the CKI p27kip1 contains an exact match to the predicted optimal binding sequence for Foxg1 (Tan et al., 2003). Moreover, in *Xenopus laevis* high level Foxg1 overexpression can repress p27

expression, repress neuronal differentiation and induce ectopic neural progenitor cells (Hardcastle and Papalopulu, 2000). Uncontrolled expression of p27 may contribute to premature neurogenesis in *Foxg1*$^{-/-}$ mouse mutants. Although deletion of p27 from a *Foxg1*$^{-/-}$ background does not rescue the proliferative defects associated with Foxg1 mutation, the authors of the study do not report on differentiation rates in the double mutants (Hanashima et al., 2002).

It should be noted at this stage that Foxg1's ability to physical bind co-repressors such as TLE factors and Plu1 does not limit Foxg1's repressive influence to genes whose regulatory elements Foxg1 binds directly. Foxg1's interactions with other transcription factors could bring both Foxg1's endogenous repressive activity as well as TLE and associated co-repressors to a wide range of DNA targets. For example Foxg1 has been shown to interact with Hes1 via TLE1 and to potentiate Hes1-mediated transcriptional repression independent of its own DNA binding (Yao et al., 2001). As discussed earlier Hes1 appears to repress neuronal differentiation. Foxg1 also interacts with FoxO and Smad transcription factors, as discussed above. Foxg1 may also have the ability to silence or even reverse FoxO- and Smad-mediated transcriptional activation to repress neuronal differentiation and promote progenitor proliferation.

In addition to Foxg1 and Hes transcription factors, other well-documented partners of TLE factors are the Tcf/Lef transcription factors. Tcf/Lef transcription factors associate with β-catenin in the nucleus to activate canonical targets of Wnt signalling (reviewed in (Reya and Clevers, 2005). TLE appears to compete with β-catenin for Tcf/Lef binding (Cavallo et al., 1998) to control whether Wnt targets are positively or negatively

regulated. Foxg1's ability to associate with TLE may bring Foxg1 into contact with Tcf/Lef factors and by this means Foxg1 may be able regulate the response of telencephalic cells to Wnt signalling. Since Wnt signalling is capable of causing a wide range of biological effects on CNS cells from the promotion of proliferation to the induction of cell cycle exit and differentiation (reviewed in (Ciani and Salinas, 2005), the potential involvement of Foxg1 in regulating the Wnt response via TLE factors may well be worthy of more investigation. Since several Wnt family members and downstream signalling molecules have been shown to be overexpressed in the Foxg1 mutant telencephalon during mid neurogenesis (Muzio and Mallamaci, 2005; Vyas et al., 2003), addressing Foxg1's involvement in regulating Wnt expression as well as Wnt signal transduction may also prove fruitful in the future.

In addition to the potential roles for Foxg1 in regulating telencephalic Wnt signalling, there is good evidence for Foxg1's involvement in Bone Morphogenetic Protein (BMP) signalling. In the telencephalon the expression of multiple BMPs is confined to the dorso-medial cortical hem (Furuta et al., 1997), an area that loses expression of Foxg1 early in telencephalic development (Furuta et al., 1997; Shimamura et al., 1997). This suggests that Foxg1 may confine BMP expression to this region. In support of this idea, multiple BMPs including BMP2,4,6 and 7 are expressed in a greatly expanded domain in the telencephalon of *Foxg1*$^{-/-}$ mutants. Various studies in vivo and in vitro suggest that BMP signalling can induce neuronal differentiation and stop progenitor proliferation in telencephalic precursors (Li et al., 1998; Mehler et al., 2000; Panchision et al., 2001). Furthermore, *Foxg1*$^{-/-}$ embryos expressing Foxg1-NHAA continue to ectopically express several BMPs in concert with increased rates of neuronal differentiation (Hanashima et

al., 2002). This finding also suggests that the DNA binding activity of Foxg1 may be required to regulate BMP expression. Although this regulation may well be indirectly mediated, it would be interesting to examine BMP promoters for Foxg1 binding sequences. Continued BMP overexpression in Foxg1-NHAA transgenic embryos without overt proliferative defects suggest that either DNA binding defective Foxg1 can antagonise the antiproliferative activities of BMP signalling (Panchision et al., 2001), or that excess BMP signalling at the level observed in these mutants has no effect on progenitor proliferation, but can promote cell cycle exit. Recently an attempt was made to test the hypothesis that excess BMP expression drives the premature differentiation of telencephalic progenitors by transgenically overexpressing a dominant negative BMP receptor (dnBMPR2B) in *Foxg1*$^{-/-}$ embryos (Shen, 2004). Despite the demonstration that this manipulation did reduce the sensitivity of *Foxg1*$^{-/-}$ telencephalic cells to exogenous BMP application, no improvement in the neurogenic or proliferative phenotypes of *Foxg1*$^{-/-}$ mutants was observed. This experiment, although it does not rule it out, suggests that ectopic BMP expression is not the major cause of premature differentiation if *Foxg1*$^{-/-}$ telencephalon.

A final possible cause of premature neurogenesis when Foxg1 is removed is the finding that increased cell cycle time alone is sufficient to promote neuronal differentiation in telencephalic precursors (Calegari and Huttner, 2003). This idea is supported by the observation that precursors undergoing neurogenic divisions exhibit a longer Tc than those undergoing symmetric, progenitor expanding, divisions (Calegari et al., 2005). This interesting hypothesis highlights the complex interaction between cell cycle kinetics and cell cycle exit. On initial inspection, if we return to the Foxg1-NHAA model, this mutant

may appear to separate progenitor proliferation rate from neuronal differentiation rate. According to the hypothesis of Calegari and colleagues, we would expect the improvement in progenitor proliferation rate (as is observed in Foxg1-NHAA mutants) to be followed by a decrease in neuronal differentiation rate. This is not reported, perhaps uncoupling the causal connection (Hanashima et al., 2002). However, the method used to assess proliferation rate lacked precision and relied on some poorly tested assumptions. Calegari and colleagues document a relatively subtle 20% increase in Tc during neurogenic divisions compared to precursor divisions during early neurogenesis (Calegari et al., 2005). It appears that an increase in Tc of this magnitude could easily have been overlooked by Hanashima et al (2002) with the result that their claimed 'rescue' of proliferation rate may be an overstatement and some degree of Tc lengthening may still be evident in Foxg1-NHAA transgenic embryos. This could, in turn, contribute to the enhanced rates of neurogenesis in combination with other factors including those discussed above.

Molecular mechanisms by which Foxg1 may regulate the onset of astrogliogenesis

Astrogliogenesis is a relatively late event in dorsal telencephalic development, largely occurring after the completion of embryonic neurogenesis in the normal mammalian brain (Reviewed in (Sauvageot and Stiles, 2002). In this chapter evidence is presented that numerous glial cells, with astrocyte-like characteristics, are produced throughout the *Foxg1*$^{-/-}$ telencephalon from E15.5. At this age equivalent cells are few in number and restricted to a small region of the dorsomedial telencephalon of wt embryos. It therefore

appears that as well as delaying the onset of neurogenesis, Foxg1 also delays the formation of telencephalic astroglia.

On a molecular level there are several interesting clues as to Foxg1's involvement in regulating glial development. Foremost among these is the aforementioned expansion of BMP expression in the *Foxg1*$^{-/-}$ telencephalon (Dou et al., 1999; Hanashima et al., 2002). The action of BMPs appears to be context dependent. Whilst BMPs appear to induce cytostasis, apoptosis or neuronal differentiation in early telencephalic precursors (Li et al., 1998; Mehler et al., 2000; Panchision et al., 2001), exogenous BMP expression has been shown to induce astrocytic differentiation in late embryonic progenitors in vitro and in vivo (Gomes et al., 2003; Gross et al., 1996; Nakashima et al., 1999). Because the program of neurogenesis is accelerated in *Foxg1*$^{-/-}$ mutants, molecular changes that shift BMP responses from pro-neural to pro-astrocytic may also be accelerated in the absence of Foxg1. At a mechanistic level BMP signalling in late telencephalic progenitors is believed to induce astrocytic development by a number of mechanisms. The synergy of BMP-activated Smad proteins with phosphorylated Stat factors and the coactivator CREB binding protein (CBP)/P300 has been characterised. Together the Smad/Stat/CBP/p300 complexes activate astrocyte specific genes such as GFAP (Nakashima et al., 1999). BMP-activated Smads also act to induce anti-neural bHLH genes of the Hes and Id families (Nieto et al., 2001). Hes and Id genes are then able to down-regulate bHLH genes such as Ngns and Mash1, which are pro-neural and have been shown to actively suppress astrocyte fate (Nieto et al., 2001; Sun et al., 2001). In summary, it seems reasonable to posit that Foxg1's ability to repress BMP expression and also its ability to interact not just with TGFβ-activated Smads, but also BMP-

activated Smad1 (Rodriguez et al., 2001), presumably to repress Smad targets, combine to provide a potential model for accelerated astrocytic development that is observed in *Foxg1*$^{-/-}$ mutants. This model is also testable in vitro or in vivo. Indeed, it would be interesting to see whether early glial differentiation is rescued in the *Foxg1*$^{-/-}$ mice over-expressing dnBMPR2b (Shen 2004).

An alternative, but perhaps entirely compatible, explanation for the early appearance of astrocytes in the Foxg1 mutant is that it represents a secondary consequence of a gross areal respecification of the mutant telencephalon to a dorso-medial fate, as has been proposed on the basis of the expression patterns of several regionally restricted genes (Muzio and Mallamaci, 2005). Since astrocyte-like GFAP-expressing cells first appear in the dorso-medial telencephalon of wildtype embryos during mid-neurogenesis, their more widespread appearance in *Foxg1*$^{-/-}$ mutants may simply represent an adoption of a dorsomedial fate. However, since Foxg1 is not expressed in the dorso-medial telencephalon at the time it generates astrocytes, understanding the mechanisms by which Foxg1 may be able to delay astrocyte differentiation elsewhere in the telencephalon, where it is expressed, are still pertinent.

Chapter 4: Foxg1 is required cell autonomously for the specification of ventral telencephalic fate.

INTRODUCTION

The initial study of mice mutant for Foxg1 revealed a failure of ventral telencephalic marker gene expression from mid-gestation onwards (Xuan et al., 1995). Xuan and colleagues posit that the telencephalon is initially specified normally, but then reduced proliferation in the ventral telencephalon from E10.5 leads to its retarded development. A subsequent study from the same group found that the morphogen Shh, which is important for ventral induction and mitogenesis throughout the neuraxis (Ericson et al., 1995a; Ingham and McMahon, 2001; Patten and Placzek, 2000), is absent from the *Foxg1*$^{-/-}$ telencephalon at E10.5 (Huh et al., 1999). They go on to suggest that this defect in Shh expression compounds the failure of ventral telencephalic development in a non-cell autonomous fashion (Huh et al., 1999).

In Chapter 3 growth defects in the telencephalon of *Foxg1*$^{-/-}$ mouse mutants were systematically analysed from early stages of telencephalic development. Although defects in proliferation rate, differentiation rate and the induction of apoptosis were observed, these defects were never initiated or specifically localised to the ventral part of the *Foxg1*$^{-/-}$ telencephalon. These findings cast serious doubt on the hypothesis that the primary defect in ventral telencephalic development is related to defective growth of a 'normally specified' ventral telencephalic anlage. A more plausible explanation for the

complete absence of ventral marker gene expression may be that Foxg1 is required for the initial specification of the ventral telencephalon.

In this chapter the hypothesis that Foxg1 is required for ventral telencephalic specification is tested. Initially, this is done by analysing the expression patterns of marker genes whose expression is limited to the early ventral telencephalon. The marker gene analysis is then extended and complemented by the construction of chimeric embryos consisting of genotypically wildtype (wt) and *Foxg1*$^{-/-}$ cells.

The use of chimeric embryos can provide a range of interesting and subtle insights into the analysis of complex gene requirements (Tam and Rossant, 2003). Foremost amongst these is the ability to test whether a gene functions cell autonomously in the regulation of a particular cellular trait. A gene can function directly, only in cells expressing that gene (cell-autonomously) or it can also exert an action on other cells which either do or do not express the gene (non-cell-autonomously). A cell autonomous role for a gene means that mutant cells maintain their mutant phenotype in any cellular context. In development this often suggests that mutant cells cannot respond appropriately to extrinsic developmental cues. Non-cell-autonomy is inferred when mutant cells are either phenotypically rescued by surrounding wt cells or, conversely, they impose a mutant phenotype on their genotypically wildtype neighbours. This issue is particularly pertinent to the study of a transcription factor such as Foxg1. The factor may be required cell autonomously, for example in the transcriptional readout of incoming developmental cues, or non-cell autonomously if the factor is required to promote or repress the expression of a molecule capable of influencing the fate of neighbouring cells. Since mutant embryos lack gene

function in all cells, it is impossible to determine whether gene functions are cell autonomous by straightforward mutant analysis. In this chapter particular attention is paid to the question of whether Foxg1 is required cell autonomously for the proper development of the ventral telencephalon.

Some of the results presented in this chapter have already been published (Martynoga et al., 2005). This paper is bound into the back of this thesis and will not be cited further in this chapter.

RESULTS

The ventral telencephalon is never specified in Foxg1$^{-/-}$ mutants

Previous authors have described an absence of gene expression associated with ventral telencephalic cell types in the telencephalon of *Foxg1* mutant embryos from E10.5 onwards (Xuan et al., 1995). This report suggested that the telencephalon was specified normally in the mutant, but proposed that the ventral telencephalon failed to expand after this early stage due to a ventral specific proliferative defect. This hypothesis was never rigorously tested, relying as it did on a BrdU labelling index experiment (the results of which we fail to replicate, see Chapter 3). Furthermore their assertion that the telencephalon is normally specified was not tested since they did not determine whether ventral cell types are present in the mutant telencephalon prior to E10.5. As detailed in the previous chapter we see no evidence for a ventral telencephalon-specific growth

impairment in terms of proliferation rate, differentiation rate or in the occurrence of programmed cell death during early telencephalic development. Given the relatively normal growth of the ventral part of the *Foxg1*$^{-/-}$ telencephalon during early development, we find it more likely that ventral telencephalic progenitor fates are never specified in the mutant. To test this hypothesis we examined the expression of a range of regionally restricted marker genes from early stages of telencephalic development to establish whether ventral telencephalic cell fate is ever specified in the absence of *Foxg1*.

At E8.5, prior to the closure of the anterior neural tube, the prosencephalic neural plate is patterned into distinct caudal (diencephalic) and rostral (telencephalic) domains. In the tangential plane the anterior neural plate is patterned into medial (ventral) and lateral (dorsal) domains in terms of gene expression. Nkx2.1 expression marks both the prospective ventral diencephalon and ventral telencephalon. In *Foxg1*$^{-/-}$ embryos at this age, the expression of Nkx2.1 does not extend rostrally into the prospective telencephalon (arrows in Fig.4.1A,B), consistent with a failure to induce the ventral telencephalon. At E9.5 there is a total absence of Nkx2.1 expression in the ventral region of the *Foxg1*$^{-/-}$ telencephalon (Fig.4.1D), but not in the ventral diencephalon (Fig.4.1H). At E9.5, Mash1, which appears in the ventralmost telencephalon of normal embryos at this age (Fig.4.1E) is also absent from the *Foxg1*$^{-/-}$ telencephalon (Fig.4.1F) but is still present in the dorsal and ventral diencephalon (Fig.4.1G). By E10.5 Gsh2 gene expression becomes detectable in the control telencephalon (Fig.4.1I) in a lateroventral domain, which will later give rise to the lateral ganglionic eminence (LGE) (Corbin et al., 2000; Corbin et al., 2003). Again, Gsh2 expression is completely absent from the *Foxg1*$^{-/-}$ telencephalon (Fig.4.1J). Expression of ventral progenitor markers Nkx2.1,

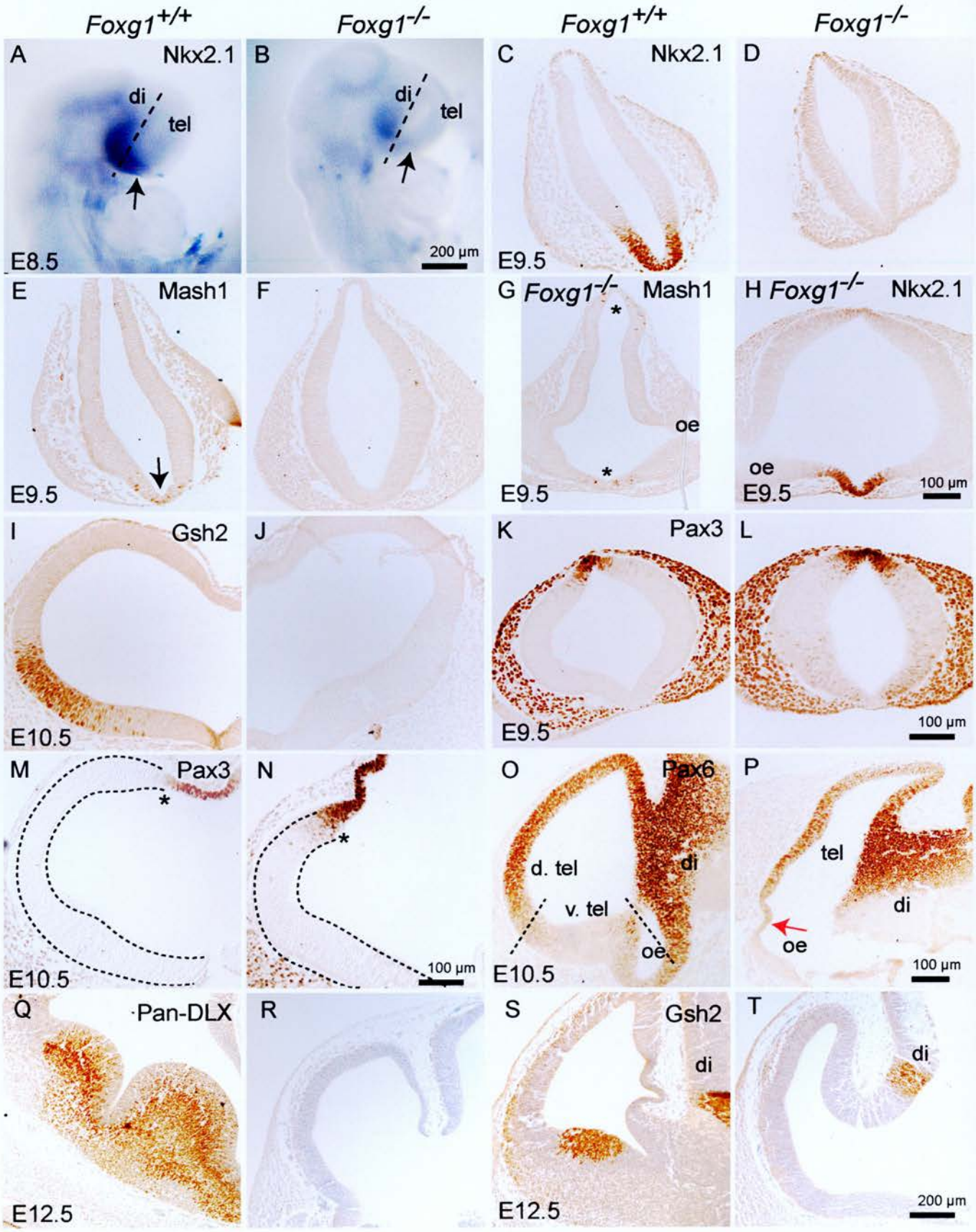
Olig2, Islet1 (Chapter 5, Fig.5.5 and 5.6), Gsh2 and Dlx (Fig.4.1Q-T) was absent in all rostrocaudal levels of the *Foxg1*$^{-/-}$ telencephalon later in development (at E12.5), demonstrating that ventral specification is not simply delayed.

Concurrent with the loss of ventral marker gene expression, dorsal cell types are over-represented when *Foxg1* is absent. Pax3, which marks the dorsal-most of telencephalic cells (Fig.4.1K), is expressed in a slightly expanded dorsal zone in *Foxg1*$^{-/-}$ telencephalon and appears ectopically in a number of laterally and ventrally positioned cells at E9.5 (Fig.4.1L). At E10.5 the roofplate Pax3 domain is clearly expanded in *Foxg1* mutants relative to control, whilst the ectopic lateral and ventral expression seen at E9.5 is no longer apparent (Fig.4.1M,N). Pax6, whose expression is normally restricted to the dorsal telencephalon (Fig.4.1O), is expanded throughout the whole of the *Foxg1*$^{-/-}$ telencephalon at E10.5 and E12.5 (Fig.4.1P and Chapter 5 Fig.5.5,5.7). Also at E12.5 an antibody that recognises LIM homeobox transcription factors 2 and 9 (Lhx2,9) marks cells throughout the dorso-ventral axis of the *Foxg1*$^{-/-}$ telencephalon, but is largely restricted to the wt dorsal telencephalon (Chapter 5 Fig.5.5). This latter finding is somewhat in contrast to the initial study on *Foxg1*$^{-/-}$ embryos (Xuan et al., 1995). Although they do report an expansion of dorsal genes Emx2 and Pax6, they describe a region in the ventral part of the mutant telencephalon that does not express these dorsal genes or markers of ventral telencephalon. In the embryos examined here, I fail to see such a territory at equivalent ages.

The gene expression studies described here provide good support for the idea that *Foxg1* is required for the induction of the ventral telencephalon and in the absence of this

transcription factor, dorsal telencephalic fates are expanded throughout the telencephalic territory.

Fig.4.1. Ventral telencephalon is not specified in *Foxg1*$^{-/-}$ mutants. (A,B) Whole mount in situ hybridisation for *Nkx2.1* in control and mutant embryos at E8.5 shows reduced anterior expression of *Nkx2.1* (compare arrows in A,B), consistent with loss ventral telencephalic induction. Dotted line marks approximate di/telencephalic boundary. (C,D) Nkx2.1 immunohistochemistry shows total absence lack of expression in *Foxg1*$^{-/-}$ telencephalon at E9.5. (E,F) Immunohistochemistry shows first appearance of Mash1 expressing cells in wildtype ventral telencephalon (arrow in E), but not in the mutant. (G,H) *Foxg1*$^{-/-}$ diencephalon appears correctly patterned with Mash1 expression in dorsal and ventral domains (Asterisks in G) and Nkx2.1 protein ventrally (H). (I,J) By E10.5 Gsh2 is detectable in control, but not *Foxg1*$^{-/-}$ ventrolateral telencephalon. (K,L,M,N) Pax3 expression in the dorsalmost telencephalic cells is slightly increased in *Foxg1*$^{-/-}$ embryos at E9.5 and E10.5 (Asterisks in M,N), consistent with an expansion of dorsal fates. (O,P) Pax6 is expressed from the most dorsal to ventral telencephalon in *Foxg1*$^{-/-}$ mutants (red arrow marks ventralmost telencephalon, which is fused directly with developing eye). (Q,R) Dlx transcription factors are expressed in the wt ventral telencephalon at E12.5, but are absent from *Foxg1*$^{-/-}$ telencephalon. (S,T) Ventral telencephalic Gsh2 expression is similarly absent in *Foxg1*$^{-/-}$ mutants demonstrating that ventral specification is not simply delayed. d.tel., dorsal telencephalon; v.tel, ventral telencephalon; di, diencephalon; oe, optic eminence. All sections are in the coronal plane, except for O and P, which are sagittal.


Foxg1+/+
Foxg1-/-
Foxg1+/+
Foxg1-/-
A
Nkx2.1
di
tel
E8.5
B
di
tel
200 µm
C
Nkx2.1
E9.5
D
E
Mash1
E9.5
F
G Foxg1-/-
Mash1
*
oe
*
E9.5
H Foxg1-/-
Nkx2.1
oe
E9.5
100 µm
I
Gsh2
E10.5
J
K
Pax3
E9.5
L
100 µm
M
Pax3
*
E10.5
N
*
100 µm
O
Pax6
d. tel
di
v. tel
oe
E10.5
P
tel
di
oe
100 µm
Q
Pan-DLX
E12.5
R
S
Gsh2
di
E12.5
T
di
200 µm

The generation of chimeric embryos to test whether Foxg1 is required cell autonomously for the development of the telencephalon

As discussed in the introduction to this chapter, the role of Foxg1 in the specification of the ventral telencephalon could be direct/cell-autonomous or via a non-cell autonomous mechanism. Chimeric embryos consisting of wt and *Foxg1*$^{-/-}$ cells provide the ideal experimental situation to answer this question. In a balanced or low percentage chimera (where mutant cells are ≤50% of cells) mutant cells will be exposed to developmental cues, produced by their neighbouring wt cells, that are required for the induction and maintenance of dorsal and ventral telencephalic territories throughout development. By this means it is possible to test the intrinsic potential of *Foxg1*$^{-/-}$ cells to contribute to the ventral telencephalic lineage when exposed to a wt environment *in vivo*.

It was decided to make chimeric embryos by the aggregation of pre-implantation embryos. From the aggregation and uterine transfer of approximately four hundred pairs of embryos, seven chimeric embryos were recovered at E12.5. Of these seven chimeras, three were *Foxg1*$^{-/-}$↔wt chimeras, three were Foxg1$^{+/-}$↔wt chimeras and one was a wt↔wt chimera. The two strains used to generate the chimeras carried different alleles of the glucose phosphate isomerase 1 gene (GPI1a and GPI1b). Electrophoretic separation of GPI1 protein isoforms allows calculation of the global chimerism of embryos. The global percentage chimerism varied from 35% to 80% contribution of the GPI1b cell type. The genotypes and levels of chimerism of each chimera generated are displayed in table 1.

Name	Genotype	β-Globin Tg?	Mean %GPI1a (wt)	Mean %GPI1b
BC6	wt↔Foxg1$^{cre/+}$	No	20	80
BC8	wt↔Foxg1$^{cre/lacZ}$	No	47	53
BC9	wt↔Foxg1$^{cre/+}$	Yes	64	36
BC10	wt↔Foxg1$^{cre/lacZ}$	No	50	50
BC26	wt↔wt	No	34	66
BC27	wt↔Foxg1$^{+/lacZ}$	No	35	65
BC28	wt↔Foxg1$^{cre/lacZ}$	Yes	29	71

Table 3.1. Genotypes and global contributions of each cell type of chimeras generated in this study.

The experiment was planned such that all GPI1b cells (derived from the intercross of Foxg1cre/+ x Foxg1lacZ/+ parents) would be heterozygous for the re-iterated β-globin transgene (Tg) (Keighren and West, 1993). The Tg transgene serves as an independent genetic marker. It can be detected by DNA:DNA in situ hybridisation to show the contribution of marked cells throughout the embryo. However, as shown in Table 1, only 2/7 chimeras were positive for Tg, suggesting that there had been a genotyping error in some members of the parental generation. Of the two Tg+ chimeras, one was *Foxg1*$^{-/-}$ ↔wt (BC28) and the other Foxg1$^{+/-}$↔wt (BC9). In situ hybridisation for the Tg marker allowed the contribution of *Foxg1*$^{-/-}$ and Foxg1$^{+/-}$ cells to each part of the telencephalon and surrounding tissues to be assessed (Fig.4.2A,B,I). *Foxg1*$^{+/-}$ cells contributed to all regions of the chimeric telencephalon and surrounding mesenchyme and ectoderm, and were freely mingled with wt cells, consistent with the lack of mutant phenotype in

Foxg1$^{+/-}$ embryos (Hebert and McConnell, 2000; Martynoga et al., 2005; Xuan et al., 1995). The telencephalic morphology of *Foxg1*$^{+/-}$↔wt and wt↔wt chimeras (henceforth called 'control chimeras') was apparently indistinguishable from wt embryos, as expected (Fig.4.2A). The *Foxg1*$^{-/-}$↔wt chimera (henceforth called '*Foxg1*$^{-/-}$ chimeras') where mutant cells were marked by the Tg transgene (BC28) consisted of 71% *Foxg1*$^{-/-}$ cells and 29% wt cells. The telencephalon of this chimera was smaller than equivalent control chimeras and had an unusual morphology, presumably due to the high contribution of *Foxg1*$^{-/-}$ cells (Fig.4.2B). The ventricular surface was undulatory but, unlike *Foxg1*$^{-/-}$ telencephalon (Fig.4.1 and Chapter3 Fig.3.1), appeared to contain ventral telencephalic structures morphologically equivalent to the ganglionic eminences (Fig.4.2B,E,F). Furthermore, *Foxg1*$^{-/-}$ mutant cells were present in all regions of the chimeric telencephalon from the ventral sectors to the dorso-medial cortical hem and choroid plexus epithelium (Fig.4.2B).

Despite the absence of the Tg transgene from several of the chimeras, all three *Foxg1*$^{-/-}$ chimeras and one Foxg1$^{+/-}$ chimera expressed the reporter gene β-galactosidase (LacZ) under the control of the Foxg1 promoter, since the nature of the targeted gene knock-out means that lacZ gene replaces all but the first 13 amino acids of Foxg1 (Xuan et al., 1995). Foxg1-driven lacZ (Foxg1-lacZ) has been shown to accurately re-capitulate the Foxg1 mRNA expression (Dou et al., 1999; Hatini et al., 1999; Xuan et al., 1995) and is expressed throughout the majority of cells in the telencephalon in Foxg1$^{+/-}$ and *Foxg1*$^{-/-}$ embryos at E12.5. Foxg1-lacZ expression appears to be absent from the dorso-medial-most cortical hem region (Chapter 3, Fig.3.1) and from early born Cajal-Retzius cells (CR cells) (Hanashima 04), but is present elsewhere and in all other telencephalic cell

types. Furthermore Foxg1-lacZ can be detected by X-gal histochemistry or immunohistochemistry. lacZ immunohistochemistry revealed that Foxg1-lacZ expression is not extinguished in *Foxg1*$^{+/-}$ and *Foxg1*$^{-/-}$ cells in the chimeric telencephalon and is, as such, a useful positive marker to reveal the presence of mutant cells within Foxg1-expressing territories. Staining for Foxg1-lacZ echoed the analysis of Tg+ cells in that Foxg1$^{+/-}$ cells mixed freely with wt cells (Fig.4.2C,D) and *Foxg1*$^{-/-}$ cells contributed to both dorsal and ventral telencephalic regions (Fig.4.2E-H). lacZ immunohistochemistry also made it possible to examine the telencephalic contribution of mutant cells in more balanced chimeras that did not carry the Tg transgene (BC8, 53% and BC10, 50%) (Fig.4.2,G,H and subsequent figures). In these chimeras gross telencephalic morphology was remarkably similar to that of wt embryos and control chimeras. *Foxg1*$^{-/-}$ cells were also identified within the ganglionic eminences of these chimeras, where they appeared to form cellular aggregates which may be actively segregated from neighbouring wt cells. Segregation between *Foxg1*$^{-/-}$ and wt cells is even more marked in BC28 (71% mutant cells), where distinct stripes and bands of alternating wt and mutant cells are readily apparent (Fig.4.2B,E,F). Given the ready mixing of *Foxg1*$^{+/-}$ and wt cells in control chimeras in both dorsal and ventral telencephalon, it seems very unlikely that the observed segregation stems from clonal growth patterns of genetically distinct progenitor patches in the ventricular zone. In regions of the brain where Foxg1 is not expressed, such as the dorsal thalamus, *Foxg1*$^{-/-}$ cells appeared to be freely mixed with wt cells in a mosaic fashion, suggesting that mutant cells are not inherently sorted from wt cells in non-telencephalic tissues, due, for example, to altered surface properties (Fig.4.2I).

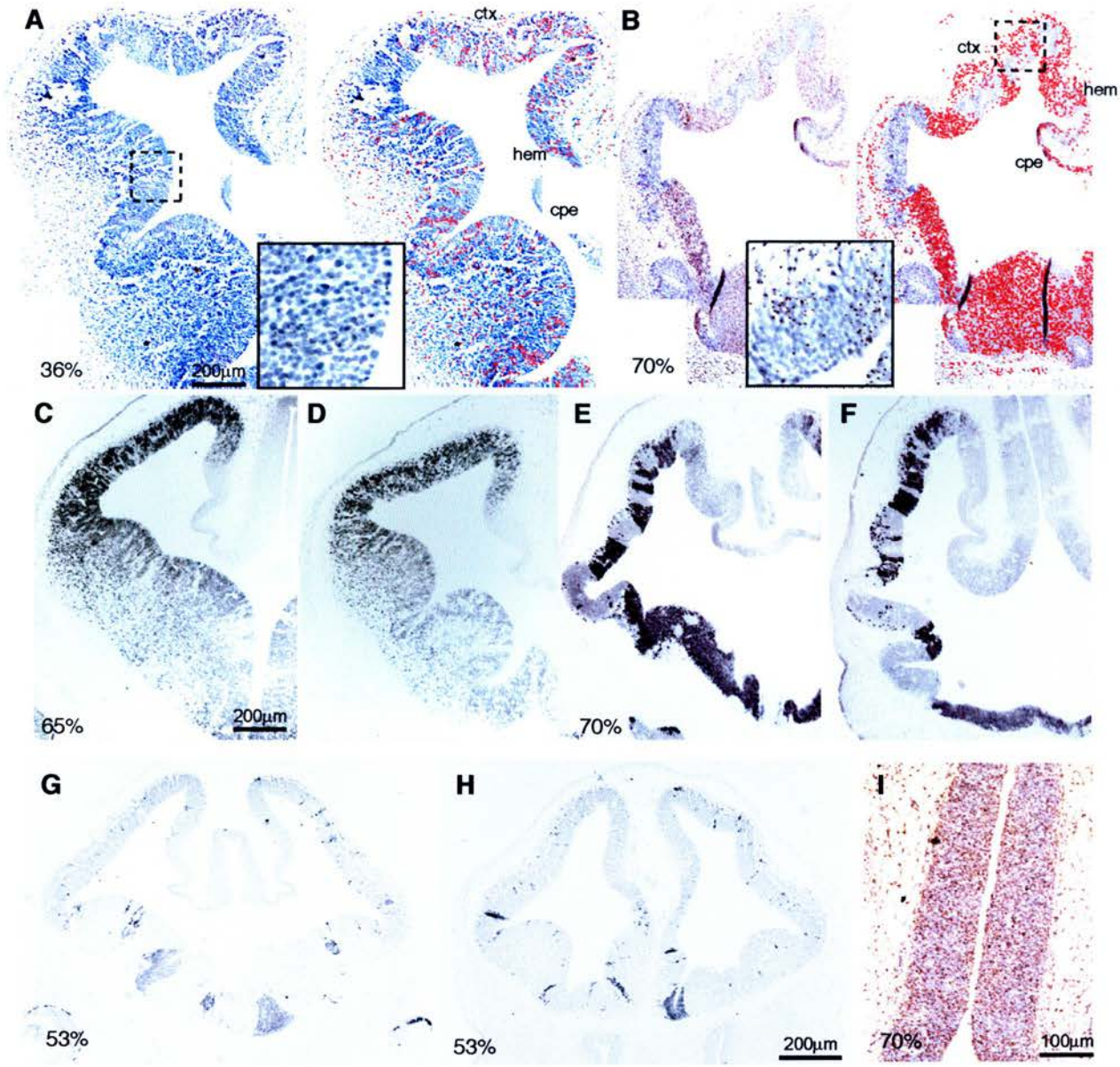


Fig.4.2. Contribution of *Foxg1*$^{+/-}$ and *Foxg1*$^{-/-}$ cells to the chimeric telencephalon. (A,B) DNA:DNA in situ hybridisation to the re-iterated β-globin transgene, an independent genetic marker of one *Foxg1*$^{+/-}$ (A) and *Foxg1*$^{-/-}$ (B) cells. Due to difficulty in visualising the hybridisation signal at low magnification (see high power insert for clearer view) positive nuclei in each chimera are marked with a red dot. (C,D) Immunohistochemical detection of LacZ expressed by *Foxg1*$^{+/-}$ cells in caudal (C) and rostral (D) sections from a control chimera. (E,F) Caudal and rostral sections from a high percentage *Foxg1*$^{-/-}$ chimera also stained for LacZ and (G,H) equivalent sections from a balanced chimera, showing the incorporation of Foxg1-/- cells into each region of the chimeric telencephalon. (I) β-globin hybridisation (brown) in the dorsal thalamus of a *Foxg1*$^{-/-}$ chimera, showing mosaic contribution of mutant cells. In each panel, the global percentage contribution from the genetically marked cellular component is indicated in the bottom left corner. ctx, neocortex; hem, cortical hem; cpe, choroid plexus epithelium. All sections are in the coronal plane.

Unfortunately, the relatively small number of chimeras generated made it impossible to carry out a quantitative analysis of the observed numerical contribution of *Foxg1*$^{-/-}$ mutant cells to each region of the telencephalon, relative to the expected contribution (ie the percentage global chimerism). However, given the assumption that Foxg1-lacZ marks the majority of *Foxg1*$^{-/-}$ cells in the mutant telencephalon, it appears qualitatively that mutant contribution to the balanced chimeras (BC8 and BC10) is much less than the global chimerism of these embryos (50% and 53%, respectively). One possible reason for a reduced contribution of mutant cells is that the proliferative defects described in the previous chapter are cell autonomous defects and mitogens from wt cells cannot rescue the reduced proliferation rate of *Foxg1*$^{-/-}$ cells. Despite this potential reduction in numerical contribution to the telencephalon, the striking conclusion from analysis of the contributions of *Foxg1*$^{-/-}$ cells to the chimeric telencephalon is that they have the potential to contribute to all the morphological areas of the E12.5 telencephalon: the ventral ganglionic eminences, the neocortex, archicortex and dorso-medial cortical hem.

Foxg1 is required cell autonomously for ventral progenitor fate

Having established that *Foxg1*$^{-/-}$ cells have the potential to physically contribute to both dorsal and ventral structures in chimeric embryos, it is important to determine whether mutant cells express the correct molecular profiles associated with each telencephalic cell lineage. To determine whether *Foxg1*$^{-/-}$ cells were able to take on ventral telencephalic progenitor fate, immuno-staining for FOXG1-LACZ expressed by mutant cells was combined with staining for a range of well-characterised ventral progenitor markers.

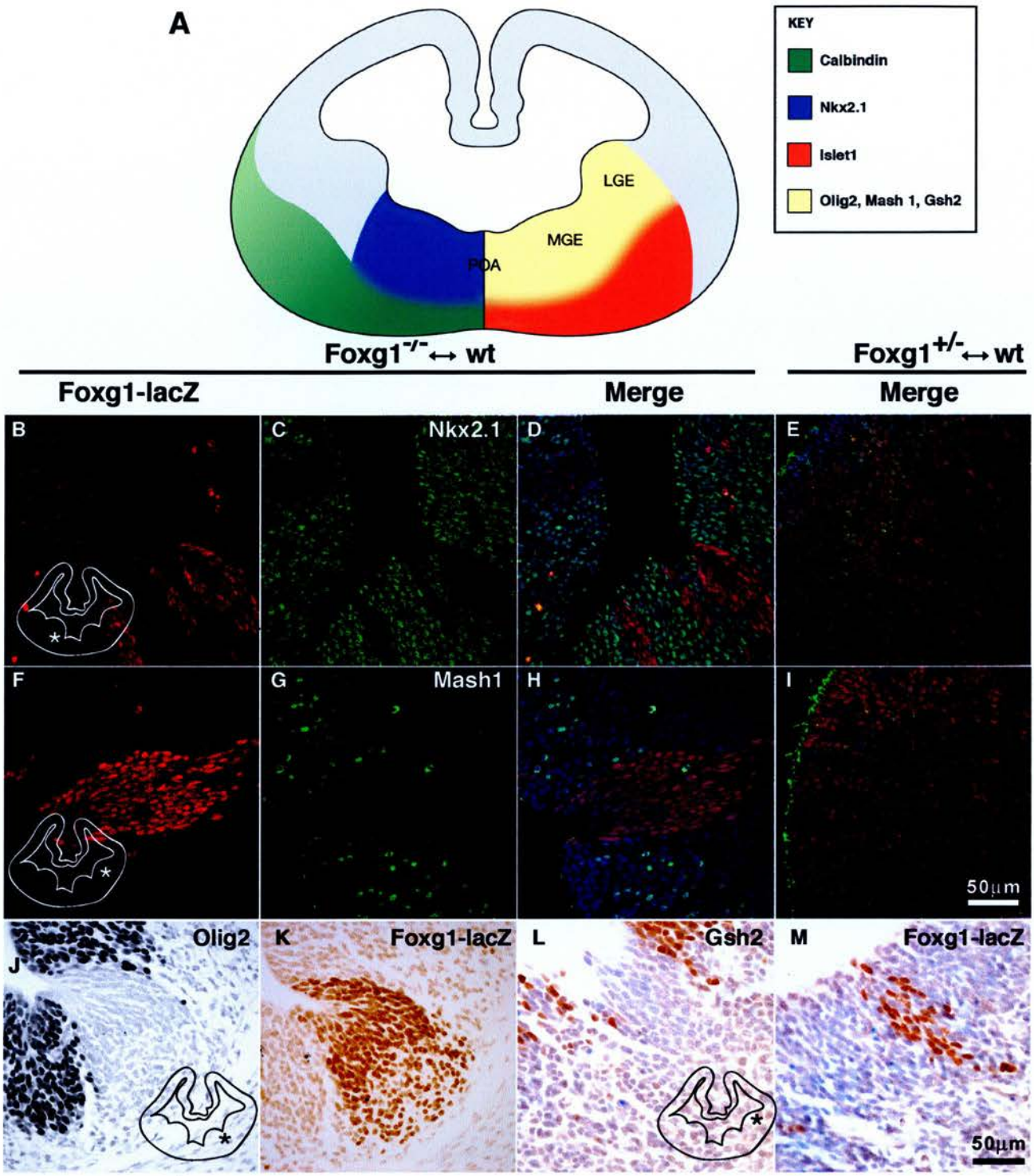


Fig.4.3. *Foxg1*$^{-/-}$ cells in chimeric telencephalon fail to express markers of ventral cell fate. (A) Schematic showing restricted expression of ventral markers Olig2, Gsh2, Mash1, Nkx2.1, Calbindin and Islet1 in wt E12.5 telencephalon. Coronal sections through experimental (*Foxg1*$^{-/-}$) (B-D,F-H,J-M) and control (*Foxg1*$^{+/-}$) (E,I) chimeras. Nkx2.1 immunofluorescence (Green) in the Mge of a control (E) and experimental (B-D) chimera. Mash1 immunofluorescence (Green) in the Lge progenitor zone of a control (I) and experimental chimera (F-H). In B,D,F,H the nuclei of *Foxg1*$^{-/-}$ cells are labelled by LacZ immunofluorescence (Red). In E and I of *Foxg1*$^{+/-}$ cells are labelled by LacZ immunofluorescence (Red). In D,E,H,I nuclei are counterstained with TO-PRO-3 (blue). Olig2 (J) and Foxg1-lacZ (*Foxg1*$^{-/-}$ cells, K) immunohistochemistry from adjacent sections from a *Foxg1*$^{-/-}$ chimera. Gsh2 (L) and Foxg1-lacZ (*Foxg1*$^{-/-}$ cells, M) immunohistochemistry from adjacent sections from a *Foxg1*$^{-/-}$ chimera. For each staining, the approximate location of field of view shown is indicated by the asterisk in the telencephalic schematics which are overlaid on selected panels.

Nkx2.1 is expressed in progenitors of the ventro-medial most telencephalic regions, the medial ganglionic eminence (Mge) and the pre-optic area, whilst Mash1, Gsh2 and Olig2, have a broader expression domain that includes the lateral ganglionic eminence (Lge) (Summarised in Fig.4.3A). Double immunofluorescence shows that Foxg1-lacZ+, *Foxg1*$^{-/-}$ cells in *Foxg1*$^{-/-}$↔wt telencephalon were never observed to express Nkx2.1 (Fig.4.3B-D) or Mash1 (Fig.4.3F-H) in ventral telencephalic progenitor zones in all chimeras analysed, whilst *Foxg1*$^{+/-}$ cells in control chimeras frequently expressed these markers (Fig.4.3E,I). Analysis of Olig2 and Gsh2 expression was more difficult since the primary antibodies were raised in the same species making double immunos very difficult. Consequently analysis was carried out on adjacent 7μm sections. Similar to Nkx2.1 and Mash1, the expression of Olig2 and Gsh2 appeared to be complementary to Foxg1-lacZ in the ventral telencephalon (Fig.4.3J-M). These findings demonstrate a cell autonomous requirement for Foxg1 in the acquisition of ventral telencephalic progenitor fate.

To determine whether *Foxg1*$^{-/-}$ cells were completely excluded from the ventral lineages of the chimeric telencephalon, markers of ventrally derived neuronal types were analysed. Calbindin expression marks the majority of γ-aminobutyric acid (GABA)-ergic interneurons destined for migration to the neocortex (Anderson et al., 1997) and Islet-1 is expressed by differentiating striatal projection neurons and cholinergic interneurons (Stenman et al., 2003; Wang and Liu, 2001). Whilst in control chimeras, Foxg1$^{+/-}$ cells frequently expressed calbindin (yellow cells in Fig.4.4D), in *Foxg1*$^{-/-}$ chimeras, *Foxg1*$^{-/-}$, Calbindin+ interneurons were never observed (Fig.4.4A-C). Also in *Foxg1*$^{-/-}$ chimeras the vast majority of Islet1+ cells were derived from wt cells

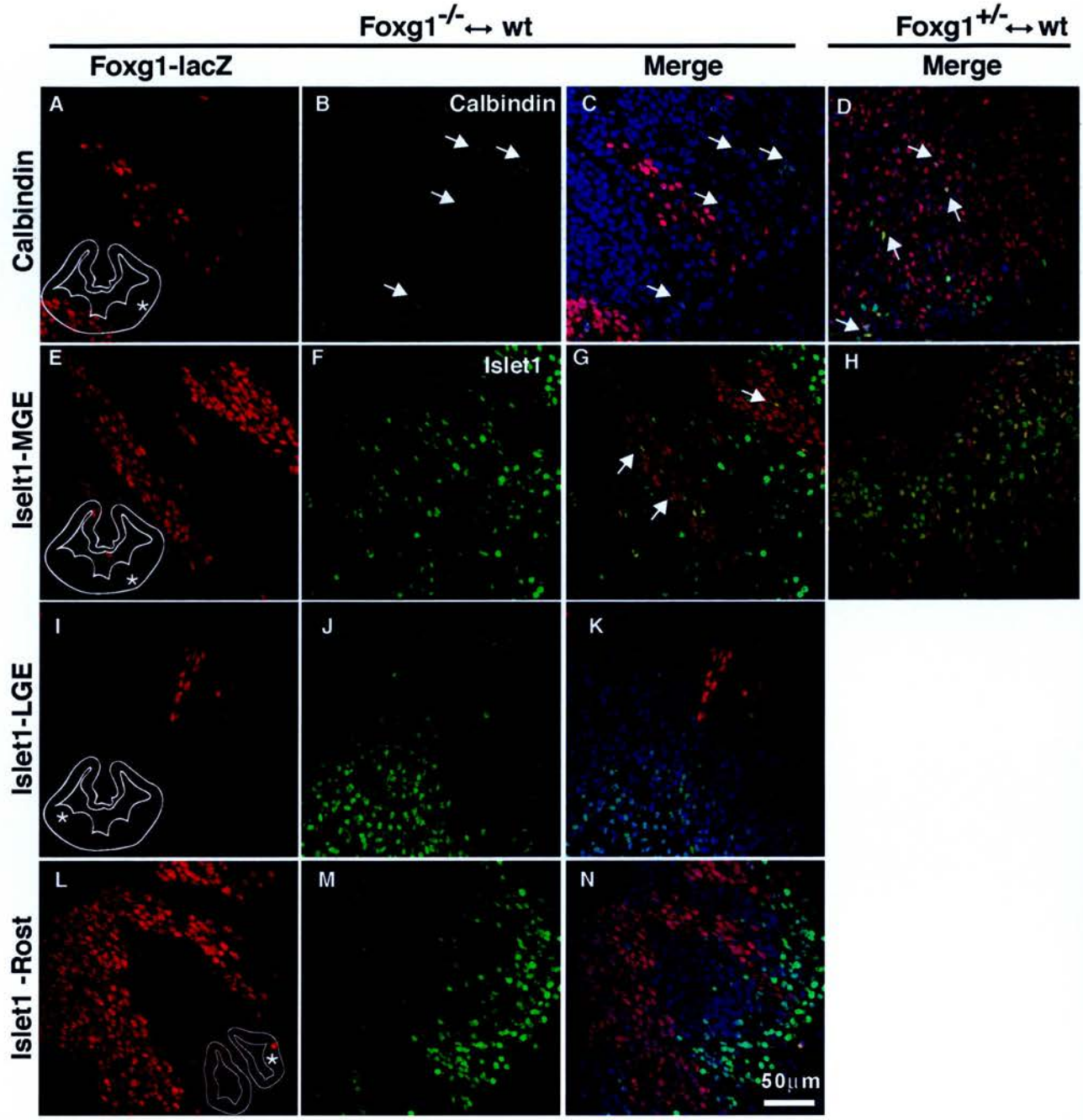


Fig.4.4. Generation of ventral neurons in chimeric telencephalon. Calbindinir immunofluorescence (green) in the mantle zone of a control (D) and experimenta (A-C) chimera). Islet1 immunofluoresence (green) in the mantle zone of a control (H and experimental (E-G, I-N) chimera. Islet1 expression (green) in Mge (E-G), Lge (I-K) and rostral (L-N) regions of the ventral telencephalon of a Foxg1-/- chimera When shown, *Foxg1*$^{-/-}$ and *Foxg1*$^{+/-}$ cells in chimeras are identified by Foxg1-Lacz immunofluorescence (Red). Colocalisation of red and green labelling (yellow) is indicated by arrowheads in selected panels. When shown, nuclei are counterstained with TO-PRO-3 (blue). For each staining, the approximate location of field of view shown is indicated by the asterisk in the telencephalic schematics which are overlaid on selected panels. Mge, medial ganglionic eminence; Lge, latera ganglionic eminence.

(Foxg1-lacZ -), however, a small number of *Foxg1*$^{-/-}$ cells expressed Islet1 (white arrows in Fig.4.4E-G). These were only seen in discrete regions of the medial mge. In more rostral, caudal and lateral portions of the chimeric ventral telencephalon *Foxg1*$^{-/-}$/Islet1+ cells were never observed (Fig.4.4I-N). In control chimeras, large numbers of Foxg1$^{+/-}$ cells expressed Islet1 (Fig.4.4H). These data demonstrate that Foxg1 is required in a direct and cell autonomous manner for the proper specification of ventral telencephalic fate and allow us to reject the hypothesis that the failure of ventral development in *Foxg1*$^{-/-}$ embryos is primarily a non-cell autonomous consequence of the loss of telencephalic Shh expression, as has been suggested previously (Huh et al, 99).

Foxg1 is required cell autonomously for the repression of dorsal telencephalic identity

As demonstrated in Figure 4.1, the whole of the *Foxg1*$^{-/-}$ telencephalon appears to be specified as dorsal telencephalon. Given the cell autonomous failure of mutant cells to adopt a ventral fate, it was of great interest to determine whether *Foxg1*$^{-/-}$ progenitors autonomously default to a dorsal telencephalic fate in the chimeric telencephalon. Pax6 and Ngn2 are transcription factors whose expression is restricted to progenitor cells in the ventricular zone of the dorsal telencephalon (Fig.4.5A). Tbr2 is expressed by basally dividing precursors and some newborn neurons in the dorsal telencephalon (Englund et al., 2005) (Fig.4.5A). Double immunofluorescence for Foxg1-lacZ and Pax6 provided a striking demonstration that *Foxg1*$^{-/-}$ cells in both the Lge and Mge of the chimeric telencephalon cell autonomously express Pax6 to detectable levels (Fig.4.5B-D, F-H). Surrounding wt cells in *Foxg1*$^{-/-}$ chimeras were negative for Pax6 and Foxg1$^{+/-}$ cells in

progenitor zones of the Lge and Mge of control chimeras (Fig.4.5E,I) were negative for Pax6. Ngn2 and Foxg1-lacZ and Tbr2 and Foxg1-lacZ expression patterns were examined by immunohistochemistry on adjacent sections. This analysis revealed that *Foxg1*$^{-/-}$ progenitors frequently expressed Ngn2 when positioned in the chimeric Lge (Fig.4.5J,K). *Foxg1*$^{-/-}$ cells positioned ventral to the Lge/Mge sulcus were not found to express Ngn2. A similar pattern was observed for Tbr2 expression. *Foxg1*$^{-/-}$ cells in the chimeric Lge frequently expressed Tbr2 (Fig.4.5N,O) whereas those positioned more ventrally did not. Wt cells in *Foxg1*$^{-/-}$ chimeras and both cell types in the ganglionic eminences of control chimeras did not express Ngn2 or Tbr2, as expected (Fig.4.5L,M).

Staining for Tbr1, a transcription factor expressed by glutamatergic neurons derived from the dorsal telencephalon (Hevner et al., 2003; Hevner et al., 2001), showed large numbers of ectopic Tbr1+, *Foxg1*$^{-/-}$ cells in the chimeric lge (Fig.4.5O,P). Whilst a small number of Tbr1+ cells were observed in the marginal zone of control chimeras (Arrows in Fig.4.5Q), these are also observed in wt embryos and are believed to be generated in the dorsal telencephalon (Hevner et al., 2003; Hevner et al., 2001). The abundance of Tbr1+ cells, their position deeper in the telencephalic wall and their close association with ectopic Tbr2 staining argue that these ectopic dorsally patterned cells in the chimeric Lge are produced *in situ* by *Foxg1*$^{-/-}$ progenitors in the ventral telencephalon, rather than migrating in from dorsal progenitor zones.

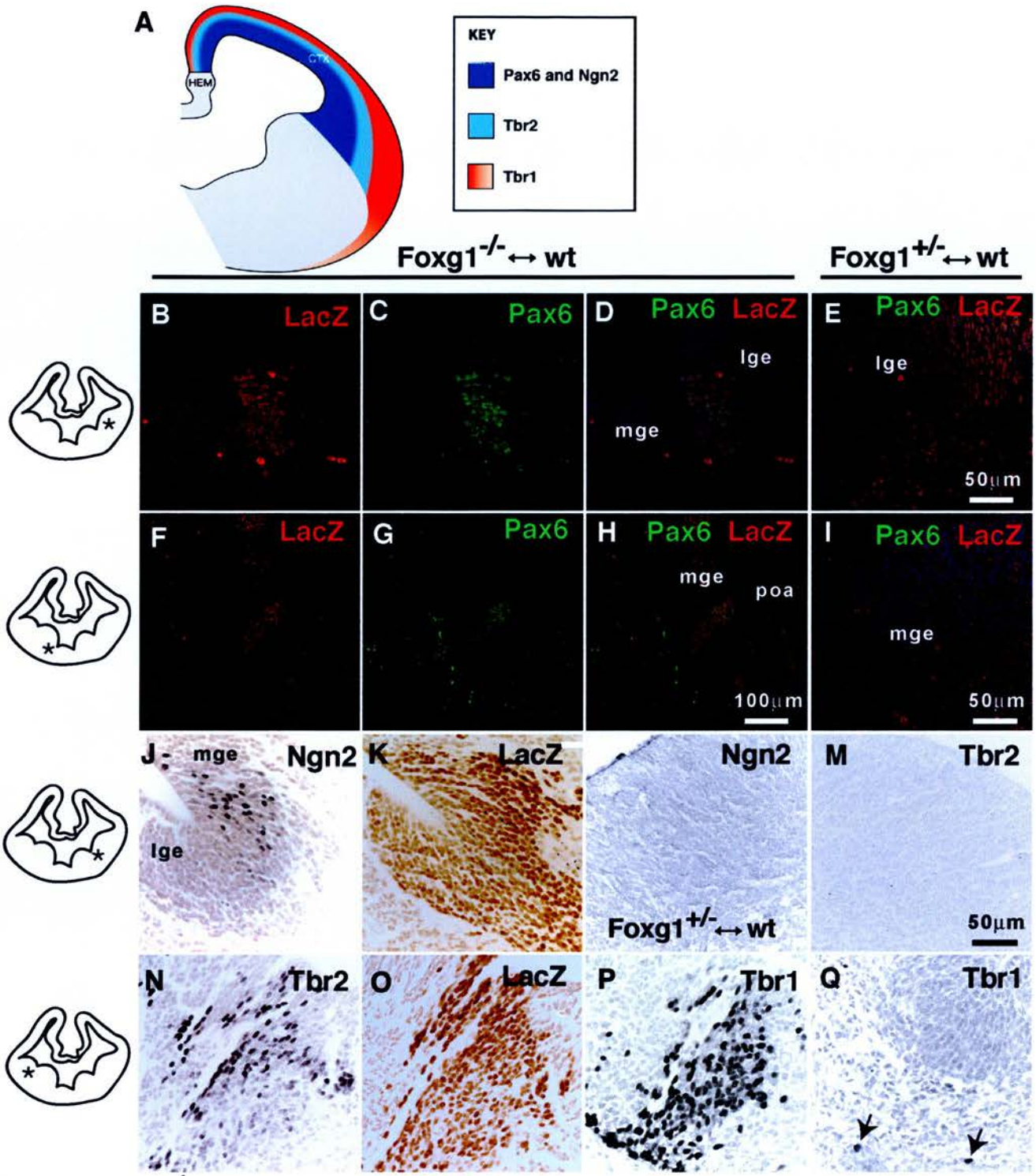


Fig.4.5. *Foxg1*$^{-/-}$ cells maintain dorsal specification in ventral regions of the chimeric telencephalon. (A) Schematic showing restricted expression of dorsal markers Pax6, Ngn2, Tbr2 and Tbr1 in wt E12.5 telencephalon. Coronal sections through experimental (*Foxg1*$^{-/-}$) (B-D,F-H,J-K,N-P) and control (*Foxg1*$^{+/-}$) (E,I,L,M,N,Q) chimeras. Pax6 immunofluorescence (Green) in the Lge (B,D,E) and Mge (F,H,I) ventral progenitor zone of a control and experimental chimera, overlaid with LacZ immunofluorescent staining (Red) of *Foxg1*$^{-/-}$ cells (C,D,F,H) and *Foxg1*$^{+/-}$ cells (E,I). Pax6 labelling in E, at the right, is within dorsal telencephalic cells at the dorso-ventral boundary. In selected panels nuclei are counterstained (Blue) with TO-PRO-3. Adjacent 7µm sections were examined to compare the contribution of *Foxg1*$^{-/-}$ cells (Brown immunohistochemistry in K,O) to the expression of Ngn2 (Black cells in J), Tbr2 (N) and Tbr1 (P) in experimental chimeras. Absence of Ngn2 (M), Tbr2 (N) and Tbr1 (Q) expression in equivalent regions of the ventral telencephalon of control chimeras. For each staining, the approximate location of field of view shown is indicated by the asterisk in the telencephalic schematic, to the left of each row.

In summary the ectopic expression of Pax6, Ngn2, Tbr2 and Tbr1 by *Foxg1*$^{-/-}$ cells in ventral telencephalon of *Foxg1*$^{-/-}$ chimeras suggest that *Foxg1*$^{-/-}$ progenitors not only fail to activate ventral patterning genes, but are defective in their ability to repress dorsal telencephalic fates. This characteristic is particularly marked in the chimeric Lge, where mutant cells appear to follow a dorsal telencephalic differentiation program, similar to that recently described by Englund and colleagues (Englund et al., 2005). More medially, in the Mge, only ectopic Pax6 persists, consistent with the idea that ventral midline cues are able to repress some aspects of dorsal fate in *Foxg1*$^{-/-}$ mutant cells.

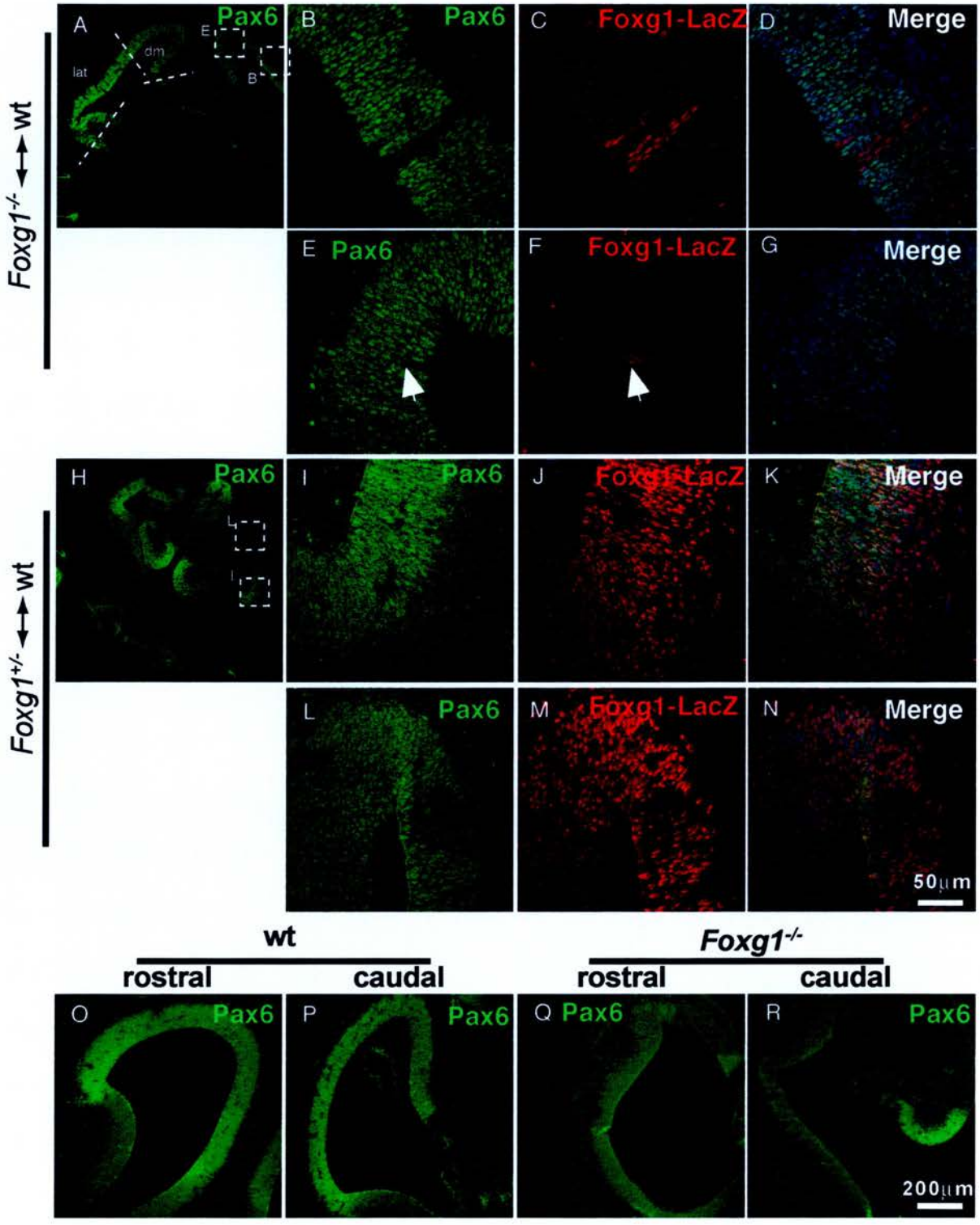
Pax6 is misregulated in a cell autonomous fashion in Foxg1$^{-/-}$ *telencephalon*

The ectopic expression of genes characteristic of dorsal lineages in ventrally positioned *Foxg1*$^{-/-}$ cells led me to examine whether dorsal genes are correctly regulated in dorsally positioned cells. In wt telencephalon Pax6 protein is expressed in the dorsal telencephalic VZ in a high lateral to low medial gradient at E12.5 (Fig.4.6O,P). Pax6 immunofluorescence in *Foxg1*$^{-/-}$ chimeric dorsal telencephalon revealed groups of cells in the lateral telencephalon which expressed Pax6 at a discernably lower level than their neighbours (Fig.4.6B). These cells proved to be *Foxg1*$^{-/-}$ on the basis of Foxg1-lacZ immunofluorescence (Fig.4.6C,D). *Foxg1*$^{-/-}$ cells in a more dorsomedial position of the same section (where Pax6 levels are generally lower) appeared to express Pax6 at a similar level to their neighbouring wt cells. In control chimeras Foxg1$^{+/-}$ cells express Pax6 at the same levels as adjacent wt cells in both lateral and dorso-medial positions

(Fig.4.6H-N). Thus *Foxg1*$^{-/-}$ cells in the chimeric telencephalon express Pax6 when in ventral (Fig.4.5), lateral and dorsomedial positions. However, mutant cells appear to be unable to upregulate Pax6 to control levels in the lateral cortex in the chimeric telencephalon.

It is possible that wt cells in the chimeras affect *Foxg1*$^{-/-}$ cells to limit their expression of Pax6 non-cell-autonomously. To examine this possibility *Foxg1*$^{-/-}$ embryos were analysed. Immunofluorescence for Pax6 on *Foxg1*$^{-/-}$ telencephalic sections and sections from wt littermates are shown in Fig.4.6Q-R. At rostral (Fig.4.6O) and caudal (Fig.4.6P) levels of the wt telencephalon the gradient in Pax6 expression is apparent. In equivalent sections rostral (Fig.4.6Q) and caudal (Fig.4.6R) from *Foxg1*$^{-/-}$ telencephalon there is no obvious gradient and Pax6 appears to be expressed at a lower level (staining was carried out with wt and mutant sections on the same slide and were scanned at identical laser settings on the confocal microscope to minimise experimental variation). To obtain a more rigorous quantification of PAX6 levels in *Foxg1*$^{-/-}$ telencephalon, protein extracts were made from dissections of *Foxg1*$^{-/-}$ telencephalon and medial and lateral regions of wt dorsal telencephalon (approximate regions dissected are indicated by dashed lines in Fig.4.6A) and western blotting for Pax6 was carried out (Fig.4.6S). Pax6 is present in two major isoforms: canonical Pax6 and Pax6(5a) (which includes an extra short exon, causing the protein to migrate more slowly, (Epstein et al., 1994)). Both of these isoforms were present in both wt and *Foxg1*$^{-/-}$ extracts. Quantification of total Pax6 levels (Pax6 + Pax6(5a)) reveals that Pax6 is expressed at higher levels in the wt lateral cortex than in the dorsomedial cortex (two sample t-test $p<0.05$, n=3 independent extracts), as expected (Fig.4.6T). Overall Pax6 levels in *Foxg1*$^{-/-}$ telencephalon are also lower than wt

lateral cortex (t-test $p<0.05$, n=3-4 independent extracts) and almost identical to Pax6 levels in the wt dorsomedial telencephalon (Fig.4.6T). In both medial and lateral parts of wt telencephalon Pax6 is more abundant than Pax6(5a) (Ratio (Log_2) of Pax6:Pax(5a) > 0) whereas in *Foxg1*$^{-/-}$ telencephalon that trend is reversed, Pax6(5a) is more abundant (Ratio (Log_2) of Pax6:Pax(5a) < 0) (Fig.4.6U).


Foxg1-/- ↔ wt
Foxg1+/- ↔ wt
A Pax6
B Pax6
C Foxg1-LacZ
D Merge
dm
lat
E
B
E Pax6
F Foxg1-LacZ
G Merge
H Pax6
I Pax6
J Foxg1-LacZ
K Merge
L Pax6
M Foxg1-LacZ
N Merge
50µm
wt
Foxg1-/-
rostral
caudal
rostral
caudal
O Pax6
P Pax6
Q Pax6
R Pax6
200µm

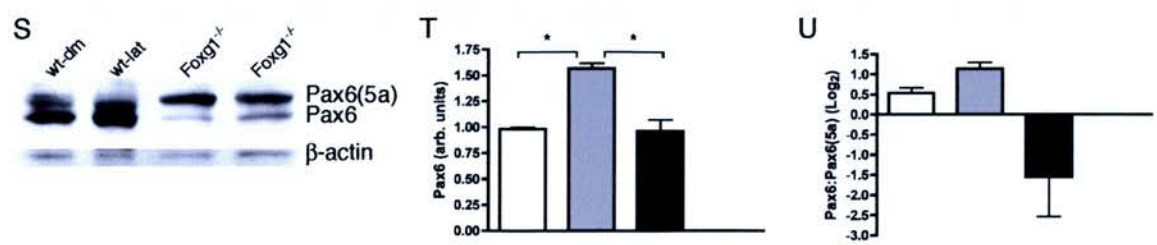

S
wt-dm
wt-lat
Foxg1-/-
Foxg1-/-
Pax6(5a)
Pax6
β-actin
T
Pax6 (arb. units)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
*
U
Pax6:Pax6(5a) (Log2)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0

Fig.4.6. Misregulation of Pax6 by *Foxg1*$^{-/-}$ telencephalic cells. Pax6 immunofluorescence in coronal sections from a *Foxg1*$^{-/-}$ (A-G) and a *Foxg1*$^{+/-}$ (H-N) chimera. Selected regions from dorso-lateral (B-D, I-K) and dorso-medial (E-G, L-N) are shown at higher magnification and co-labelled for Foxg1-lacZ expressed by *Foxg1*$^{-/-}$ (C,D,F,G) or *Foxg1*$^{+/-}$ (J,K,M,N) cells. Nuclei are counterstained (Blue) with TO-PRO-3. Pax6 immunofluorescence in rostral (O,Q) and caudal (P,R) sections from wt (O,P) and *Foxg1*$^{-/-}$ (Q,R) embryos at E12.5. (S) Western blot showing Pax6 and β-actin (loading control) protein expression in wt and *Foxg1*$^{-/-}$ telencephalic extracts. (T) Quantification of Pax6 levels (normalised to b-actin) in wt dorso-medial (white bar), dorso-lateral (grey bar) and *Foxg1*$^{-/-}$ (black bar) telencephalic extracts. Values are means ±SEM from 3-4 independent extracts. (U) Ratio (Log_2±SEM from 3-4 independent extracts) of Pax6:Pax6(5a) isoforms in wt dorso-medial (white bar), dorso-lateral (grey bar) and *Foxg1*$^{-/-}$ (black bar) telencephalic extracts.

Ngn2, Tbr2 and Tbr1 appear to be correctly regulated in dorsal telencephalon of chimeric embryos

In the light of the cell autonomous misregulation of Pax6 (the defect appeared to be the same in *Foxg1*$^{-/-}$ and chimeric embryos), the expression of further dorsal telencephalic markers was examined in *Foxg1*$^{-/-}$ chimeras. Ngn2 is expressed in a subset of cells scattered through the dorsal telencephalon. In both balanced and high percentage chimeras, *Foxg1*$^{-/-}$ cells were able to express Ngn2 at levels that appeared identical to their wt neighbours (Fig.4.7.A-D). Similarly no overt defects were observed in the expression of Tbr2 (Fig.4.7E-H) or Tbr1 (Fig.4.7I-L) in wt or *Foxg1*$^{-/-}$ cells within *Foxg1*$^{-/-}$ chimeras. It must be noted, however, that Tbr1 and Tbr2 are expressed mosaically in sub-populations of cells and since mosaic Foxg1-lacZ expression had to be visualised on adjacent sections, it is impossible to rule out more subtle defects in the expression of these proteins in *Foxg1*$^{-/-}$ cells.

Foxg1 represses p21^{cip1} expression in a non-cell autonomous fashion

The expression domain of the cyclin dependent kinase inhibitor (CKI) p21^{cip1} is expanded in *Foxg1*$^{-/-}$ telencephalon ((Seoane et al., 2004) and Chapter 3 Fig.3.7). To determine whether this increased tendency to upregulate p21^{cip1} is a cell autonomous effect of the lack of Foxg1, p21^{cip1} immunohistochemistry was carried out on adjacent sections to those reacted for Foxg1-lacZ. In *Foxg1*$^{-/-}$ chimeras p21^{cip1} expression was restricted to a

relatively small number of cells in the marginal zone of the dorso-medial telencephalon (Fig.4.7.N,P), in a pattern very similar to that observed in wt telencephalon (Chapter 3 Fig.3.7). Despite the presence of numerous *Foxg1*$^{-/-}$ cells in ventricular and marginal zones of the same telencephalic region, p21^{cip1} expression was not associated with Foxg1-lacZ staining. This suggests that p21^{cip1} overexpression is rescued by the presence of wt telencephalic cells, implying that this CKI is non-cell autonomously regulated by Foxg1.

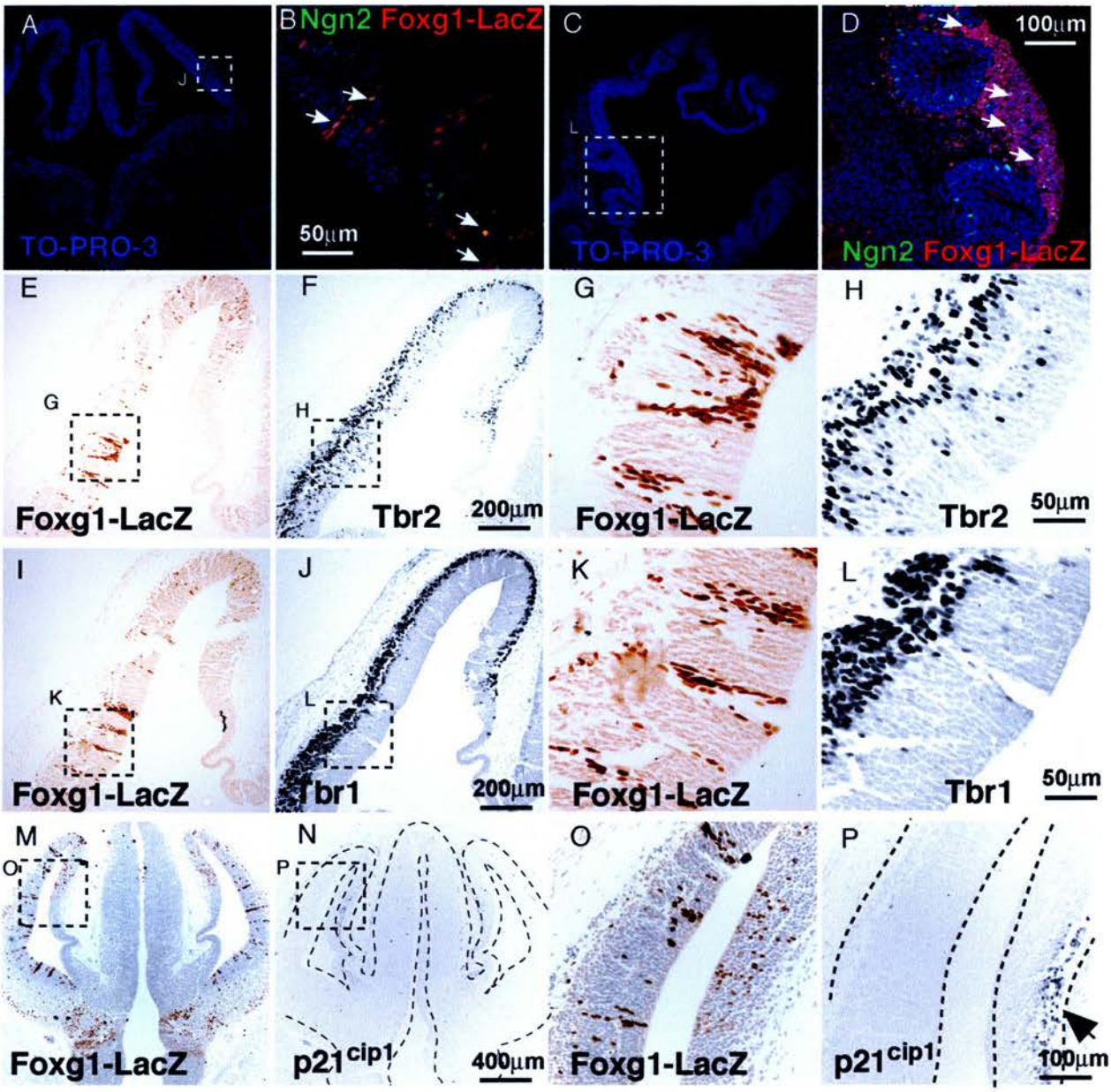


Fig.4.7. Expression of dorsally restricted genes in the dorsal telencephalon of *Foxg1*$^{-/-}$ chimeras. (A-D) Double immunofluorescence for Ngn2 and Foxg1-lacZ in sections from balanced (A,B) and high percentage *Foxg1*$^{-/-}$ chimeras (C,D). In both types of chimera Ngn2+,Foxg1-LacZ+ cells are observed (yellow cells, some of which are indicated by white arrows). Nuclei are counterstained (blue) with TO-PRO-3. Expression of dorsal markers Tbr2 (E-H), Tbr1 (I-L) and p21^{cip1} (M-P) in the dorsal telencephalon of balanced chimeras. For each marker, Foxg1-lacZ immunohistochemistry labels *Foxg1*$^{-/-}$ cells in the adjacent 7µm section.

DISCUSSION

Foxg1 is required cell autonomously for ventral telencephalic fate specification

The experiments described in this chapter come together to provide strong support to the hypothesis that Foxg1 is required for the specification of ventral telencephalic cell fate. Moreover, the failure to acquire ventral identity is maintained when *Foxg1*$^{-/-}$ cells are exposed to developmental cues produced by wt cells in chimeric embryos. Thus Foxg1 is required cell autonomously for proper specification of ventral telencephalic fate. The only minor exception to this rule observed here is the appearance of a small number of *Foxg1*$^{-/-}$ cells that express the ventral neuronal marker Islet1 in the *Foxg1*$^{-/-}$↔wt telencephalon. The cell autonomous requirement for Foxg1 in ventral *specification* falsifies previous hypotheses that the primary defects in the ventral telencephalic development of *Foxg1*$^{-/-}$ mutants are in progenitor proliferation (Xuan et al., 1995) and in the regulation of Shh expression (Huh et al., 1999).

Further corroborative evidence in support of a direct and early requirement for Foxg1 in ventral telencephalon comes from experiments carried out by another group (Hanashima et al., 2002). These researchers examined the ability of various forms of the Foxg1 gene to rescue the defects associated with null mutation of Foxg1. To achieve this they used the tetracycline-driven transactivator system. By inserting the sequence encoding the

tetracycline transactivator (tetTA) protein into the endogenous Foxg1 locus, Foxg1 promoter-driven tetTA protein was used to activate the expression of a randomly incorporated transgene encoding Foxg1 under the control of the tetracycline operator (tetO), on a *Foxg1*$^{-/-}$ background. For control experiments wt Foxg1 was expressed by this means. However, despite the reported rescue of dorsal telencephalic proliferation and differentiation phenotypes by wt Foxg1, the ventral telencephalon was notably absent (Hanashima et al., 2002). The most parsimonious explanation for this is that early in development, following Foxg1 promoter activation TetA protein gradually builds up to levels where TetO-driven Foxg1 expression is activated. Due to the two-step nature of this process, a lag between Foxg1 promoter activation and Foxg1 protein synthesis is therefore anticipated. If Foxg1 is required for ventral induction during this time period, induction will not be achieved in these embryos, as is observed.

Foxg1 is required to suppress dorsal gene expression in ventral telencephalic territories

The experiments in this chapter also reveal a cell autonomous requirement for Foxg1 to repress dorsal gene expression in ventral telencephalic territories, a logical requirement for ventral lineage commitment. Whilst some dorsal genes, namely Ngn2, Tbr1 and Tbr2, were partially repressed (eg. in ventro-medial regions), Pax6, a gene that is required for proper specification and maintenance of dorsal telencephalon (Manuel and Price, 2005), was expressed by *Foxg1*$^{-/-}$ cells in progenitor zones throughout the telencephalon. It is possible that Pax6 expression in ventrally positioned *Foxg1*$^{-/-}$ cells is incompatible with the acquisition of ventral telencephalic identity. A parallel to this in

more caudal regions of the neural tube is that Pax6 is thought to repress expression of the ventral marker Nkx2.2 in the spinal cord due to the expansion of Nkx2.2 in Pax6 mutants (Briscoe et al., 1999; Ericson et al., 1997) and the repression of Nkx2.2 by ectopic expression of Pax6 (Muhr et al., 2001). In the telencephalon Pax6 is required for proper development of dorsal cell types, and in its absence, there is a clear expansion of ventral identities, consistent with a role in repressing ventral cell fates (Manuel and Price, 2005).

Foxg1$^{-/-}$ cells appear to segregate from wt cells in the chimeric telencephalon

Although it could not be quantified here, qualitatively there was a repeated trend for *Foxg1$^{-/-}$* cells to be segregated from wt cells in the chimeric telencephalon, whereas the contribution of *Foxg1$^{+/-}$* cells was much more mosaic. This effect was particularly marked in ventral regions. Since *Foxg1$^{-/-}$* cells express a different profile of molecular fate determinants to their wt neighbours (for example, mutant cells in the ventral telencephalon express Pax6), the segregation may reveal the existence of an important developmental biological principle: that cells of different lineages often actively repel one another, to allow the independent development of morphologically and molecularly distinct progenitor domains or compartments. A similar effect was observed in a recent study of the spinal cord *Smo$^{-/-}$*↔wt chimeras (Wijgerde et al., 2002). Smo$^{-/-}$ cells are cell autonomously unable to respond to Hh signals and take on a dorsal fate, even in ventral regions, and tend to segregate away from wt ventral cells, whereas Smo$^{+/-}$ cells contributed in a genuinely mosaic fashion (Wijgerde et al., 2002).

Another interesting aspect of the mis-regulation of Pax6 in the absence of Foxg1 is the altered isoform ratio observed in the *Foxg1*$^{-/-}$ telencephalon. Canonical Pax6 has two DNA-binding domains: a paired domain and a homeobox domain, each of which binds unique target sequences (reviewed (Simpson and Price, 2002)) and is the most abundant isoform in the developing wt telencephalon. The other major isoform, Pax6(5a), contains a 14 amino acid insertion into the paired domain that negatively affects its DNA-binding and transactivation properties and is less abundant (reviewed by (Simpson and Price, 2002)). In *Foxg1*$^{-/-}$ telencephalon, Pax6(5a) is more abundant relative to Pax6. In *Foxg1*$^{-/-}$ chimeras it is currently technically impossible to establish whether the alteration in isoform ratio is a cell autonomous phenotype, but given that the reduction in Pax6 levels relative to lateral wt cortex is cell autonomous, it may well be. It is interesting to consider whether this finding may impact on the dorso-ventral patterning defects in *Foxg1*$^{-/-}$ and *Foxg1*$^{-/-}$↔wt telencephalon and also contribute to the proliferative and differentiation defects described in the previous chapter. A recent paper comparing the effect of various Pax6 mutations in the telencephalon failed to find an essential role for Pax6(5a) in dorso-ventral patterning or the regulation of proliferation and differentiation (Haubst et al., 2004). The production of a mouse mutant expressing only Pax6(5a) would provide an interesting parallel to the *Foxg1*$^{-/-}$ telencephalon and perhaps test whether unusually high levels of Pax6(5a) at the expense of Pax6 recapitulates any aspects of the *Foxg1*$^{-/-}$ phenotype.

Dorsalisation of Foxg1$^{-/-}$ telencephalic cells may be caused by inappropriate regulation of dorsalising pathways

In addition to the potential inability of *Foxg1*$^{-/-}$ telencephalic progenitor cells to repress Pax6 and to correctly regulate Pax6 splicing, there are several other interesting routes by which Foxg1 may regulate dorso-ventral patterning of the telencephalon. As discussed in the previous chapter several members of the BMP and Wnt families have been shown to be ectopically expressed in the *Foxg1*$^{-/-}$ telencephalon (Dou et al., 1999; Hanashima et al., 2002; Muzio and Mallamaci, 2005; Vyas et al., 2003). In several regions of the neural tube there is good evidence that both BMP and Wnt signalling are required for proper induction of dorsal fates and suppression of ventral fates (reviewed in (Chizhikov and Millen, 2005; Ciani and Salinas, 2005). In the telencephalon *in vitro* (Furuta et al., 1997; Monuki et al., 2001) and *in vivo* (Panchision et al., 2001) approaches have shown that exogenous BMP signalling can induce dorso-medial identities and repress more lateral identities (including *Foxg1* and *Lhx2* expression, two genes which are not expressed in the dorsomedial-most telencephalon). However, mice mutant for BMP receptor 1A (*BMPR1A*) only exhibit a loss of the dorsal-most telencephalic structure, the choroid plexus of the third ventricle, suggesting a limited, local requirement for high level BMP signalling in dorso-ventral patterning of the telencephalon (Hebert et al., 2002). Removal of further BMP receptors, which may compensate for BMPR1A loss of function, might reveal wider requirements for BMP signalling in dorsal telencephalic development.

There is good evidence from chick explant studies that Wnt signalling in the early forebrain is necessary and sufficient for the induction of dorsal fates, and that Wnts may

have a broader role than BMPs in dorsal induction (Gunhaga et al., 2003; Marklund et al., 2004). There is also in vivo evidence that exogenous Wnt antagonists can block dorsal telencephalic fate (Houart et al., 2002) and it has also been shown that *Wnt3a* and *Lef1* (a downstream mediator of wnt signalling) (Galceran et al., 2000; Lee et al., 2000) mutant mice have serious defects in hippocampal development.

Consequently it seems entirely possible that increased BMP and/or Wnt signalling early in telencephalic development may be capable of inducing ectopic dorsal identity and suppressing ventral identity. However, in the case of *Foxg1*$^{-/-}$ mutants it seems unlikely that ectopic expression of Bmp and Wnt ligands is the primary cause of dorso-ventral patterning defects in the ventral telencephalon. The main reason for this is that the documented increase in expression of BMP and Wnt ligands does not appear to occur, at least in a dramatic fashion, until at least E11.5, by which time dorsal and ventral lineages are well established in the telencephalon. Prior to E11.5, Wnt8b and BMP signalling (as assessed by staining for BMP Receptor phosphorylated Smads) are not ectopically activated (chapter 3, Fig.3.9) and ventral cell types are clearly absent (Fig.4.1). It is, however, possible that Wnt's other than *Wnt8b* are ectopically activated in the early *Foxg1*$^{-/-}$ telencephalon and contribute to the suppression of ventral identity.

The BMP and Wnt upregulation in the *Foxg1*$^{-/-}$ telencephalon (and potentially in *Foxg1*$^{-/-}$ cells in the chimeric telencephalon) seems to go hand in hand with a progressive re-specification of the mutant telencephalon towards a dorso-medial, cortical hem-like fate, as has been suggested recently (Muzio and Mallamaci, 2005). It is possible that other features of a dorso-medial identity are capable of suppressing ventral fate. However, the

impact of expanded cortical hem identity (including BMP and Wnt expression) on ventral telencencephalic patterning may not be very great since in mice mutant for Lhx2, cortical hem fates are greatly expanded and various Wnt's and BMP's are expressed in a greatly expanded domain, yet ventral telencephalic development proceeds normally (Bulchand et al., 2001; Monuki et al., 2001). In any case, other than a slight increase in Pax3 expression there is no evidence for an early re-specification to a dorso-medial fate in Foxg1 mutants. Also, if the mutant telencephalon was totally re-specified as cortical hem, we would not expect to observe Foxg1 driven expression of the LacZ reporter since Foxg1-lacZ is not expressed in the hem region of *Foxg1*$^{+/-}$ heterozygotes and this distinction is maintained in the Fox$^{-/-}$ telencephalon (see Chapter 3, Figure 3.1).

Further to this point, it would be interesting to know whether the upregulation of BMP and Wnt ligands and other dorso-medial telencephalic markers is a cell autonomous defect in Foxg1 mutants. Chimeric embryos of the type generated in this study provide the test for this idea. Lack of suitable reagents and time meant this issue was not pursued. If these genes were upregulated by mutant cells in the chimeric telencephalon, it may be expected that these diffusible signals would have non-cell autonomous effects on surrounding wt cells. In terms of both dorsal and ventral gene expression patterns, no evidence for such an effect on wt components of the chimeras was observed. Also related to this point, it has been shown that exposure of telencephalic cells to high levels of exogenous BMP4 or consitutively active BMPR1A represses Foxg1 transcription (Furuta et al., 1997; Panchision et al., 2001), since mutant cells in chimeras and *Foxg1*$^{-/-}$ embryos clearly express Foxg1-lacZ, it is unlikely that BMPs are overexpressed to a high degree.

An alternative, and perhaps more likely, link between BMP and Wnt signalling and the cell autonomous dorsalisation of *Foxg1*$^{-/-}$ progenitors is the idea that they may be hypersensitive to the putative dorsalising activities of these morphogens. Early exposure of *Foxg1*$^{-/-}$ cells to levels of Wnt and or BMP signals that would not normally affect wt cells in both *Foxg1*$^{-/-}$ and chimeric embryos may be sufficient to repress ventral and activate dorsal pathways. A precedent for this may be provided by the study of mice mutant for both chordin and noggin (*Chrd* and *Nog*), two secreted antagonists of Bmp signalling (Anderson et al., 2002). In *Chrd*$^{-/-}$;*Nog*$^{+/-}$ embryos, rostral neural cells are hyper-sensitive to exogenous application of Bmps and express rostral Fgf8 and ventral Shh at much reduced levels, similar to Foxg1 mutants. Unfortunately, the expression of markers of D/V patterning were not examined in these mutants. It is therefore difficult to determine whether increased sensitivity to BMPs necessarily leads to dorsalisation of telencephalic progenitors in a direct fashion. If it does, this could also be an indirect consequence of the collapse of Fgf and Shh-producing signalling centres as observed in *Chrd*$^{-/-}$;*Nog*$^{+/-}$ telencephalon.

Experiments in vitro could test whether defective responses to dorsalising cues underlying D/V patterning defects in *Foxg1*-/- telencephalon by examining the response of *Foxg1*$^{-/-}$ telencephalic cells or explants to exogenous positive and negative mediators of BMP and Wnt signalling in terms of dorsal and ventral gene expression. In vivo, the combination of Foxg1 null mutation with loss of function mutations in the BMP and Wnt signalling pathways, or the expression of dominant negative components of these pathways, may rescue the dorsalisation phenotype and support the idea that *Foxg1*$^{-/-}$ progenitors are unusually sensitive to dorsalising signals.

Foxg1 may be required for telencephalic cells to respond to ventralising signals

Moving on from possible defects in pro-dorsal signalling, the other major possible explanation for the cell autonomous failure of ventral fate specification in the absence of Foxg1 is that Foxg1 is required for telencephalic cells to respond appropriately to ventralising signals. The best characterised ventral morphogen is sonic hedgehog (Shh), a member of the hedgehog (Hh) family of secreted signalling molecules. Shh is strongly implicated in ventral induction at all levels of the neural tube, including the telencephalon (Ericson et al., 1995a; Ingham and McMahon, 2001; Patten and Placzek, 2000) (Zaki, Martynoga et al 2005). The possible involvement of Foxg1 in regulating the Hh response in the telencephalon is investigated in the following chapter.

Chapter 5: Foxg1 and Hh/Gli signalling in D/V patterning of the telencephalon

INTRODUCTION

Results described in Chapter 4 of this thesis demonstrate that Foxg1 is required cell autonomously for the specification of ventral telencephalic fate. One hypothesis that might explain this fundamental reliance on Foxg1 for the development of the ventral telencephalon is that Foxg1 is required for telencephalic cells to receive and transduce signals that promote ventral fate. The morphogen sonic hedgehog (Shh), a member of the hedgehog (Hh) family of secreted proteins, is deeply involved in ventral cell fate specification in all regions of the neural tube from caudal-most to rostral-most (Ericson et al., 1995a; Ingham and McMahon, 2001; Patten and Placzek, 2000), including telencephalon (reviewed in Zaki, Martynoga et al 2005).

The Hh/Gli signalling pathway

The basic components of the Hh signalling pathway are highly conserved and have been best characterised in *Drosophila melanogaster* (reviewed in (Huangfu and Anderson, 2006; Ingham and McMahon, 2001)). Our understanding of vertebrate Hh signalling is growing at a fast pace (Huangfu and Anderson, 2006). Patched 1 (Ptc1) is the major cell

surface receptor for Hh ligands in the mouse embryo (Ingham and McMahon, 2001). Whilst Patched 2 and hedgehog interacting protein 1 (Hip1) also function as Hh receptors, Hip1 is not expressed in the telencephalon (Chuang and McMahon, 1999) and the presence of Ptc2 cannot compensate for the severe phenotype of mouse *Ptc1*$^{-/-}$ mutants (Goodrich et al., 1997), consequently these genes are unlikely to play an important role in Hh. Gli signalling in the telencephalon. Ptc1 is required to relieve Ptc1-mediated inhibition of the transmembrane protein Smo[2]. De-repression of Smo then leads to Smo-dependent activation of members of the Gli family of zinc finger transcription factors, which are believed to mediate all Hh signalling (Bai et al., 2004; Lei et al., 2004; Motoyama et al., 2003). The expression of three vertebrate Gli genes, Gli1, Gli2 and Gli3, all of which have been implicated in the transduction and regulation of Hh signalling provides a range of responses to the Hh ligands (reviewed in (Jacob and Briscoe, 2003)). The net result of positive Hh signalling in *Drosophila* appears to be determined by the overall balance between activator and repressor forms of cubitus interuptus (Ci), the single fly homolog of the Gli factors. A similar model appears to be applicable to the developing vertebrate embryo. The activator and repressor functions of Ci are thought to be distributed between the 3 Gli factors. Gli1 and Gli2 appear to be the major activators of positive hedgehog signalling. Gli1 activity is capable of inducing a range of Hh target genes that include Gli1 itself (indeed, unlike Gli2, Gli1 expression *depends* on Hh signalling (Bai et al., 2002)) and Ptc1. Although *Gli2* transcription may not be Shh regulated, available evidence suggests that Gli2 may mediate the initial Gli activator functions of Hh signalling (Bai et al., 2002). Gli3's position in the Hh signalling

[2] The most striking evidence for this probably comes from mouse *Ptc1*$^{-/-}$ mutants, which are severely ventralised at all levels of the neuraxis due to de-repession of the Hh signalling pathway (Goodrich et al 1997).

pathway is more complex. Like Ci, the protein exists in two forms: a full-length form (Gli3-FL) which seems to be primarily a transcriptional activator[3] and a cleaved form (Gli3-CL), which is primarily a transcriptional repressor (reviewed (Jacob and Briscoe, 2003))[4]. In the absence of Hh signalling, Gli3-CL is more abundant than Gli3-FL and appears to function as a negative regulator of Hh signalling. As has been well characterised in *Drosophila*, it appears that the balance of positive and negative Gli signalling in the developing vertebrate neural tube determines whether downstream targets of Hh signalling are activated or repressed. Positive Gli activator functions result from Hh signalling and are associated with the antagonism of Gli repressor functions and the induction of ventral cell types (Lei et al., 2004; Stamataki et al., 2005). In mutants with impaired Hh signalling, Gli activator functions are compromised and Gli repressor functions dominate, with the result that ventral Hh target genes are not expressed.

Hh/Gli pathway patterns the D/V axis of the telencephalon

Direct evidence for Hh/Gli signalling playing an important role in D/V patterning of the telencephalon comes from numerous sources utilisation a wide range of experimental apporoaches. Application of Shh protein to early forebrain explants is able to induce ventral telencephalic markers, suggesting that Shh is sufficient for ventral induction (Ericson et al., 1995b; Gunhaga et al., 2000; Shimamura and Rubenstein, 1997).

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[3] Although *Gli3*$^{-/-}$ mutants do not have defects in the activation of ventral Hh targets, in the absence of *Gli2* and *Gli1*, the activator functions of Gli3 seem to be required for ventral development of the neural tube, suggesting it may have partially redundant Hh/Gli activator functions in vivo (Bai et al 2004, Lei et al 2004, Motoyama et al 2003).

[4] It is of interest to note that some studies predict that Gli2 may also be processed in a similar manner to Gli3 (Wang et al 2000). However, cleaved forms of Gli2 have never been observed and only Gli2 activator functions are required *in vivo* (Bai et al 2001).

Conversely, *Shh*$^{-/-}$ mice are holoprosencephalic and have a small and strongly dorsalised telencephalon (Aoto et al., 2002; Chiang et al., 1996; Corbin et al., 2003; Ohkubo et al., 2002; Pabst et al., 2000; Rallu et al., 2002b). The *Shh*$^{-/-}$ telencephalon does, however, still contain a small number of ventrally patterned cells, expressing markers characteristic of the wt lateral ganglionic eminence (Lge) (Corbin et al., 2003; Rallu et al., 2002b). This finding demonstrates that although Shh is sufficient for the induction of ventral identity, it is not absolutely required for ventral telencephalic development. Residual ventral pattern may best be explained by the presence of low level of signalling from other Hh family members reaching the early telencephalic anlage. Indian hedgehog (Ihh) is expressed in the gastrulating embryo and the gut endoderm underlying the neural plate (Bitgood and McMahon, 1995; Zhang et al., 2001) and may partially compensate for Shh in ventral telencephalic specification. In support of this possibility, telencephalon-specific conditional knockouts of *Smo*[5], a transmembrane protein required for all Shh signalling, lack all trace of ventral telencephalic identity from early stages of telencephalic development (Fuccillo et al., 2004). The telencephalon of these embryos expresses a range of dorsal markers throughout. These mutants clearly demonstrate that in the absence of Hh signalling, ventral telencephalic cell fates are not specified. The severity of ventral defects and the morphology of telencephalon-specific *Smo* knock-outs are strikingly similar to those of the Foxg1 mutant. This raises the possibility that Foxg1 regulates dorso-ventral patterning of the telencephalon by regulating Hh signalling.

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[5] Mice mutant for *Smoothened* (*Smo*) lack all signs of Hh pathway activity and die between E8.75-E9.0 (Zhang et al., 2001), soon after the formation of the telencephalic vesicle. Dorso-ventral patterning in the telencephalon of these mutants has not been described, but the caudal neural tube lacks all ventral cell types (Wijgerde et al., 2002). The severe *Smo*$^{-/-}$ phenotype is also phenocopied by compound *Shh*$^{-/-}$;*Ihh*$^{-/-}$ embryos (Zhang et al., 2001).

Shh expression is induced in the wild type presumptive ventral telencephalon between E9.5 and E10 (Platt et al., 1997). However, as demonstrated in the previous chapter and by other authors (Rubenstein et al., 1998; Shimamura et al., 1995), rudimentary ventral telencephalic pattern (including the induction of the transcription factor Nkx2.1) is already in place by this stage of development. It is therefore clear that, assuming Shh is the major inducer of ventral telencephalon, sources of Shh outside of the telencephalon must be responsible for the initial induction of ventral fate. For this reason it appears logical that the failure of telencephalic induction observed in *Foxg1*$^{-/-}$ mutants (Huh et al., 1999) is a consequence, rather than a cause of impaired ventral telencephalic specification.

Foxg1 may be required for ventral telencephalic development downstream of Hh/Gli pathway activation

Various extra-telencephalic sources of Shh have been implicated in the initial specification of ventral telencephalic fate, including Hensen's node and the anterior primitive streak during gastrulation (Gunhaga et al., 2000), the prechordal mesendoderm (Pera et al., 2003; Shimamura and Rubenstein, 1997) and the ventral diencephalon (Ericson et al., 1995b). Although the authors did not look as early as gastrulation stages, sources of Shh in the prechordal mesendoderm and ventral diencephalon in *Foxg1*$^{-/-}$ embryos are very similar to wt embryos, prior to the failure of telencephalic Shh expression (Huh et al., 1999). This implies that defective expression of Shh in non-telencephalic sources is unlikely to underpin the primary defect in ventral induction.

Furthermore, the demonstration in the previous chapter that Foxg1 is required cell autonomously for the specification of ventral telencephalon makes it very unlikely that defective specification stems from a failure in the expression of Hh ligands, even at very early stages of development.

It is also interesting to note that the phenotype of *Foxg1*$^{-/-}$ cells in the mosaic context of the chimeric telencephalon is highly analogous to a range of experimental manipulations that impair Hh signalling in a cell autonomous fashion. *Smo*$^{-/-}$ cells in *Smo*$^{-/-}$↔wt chimeras (Wijgerde et al., 2002) and cells mosaically overexpressing a dominant negative version of the Shh receptor Patched (see below) (Briscoe et al., 2001) or a dominant negative Gli repressor construct which negates positive Hh signalling (Meyer and Roelink, 2003; Persson et al., 2002) in the spinal cord all have very similar phenotypes. The common motif is that cells in the ventral part of the neural tube fail to express ventral genes and upregulate dorsal genes, just as was observed in *Foxg1*$^{-/-}$↔wt chimeric telencephalon (please refer to chapter 4 of this thesis). On an evolutionary note, it has also been suggested that the *Drosophila* orthologues of *Foxg1*, the *slp* genes, have also been implicated in the transduction of hedgehog signalling (Cadigan et al., 1994). For these reasons it was hypothesised that a defect in the ability of telencephalic cells to respond to the ventralising influence of Hh ligands may, therefore, be the most parsimonious explanation for the observed phenotypes in *Foxg1*$^{-/-}$ embryos.

In this chapter a range of approaches are taken to examine the potential involvement of Foxg1 in regulating the response of telencephalic cells to Shh signalling. Some of the

immunohistochemical and western blotting experiments described in this chapter were completed with the aid of Martine Manuel, Tian Yu and Louise Leon.

RESULTS

Foxg1$^{-/-}$ telencephalon expresses components of the Shh signalling pathway

An inability to respond to Shh signalling by telencephalic cells could easily be explained by the lack of expression of crucial nodes in the Hh/Gli signalling pathway in *Foxg1*$^{-/-}$ telencephalic cells. The expression of several crucial components of this pathway were examined by RT-PCR of cDNA synthesised from mRNA extracted from *Foxg1*$^{-/-}$ and wt telencephalon at E12.5 (Fig.5.1A). *Ptc1* was efficiently amplified in wt ventral telencephalon and less easily detected in *Foxg1*$^{-/-}$ telencephalon and wt dorsal telencephalon, suggesting that all three tissues express the principle Hh receptor. *Smo* was also expressed by *Foxg1*$^{-/-}$ and wt telencephalon. *Gli1* transcripts were readily amplified in wt-ventral and *Foxg1*$^{-/-}$ telencephalon, but barely detectable in wt-dorsal

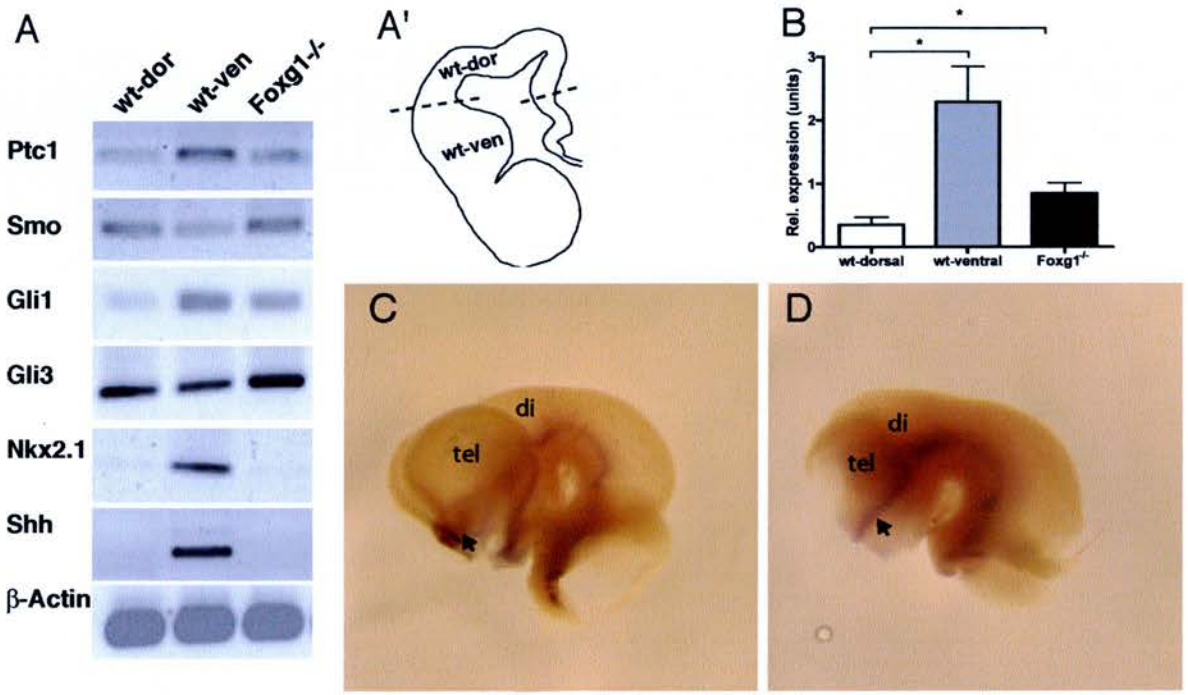


Fig.5.1. Expression of Hh signalling pathway components in *Foxg1*$^{-/-}$ telencephalon. (A) RT-PCR analysis of components of Shh signalling pathway and Shh target genes in mRNA extracted from wt-dorsal (wt-dor) and wt-ventral (wt-ven) telencephalon and from *Foxg1*$^{-/-}$ whole telencephalon. Approximate regions dissected are shown in A'. All three tissues contain detectable levels of mRNA for Shh receptor *Ptc1* and the transmembrane protein *Smo*, both of which are required for proper Hh signal transcduction. *Gli1* transcripts are barely detected in wt-dor cDNA, but are more abundant in wt-ven and *Foxg1*$^{-/-}$ cDNA. *Gli3* mRNA is also present in all three extracts. As expected, *Nkx2.1* and *Shh* are expressed in wt-ven but not wt-dor extracts. Consistent with in situ hybridisation and immunohistochemistry data (Huh, et al, 1999, Martynoga, et al, 2005), *Nkx2.1* and *Shh* were not detected in *Foxg1*$^{-/-}$ telencephalon. (B) Quantitative RT-PCR shows that Ptc1 is expressed at very low levels in wt-dor (white bar), at high levels in wt-ven (grey bar) and intermediate levels in *Foxg1*$^{-/-}$ telencephalon (black bar). Expression in wt-ven and *Foxg1*$^{-/-}$ is significantly greater than expression in wt-dor (t-test, $p<0.05$, n=4 independent extracts). (C,D) Lateral views of in situ hybridisations for *Ptc1* in dissected E12.5 telencephalon from wt (C) and *Foxg1*$^{-/-}$ (D) embryos show expression of *Ptc1* in the ventromedial region of both wt and *Foxg1*$^{-/-}$ telencephalon (Arrows in C,D. Rostral is to the left and dorsal up). Tel, telencephalon; Di, diencephalon.

telencephalon, even after 32 PCR cycles. *Gli3* was also expressed by all three tissues. Nkx2.1, which is rapidly induced by ectopic Hh signalling in early telencephalic explants suggesting it might be a direct target of positive Shh signalling (Ericson et al., 1995b; Gunhaga et al., 2000), was only expressed by wt ventral telencephalon, consistent with *in situ* analyses described in Chapter 3 of this thesis. Similarly, *Shh* was only expressed by wt ventral telencephalon.

As a crucial component of Hh signalling and a target of Hh signalling itself, the expression of *Ptc1* by *Foxg1*$^{-/-}$ telencephalon was interesting and deemed worthy of further investigation. Quantitative RT-PCR (qRTPCR) confirmed that *Ptc1* was expressed at high levels by wt ventral telencephalon and at significantly lower levels by wt dorsal telencephalon (t-test, $p<0.05$, n=4 independent mRNA extracts) (Fig.5.1B). In *Foxg1*$^{-/-}$ telencephalon, whilst not matching that of wt ventral telencephalon, *Ptc1* was expressed at a significantly higher level than in wt dorsal telencephalon (t-test, $p<0.05$, n=4 independent mRNA extracts) (Fig.5.1B). *In situ* hybridisation provided further support to this finding, revealing a small region in the ventral part of the *Foxg1*$^{-/-}$ telencephalon that expressed *Ptc* to detectable levels (Fig.5.1C). In wt controls, telencephalic *Ptc1* expression, as assessed by this technique, was limited to the Mge (Fig.5.1C). These expression analyses demonstrate that several obligate components of positive Hh signalling are expressed by the *Foxg1*$^{-/-}$ telencephalon. Beyond this, the expression of Shh targets *Ptc1* and *Gli1* also raise the possibility that the *Foxg1*$^{-/-}$ telencephalon is receiving a low degree of positive Hh signalling that may be responsible for the increased expression of these genes relative to the dorsal telencephalon of wt embryos. Since *Shh* is not expressed in the *Foxg1*$^{-/-}$ telencephalon ((Huh et al., 1999) and

RT-PCR analysis here), *Ptc1* and *Gli1* activity must be driven by extra-telencephalic sources of Shh and perhaps Ihh. Shh expression in the ventral diencephalon and rostral mesendoderm is observed in *Foxg1* mutants (Huh et al., 1999) and although it has not been analysed in the *Foxg1*$^{-/-}$ embryos, Ihh is expressed in the gut endoderm that underlies the neural plate at early stages of development (Zhang et al., 2001).

Foxg1$^{-/-}$ *telencephalic cells upregulate Hh target genes in response to Shh signalling*

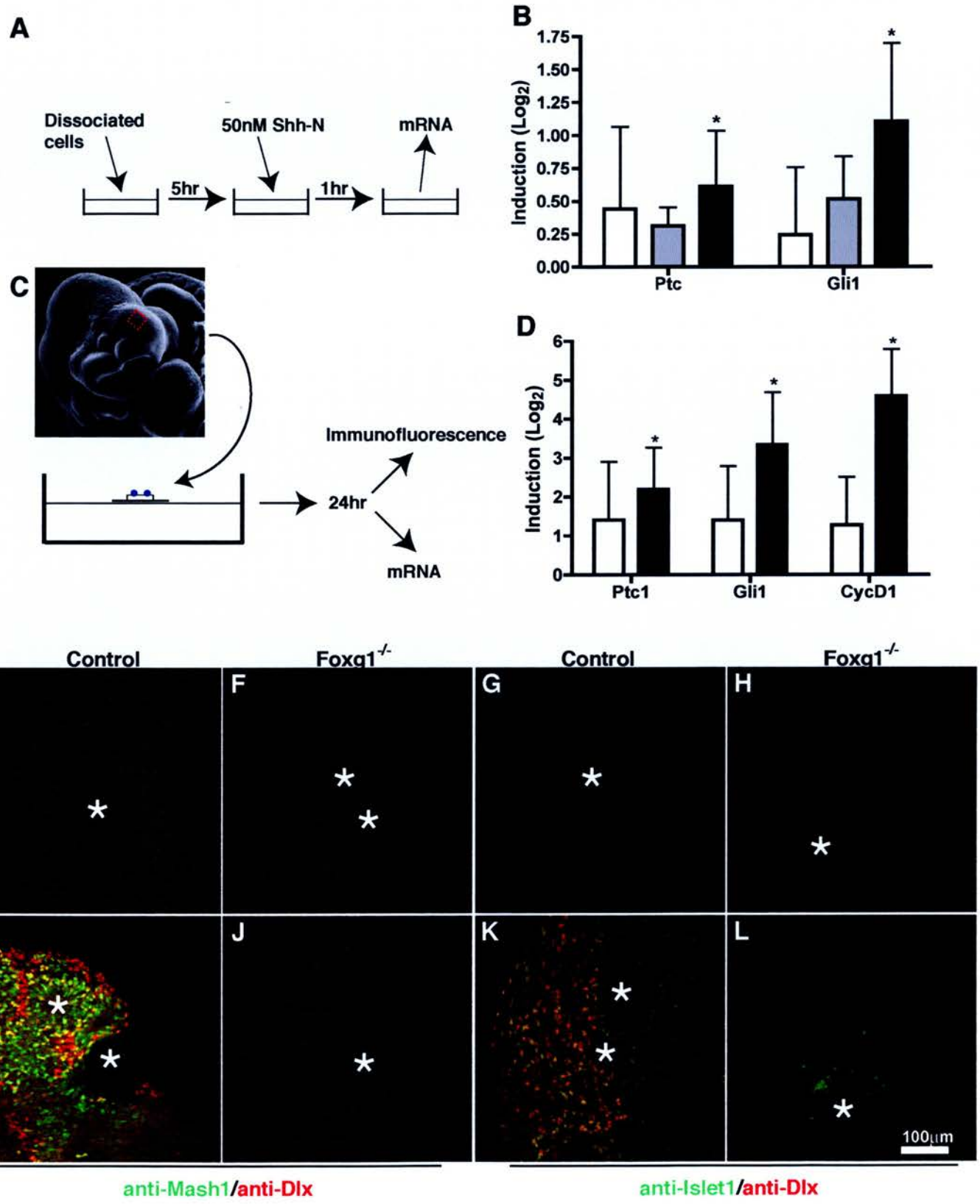
To determine whether the Hh signalling components expressed by *Foxg1*$^{-/-}$ cells are functionally active, an *in vitro* approach was taken to assess the early transcriptional response of mutant cells to exogenously applied Shh protein. Cells from E12.5 *Foxg1*$^{-/-}$ telencephalon and the dorsal and ventral telencephalic regions of littermate controls were dissociated and plated in tissue culture dishes. After 5hrs in culture, by which time the cells had settled and adhered, Shh N-terminal peptide (SHH-N) or a bovine serum albumin (BSA) control was added to the cultures (see Fig.5.2A). After one hour cells were lysed and mRNA extracted. qRTPCR was then used to examine the expression of Shh target genes *Ptc1* and *Gli1* in the cultures. The induction of *Ptc1* and *Gli1* expression in wt dorsal and ventral, and *Foxg1*$^{-/-}$ telencephalic cells, relative to untreated controls is plotted (mean ratio of SHH-N-treated to BSA-treated (Log_2)±SEM from 4 independent experiments) (Fig.5.2B). Both genes were positively regulated (Ratio $\log_2>0$) by Shh treatment, but only in *Foxg1*$^{-/-}$ cells was the induction statistically significant (one sample t-test, $p<0.05$, n=4 independent experiments). These data show that *Foxg1*$^{-/-}$ telencephalic cells are responsive to exogenous Shh signalling and that even a short exposure to a

relatively low dose of Shh protein can induce expression of two well-characterised Shh target genes.

Shh cannot re-specify *Foxg1*$^{-/-}$ telencephalic explants to a ventral fate.

Dorsal telencephalic explants from E10.5 mouse telencephalon can be re-specified to a ventral fate by the application of Shh-soaked beads (Kohtz et al., 2001; Kuschel et al., 2003). We were able to repeat these experiments and robustly induce Dlx transcription factors, Mash1 and Islet1, all markers of ventral fate. This system therefore provided a good model to test directly whether the Shh can induce ventral telencephalic fate in *Foxg1*$^{-/-}$ explants. In these experiments SHH-N-soaked beads at concentrations that reliably induced Dlx, Mash1 and Islet1 in control explants (Fig.5.2I,K, 17/18 explants) were not able to induce Mash1 or Dlx to detectable levels in *Foxg1*$^{-/-}$ explants (Fig.5.2J,L, 16/16 explants). In half of the SHH-N-treated *Foxg1*$^{-/-}$ explants examined for Islet1 expression (4/8), Islet1 was induced with low efficiency in a small number of cells. Mash1, Dlx and Islet1 were not expressed by control explants with BSA-soaked beads from control and *Foxg1*$^{-/-}$ embryos (Fig.5.2E-H) demonstrating that the inductive effect was due to SHH-N and that the regions dissected did not include ventral telencephalic cells. Despite the near total inability of exogenous SHH-N to re-specify dorsal *Foxg1*$^{-/-}$ explants to a ventral fate, the Hh pathway did appear to be activated in mutant explants treated with SHH-N, since the expression, as measured by qRTPCR, of the early Hh target genes *Ptc1*, *Gli1* and *CyclinD1* (Kenney and Rowitch, 2000; Oliver et al., 2003) was induced to significant levels (Fig.5.2D, one sample t-test, $p<0.05$, n=4 independent mRNA extractions).

Fig.5.2. Early targets of Shh signalling are activated by Shh in *Foxg1*$^{-/-}$ cells, but ventral progenitor fate cannot be induced. (A) Schematic showing experimental technique used to analyse transcriptional response to Shh-N in dissociated telencephalic cells in culture. (B) qRT-PCR for Ptc1 and Gli1 on mRNA extracted from dissociated cell cultures treated with Shh-N at 50nM for one hour. *Ptc1* and *Gli1* induction relative to untreated control (ratio, $\log_2$) is plotted for wt-dorsal (white bars), wt-ventral (grey bars) and *Foxg1*$^{-/-}$ (black bars) telencephalic cells. Values plotted are means of 4 independent experiments ±SEM. Ptc1 and Gli1 are significantly induced above baseline in *Foxg1*$^{-/-}$ telencephalic cells (one sample t-test, $p<0.05$, n=4 experiments). (C) Schematic showing experimental technique used to analyse effects of control and Shh-N-soaked beads on E10.5 telencephalic explants. (D) qRT-PCR for Hh target genes *Ptc1*, *Gli1* and *CyclinD1* in telencephalic explants treated with Shh-N beads. Induction relative to PBS-bead treated controls is plotted for wt dorsal (white bars) and *Foxg1*$^{-/-}$ explants. Values plotted are means of 4 independent experiments ±SEM. *Ptc1*, *Gli1* and *CyclinD1* are significantly induced above baseline in *Foxg1*$^{-/-}$ telencephalic cells (one sample t-test, $p<0.05$, n=4 independent extracts). (E-L) Dorsal telencephalic explants from E10.5 control and *Foxg1*$^{-/-}$ embryos cultured with Shh or BSA-soaked beads and stained for markers of ventral fate. In control explants cultured with Shh-N, Mash1+ (green), Dlx+ (red) and Islet1+ (green) and double labelled (yellow) cells were reliably induced by Shh-N (I,K), but not BSA (E,G). In *Foxg1*$^{-/-}$ explants Mash1 and Dlx were never induced (F,J,H,L), but in 4/8 explants examined, a small number of Islet1+ cells were induced (K). Asterisks mark the position of protein-soaked beads. Electron micrograph of E10.5 mouse embryos in C is from http://www.med.unc.edu/embryo_images/.


A
Dissociated cells
50nM Shh-N
mRNA
5hr
1hr
B
Induction (Log2)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Ptc
Gli1
C
Immunofluorescence
24hr
mRNA
D
Induction (Log2)
6
5
4
3
2
1
0
Ptc1
Gli1
CycD1
Control
Foxg1-/-
Control
Foxg1-/-
E
F
G
H
BSA
I
J
K
L
SHH-N
100μm
anti-Mash1/anti-Dlx
anti-Islet1/anti-Dlx

Foxg1$^{-/-}$ cells are capable of expressing the Shh target CyclinD1 in a chimeric context

To examine whether the induction of Shh target genes in *Foxg1*$^{-/-}$ cells in vitro reflected a response to non-physiological levels of Hh pathway activation (although this seemed unlikely given the low level response of wt dissociated cells to soluble SHH-N), CyclinD1 expression was examined in wt and *Foxg1*$^{-/-}$ embryos and in *Foxg1*$^{-/-}$ chimeras. Studies of cerebellar granule precursor cells have documented rapid and specific upregulation of CyclinD1 in response to exogenous Shh, showing that CyclinD1 is an early target of Shh signalling in these (Kenney and Rowitch, 2000; Oliver et al., 2003). In the wt telencephalon at E12.5, CyclinD1 is expressed in a clear gradient running from high in the ventral telencephalon to low in the dorsal telencephalon (Fig.5.3A). Since Shh expression in the telencephalon is highest in the Mge at this time (Nery et al., 2001), this expression pattern is consistent with CyclinD1 being a positively regulated target of Shh signalling in the telencephalon too. Also consistent with this view is the finding that CyclinD1 is greatly down-regulated in the *Foxg1*$^{-/-}$ telencephalon (Fig.5.3B), where Shh is not expressed and signs of Hh pathway activation are much lower than in the wt ventral telencephalon (Fig.5.1). *Foxg1*$^{-/-}$ cells in ventral regions of balanced *Foxg1*$^{-/-}$ chimeras are presumably exposed to physiological levels of Shh similar to those of the wt ventral telencephalon and higher than those of the *Foxg1*$^{-/-}$ telencephalon, and therefore provide an opportunity to examine Hh pathway responses in this context. To this end, ventrally positioned *Foxg1*$^{-/-}$ cells were frequently observed to express CyclinD1 at levels that appeared to be equivalent to their wt neighbours. Since

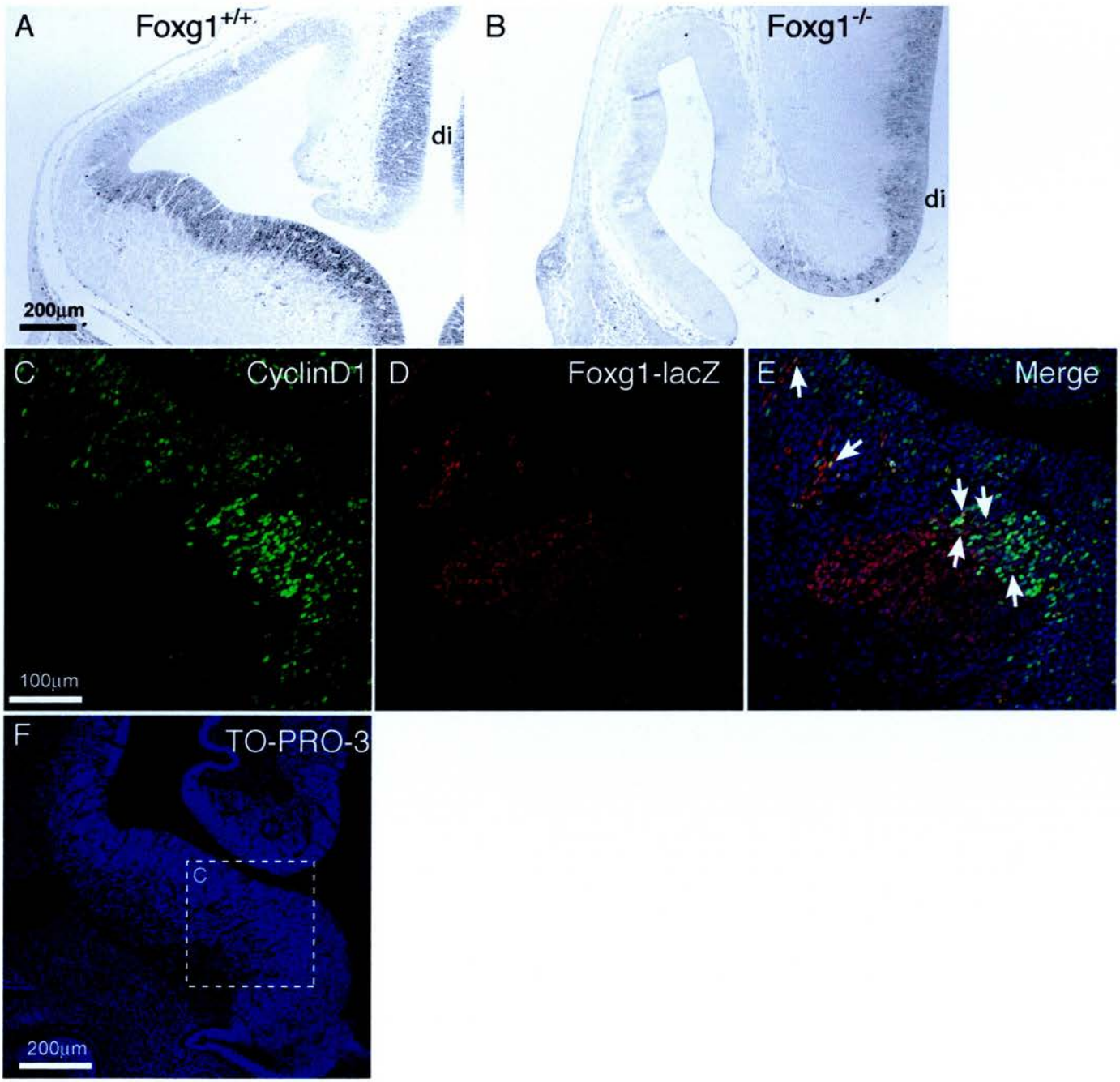


Fig.5.3. CyclinD1 is expressed by *Foxg1*$^{-/-}$ cells in ventral regions of chimeric embryos. (A) Immunohistochemical staining for CyclinD1 in wt telencephalon reveals that it is expressed in a clear high ventral to low dorsal gradient at E12.5. (B) In *Foxg1*$^{-/-}$ telencephalon, CyclinD1 is not detectable. (C-F) In *Foxg1*$^{-/-}$ chimeras, a number of ventrally located mutant cells express CyclinD1 (yellow co-localisation of Foxg1-lacZ (red) and cyclinD1 (green). (G) Low magnification view showing areas portrayed in C-F, nuclei are counterstained (blue) with TO-PRO-3.

Foxg1$^{-/-}$ cells in this position were never observed to express ventral telencephalic markers (Chapter 4), CyclinD1 expression is not a secondary consequence of ventral specification and may stem from increased Hh pathway activity in *Foxg1*$^{-/-}$ cells due to their proximity to a Shh source (wt cells in the Mge).

Despite the low level expression of positive markers of Hh signalling in *Foxg1*$^{-/-}$ mutants in vivo and the ability to further induce the Hh targets in *in vivo* (chimeric) and *ex vivo* (explant and dissociated cell cultures) contexts, *Foxg1*$^{-/-}$ cells are cell autonomously unable to transform Hh pathway activity into ventral telencephalic lineage commitment. Thus Foxg1 is required for telencephalic cells to respond appropriately to Shh signalling and this requirement appears to be downstream of the initial transcriptional induction of at least one positive activator of Hh signalling, namely Gli1.

Foxg1 is required for regulation of Gli3 expression and proteolytic processing

As discussed earlier, the end result of Hh signalling appears to reflect the final balance between the activity of Gli activator and Gli repressor functions. Experimental interventions that alter this balance in the neural tube have predictable consequences. Artificially increasing the level Gli activator function results in increased expression of ventral marker genes at the expense of dorsal genes (Lei et al., 2004; Stamataki et al., 2005), whilst overexpression of a truncated, repressor, form of Gli3 results in cell autonomous repression of ventral identities and the ectopic appearance of dorsal markers (Meyer and Roelink, 2003; Persson et al., 2002). The result of the latter manipulation is

strikingly similar to the behaviour of *Foxg1*$^{-/-}$ telencephalic cells in chimeras and mutant embryos, raising the possibility that the inability of *Foxg1*$^{-/-}$ cells to adopt a ventral fate results from an increased influence of Gli repressor forms, which may repress ventral identity even when the Gli1 activator is expressed.

Although the protein does have a transactivation domain and is required as a positive mediator of Hh signalling in the absence of Gli1 and Gli2 (at least in the caudal neural tube) (Bai et al., 2004; Lei et al., 2004; Motoyama et al., 2003), Gli3 appears to act primarily as a transcriptional repressor and a negative regulator of ventral, Hh activated, target genes. The main evidence for this comes from the ectopic expression of human GLI3 in *Drosophila melanogaster* and a variety of *in vitro* experiments (Dai et al., 1999; Ruiz i Altaba, 1998; Sasaki et al., 1997; Sasaki et al., 1999; Wang et al., 2000). As well as having the ability to repress ventral identities, Gli3 seems to be required to promote dorsal identities. *Gli3*$^{-/-}$ mutants lack the dorsal-most telencephalic structures, the choroid plexus and cortical hem and the residual neocortex is also reduced in size and at least partially ventralized (Aoto et al., 2002; Rallu et al., 2002b; Theil et al., 1999; Tole et al., 2000).

It is possible that Shh signalling cannot induce markers of ventral fate if *Foxg1*$^{-/-}$ cells because the levels and/or activity of Gli3 are misregulated in the absence of *Foxg1*. To investigate this possibility the expression of Gli3 mRNA and protein was analysed. Gli3 is expressed at higher levels in the wt dorsal telencephalon than in the ventral telencephalon, as demonstrated here by qRTPCR (Fig.5.4A) and previously by in situ hybridisation (V. Fotaki, unpublished observations). In the *Foxg1*$^{-/-}$ telencephalon *Gli3*

mRNA levels are very similar to those of the wt dorsal telencephalon and greater than those of wt ventral telencephalon (Fig.5.4A). Shh signalling has been shown to repress *Gli3* transcription in various systems (Marigo et al., 1996; Ruiz i Altaba, 1998), consistent with reduced *Gli3* expression in the ventral telencephalon, which is a source of Shh (Nery et al., 2001; Platt et al., 1997). In the explant experiments described above, treatment of E10.5 wt dorsal telencephalic explants with Shh caused a significant down-regulation of *Gli3* expression (as measured by qRTPCR) relative to controls (Fig.5.4B, one sample t-test, $p<0.05$, n=4 independent extracts). In *Foxg1*$^{-/-}$ explants, Shh treatment did not cause down-regulation of *Gli3* transcription, suggesting that Foxg1 may be required for Shh-mediated repression of *Gli3* expression in the telencephalon.

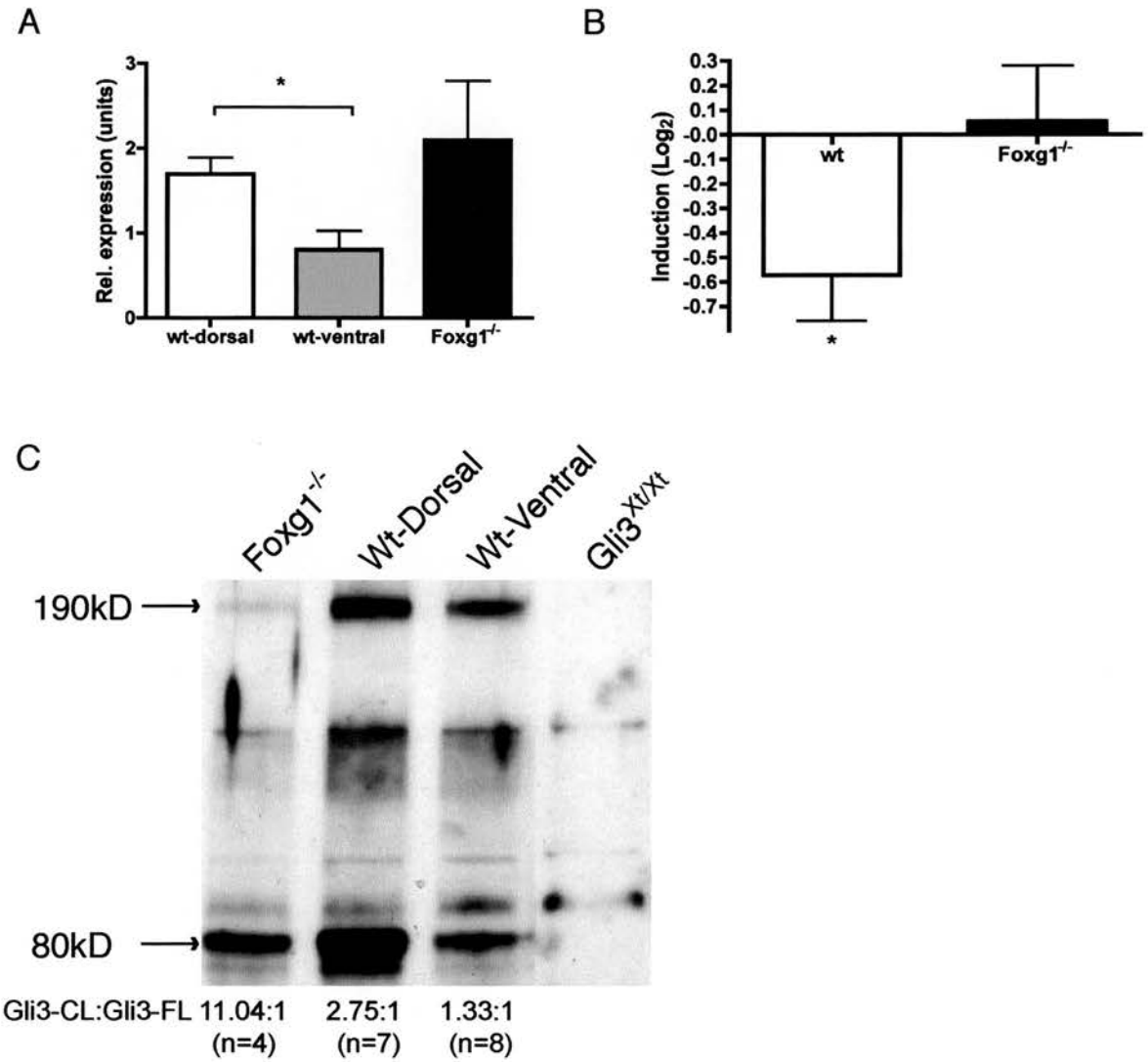


Fig.5.4. Shh-mediated repression of Gli3 and post-translational processing of Gli3 are defective in *Foxg1*$^{-/-}$ telencephalon. (A) qRT-PCR for *Gli3* mRNA in extracts from wt dorsal (white bar) and ventral (grey bar) telencephalon and *Foxg1*$^{-/-}$ (black bar) telencephalon at E12.5. *Gli3* expression in wt ventral cDNA is significantly lower than wt dorsal, whilst there is no difference between wt dorsal and *Foxg1*$^{-/-}$ extracts (two sample t-test, $p<0.05$, n=4 independent extracts). (B) *Gli3* expression (as measured by qRT-PCR) is suppressed by Shh signalling in control explants treated with Shh-N (white bar) (one sample t-test, $p<0.05$, n=4), but expression is not changed in *Foxg1*$^{-/-}$ explants (black bar) ($p>0.05$). Data are expressed as the mean ratio (log_2) of normalised *Gli3* mRNA levels in treated versus untreated explants ±SEM (n=4 independent extracts). (C) Western blotting of Gli3 protein in wt dorsal, wt ventral and *Foxg1*$^{-/-}$ protein extracts. Gli3 is present in its full-length ~190kD form (Gli3-FL) and its cleaved, ~80kD form (Gli3-CL) in all lanes, except *Gli3*$^{Xt/Xt}$ (lane 4), which shows reactivity is specific for Gli3.

At the protein level, Gli3 is present as a full-length form (Gli3-FL) and a C-terminally truncated form, cleaved-Gli3 (Gli3-CL). The two forms can be separated by electrophesis of protein extracts and visualised by western blotting (Fig.5.4C). In the wt telencephalon at E12.5 both forms of Gli3 are present dorsally and ventrally. Consistent with the idea that moderate Shh signalling causes stabilisation of Gli3-FL at the expense of Gli3-CL, the ratio of Gli3-CL:Gli3-FL is greater in wt dorsal extracts (2.75:1, n=7 independent extracts) than in wt ventral extracts (1.33:1, n=8 independent extracts). In *Foxg1*$^{-/-}$ telencephalon the ratio of Gli3-CL:Gli3-FL is even more skewed in favour of the cleaved form (ratio 11.04:1, n=4 independent extracts).

In summary, these data show that Foxg1 is required in the telencephalon to mediate Shh-dependent repression of Gli3 at the transcriptional level and it may also inhibit the proteolytic cleavage of Gli3-FL, a process which is regulated by Shh signalling (Wang et al., 2000).

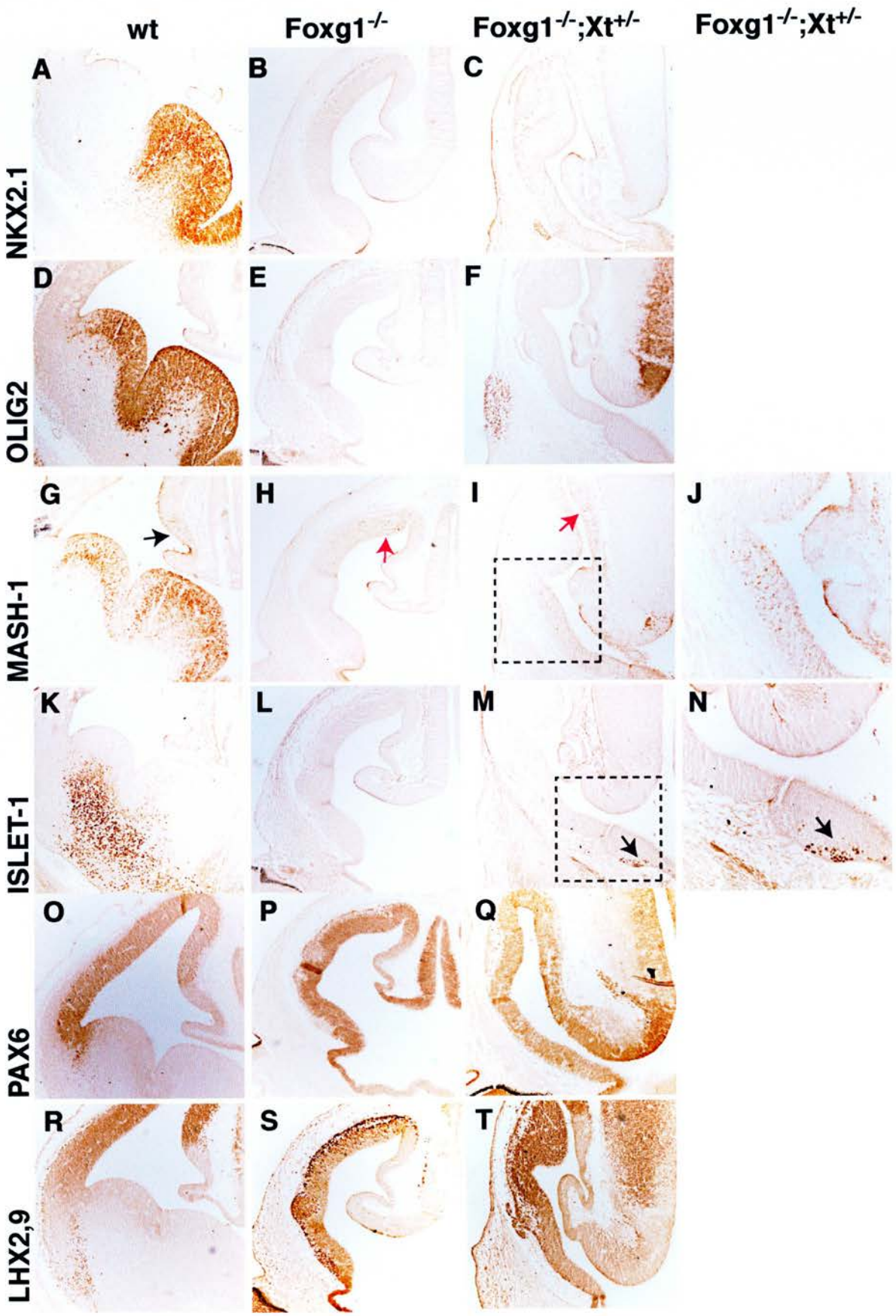
Removal of Gli3 on a Foxg1$^{-/-}$ *background partially rescues expression of ventral marker genes*

In *Shh* mutants (Chiang et al., 1996) and *Smo* mutants (Fuccillo et al., 2004; Wijgerde et al., 2002), the neural tube is severely dorsalised and ventral cells are either absent or greatly reduced in number. Removal of Gli3 on *Shh*$^{-/-}$ and *Smo*$^{-/-}$ backgrounds results in a dramatic recovery of ventral cell specification in both the spinal cord (Litingtung and Chiang, 2000; Wijgerde et al., 2002) and the telencephalon (Rallu et al., 2002b). The

most parsimonious explanation for these results is that, in the absence of Hh signalling, Gli3 dominantly represses ventral fates and that positive Hh signalling is primarily required to antagonise Gli3 activity and relieve ventral gene expression from Gli3-mediated repression. As described above, telencephalic cells lacking *Foxg1* are unable to achieve Shh-mediated repression of Gli3 transcription and contain unusually high levels of Gli3-CL. To examine whether Foxg1 is required to antagonise Gli3 function to achieve ventral gene expression, Gli3 was removed genetically from $Foxg1^{-/-}$ embryos.

In $Shh^{-/-}$ embryos, the removal of just one copy of Gli3 is sufficient for a rather dramatic rescue of ventral telencephalic patterning (Rallu et al., 2002b). Indeed, in $Shh^{-/-};Gli3^{+/-}$ mutants distinct patterns of Lge and Mge-like gene expression are observed and the expression patterns are appropriately nested. In contrast to this, only a very subtle rescue of ventral gene expression is observed in $Foxg1^{-/-};Gli3^{+/-}$ relative to $Foxg1^{-/-};Gli3^{+/+}$ embryos. Like $Foxg1^{-/-};Gli3^{+/+}$ telencephalon, *Foxg1* mutants heterozygous for *Gli3* lacked telencephalic expression of the ventral markers Nkx2.1 and Olig2 (Fig.5.5A-F). In the wt telencephalon Mash1 is expressed at high levels by ventral telencephalic precursors and at lower levels by a small number of cells in the cortical hem region (arrow in Fig.5.5G). In $Foxg1^{-/-};Gli3^{+/+}$ telencephalon at E12.5 the dorso-medial expression of Mash1 is maintained (red arrow in Fig.5.5H), and perhaps enlarged, but the ventro-lateral regions are negative for Mash1 (Fig.5.5H). In $Foxg1^{-/-};Gli3^{+/-}$ telencephalon there is similar dorso-medial expression of Mash1 (red arrow in Fig.5.5I), but also evident is a region in the ventro-lateral telencephalon that expresses a low, but easily detectable, level of Mash1 protein (Fig.5.5I,J). The fact that this ventral Mash1

Fig.5.5. Limited recovery of Mash1 and Islet1 expression in *Foxg1*$^{-/-}$*;Gli3*$^{+/-}$ telencephalon. Immunohistochemistry for Nkx2.1 (A-C), Olig2 (D-F), Mash1 (G-J), Islet1 (K-N), Pax6 (O-Q) and Lhx2/9 (R-T) in wt (A,D,G,K,O,R), *Foxg1*$^{-/-}$*;Gli3*$^{+/+}$ (B,E,H,L,P,S) and *Foxg1*$^{-/-}$*;Gli3*$^{+/-}$ (C,F,I,J,M,N,Q,T) telencephalon at E12.5. In wt telencephalon Nkx2.1 (A) is restricted to progenitors of the Mge and Olig2 (D) is expressed in progenitors of the Mge and the Lge. Both these genes are completely absent from the *Foxg1*$^{-/-}$*;Gli3*$^{+/+}$ and *Foxg1*$^{-/-}$*;Gli3*$^{+/-}$ telencephalon (B,C,E,F). In wt telencephalon (G) Mash1 is expressed in ventral progenitors and at a low level in dorsal cells in the cortical hem (black arrow). In *Foxg1*$^{-/-}$*;Gli3*$^{+/+}$ embryos (H) ventral Mash1 expression is completely absent, but dorsal, hem-specific, expression is maintained (red arrow). In *Foxg1*$^{-/-}$*;Gli3*$^{+/-}$ telencephalon (I,J) a low level of Mash1 protein is also observed in ventro-lateral telencephalic progenitors. Islet1 labels a subtype of ventrally-derived neurons in wt telencephalon (K), these cells are completely absent from *Foxg1*$^{-/-}$*;Gli3*$^{+/+}$ embryos (L), but a small number are generated in *Foxg1*$^{-/-}$*;Gli3*$^{+/-}$ telencephalon (M,N, see arrow). Pax6 and Lhx2/9 protein expression is restricted to the wt dorsal telencephalon (O,R), but are expressed throughout the dorso-ventral aspect of the *Foxg1*$^{-/-}$*;Gli3*$^{+/+}$ and *Foxg1*$^{-/-}$*;Gli3*$^{+/-}$ telencephalon (P,Q,S,T).


wt
Foxg1-/-
Foxg1-/-;Xt+/-
Foxg1-/-;Xt+/-
A
B
C
NKX2.1
D
E
F
OLIG2
G
H
I
J
MASH-1
K
L
M
N
ISLET-1
O
P
Q
PAX6
R
S
T
LHX2,9

expression domain is separated from dorso-medial Mash1 expression by a Mash1-negative territory suggests that it does not represent an expansion of dorsal Mash1 expression, although this possibility cannot be ruled out. It may, however, represent a limited rescue of ventral telencephalic Mash1 expression due to decreased *Gli3* gene dosage. The ventral region of *Foxg1*$^{-/-}$;*Gli3*$^{+/-}$ telencephalon also contained a small number of Islet1+ cells (arrow in Fig.5.5M,N), which are completely absent from the *Foxg1*$^{-/-}$;*Gli3*$^{+/+}$ telencephalon (Fig.5.5L) and abundant in the wt ventral telencephalon at this age (Fig.5.5K). Despite this very limited rescue of ventral markers, the dorsalisation of the *Foxg1*$^{-/-}$ telencephalon, as revealed by the widespread expression of Pax6 and Lhx2/9 (Fig.5.5P,S), was perpetuated in *Foxg1*$^{-/-}$;*Gli3*$^{+/-}$ mutants (Fig.5.5Q,T).

Since the ventral telencephalon is completely absent in *Foxg1*$^{-/-}$ mutants and the dorsal telencephalon is greatly reduced in size in *Gli3*$^{-/-}$ mutants, one possibility was that these effects would combine in an additive fashion to cause the loss of telencephalon in *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ embryos. This proved not to be the case. In all double homozygous mutants studied, telencephalic tissue, as measured by expression of *Foxg1*-promoter driven lacZ and its apposition to diencephalic tissues, was present (Fig.5.6D). The *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ telencephalon did, however, vary somewhat in size and morphology. Nevertheless, the range of molecular markers expressed was largely consistent between the *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ embryos analysed (n=3). In terms of gene expression, the most striking feature of the double mutant telencephalon was the widespread expression of the bHLH gene Olig2 (Fig.5.6F,G). In wt embryos Olig2 expression is restricted to progenitors of the Lge and Mge (Fig.5.6E, 5.5D) and it is completely absent from *Foxg1*$^{-/-}$ and *Foxg1*$^{-/-}$;*Gli3*$^{+/-}$ mutants (Fig.5.6F, 5.5E,F). In *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$, Olig2+ cells were observed throughout the

D/V axis of the telencephalon. Similarly, Mash1 protein was observed in the majority of ventricular zone cells in the double mutant telencephalon (Fig.5.6L). It must, however be noted that Mash1 was expressed at a lower level than it is in the wt or *Gli3*$^{-/-}$ ventral telencephalon (Fig.5.6I,K). Also present were a small number of Islet1+ cells (Fig.5.6P), which are also absent from the *Foxg1*$^{-/-}$ telencephalon (Fig.5.6N, 5.5). Unlike Olig2, Mash1 and Islet1, Nkx2.1 expression was completely absent from the *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ telencephalon (Fig.5.6T), unlike wt (Fig.5.6Q) and *Gli3*$^{-/-}$ (Fig.5.6Q) ventral telencephalon.

It therefore appears that removing *Gli3* on a *Foxg1*$^{-/-}$ background is sufficient to regain several features of ventral telencephalic gene expression that are completely absent in *Foxg1*$^{-/-}$ mutants. The genes whose expression was recovered (Olig2, Mash1 and Islet1) are expressed throughout the wt ventral telencephalon, whereas Nkx2.1, which is only expressed in the ventro-medial telencephalon (Mge), was not expressed at all. Thus the *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ telencephalon had features of ventro-lateral (Lge and Cge) telencephalic gene expression, but given the weak expression of Mash1, the sporadic expression of Islet1 and the dorsal defects described below, this fate was not fully acquired. To investigate this issue further, the expression of a range of dorsal telencephalic markers was analysed.

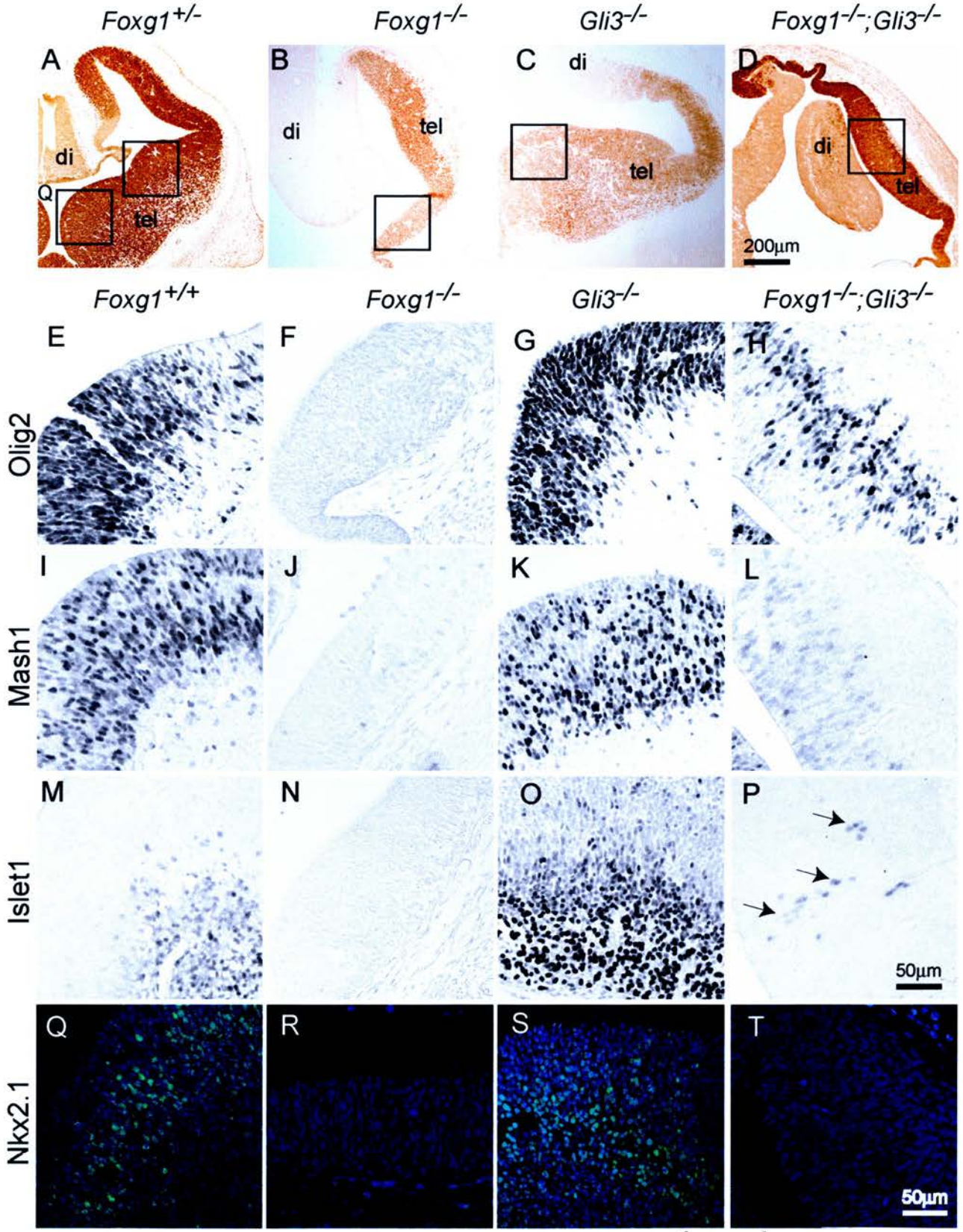


Fig.5.6. Partial rescue of ventro-lateral fates in *Foxg1$^{-/-}$;Gli3$^{-/-}$* telencephalon. Immunohistochemistry for Foxg1-lacZ marks telencephalic territories in *Foxg1$^{+/-}$* (A, control), *Foxg1$^{-/-}$* (B) and *Foxg1$^{-/-}$;Gli3$^{-/-}$* (D). Foxg1 immunohistochemistry labels the telencephalic territory in *Gli3$^{-/-}$* forebrain (C). Immunohistochemistry for Olig2 (E-H), Mash1 (I-L), Islet1 (M-P) and Nkx2.1 (Q-T) in wt (E,I,M,Q), *Foxg1$^{-/-}$* (F,J,N,R) and *Gli3$^{-/-}$* (G,K,O,S) ventral telencephalon and *Foxg1$^{-/-}$;Gli3$^{-/-}$* (H,L,P,T) lateral telencephalon. Olig2, Mash1 and Islet1, which are absent from *Foxg1$^{-/-}$* telencephalon are rescued, to varying extents, when *Gli3* is removed. Nkx2.1 is not rescued in any cells. tel, telencephalon; di, diencephalon.

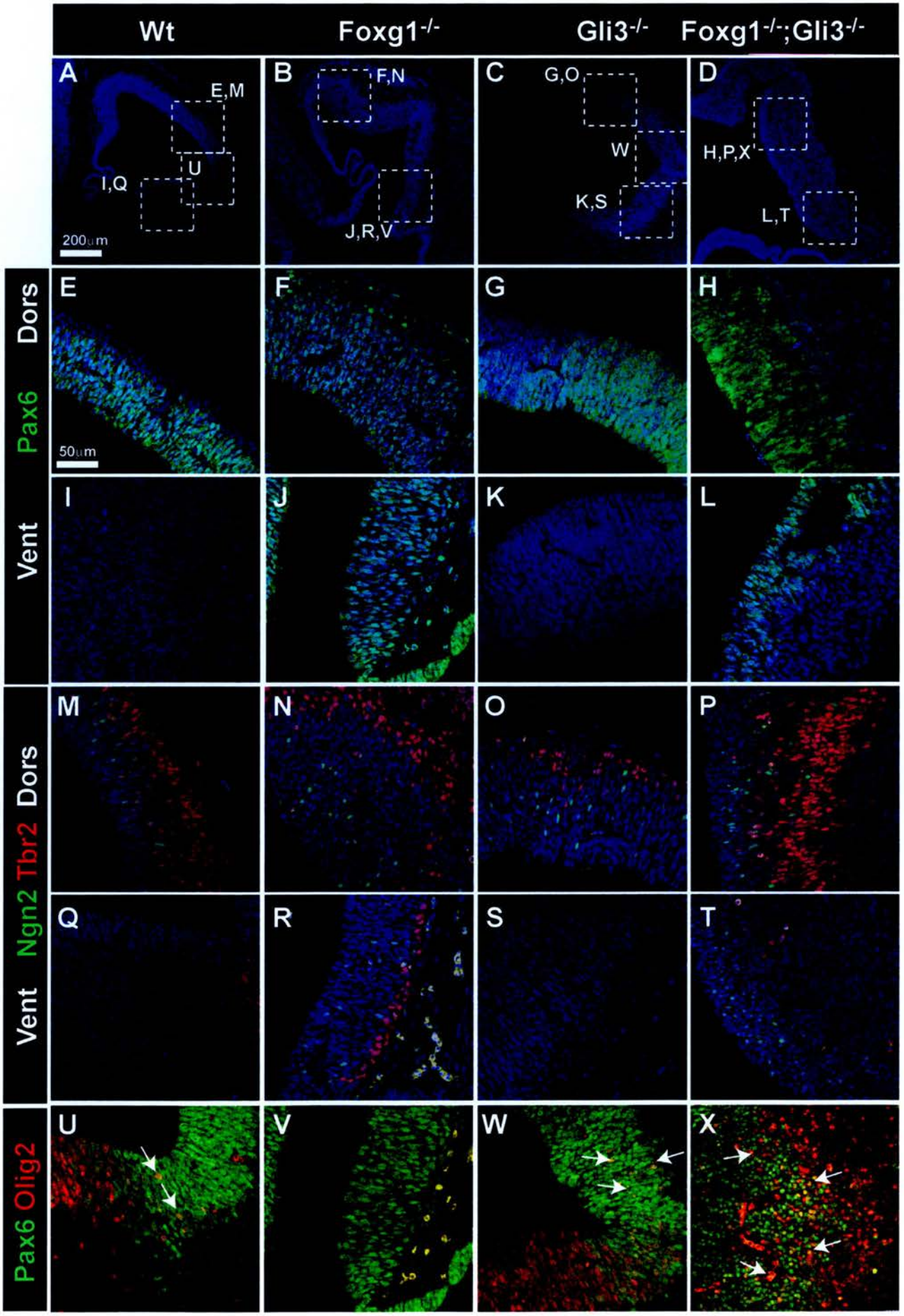

Wt
Foxg1-/-
Gli3-/-
Foxg1-/-;Gli3-/-
A
B
C
D
E,M
U
I,Q
200µm
F,N
J,R,V
G,O
W
K,S
H,P,X
L,T
Dors
Pax6
50µm
E
F
G
H
Vent
I
J
K
L
Dors
Ngn2 Tbr2
M
N
O
P
Vent
Q
R
S
T
Pax6 Olig2
U
V
W
X

Fig.5.7. Persistent dorsalisation of *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ telencephalon. Immunofluoresence for Pax6 (Green, E-L,U-X), Ngn2 (Green, M-T), Tbr2 (Red, M-T) and Olig2 (Red, U-X) in dorsal and ventral regions of wt (A,E,I,M,Q,U), *Foxg1*$^{-/-}$ (B,F,J,N,R,V), *Gli3*$^{-/-}$ (C,G,K,O,S,W) and *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ (D,H,L,P,T,X) telencephalon. Pax6, Ngn2 and Tbr2 are confined to dorsal telencephalon in wt and *Gli3*$^{-/-}$ telencephalon. In *Foxg1*$^{-/-}$ mutants dorsal markers are also expressed in ventral regions and this phenotype in maintained in *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ telencephalon, except for Tbr2 which is excluded from a small ventral region. Olig2 and Pax6 meet at the cortico-striatal boundary in wt (U) with minimal overlap. In telencephalon, *Gli3*$^{-/-}$(W) the boundary is partially impaired, but in *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ telencephalon there is no obvious boundary and many cells co-express Pax6 and Olig2 (X). Positions of some double-labelled cells (yellow) are highlighted with white arrows.

Gli3 is not required for the expression of dorsal markers in Foxg1$^{-/-}$ telencephalon

As described in detail in Chapter 4, Foxg1 is required cell autonomously for telencephalic cells to repress dorsal gene expression in ventral telencephalic territories. *Foxg1$^{-/-}$;Gli3$^{-/-}$* double mutants therefore made it possible to determine whether the action of Gli3 drives dorsal gene expression in *Foxg1$^{-/-}$* cells. In wt and *Gli3$^{-/-}$* embryos, the dorsal progenitor markers Pax6, Ngn2 and Tbr2 are restricted to the dorsal telencephalon (Fig.5.7E,G,I,K,M,O,Q,S). In *Foxg1$^{-/-}$* mutants these genes are expressed in both dorsal and ventral regions of the telencephalon (Fig.5.7F,J,N,R). When these markers were analysed in *Foxg1$^{-/-}$;Gli3$^{-/-}$* double mutants, it was immediately obvious that Pax6 and Ngn2 were robustly expressed in both dorsally and ventrally positioned cells (Fig.5.7H,L,P,T). Tbr2 was also expressed in a large number of cells in the double mutant telencephalon (Fig.5.7P), but unlike Pax6 and Ngn2, the ventral-most portion of the telencephalic territory lacked Tbr2 expression (Fig.5.7T).

The gene expression analyses described above demonstrate that despite the partial rescue of ventral telencephalic marker expression in *Foxg1$^{-/-}$;Gli3$^{-/-}$* embryos, the telencephalon of these mutants is still heavily dorsalised. Moreover the expression of the dorsal markers analysed does not depend on Gli3 activity. It is also of note that the cells expressing ventral markers appear to occupy the same territory as those expressing dorsal markers. To examine whether the same cells were able to express markers of both dorsal and ventral territories double immunofluorescence for Pax6 and Olig2 was performed. In

wt telencephalon Pax6 and Olig2 expression meet at the cortico-striatal boundary (csb) with minimal overlap (Fig.5.7U). In *Gli3*$^{-/-}$ telencephalon, the csb is at least partially impaired, as has been described previously (Rallu et al., 2002b). Pax6 and Olig2 expressing cells are interspersed, but only a very small number co-express these two markers in the embryos analysed here (Fig.5.7W). In *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ double mutants numerous double labelled cells are observed and the normal division between dorsal, Pax6-expressing, and ventral, Olig2 expressing telencephalic territories is lost (Fig.5.7X).

Over-production of Cajal-Retzius cells in Foxg1$^{-/-}$ *telencephalon requires Gli3*

Another interesting facet of the *Foxg1*$^{-/-}$ phenotype is the finding that, at least during the second half of embryogenesis, there is a gradual re-specification of telencephalic cell identities towards a dorso-medial telencephalic fate (Muzio et al 05). It has also been shown that early born Cajal-Retzius (CR) neurons are over-produced in the *Foxg1*$^{-/-}$ telencephalon ((Hanashima et al., 2004; Muzio and Mallamaci, 2005) and Chapter 3 of this thesis). The predominant site of CR neuron production appears to be in the dorso-medial telencephalon (Muzio and Mallamaci, 2005; Takiguchi-Hayashi et al., 2004; Yoshida et al., 2006), although other telencephalic areas do produce some of these cells (Bielle et al., 2005). Consistent with this, in Gli3 mutants, where the dorso-medial telencephalon appears to be absent (Grove et al., 1998; Theil et al., 1999; Tole et al., 2000), CR-cells are under-produced (Theil, 2005).

I was then motivated to determine whether the over-production of CR cells in *Foxg1*$^{-/-}$ mutants was mediated by Gli3. To achieve this the expression of Reelin, a marker of CR neurons, was analysed in combination with Tbr1, a transcription factor expressed in most dorsal telencephalon-derived neurons, including CR cells (Hevner et al., 2003; Hevner et al., 2001). In wt telencephalon at E12.5 Reelin expression is limited to a small number of Tbr1+ cells at the pial surface of the telencephalon in both dorsal and ventral regions (Fig.5.8E,I). In *Foxg1*$^{-/-}$ telencephalon at this age, most but not all Tbr1+ cells appear to express Reelin and such cells are abundant in dorsal and ventral regions (Fig.5.8F,J). In *Gli3*$^{-/-}$ telencephalon a small number of Reelin+/Tbr1+ cells were detected, as described previously (Theil, 2005), and were again distributed between dorsal and ventral regions (Fig.8G,K). In *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ mutant telencephalon Reelin+ cells were extremely scarce and only found dorsally (Fig.5.8H,L). Also of note was the finding that Tbr1+ cells, which were abundant through the majority of the double mutant telencephalic territory, were excluded from a small region in the ventral part of the telencephalon (Fig.5.8L). This expression pattern was similar to that of Tbr2, but in contrast to those of Pax6 and Ngn2, which were expressed throughout (Fig.5.7).

The finding that Reelin+/Tbr1+ cells are greatly reduced in number in the *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ telencephalon suggests that the exuberant production of these cells relies on the activity of Gli3. At this stage it is difficult to determine whether this is a direct consequence of the lack of Gli3, or an indirect consequence of the lack of dorso-medial telencephalic structures and cell types in the *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ telencephalon.

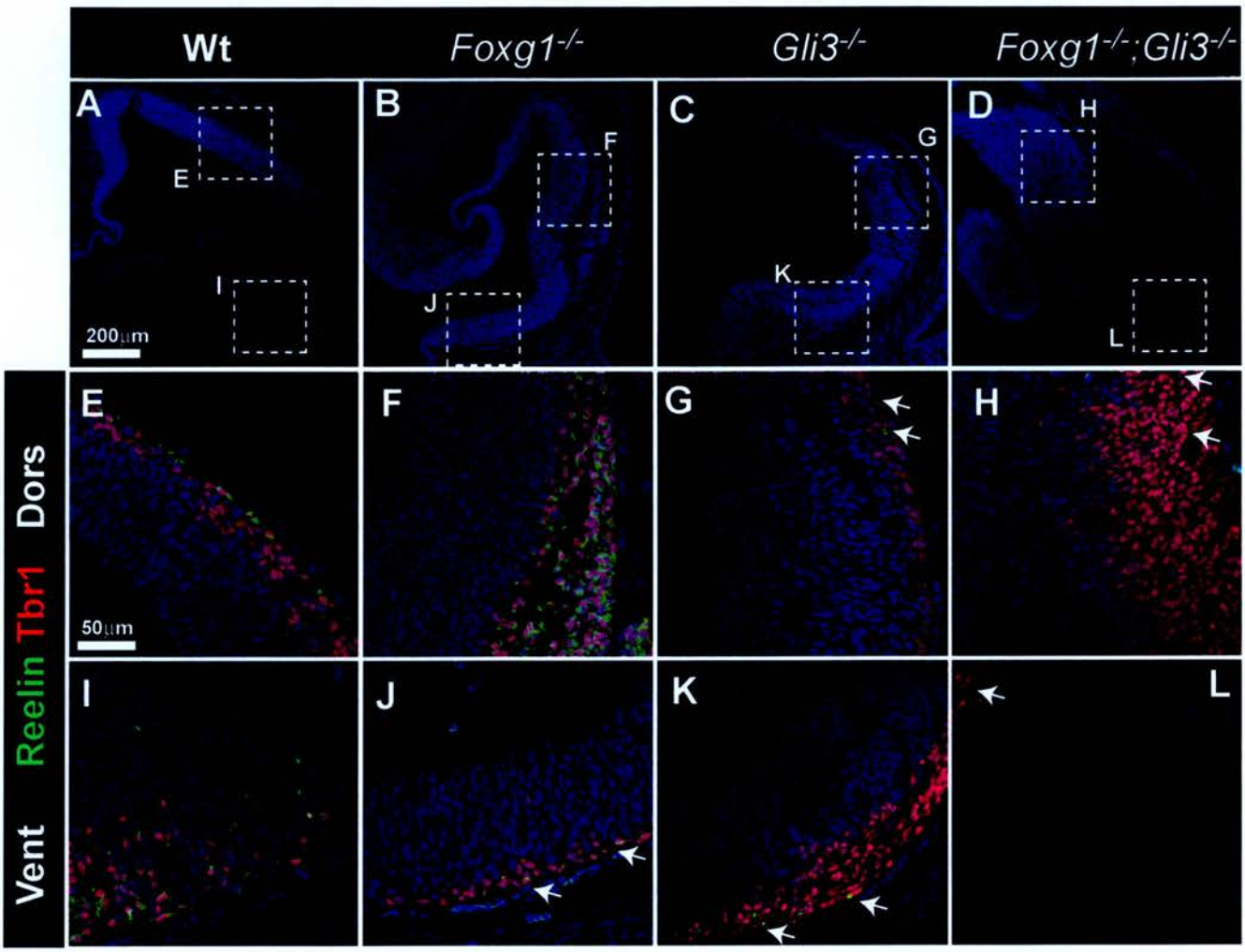


Fig.5.8. Ectopic Cajal-Retzius cell production is reversed in *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ telencephalon. Immunofluorescence for Reelin (green, E-L), which marks Cajal-Retzius (CR) cells, and Tbr1 (red, E-L), which marks dorsally-generated glutamatergic neurons (including CR cells), in dorsal and ventral regions of wt (A,E,I), *Foxg1*$^{-/-}$ (B,F,J), *Gli3*$^{-/-}$ (C,G,K) and *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ (D,H,L) telencephalon. CR cells are present in the marginal zone of wt telencephalon (E,I), and are produced in excess in *Foxg1*$^{-/-}$ telencephalon (F,J). CR cells are greatly reduced in numbers in *Gli3*$^{-/-}$ telencephalon (G,K) and even more depleted in *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ telencephalon (H,L).

DISCUSSION

As set out in the introduction to this chapter, the aim of the experiments described here was to determine whether defects in D/V patterning of *Foxg1*$^{-/-}$ telencephalon (as described in Chapter 4) stem from mis-regulation of the Hh/Gli signalling pathway. The motivation behind this was twofold. Firstly, there is a large and still growing body of work implicating Hh/Gli signalling in patterning the telencephalon, in a fundamental and evolutionarily conserved fashion (reviewed in Zaki, Martynoga et al 2005). Second was the striking similarities in fate and behaviour between *Foxg1*$^{-/-}$ cells and cells with compromised Hh/Gli signalling in both whole embryos (for example *Shh*$^{-/-}$ mutants (Chiang et al., 1996) and telencephalic *Smo*$^{-/-}$ conditional mutants (Fuccillo et al., 2004)) and mosaic contexts (for example *Smo*$^{-/-}$ cells in *Smo*$^{-/-}$↔wt chimeras (Wijgerde et al., 2002) and cells ectopically overexpressing dominant negative components of Hh/Gli signalling in the chick neural tube (Briscoe et al., 2001; Meyer and Roelink, 2003; Persson et al., 2002)). The most striking of these features are a cell autonomous inability to express ventral telencephalic marker genes and the inappropriate expression of dorsal genes in ventral territories.

Foxg1$^{-/-}$ *telencephalic cells are responsive to Shh*

As a first step I confirmed that *Foxg1*$^{-/-}$ telencephalon expressed important components of the Hh signalling pathway, including cell surface transducers (Ptc1 and Smo) and

intracellular mediators of Hh signalling (Gli1 and Gli2). Furthermore Ptc1 and Gli1 are transcriptional targets of Hh signalling (indeed Gli1 expression depends on Hh pathway activation (Bai et al., 2002)). The presence of these transcripts at increased levels relative to wt dorsal telencephalon suggests that the *Foxg1*$^{-/-}$ telencephalon may actually receive a low level of Hh signalling during its development and be able to respond appropriately at the transcriptional level. In support of this idea, Ptc1 and Gli1 were robustly induced by the application of Shh protein to *Foxg1*$^{-/-}$ telencephalic cells in culture. This was true after just one hour of exposure to a relatively low concentration of Shh suggesting that this response was a *bona fide* transcriptional response to Hh pathway activation.

Foxg1 is required downstream of Hh/Gli pathway activation in the specification of ventral telencephalic fate

Despite the induction of Hh target genes, application of Shh protein at high concentrations could not induce ventral marker gene expression in explant culture (the only slight exception to this being the inefficient induction of Islet1). In the same experiments wt dorsal explants were reliably induced to express ventral markers characteristic of an Lge fate (Mash1, Dlx and Islet1), as has been demonstrated previously (Kohtz et al., 1998; Kohtz et al., 2001; Kuschel et al., 2003). Results from the *Foxg1*$^{-/-}$↔wt chimeras generated for experiments in Chapter 4 of this thesis provide corroborative evidence for this finding. *Foxg1*$^{-/-}$ cells in ventral regions of the chimeric telencephalon were presumably exposed to physiologically relevant levels of Shh (and have been throughout their development) and responded by expressing the putative Hh

target CyclinD1 (Kenney and Rowitch, 2000; Oliver et al., 2003), but were unable to express markers of ventral fate.

This situation where Hh target genes, including the activator Gli, Gli1, are activated but are not sufficient to induce ventral telencephalic fates is reminiscent of results from a Hh gain of function study described recently (Rallu et al., 2002b). In these experiments E9.5 embryos were infected with a retrovirus encoding a constitutively active version of Smo (actSmo). When analysed later in development, cells infected with actSmo expressed Gli1 and Ptc1, consistent with cell autonomous activation of the Hh/Gli pathway. Infected cells in lateral and many dorsal regions of the telencephalon responded to Hh pathway activity by expressing pan-ventral markers Gsh2 and Dlx2, and by repressing Pax6 and the basic helix-loop-helix (bHLH) genes Ngn2, NeuroD and Math2 in dorsal territories. Nkx2.1, on the other hand, was only induced in ventro-lateral portions of the telencephalon. It was also particularly striking that infected cells in dorso-medial and caudo-medial regions failed to activate Gsh2 and Dlx2, despite the expression of Gli1. When the same group retrovirally over-expressed Shh a day earlier, at E8.5, Nkx2.1 and Dlx2 were both induced in both lateral and dorsal regions (Gaiano et al., 1999). These experiments show in vivo an effect that has been observed in vitro (Gunhaga et al., 2000; Kohtz et al., 1998), whereby the response to Shh (and presumably other morphogenetic signals) changes in space and time during telencephalic development. It is therefore clear that even relatively early in telencephalic development, some cells cannot be re-patterned to a ventral identity by Hh signalling. There are two major possibilities for how this may be explained, and these are not mutually exclusive. Either intracellular factors are required to synergize with Hh/Gli pathway activation to induce ventral fates, or

antagonistic factors are expressed that are sufficient to block Hh-induced ventral patterning. Ultimately the expression of these, as yet unidentified factors, must be under the control of either intrinsic 'timer-like' mechanisms or extrinsic morphogenetic signals (either ventralising cues which converge with Hh signals to activate ventral targets or dorsalising cues that induce antagonistic factors). I therefore propose that similar factors must be expressed in, or be absent from, the *Foxg1*$^{-/-}$ telencephalon. The identification of such factors would enhance our understanding of the control of the Hh/Gli signalling pathway and, in particular, improve our comprehension of the way a single ligand can have multiple effects in the developing embryo in distinct spatiotemporal circumstances. Since a relatively small number of signalling molecules appear to be re-used again and again across phyla and within different developing tissues, this latter goal is a priority for modern developmental biology.

Foxg1 is required for the appropriate regulation of Gli3 expression

As suggested above, it is possible that the reason *Foxg1*$^{-/-}$ telencephalic cells (and dorso-medial cells in the wt telencephalon[6]) are refractory to the ventralising influences of the Hh/Gli pathway is that they express an antagonistic factor. The best-characterised negative regulator of Hh/Gli signalling is a Gli family transcription factor itself, namely Gli3. Experiments primarily carried out in the caudal neural tube and developing limb

[6] It is interesting to note that some of the cells in the dorsal telencephalon which are not re-patterned by actSmo in the experiments of Rallu and colleagues (2002) are the very cells that that down-regulate Foxg1 first in the telencephalon (Shimamura et al., 1995; Dou et al., 1999). Consequently Foxg1 itself may be required directly for Hh/Gli-mediated ventralisation or indirectly for the regulation of crucial positive and negative modulators and/or co-factors of Hh/Gli signalling.

demonstrate that Gli3, and specifically the C-terminally cleaved repressor forms of Gli3, negatively regulate Hh target genes and ventral neural fates (reviewed in (Jacob and Briscoe, 2003)). Furthermore, genetic evidence strongly suggests that in the absence of positive Hh/Gli pathway activation, Gli3 strongly represses ventral gene expression in the spinal cord (Litingtung and Chiang, 2000; Wijgerde et al., 2002) and in the telencephalon (Rallu et al., 2002b). The logical conclusion from these studies is that the primary function of positive Hh signalling in the telencephalon is to antagonise the repressive activities of Gli3. It appears that Hh signalling achieves this antagonism on two levels. Firstly, high exposure to Shh represses Gli3 transcription. Secondly, Shh promotes the stabilisation of Gli3-FL, a protein that may in some contexts be an activator of Hh signalling (Bai et al., 2004; Lei et al., 2004; Motoyama et al., 2003), by preventing its proteolytic cleavage to Gli3-CL, the truncated protein believed to be the main antagonist of Hh/Gli signalling. It seemed possible that the inability of Shh to ventralise *Foxg1*$^{-/-}$ telencephalic cells may be due to altered levels or activity of Gli3.

Although Gli3 was expressed at a similar level to that of the wt dorsal telencephalon at the mRNA level, at the protein level there was a marked increase in the relative abundance of Gli3-Cl. This was true despite the signs of Hh pathway activity in the *Foxg1*$^{-/-}$ telencephalon, as discussed above, that would normally lead to stabilisation of Gli3-FL. From the explant culture model it also became apparent that Foxg1 might be required for the Shh-mediated repression of Gli3 transcription. Treatment with Shh protein caused a significant downregulation of Gli3 expression in control explants, but did not alter Gli3 transcription in *Foxg1*$^{-/-}$ explants. These experiments suggest that Foxg1 may be required to regulate Gli3 activity downstream of Hh/Gli pathway

activation by facilitating the repression of Gli3 transcription and by antagonising the proteolytic processing of Gli3. The net result of these defects is an unusually high level of Gli3-CL, which may mediate the dorsalisation of *Foxg1*$^{-/-}$ telencephalon. Unfortunately, suitable reagents are not available to assess the expression of the two versions of Gli3 in situ, otherwise it would have been extremely interesting to determine whether the increased expression of Gli3-CL is a cell autonomous feature of *Foxg1*$^{-/-}$ cells, by looking at Gli3 in *Foxg1*$^{-/-}$↔wt chimeras.

One question that arises from this result is why the unusual abundance of Gli3-CL does not quash the expression of Hh target genes in *Foxg1*$^{-/-}$ telencephalic cells? Studies in various systems have shown that Hh targets such as Ptc1 and Gli1 are under negative regulation by Gli3. However, it remains possible that a different mechanism is in place in the telencephalon. In support of the idea that Gli1 and Ptc1 are regulated independently of Gli3, ventral telencephalic expression of these genes is indistinguishable between wt and *Gli3*$^{-/-}$ embryos (Theil et al., 1999; Tole et al., 2000). Also, since Gli3-CL and Gli1 are co-expressed in the wt ventral telencephalon (results in Fig5.4 show that in this region of the telencephalon, nearly half of Gli protein is in the repressor form Gli3-CL), and consequently there can be no absolute consensus on the ability for Gli3-Cl to repress Gli1 activation in the telencephalon. Even if Gli3 does negatively regulate these promoters in the telencephalon it is also possible that Foxg1 might mediate aspects of this Gli3-mediated repression. Indeed, Foxg1 has been described as a transcriptional repressor and associates with co-repression complexes including Groucho homologues and Histone deacetylases (Sonderegger and Vogt, 2003; Yao et al., 2001) and might, therefore, contribute directly to this process.

Gli3 partially mediates repression of ventral markers in Foxg1$^{-/-}$ telencephalon

To investigate whether the unusual expression dynamics and post-translational processing of Gli3 observed in *Foxg1*$^{-/-}$ telencephalic cells are a causal factor in the absence of ventral gene expression, *Gli3* was removed genetically on a *Foxg1*$^{-/-}$ background. Unlike *Shh*$^{-/-}$ mutants heterozygous for a null mutation in *Gli3*, which have a dramatic rescue of ventral telencephalic pattern, *Foxg1*$^{-/-}$;*Gli3*$^{+/-}$ mice exhibited only very subtle evidence of ventral pattern and were still severely dorsalised. In *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ double mutants the situation was slightly different. Most strikingly, the ventrally resticted bHLH gene *Olig2* was expressed in a large number of cells through the D/V axis of the presumptive telencephalon. A low level of Mash1 was also induced in most VZ cells and several Islet1+ cells were observed. In contrast, the Mge marker Nkx2.1 was not induced at all. Thus, in the absence of *Foxg1* and *Gli3*, the telencephalic cells are able to express some markers characteristic of a generic ventral telencephalic identity. However, neither the number of cells expressing these markers nor the level at which they are expressed (as far as this can be judged by immunohistochemistry) are equal to that of the wt ventral telencephalon. It therefore appears that in addition to antagonising Gli3 action (presumably downstream of Hh/Gli pathway activation), Foxg1 has further roles in promoting full acquisition of ventral telencephalic pattern, in particular in the induction of Mge fates.

In addition to the induction of ventral gene expression programs, dorsal gene expression must be extinguished for proper development of the ventral telencephalon. As evidenced by the severe dorsalisation of *Shh* and *Smo* mutants, Hh signalling is *necessary* for this process. Exogenous Hh signalling is also *sufficient* to suppress a range of dorsal genes (Kohtz et al., 1998; Kuschel et al., 2003; Rallu et al., 2002b). As described in Chapter 4 of this thesis, like Hh signalling, Foxg1 is required in a cell-autonomous fashion for this process to occur properly. Examination of dorsal gene expression patterns in *Foxg1*$^{-/-}$*;Gli3*$^{-/-}$ mutants allowed me to determine whether Foxg1-mediated antagonism of Gli3 action is required for the repression of dorsal markers in the developing telencephalon[7]. However, the double mutant telencephalon retains several hallmarks of dorsal telencephalic patterning. Pax6 and Ngn2 are strongly expressed in the whole of the telencephalic VZ and Tbr1 and Tbr2 are expressed in a large number of cells in all but a small ventral region of the double mutant telencephalon. The absence of Tbr1+ and Tbr2+ cells in the ventral-most region of the double mutant telencephalon is reminiscent of the repression of these markers in *Foxg1*$^{-/-}$ cells in Mge regions of the *Foxg1*$^{-/-}$↔wt chimeric telencephalon (Chapter 4 Fig.4.3). Thus it appears that sustained high level Hh/Gli pathway activation in *Foxg1*$^{-/-}$ cells, as simulated by the genetic removal of Gli3's repressive action (see below for a discussion of whether this is a reasonable premise) or

[7] Although Rallu and colleagues did not illustrate the expression of dorsal marker genes in *Shh*$^{-/-}$*;Gli3*$^{-/-}$ telencephalon, they state that D/V patterning was essentially normal (Rallu et al., 2002). Consequently we must assume that dorsal gene expression was properly positioned in *Shh*$^{-/-}$ when *Gli3* was removed, suggesting that Gli3 mediates the dorsalisation of the ventral regions of the *Shh*$^{-/-}$ telencephalon. A subsequent study does indeed show the restricted of Pax6 expression to the dorsal telencephalon in *Shh*$^{-/-}$*;Gli3*$^{-/-}$ mutants, in support of this (Aoto et al., 2002).

by the presence of *Foxg1*$^{-/-}$ cells within a Shh source (the Mge) may be sufficient to repress some aspects of dorsal identity (for example, Tbr1 and Tbr2 expression), but not others (for example, Pax6 expression). Consequently Foxg1's requirement to antagonise Gli3 function downstream of, or in parallel to Hh/Gli signalling is only one aspect of Foxg1's ability to repress dorsal telencephalic markers in ventral territories.

In the absence of Foxg1 and Gli3 dorsal and ventral telencephalic identities are inter-mingled

As described above, the telencephalon of *Foxg1*$^{-/-}$*;Gli3*$^{-/-}$ embryos expressed aspects of both dorsal and ventral telencephalic pattern. However, unlike in the wt telencephalon, dorsal and ventral cells were not compartmentalised into distinct regions of the developing telencephalon. Rather, the expression patterns overlap. It also appears that individual cells express markers of both dorsal and ventral fate in a way that is never seen in the wt telencephalon. The presence of a large number of cells co-expressing Pax6 and Olig2 provide direct evidence for this. Thus many cells of the *Foxg1*$^{-/-}$*;Gli3*$^{-/-}$ telencephalon have a confused dorsal/ventral hybrid identity.

Little is known about the mechanisms by which dorsal and ventral markers are segregated in the telencephalon such that cells join either the dorsal or ventral lineages. Since even at early stages of telencephalic development very few cells co-express dorsal and ventral markers (Corbin et al., 2003; Yun et al., 2001), the decision between dorsal and ventral seems to occur early and be an absolute decision. Equivalent mechanisms are better understood in the developing spinal cord where cross-repressive and inductive

interactions between patterning transcription factors seem to lead to the segregation of distinct progenitor domains along the D/V axis (reviewed in (Briscoe and Ericson, 2001)). These mechanisms are largely maintained in *Gli3*$^{-/-}$;*Shh*$^{-/-}$, *Gli3*$^{-/-}$;*Smo*$^{-/-}$, and *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ mutants where discrete cell types, characteristic of most of the D/V domains are represented. Although the different cell types are physically intermingled, individual cells do seem to have a unique identity, suggesting that Hh/Gli signalling is not required for this process. Things may be slightly different in the telencephalon (for example, at least in anterior regions of *Gli3*$^{-/-}$ mutants some telencephalic cells co-express dorsal and ventral markers (Rallu et al., 2002b), but I am not aware of any studies that reveal quite the same level of intermixing of identity as that observed in *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ mutants. Thus Foxg1 appears to be required in combination with Gli3 for the proper selection of dorsal versus ventral lineage commitment in the developing telencephalon.

Foxg1 has Hh/Gli independent roles in D/V patterning of the telencephalon

As described above, the defects in the activation of ventral gene expression and in the appropriate repression of dorsal gene expression in *Foxg1*$^{-/-}$ mutants are only partially rescued by the removal of Gli3. Given that the primary role of positive Hh/Gli signalling appears to be the antagonism of Gli3 function, this result is strongly suggestive of a crucial role for Foxg1 in D/V fate specification in the telencephalon independent of the Hh/Gli axis. The evidence for this is that once Gli3 taken out of the system, the presence of activator Gli functions appears to be truly dispensable for most aspects of D/V patterning in the vertebrate neural tube.

This has been inferred both indirectly and directly by the analysis of various Hh/Gli pathway mutants. As already described, the telencephalon (Rallu et al., 2002b) and spinal cord (Litingtung and Chiang, 2000; Wijgerde et al., 2002) of *Shh*$^{-/-}$;*Gli3*$^{-/-}$ and *Smo*$^{-/-}$;*Gli3*$^{-/-}$ mutants contain most ventral cell types that are absent or hugely reduced in *Shh*$^{-/-}$ and *Smo*$^{-/-}$ mutants and demonstrate surprisingly normal D/V pattern. In these compound mutants it is inferred, but not actually proven, that activator Gli functions are absent or greatly reduced, due to the reduction in positive Hh pathway input and the predominance of Gli3-repressor. However, Gli2 does not depend on Hh signalling for its expression (Bai et al., 2002) and may theoretically carry out Hh/Gli pathway-independent activator and/or repressor functions even in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ and *Smo*$^{-/-}$;*Gli3*$^{-/-}$ embryos. However, recent studies of *Gli3*$^{-/-}$;*Gli2*$^{-/-}$ mouse mutants suggested that this is unlikely to be the case. In these mutants Gli1 is not expressed in any region of the embryo and consequently all Gli repressor and activator functions appear to be lost (Bai et al., 2004; Lei et al., 2004). Interestingly, the patterning of the spinal cord of these mutants is very similar to that of *Shh*$^{-/-}$;*Gli3*$^{-/-}$ and *Smo*$^{-/-}$;*Gli3*$^{-/-}$ mutants: all dorsal and most ventral (floorplate and ventral-most interneuron populations are still absent) cell types are present and express appropriate marker genes. Unfortunately telencephalic patterning has not been examined in these mutants. On the basis of the similarities between the D/V patterning of *Gli3*$^{-/-}$;*Shh*$^{-/-}$ and *Gli3*$^{-/-}$;*Smo*$^{-/-}$ embryos in caudal and rostral regions of the neural tube and that facto that mice mutant for both *Gli1* and *Gli2* (Park et al., 2000) have now overt telencephalic phenotype, I would predict that mutants lacking all Gli function have a similar phenotype in the telencephalon as in the spinal cord. The initially unexpected, but inescapable, conclusion from these studies is that Hh/Gli pathway

activation is not required at all for the induction of most aspects of D/V patterning in the neural tube. In the absence of Hh/Gli signalling other parallel or compensatory pathways are sufficient for most features of D/V patterning in the telencephalon[8] and beyond.

It then follows that if Foxg1 functioned solely downstream of Hh Gli signalling for the induction of ventral telencephalon, then in *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ mutants, even if Gli activator functions are compromised, ventral induction should proceed normally, (as it appears to in *Gli3*$^{-/-}$ mutants (Theil et al., 1999; Tole et al., 2000), since Gli3 function is completely removed. However ventral induction is still highly defective in *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ telencephalon. It therefore appears that the residual defects in D/V patterning in *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ telencephalon must stem from Hh/Gli-independent functions of Foxg1 in co-ordinating the induction of D/V pattern in the telencephalon. Further analysis of Foxg1 mutants and the role of the Foxg1 protein in telencephalic development may provide an excellent portal through which to identify the nature of the pathways that regulate telencephalic development independent of Hh/Gli signalling. In the following Chapter, the nature of candidate pathways are discussed, Foxg1's potential involvement in them considered, and some experiments to test these ideas are proposed.

Gli3 may effect the over-production of Cajal-Retzius cells in the Foxg1$^{-/-}$ *telencephalon*

[8] One exception to this being the induction of the dorso-medial telencephalon which is defective in both *Shh*$^{-/-}$ and *Gli3*$^{-/-}$ embryos and is absent in double mutants, suggesting that Shh and Gli3 may actually co-operate there (Rallu et al., 2002).

Another interesting finding from the analysis of *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ mutants was that the over-production of CR neurons that has been documented in *Foxg1*$^{-/-}$ embryos (Hanashima et al., 2004; Muzio and Mallamaci, 2005), was almost totally rescued by the removal of Gli3. Whilst this may be a secondary consequence of widespread changes in the expression of D/V patterning genes in these mutants, it is also possible that Gli3 may promote CR cell fate directly and the mis-regulation of Gli3 expression in *Foxg1*$^{-/-}$ telencephalon leads to enhanced CR cell generation. The resumption of CR cell generation by telencephalic progenitors during mid-neurogenesis (a time when they never normally produce CR cells) when *Foxg1* is conditionally ablated (Hanashima et al., 2004) may possibly be related to changes in the expression and or protein-processing of Gli3.

Since dorso-medial telencephalic fates and the associated gene expression of that region are lost in *Gli3*$^{-/-}$ mutants (Grove et al., 1998; Theil et al., 1999; Tole et al., 2000) and variably present or over-represented in *Foxg1*$^{-/-}$ mutants (Muzio and Mallamaci, 2005; Vyas et al., 2003), it will be interesting to determine how these genes interact to direct development of the dorso-medial telencephalon. Unfortunately lack of time and appropriate reagents precluded analysis of molecular markers of dorso-medial telencephalon in *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ double mutants. At this stage it remains possible that the lack of CR cells in these mutants is due to a failure of development of dorso-medial cell types due to lack of Gli3 action. It is also possible that dorso-medial cell types are recovered (relative to Gli3-/- telencephalon) and dorsalising signals produced by these signals are sufficient to contribute to the persistent dorsalisation of the *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ telencephalon, but cannot induce CR cell fates in the absence of Gli3?

Shh and the regulation of proliferation and survival in Foxg1$^{-/-}$ telencephalon

Although this Chapter of the thesis has been focused on the role of Hh/Gli signalling in D/V patterning, the same pathway appears to regulate both proliferation and cell survival in the developing telencephalon. Evidence from both gain and loss of function studies supports this idea. Firstly, various *Shh* and *Smo* mutants exhibit hypoplastic cerebral hemispheres (reviewed in Zaki, Martynoga et al 2005). Increased cell death (Fuccillo et al., 2004; Ohkubo et al., 2002) and reduced proliferation (Dahmane et al., 2001) have been documented in the same mutants. Secondly, ectopic exposure to Shh in vitro (Dahmane et al., 2001) has been shown to increase telencephalic precursor proliferation and in vivo overexpression of Shh causes overgrowth of the telencephalon (Gaiano et al., 1999; Rallu et al., 2002b). Interestingly, the finding that Shh treatment appears to act like a mitogen by promoting progenitor identity and growth of telencephalic explants, even when the explants are not competent to be re-specified to a ventral fate (Kohtz 98), gave an early indication that mitogenic and inductive actions of Hh signalling may be independently mediated within the cell.

Although focussed on the caudal neural tube, a recent study has examined in some detail the influence of the Hh/Gli pathway on patterning, proliferation and surival (Cayuso et al., 2006). The conclusion from this study is that Shh does indeed act to promote proliferation and survival in the neural tube and, like its roles in D/V patterning, this is achieved primarily via the regulation of the Gli transcription factors. The authors do,

however, demonstrate that these diverse actions of Hh signalling are separable and therefore presumably under a degree of independent control. Assuming similar mechanisms are in place in the telencephalon, it seems possible that *Foxg1*$^{-/-}$ cells, although refractive to the ventralising influences of Shh, may be able to respond to the mitogenic activities of Hh/Gli pathway activation. Although this was not investigated directly, some evidence presented in this thesis corroborates this idea and suggests that it may be worthy of further investigation. Firstly, *Foxg1*$^{-/-}$ cells were able to express CyclinD1 *in vivo,* when positioned close to a Shh source in the chimeric telencephalon. Secondly, Shh treatment led to the induction of CyclinD1 in vitro. Since cyclinD1 promotes S-phase entry by its association with CDK's, the induction of this protein in response to Shh may promote the proliferation of *Foxg1*$^{-/-}$ cells. On a more speculative level, it appeared that patches of *Foxg1*$^{-/-}$ cells in ventral regions of *Foxg1*$^{-/-}$↔wt telencephalon appeared to be larger than equivalent patches in dorsal regions. Although this may be partly explained by increased segregation of mutant cells away from wt cells, it may also reflect increased proliferation due to proximity to Shh expressing cells. The generation of more chimeras and the quantification of the relative contributions of *Foxg1*$^{-/-}$ cells to dorsal and ventral domains would shed light on this issue. Reduced Shh expression may, therefore, contribute to the reduced proliferation of the *Foxg1*$^{-/-}$ telencephalic progenitors, as described in Chapter 3, in a non cell-autonomous fashion. It is also interesting to note that apoptosis is not increased in the *Foxg1*$^{-/-}$ telencephalon (Chapter 3), but is increased in *Shh*$^{-/-}$ (Ohkubo et al., 2002) and telencephalic *Smo*$^{-/-}$ (Fuccillo et al., 2004) mutants. In the light of the suggestion by Cayuso and colleagues that a low level of Gli activator activity is necessary and sufficient to suppress apoptosis (Cayuso et al., 2006), this finding may reflect the observation that Gli1 is indeed

expressed in *Foxg1*$^{-/-}$ telencephalon (Fig.5.1), perhaps at a greater level than in Hh pathway mutants and at a level sufficient to suppress excess cell death.

Chapter 6: Discussion

In this thesis a range of experimental approaches were taken to further the understanding of the role of Foxg1 in the development of the telencephalon. Mice mutant for *Foxg1* exhibit a wide range of developmental defects specific to the telencephalon, demonstrating that Foxg1 is essential for telencephalic development. Many questions remain regarding the precise roles of Foxg1 in forebrain development. As summarised below, some of these questions were addressed in this work and previously uncharacterised functions of Foxg1 are proposed.

The signalling centre model of telencephalic development

As set out in the introduction to this thesis, our undertanding of the development of the telencephalon, like that of several other developing tissues, is best synthesised by the signalling centre model. The essence of this model is that the action of discrete groups of cells (signalling centres) at the margins of the telencephalic territory progressively convert a relatively homogeneous sheet of neuro-epithelial progenitors into a complex and multi-functional brain consisting of numerous cell types. Cells in signalling centres exert their influence on cells in their receptive fields via the production of extracellular morphogenetic signalling molecules[9] that control regional pattern in developing tissues

[9] Although the designation 'morphogen' has a specific definition as a molecule that controls gene expression and cell fate specification in a graded, concentration dependent fashion (for a recent review of this concept, see Ashe and Briscoe 2006), here the term 'morphogenetic signal' will be used in the broader sense to include any extracellular

and control parameters of tissue proliferation, survival and differentiation. It is believed that these morphogenetic signals principally effect their diverse and potent influences by controlling the expression and activity of developmentally regulated transcription factors which co-ordinate gene expression patterns more globally, thus controlling the behaviour of signal-receiving cells. Since many signalling molecules are believed to be soluble molecules capable of an action at some distance from their source (reviewed in (Ashe and Briscoe, 2006)), a given cell at a given stage of development is likely to receive a unique and combinatorial range of signalling inputs. Furthermore, the output of signal-receiving cells, in terms of genes expressed and behaviour induced, will be modulated by their local environment (i.e. the signals received at that moment) and also their history of exposure to developmental cues. By these mechanisms, a relatively small number of signalling centres, producing a relatively small range of morphogenetic molecules can co-ordinate a very large number of cellular responses and by this means orchestrate the development of hugely complex biological structures, such as the mammalian telencephalon. It also seems clear that whilst extrinsic signals are crucial for the induction of initial pattern in developing tissues, in many cases, cells within these tissues gradually change their response to these signals and become progressively more dependent on cell-intrinsic programs of differentiation, and less dependent on signalling centres (this concept is reviewed in (Edlund and Jessell, 1999)).

Since Foxg1 is a developmentally regulated transcription factor, expressed specifically in the telencephalon, the signalling centre model provides an excellent framework through

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signalling molecule capable of influencing the behaviour and fate of receiving cells, principally by promoting changes in gene expression.

which to investigate the role of Foxg1 in early telencephalic development. It is of particular interest to determine which aspects of the Foxg1 mutant phenotype stem from defects in the expression of signalling molecules (upstream of the signals) and which aspects of the phenotype stem from requirements for Foxg1 in controlling the response to morphogenetic molecules (downstream of the signals).

As discussed in the preceding chapters, there is evidence that Foxg1 is required both upstream and downstream of the expression and action of a range of morphogenetic molecules. Some of these activities have been proposed and investigated by other researchers and some are suggested for the first time in the current study. In the following pages, the proposed roles of Foxg1 that have been investigated in this thesis will be briefly summarised and several potential avenues for future work on the role of Foxg1 in telencephalic development will be proposed.

Foxg1 controls the growth of the telencephalon during the neurogenetic period

In *Foxg1*$^{-/-}$ mouse embryos, the telencephalic territory is specified, demonstrating that although it is an early marker of telencephalic fate, Foxg1 is not required for the induction of the telencephalon. It also appears that Foxg1 is not required for the early growth of the telencephalon since the size and precursor proliferation rate of the *Foxg1*$^{-/-}$ telencephalon is indistinguishable from wt until after E9.5 (Chapter 3 and (Martynoga et al., 2005)). By E10.5, the stage when neurogenesis is initiated (McConnell, 1995), the *Foxg1*$^{-/-}$ telencephalon is notably smaller than wt controls and the discrepancy in size

continues to increase through the second half of gestation. Previous groups have demonstrated roles for Foxg1 in promoting progenitor fate, suppressing neuronal fate and in promoting proliferation of telencephalic progenitors (Bourguignon et al., 1998; Dou et al., 1999; Hanashima et al., 2002; Hardcastle and Papalopulu, 2000; Xuan et al., 1995). One study also suggested that chick Foxg1 may be required to suppress apoptosis in the telencephalon (Ahlgren et al., 2003). Thus, at the stage that this thesis was commenced there was good evidence that Foxg1 is a crucial regulator of telencephalic growth during the neurogenetic interval.

However, as explored in Chapter 3, there were several questions left unanswered regarding the actual means by which Foxg1 was capable of promoting progenitor fate and proliferation rate. Some of these questions were addressed and new facets of Foxg1's role in regulating telencephalic growth were revealed. I found that decreased progenitor proliferation rates were first apparent at E10.5, where they were specifically localised to the rostro-dorsal regions of the telencephalon. In the same region and at the same time, it was shown that apoptosis was reduced. These results suggested two novel findings. Firstly it appears that Foxg1 may be required to promote telencephalic midline apoptosis, rather than suppress it, as has been suggested previously (Ahlgren et al., 2003). Secondly, the highly localised initiation of changes in proliferation rate and cell death at E10.5 in the rostro-dorsal telencephalon suggested a defect in the expression of, or response to, a locally expressed signalling molecule. I then went on to demonstrate that Fgf8 expression, which is expressed in precisely the rostro-dorsal territory where these defects are nucleated, was greatly reduced at E10.5 in *Foxg1*$^{-/-}$ mutants. It is therefore

proposed that this decrease in Fgf8 expression may cause at least some of the defects in telencephalic growth and that Foxg1 is required to maintain Fgf8 expression.

Following the initial, localised defects in proliferation and apoptosis, proliferation rates were reduced more globally and the results of increased neuronal differentiation became apparent. The initiation of these more diffuse defects are temporally correlated with an increase in the expression of Bmp and Wnt signalling molecules from about E11.5 ((Dou et al., 1999; Hanashima et al., 2002; Muzio and Mallamaci, 2005; Vyas et al., 2003) and results in Chapter 3) that may cause further changes in proliferation and differentiation rates. The more diffuse nature of these defects may also be accountable to a cell intrinsic role for Foxg1 in promoting telencephalic growth during neurogenesis, a function that may be more independent from localised signalling centres. On a mechanistic level, this aspect of the *Foxg1*$^{-/-}$ phenotype may partly have its root in Foxg1's role as a transcriptional repressor. As clearly demonstrated by the functions of the transcription factor REST (Ballas et al., 2001; Ballas and Mandel, 2005) and bHLH factors of the Hes family (Ross et al., 2003), both of which are potent transcriptional repressors, transcriptional repression is an important mechanism for the prevention of premature neuronal differentiation (Guillemot, 2005). Foxg1 has a basal repressor activity in functional in vitro assays and this activity is greatly accentuated by its associated with the well-characterised co-repressor proteins of the TLE/Gro family (Li et al., 1995; Li et al., 1997; Marcal et al., 2005; Sonderegger and Vogt, 2003; Yao et al., 2001). TLE-mediated repression is implicated in preventing neuronal differentiation in the telencephalon (Nuthall et al., 2002a; Nuthall et al., 2002b; Nuthall et al., 2004; Yao et al., 2000), in part through its association with anti-neural Hes proteins ((Ross et al., 2003)).

It appears that Foxg1 may play a similar role to Hes proteins in recruiting the repressor activities of TLE proteins to the promoters of pro-neural factors (Marcal et al., 2005; Yao et al., 2001).

Despite this strong evidence for a transcriptional role for Foxg1, it is unfortunate that no direct transcriptional targets of Foxg1 have yet been identified. Looking for direct targets could, however, be an exciting project for the future and would certainly provide a great deal more information about the actual functions of the gene at the molecular level. Modern approaches including the use of micro-arrays to compare global gene expression patterns in cDNAs from wt telencephalic regions and telencephalon with loss and gain of Foxg1 function could be fruitful first step in identifying candidate targets. Chromatin immunoprecipitation could also be used to test whether Foxg1 does actually bind candidate promoters and also as strategy to identify further transcriptional targets (Sikder and Kodadek, 2005). Bioinformatics approaches are also growing in power (Vavouri and Elgar, 2005) and may be informative, especially given the publication of a putative Foxg1 consensus binding sequence (Li et al., 1997).

Independent of its DNA-binding activity, Foxg1 also appears to have many potent biological functions, presumably mediated via its protein-protein interactions (Hanashima et al., 2002). Best characterised of these is the role of Foxg1 in antagonising the anti-proliferative actions of TGFβ signalling (Dou et al., 2000; Rodriguez et al., 2001; Seoane et al., 2004). Since TGFβ ligands appear to be fairly broadly expressed in the telencephalic VZ and surrounding tissues (Flanders et al., 1991; Miller, 2003; Seoane et al., 2004), this function of Foxg1, like its association with TLE proteins (which are

also widely expressed in the telencephalon (Marcal et al., 2005), may be an intrinsic action, not directly related to the action of localised telencephalic signalling centres. It is also interesting to consider that if this is indeed the case, why do these two defects (i.e. reduced proliferation and increased differentiation) only appear during the neurogenic period? Presumably factors other than Foxg1 prevent precocious neuronal differentiation and promote progenitor proliferation prior to the onset of neurogenesis. Molecular mechanisms regulating the shift from progenitor expansion phases to neuron-producing phases of telencephalic development remain poorly understood. It will be very interesting to establish which factors are important during this transition and how Foxg1 interacts with them.

It is therefore clear that Foxg1 has been implicated in a wide range of molecular pathways that regulate telencephalic growth and differentiation. In many cases, however, it is difficult to determine the level at which Foxg1 functions, whether it is required to control the expression of a signalling molecule or if it is required for the appropriate response to a signalling molecule. It is also very difficult to determine which of the various molecular correlates of telencephalic defects in *Foxg1*$^{-/-}$ mutants are primary defects, and which are secondary defects that arise as downstream consequences of the primary defects. This problem is particularly difficult to address in *Foxg1*$^{-/-}$ null mutant embryos, since all cells lack Foxg1 and the defects present at the time of analysis represent the culmination of all previous defects and their secondary consequences. The issues discussed above are of particular pertinence to telencephalic development due to the nature of the signals that regulate early telencephalic development. Inherent in our understanding of the signalling centre model of telencephalic development is the idea

that the signals emanating from midline signalling centres are in some circumstances cross-repressive and in others mutually supportive: changing the activity of one signalling centre invariably causes corresponding changes in the activity of others (Aoto et al., 2002; Kuschel et al., 2003; Ohkubo et al., 2002; Shimogori et al., 2004). Furthermore, the presence of inputs from one signalling pathway can change the response of a cell to inputs from another pathway[10].

A model to integrate the various roles for Foxg1 in controlling telencephalic growth

Given all these caveats and words of caution, I have attempted to synthesise a model that sums up the available evidence regarding Foxg1's role in controlling telencephalic growth. This model is presented in the following paragraph and following that, some ways to start testing the model are proposed.

Two of the earliest molecular correlates of impaired telencephalic growth of the *Foxg1*$^{-/-}$ telencephalon are the failure of telencephalic Shh expression (Huh et al., 1999), and the near total loss of Fgf8 expression (Chapter 3, Martynoga et al 2005). The local loss of Fgf8 and Shh[11] may then lead to the initial reduction in proliferation and cell death. Loss

[10] An example of this effect is the observation that the absence of Fgf signalling alters the response of telencephalic progenitors to Wnt signalling from a proliferative to a differentiative response (Israsena et al 2004). This example also shows that although Wnts are classically thought of as mitogens there is evidence that in some circumstances they can promote neuronal differentiation (reviewed in Ciani and Salinas 2005).

[11] *Nkx2.1*$^{-/-}$ mutants (Sussel et al 1999) and conditional nestin-cre mediated *Shh*$^{-/-}$ mutants (Machold et al 2003) both lack telencephalic Shh expression from E10.5 and both seem to have normal proliferation and cell death rates in the telencephalon, suggesting that Shh

or reduction of Shh and Fgf signalling may be an early event that then clears the path for the later expansion of Wnt and Bmp expression. Loss of the mitogenic activities of Fgf and Shh signalling may then combine with anti-mitotic and pro-neural activities of Bmp and Wnt (see footnote 2) signalling to limit telencephalic growth. These effects may then be coupled with less localised failures in the intracellular antagonism of TGFβ signalling (Dou et al., 2000; Rodriguez et al., 2001; Seoane et al., 2004) and a failure of Foxg1/TLE-mediated repression of neural differentiation-promoting factors, which become crucial functions for Foxg1 only after the onset of telencephalic neurogenesis. The combined result of these molecular changes is that progenitors proliferate more slowly and are more prone to differentiate and consequently the telencephalon becomes increasingly hypoplastic.

One way to start testing this model would be to generate more *Foxg1*$^{-/-}$↔wt chimeras, in the same way as those generated for experiments in Chapters 4 and 5, to try to determine whether increased differentiation rates and reduced proliferation rates are cell autonomous facets of the *Foxg1*$^{-/-}$ phenotype. This would help separate the aspects of the phenotype that stem from alterations in the expression of signalling molecules, and which stem from an inability to respond to environmental cues.

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expression within the telencephalon itself may not at this stage have a crucial mitogenic role in the telencephalon.

Machold, R., Hayashi, S., Rutlin, M., Muzumdar, M. D., Nery, S., Corbin, J. G., Gritli-Linde, A., Dellovade, T., Porter, J. A., Rubin, L. L., Dudek, H., McMahon, A. P., and Fishell, G. (2003). Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. *Neuron* **39,** 937-50,

Sussel, L., Marin, O., Kimura, S., and Rubenstein, J. L. (1999). Loss of Nkx2.1 homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum. *Development* **126,** 3359-70.

If one or both of these defects prove to be non-cell autontomous, specific questions could then be asked about whether Foxg1 is required cell autonomously to express Shh, maintain Fgf8 expression and to repress Bmp and Wnt expression in the telencephalon. If cell autonomous defects in the expression of one or more signalling molecule are identified, then it seems that conducting these experiments in low percentage, or balanced chimeras may help determine whether the change is a primary defect. The rationale for this is that the expression of these molecules will only be altered locally, in *Foxg1*$^{-/-}$ cells, and it might therefore be expected that surrounding wt cells will buffer the mutant cells from signals from other regions and stop or reduce secondary compensation. It could also be interesting to determine whether the DNA-binding defective form of Foxg1, Foxg1-NHAA (Hanashima et al., 2002), can rescue the expression of particular molecules whose expression is cell autonomously altered in *Foxg1*$^{-/-}$ cells. This would help determine whether the regulation is direct, through direct DNA-binding, or whether Foxg1 regulates expression, indirectly through the interaction with other proteins, and particularly other transcription factors.

If, on the other hand, the chimeras suggest that proliferative and/or differentiative defects are primarily cell autonomous in origin, then gain and loss of function approaches could be taken in vivo and in vitro to investigate the response of *Foxg1*$^{-/-}$ cells to specifically defined developmental signals. By careful experimental planning, attempts could be made to control for secondary, compensatory defects in the expression of further signalling molecules. For example, if the proliferative defects were to prove to be cell autonomous, then it may be that Foxg1 is primarily required for the response to Fgf

signalling, rather than for the expression of Fgf ligands. Exposure of *Foxg1*$^{-/-}$ cells to exogenous Fgf signalling activity in the highly defined conditions of the tissue culture dish, or in the more complex situation of the developing embryo *in vivo*, could determine whether or not Fgf signalling can rescue the proliferative defects. Similar experiments, replacing and removing aspects of molecular pathways to attempt rescue of the *Foxg1*$^{-/-}$ phenotype, could eventually shed light on the primary functions for Foxg1 in regulating telencephalic growth.

Foxg1 is required cell autonomously for ventral telencephalic specification

Although Foxg1 is not required during early development for specification of the telencephalon as a whole from anterior neuro-ectoderm, it does appear to be required for the specification of the early ventral sub-division of the telencephalon. From early stages of telencephalic development (E8.5) I showed that molecular markers of ventral telencephalic fate are not expressed at all in the *Foxg1*$^{-/-}$ telencephalon. With the lack of ventral marker expression, there is a corresponding expansion of dorsal telencephalic marker gene expression throughout the D/V axis of the mutant telencephalon (Chapter 3 and Martynoga et al 2005).

The construction of *Foxg1*$^{-/-}$↔wt chimeric embryos demonstrated clearly that Foxg1 is required cell autonomously for the acquisition of ventral telencephalic fate. In all the chimeras analysed, *Foxg1*$^{-/-}$ cells failed to express ventral progenitor markers and regardless of their position in the D/V axis of the chimeric telencephalon, mutant cells

expressed markers of dorsal telencephalic fate. These important findings greatly increased our understanding of the role of Foxg1 in D/V patterning of the telencephalon. The chimeric analysis refuted previous suggestions that the primary ventral defect was a proliferative one (Xuan et al., 1995) and due to a failure in the expression of Shh (Huh et al., 1999). Instead these experiments imply that Foxg1 is primarily required for telencephalic cells to integrate and decipher the developmental signals that pattern the early telencephalon into dorsal and ventral domains. This interpretation may also help us to understand a long-standing question about the role of Foxg1 in telencephalic development: if Foxg1 is expressed throughout the early telencephalon, then why are the defects in *Foxg1*$^{-/-}$ mutants so much more severe in the ventral telencephalon? If developmental signals that induce ventral telencephalon must act through Foxg1, then the presence of Foxg1 may be an obligate permissive factor for ventral induction. In the wt telencephalon the extent of ventral induction may then be determined by the range of action of ventralising cues and the extent to which they are antagonised by dorsalising signals and the resultant interactions between downstream transcription factors. According to this interpretation, then in the *Foxg1*$^{-/-}$ telencephalon, even if ventralising signals are present, then they will not be able to induce ventral fate since Foxg1 is required for their action.

Foxg1 is required downstream of Hh/Gli pathway activation for ventral fate specification in the telencephalon

Of the mouse mutants published to date where telencephalon is successfully specified, the only mutants that match the severity of ventral defects observed in the telencephalon

of *Foxg1*$^{-/-}$ mutants are those with defects in positive Hh/Gli signalling. Like *Foxg1*$^{-/-}$ mutants, telencephalic *Smo*$^{-/-}$ mutants lack all trace of ventral telencephalic lineages (Fuccillo et al., 2004). Largely for this reason, I hypothesised that Foxg1 may be required cell autonomously for telencephalic cells to respond to positive Hh/Gli signalling to induce ventral fate.

The first step in testing this hypothesis was to examine the response of *Foxg1*$^{-/-}$ telencephalic cells to exogenous Shh protein. Although the Hh/Gli pathway could be reliably activated in mutant cells, Shh was not sufficient to induce ventral marker expression. It was therefore concluded that Foxg1 was required downstream of Hh/Gli pathway activation for ventral fate specification. My attention was then focussed on the role of Gli3, the major antagonist of positive Hh/Gli signalling. I showed that the cleaved, repressor form of Gli3 was super-abundant in *Foxg1*$^{-/-}$ telencephalic cells and that Shh-mediated repression of Gli3 transcription was defective in *Foxg1*$^{-/-}$ cells. Thus it seemed that Foxg1 might be involved in the antagonism of Gli3 function at both the transcriptional and post-translational levels. Consequently it seemed possible that Foxg1 was required for positive Hh/Gli signalling by controlling the activity of the major repressor of this pathway, Gli3. This requirement for Foxg1 was then tested by genetically removing Gli3 from *Foxg1*$^{-/-}$ mutants, as discussed in the next section.

Foxg1 regulates D/V patterning partially via the transduction of Hh/Gli signalling, but also has Hh/Gli independent functions

In *Gli3* mutants, when all positive Hh/Gli activities are compromised by the genetic removal of *Shh* or *Smo* expression (*Shh*$^{-/-}$;*Gli3*$^{-/-}$ and *Smo*$^{-/-}$;*Gli3*$^{-/-}$ double mutants), D/V patterning of the telencephalon and the caudal neural tube proceeds remarkably normally (Litingtung and Chiang, 2000; Rallu et al., 2002b; Wijgerde et al., 2002). These data demonstrate that the primary function of positive Hh/Gli signalling appears to be to antagonize the action of Gli3, and once this is achieved most or all Hh/Gli activator functions are dispensible. Genetic removal of Gli3 is, therefore, somewhat analogous to a Hh/Gli gain of function phenotype. This logic was used to test whether the primary requirement for Foxg1 in D/V patterning of the telencephalon really was via the intracellular regulation of the Hh/Gli pathway response. If Foxg1 was solely required for the induction of ventral telencephalic fates via the antagonism of Gli3, downstream of, or in parallel to, Hh/Gli signalling, we would expect full recovery of ventral telencephalic specification in *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ embryos.

Importantly, upon this genetic manipulation, some aspects of ventral telencephalic fate were recovered. Molecular markers of ventro-lateral fate were recovered to a rather variable extent. This demonstrated that Foxg1 does have an important role in antagonising Gli3 function to promote ventral fates. Nevertheless, removing Gli3 is not sufficient for full ventral rescue. Dorsal fates were still greatly expanded and over-lapped with the rescued ventro-lateral marker expression. Also, ventro-medial fates, as measured by Nkx2.1 expression, which appear to be the first ventral cell types induced in the telencephalon (Ericson et al., 1995b; Kohtz et al., 1998; Shimamura et al., 1997) were not present at all in double mutants.

These data are strongly suggestive of roles for Foxg1 in setting up telencephalic D/V patterning, and in particular in inducing ventral fate, that are independent of the Hh/Gli signalling axis. The presence and capabilities of such pathways are revealed, as noted above, by the relatively normal D/V patterning of the *Shh*$^{-/-}$;*Gli3*$^{-/-}$ and *Smo*$^{-/-}$;*Gli3*$^{-/-}$ telencephalon (Rallu et al., 2002b). The implication that Foxg1 plays a role in these Hh/Gli-independent pathways is an exciting one, not least because relatively little progress has been made in elucidating the nature of these pathways and the mechanisms by which they are integrated with Hh/Gli signalling. As described in recent reviews (Hebert, 2005; Lupo et al., 2006; Rallu et al., 2002a) there are several candidate pathways that may be crucial for inducing ventral telencephalic identity in combination with Hh/Gli signalling. These candidate pathways will be briefly covered in the following pages, and any potential links between them and the action of Foxg1 will be addressed.

Hh/Gli independent pathways to ventral telencephalic fate

i) Ventral default model of telencephalic induction

One possible explanation for the widespread re-appearance of ventral telencephalic fates in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants is that the ventral state is a default. In other words, telencephalon is specified with a ventral identity. According to such a model the prospective telencephalon would be acted upon by dorsalising and lateralising signals to induce dorsal (pallial) and lateral (Lge) domains and ventralising signals (e.g. Shh) would be required to maintain ventral identity. Such a model has been proposed in papers published by the

Edlund and Jessell laboratories (Ericson et al., 1995b; Gunhaga et al., 2000; Gunhaga et al., 2003; Marklund et al., 2004), which use experiments on chick explants in culture to provide good support.

If such a model were to be substantiated, then the evidence published in this thesis would place Foxg1 in a key position in the maintenance of ventral fate from very early stages of telencephalic development. In the absence of Foxg1, ventral fate could not be maintained and dorsal identities would rapidly take over. It should be added, however, that it may be slightly misleading to describe this as a default model, since ventralising signals are still required to maintain ventral identity, although perhaps indirectly via the antagonism of dorsalising molecules. Consequently it is still very important to consider possible roles for Foxg1 in a wide range of pro-ventral pathways. This is done in the following pages.

It should also be added that there is some counter-evidence that may refute such a model. Firstly, Nkx2.1 is one of the earliest and best markers of ventral forebrain fate and the molecule proposed by Edlund and Jessell to be a crucial hallmark of early telencephalic fate. According to their model, all telencephalic cells would go through an Nkx2.1-expressing phase. Consequently fate mapping Nkx2.1 expression, by crossing an Nkx2.1-driven Cre-recombinase mouse to a reporter mouse should reveal reporter expression throughout the telencephalon. This is not observed, and in fact patterns of recombination are more limited to

the Mge at E12.5 (Kessaris et al., 2006). This pattern is more compatible with induction of Nkx2.1 expression just in the ventro-medial telencephalon.

ii) Nodal signalling

Nodal signals belong to the TGFβ family of signalling molecules and are important for ventral neural induction throughout the neural tube (reviewed in (Schier and Shen, 2000)). Since mice with mutations in nodal pathway genes tend to die very early in development, during gastrulation, they have not been informative about nodal signalling in ventral CNS development. Consequently, more progress has been made in this area with the zebrafish model system. Various fish nodal ligands and pathway components are required for development of the ventral telencephalon (reviewed in (Lupo et al., 2006)). Interestingly, the forkhead box gene *Foxh1,* in association with Smad2, is an important intracellular effector of nodal signalling, and *Foxg1* has been shown to physically interact with both Foxh1 and Smad2 *in vitro* and alters the transcriptional activity of the complex (Dou et al., 2000; Rodriguez et al., 2001). This raises the interesting possibility that Foxg1 might be required for transduction of nodal signals. However, the fact that Foxg1 has never been shown to be co-expressed with Foxh1 casts doubt on the idea that the Foxg1:Foxh1 association is a meaningful biological interaction. Also, the fact that the lack of ventral telencephalon in nodal mutants can be rescued by ectopic Hh signalling in zebrafish (Rohr et al., 2001) shows that nodal acts upstream of Hh/Gli signalling in ventral telencephalic specification. If Foxg1 was required

for transduction of nodal signalling upstream of Hh, then I would also expect Shh to rescue ventral specification in *Foxg1*$^{-/-}$ cells. This was not observed.

iii) Fgf signalling

In recent years, an increasing canon of data supports an important role for Fgf signalling in the induction of ventral telencephalic fate. Direct evidence for this has again been more forthcoming in the zebrafish model. Various genetic, biochemical and chemical manipulations that attenuate Fgf signalling cause serious defects in ventral telencephalic marker expression (Shinya et al., 2001; Walshe and Mason, 2003). Also mutation and morpholino knock-down of various Fgf ligands, demonstrate that some Fgf family members are particularly important for telencephalic development. Zebrafish ace (Fgf8) mutants and knock-down of Fgf3 and Fgf8 expression demonstrate unique and combinatorial functions for these two Fgf's in ventral specification (Miyake et al., 2005; Shanmugalingam et al., 2000; Shinya et al., 2001; Walshe and Mason, 2003). Zebrafish Fgf19 (the orthologue of mouse Fgf15) may have an even more important role in ventral telencephalic induction, in addition to its role in promoting telencephalic growth (Miyake et al., 2005). Another interesting aspect to this study was the finding that ectopic expression of Fgf19 could partially rescue the loss of ventral telencephalic fates caused by chemical inhibition of the Hh/Gli pathway. Thus it appears that Fgf signalling, like Foxg1, may act downstream of Hh/Gli pathway activation in the induction of ventral telencephalon. Work in the *Xenopus* system also indicates that Fgf signalling is necessary and sufficient for ventral telencephalic development

(Lupo et al., 2002). Also, in chick, Fgf8-soaked beads implanted in the telencephalon can induce ectopic dorsal expression of ventral genes (Crossley et al., 2001). Further experiments on chick neural explants indicate that Fgf signalling can promote Mge fate in prospective Lge explants (Marklund et al., 2004), perhaps indicating that Fgf signalling is particularly important for ventro-medial fate specification.

In mouse progress in this area has been slower, presumably due to redundancy between ligands and receptors, and also early lethality of mutants for some Fgf ligands[12]. However, there is some good supportive evidence that give us confidence that this increasingly well-characterised pathway in fish, may be conserved in mammals. Firstly, it has been suggested that in addition to its role in antagonising Hh signalling, Gli3 may also antagonise telencephalic Fgf signalling. Consistent with this, telencephalic Fgf8 expression is greatly expanded in *Gli3*$^{-/-}$ embryos (Kuschel et al., 2003). The authors of this study go on to propose that de-repression of Fgf signalling contributes to the ventralisation of the anterior part of the telencephalon observed in *Gli3* mutants. They support this claim by demonstrating that Fgf8 protein can induce ventral markers in early wt dorsal telencephalic explants. Importantly they demonstrated that this instructive action of Fgf8 was maintained even in the presence of the Hh pathway inhibitor cyclopamine. Thus it appears that, like in

[12] An example of this is the lethality of Fgf8 mutation during gastrulation (Sun et al 1999).

Sun, X., Meyers, E. N., Lewandoski, M., and Martin, G. R. (1999). Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. *Genes Dev* **13,** 1834-46.

the fish, Fgf signalling may function independently of, or downstream of, Hh/Gli signalling to induce ventral telencephalic fate in the mouse telencephalon. To this end it is also interesting to consider whether the dramatic rescue of ventral telencephalic fate in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ telencephalon (Rallu et al., 2002b) might partly stem from de-repression of the Fgf pathway, since Fgf8 expression is lost or reduced in *Shh*$^{-/-}$ mutants but expanded in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants (Aoto et al., 2002).

Apart from my demonstration that Foxg1 is required to maintain Fgf8 expression in the telencephalon, there is, as yet no direct link between Foxg1 and the regulation of Fgf signalling. Also, because Foxg1 is required cell autonomously for ventral fate specification, the failure to express Fgf8 properly is unlikely to be the primary defect. A failure in response to Fgf signalling would be much more compelling and would help explain aspects of the *Foxg1*$^{-/-}$ phenotype. If, as suggested above, Fgf is required downstream of, or in parallel to Hh/Gli signalling, this could explain why Shh cannot rescue ventral specification, despite pathway activation. Such a role for Foxg1 may also help to explain why ventral specification in *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ embryos is so much more limited than that of the *Shh*$^{-/-}$;*Gli3*$^{-/-}$ telencephalon (Rallu et al., 2002b). As suggested earlier, an impairment in the response to Fgf signals could also help explain some of the growth defects of the *Foxg1*$^{-/-}$ telencephalon. For these reasons it appears that a role for Foxg1 is transducing telencephalic Fgf signalling would fit the available data and, furthermore, would be an excellent avenue for future investigation.

iv) Antagonism of BMP signalling

Another hugely important requirement for ventral fate specification appears to be the antagonism of dorsalising pathways. In the spinal cord there is good evidence that BMPs from dorsal sources are required for dorsal specification and form a counter-gradient of signalling activity to that of ventrally-derived Shh signalling (reviewed in (Chizhikov and Millen, 2005; Liu and Niswander, 2005)). Secreted BMP antagonists such as chordin, noggin and follistatin are also expressed in ventral regions and are believed to be important to insulate ventral cells from the dorsalising activities of BMP signals (reviewed in (Lupo et al., 2006)). Similar mechanisms may be at work in the telencephalon. Consistent with this, mice mutant for *Megalin* (also known as low-density lipoprotein-related protein 2), a gene that encodes a cell surface protein thought to be important for internalisation and degradation of BMP ligands have a strongly dorsalised telencephalon (Spoelgen et al., 2005). Many molecular hallmarks of *Megalin*$^{-/-}$ mutants are shared by *Chordin*$^{-/-}$;*Noggin*$^{+/-}$ mutants, which are hyper-sensitive to BMP signalling (Anderson et al., 2002). Whilst interpretation of these mutants is complicated since the disinhibition of BMP signalling causes decreases in Shh and Fgf8 expression, these data are consistent with a role for BMP antagonism in the specification of ventral telencephalon. It seems unlikely, however, that inhibition of BMP signalling is sufficient for ventral induction, since in early chick prospective dorsal telencephalic explants, inhibition of BMPs did not result in ventral gene expression, nor did it block dorsal gene expression (Gunhaga et al., 2003).

Given its role as an intracellular antagonist of TGFβ signalling (Dou et al., 2000; Rodriguez et al., 2001; Seoane et al., 2004), it seems possible that Foxg1 may also be able to block aspects of the closely related BMP intracellular signalling cascade. Consistent with this, Foxg1 has been shown by one lab to physically associate with Smad1, a BMP-receptor activated Smad (Rodriguez et al., 2001). Foxg1 is also required for the repression of the expression of various BMP ligands in the lateral telencephalon (Dou et al., 1999; Hanashima et al., 2002). Thus a failure to antagonize BMP signals via Foxg1 activity may also underlie aspects of the reduced expression of ventral markers in *Foxg1*$^{-/-}$ and *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ embryos. Combining pro-ventral Hh and Fgf signalling with exogenous BMP antagonists such as noggin in vitro or in vivo, may be sufficient to rescue more widespread ventral marker expression in *Foxg1*$^{-/-}$ telencephalic cells. It would also be very interesting to look in *Foxg1*$^{-/-}$ cells in chimeric embryos to determine whether hallmarks of BMP pathway activation (for example Msx gene activation (Liu and Niswander, 2005)) and BMP ligand expression are cell autonomously upregulated.

v) *Antagonism of Wnt signalling*

Like BMP signalling, there is good evidence that signalling through the Wnt pathway is important for dorsal specification throughout the CNS (reviewed in (Chizhikov and Millen, 2005; Ciani and Salinas, 2005)). Wnt antagonists such as tlc are important for specification of the telencephalon and since they are expressed at highest levels in the anterior-most neural plate that gives rise to the

ventral telencephalon, may also be required for ventral development (Houart et al., 2002). Also, in chick telencephalic explants and cultured chick embryos, Wnt3a can repress ventral identity and promote Pax6 expression in ventral territories (Gunhaga et al., 2003). Conversely, exposure to a dominant negative version of a Wnt receptor repressed dorsal gene expression and activated ventral markers in the same system (Gunhaga et al., 2003). In the mouse further supportive evidence for a role of Wnts in promoting dorsal differentiation and in repressing ventral comes from gain and loss of function mutants of β-catenin, an important downstream effector of Wnt signalling (Backman et al., 2005). Enhanced β-catenin activity early in telencephalic development expanded dorsal gene expression at the expense of ventral, whilst β-catenin loss of function had the opposite effect.

Since various Wnt ligands and downstream Wnt effectors are upregulated in *Foxg1* mutants (Muzio and Mallamaci, 2005; Vyas et al., 2003), it is possible that Foxg1 may be required to promote ventral development and repress dorsal development via the antagonism of Wnt expression and Wnt activity. It is also possible, however that the observed changes in Wnt pathway activation in *Foxg1*$^{-/-}$ telencephalon are secondary to other changes in rostral and ventral signalling centres. Analysis of chimeric embryos and careful in vitro studies could again shed some light on whether Foxg1 can act as a context-dependent antagonist of Wnt signalling.

vi) Retinoic acid signalling

Signalling activity of the vitamin A derivative retinoic acid (RA) has also been receiving recent attention as another signalling pathway likely to contribute to D/V patterning in the telencephalon. Like in the spinal cord (Pierani et al., 1999), it appears that RA can induce ventro-lateral identities (prospectve Lge) in the telencephalon, apparently in an Hh-independent fashion (Marklund et al., 2004). Interestingly RA also converted prospective Mge into Lge, the authors propose that Fgf8 may be required to maintain Mge identity against the negative influence of RA signalling (Marklund et al., 2004). Thus RA signalling may contribute to rescued Lge fates in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ telencephalon (Rallu et al., 2002b). Due to the fact that the ventral markers recovered in *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ were characteristic of Lge, rather than Mge, it seems that RA signalling may still be functioning in these mutants. Consequently, whilst a role for Foxg1 in transducing RA signalling cannot be ruled out, I would not make it a priority for future investigation.

Foxg1 and the development of dorso-medial telencephalon

In addition to its role in promoting ventral telencephalic development, it also seems clear that Foxg1 has an interesting role in the development of the dorso-medial telencephalon. The dorso-medial telencephalon includes the cortical hem, an important source of Wnt and BMP ligands in the telencephalon (Grove et al., 1998). As noted above, it appears that Foxg1 may repress dorso-medial telencephalic identity and antagonise dorsalising signals derived from this region (Dou et al., 2000; Hanashima et al., 2002; Muzio and Mallamaci, 2005; Vyas et al., 2003). Interestingly, Gli3 appears to have an opposite

function in this region. In *Gli3*$^{-/-}$ mutants the cortical hem is absent and many Wnt and BMP genes are not expressed (Grove et al., 1998; Theil et al., 1999; Tole et al., 2000). Consequently, the *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ double mutants generated in Chapter 4 would provide an excellent opportunity to evaluate the epistatic relationship between *Foxg1* and *Gli3* in the development of the dorso-medial telencephalon. One hypothesis would be that high levels of Gli3-CL repress Foxg1 in the dorso-medial telencephalon and therefore relieve hem markers such as Wnt and BMP ligands from Foxg1-mediated inhibition. If this were to be true then we would expect double mutants to have expanded hem identities, like the Foxg1 mutant. If this were to be the case, this could also help explain the limited ventral rescue in *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ mutants. In *Shh*$^{-/-}$;*Gli3*$^{-/-}$, Foxg1 may continue to suppress Wnt and BMP expression and thus facilitate the wider rescue of ventral markers.

An alternative interaction between Foxg1 and Gli3 would be that Foxg1 is required to antagonise Gli3 upstream of its activity in promoting dorso-medial telencephalon. In this situation, I would expect *Foxg1*$^{-/-}$;*Gli3*$^{-/-}$ telencephalon to lack cortical hem fates and associated expression of dorsalising signals.

To my mind establishing the relationship between Foxg1 and Gli3 in the development of the dorso-medial telencephalon is an important next step and would greatly enhance our understanding of the fundamental mechanisms that pattern the D/V axis of the telencephalon.

The importance of Foxg1's role as a transcriptional repressor in D/V patterning?

As well as understanding the relative roles for Foxg1 in regulating the expression and response to developmental signals at the phenomenological level, there is a great deal to be discovered about the function of Foxg1 at the molecular level, within the cell and how it directs gene expression to contribute to D/V pattern.

Foxg1's primary function as a transcriptional repressor and its association with TLE proteins bears comparison with a paper published by Muhr and colleagues (Muhr et al., 2001). In this study they demonstrated that numerous homeobox genes of the *Nkx*, *Pax* and *Dbx* gene families interact with TLE genes to act as transcriptional repressors. Furthermore, many of these genes have crucial roles in setting up D/V patterning of the spinal cord and, even more intriguingly, in many cases these functions were wholly reliant on the association with TLE co-repressors. The conclusion from this study is that complex repressive interactions are crucial for patterning the spinal cord. It will be very interesting to determine whether similar mechanisms are at play in the rostral neural tube and, specifically, what role Foxg1 plays in them. As suggested earlier, a comprehensive screen for transcriptional targets of Foxg1 would be hugely informative.

In addition to *Gli3*, one transcription factor that it seems Foxg1 might repress in the context of the ventral telencephalon is *Pax6*. Since Pax6 appears to promote dorsal identities in the telencephalon, then repression of *Pax6* may be a necessary step in achieving ventral gene expression. If Foxg1 was required to repress *Pax6* then failure to do so may cause some of the observed defects in ventral specification. Genetically removing Pax6 from *Foxg1*$^{-/-}$ telencephalon may recover aspects of ventral gene

expression. If this was the case then more molecular approaches could be taken to explore the basis of Foxg1's regulation of Pax6 expression. This experiment is now underway in the laboratory of David Price and John Mason.

DNA binding independent functions of Foxg1 in D/V patterning?

As described above, the construction of mice expressing a DNA binding defective version of Foxg1 (Foxg1-NHAA) in the telencephalon provides good evidence that Foxg1 has many functions independent of its direct DNA binding during neurogenesis (Hanashima et al., 2002). Unfortunately, the way this experiment was conducted meant that early telencephalic development lacked Foxg1 expression altogether (as discussed in Chapter 3 and Shen (2004)). Since this is the period when initial D/V pattern is set up, we cannot infer whether Foxg1-NHAA can rescue any of the D/V patterning defects of *Foxg1*$^{-/-}$ mutants. Repeating the experiment by placing Foxg1-NHAA in the endogenous Foxg1 locus would be very interesting. This would help determine whether Foxg1's roles in regulating the expression of a wide range of morphogenetic signalling molecules and downstream transcription factors are mediated via direct DNA interactions. If Foxg1-NHAA can rescue aspects of D/V pattern, it would be very interesting to attempt to identify further proteins that Foxg1 interacts with. Immunoprecipitation studies combined with mass spectrometry and analysis of candidate protein binding could be a good starting point in such an endeavour.

CONCLUDING PERSPECTIVE: FOXG1 AS AN INTEGRATOR OF MULTIPLE DEVELOPMENTAL SIGNALLING PATHWAYS IN TELENCEPHALIC DEVELOPMENT

The available evidence suggests that Foxg1 has many wide-ranging and important roles in the development of the vertebrate telencephalon. These functions involve both the expression of, and the response to, multiple morphogenetic signals. Although the roles of Foxg1 in regulating the progression of neurogenesis and gliogenesis and in regulating D/V patterning have been largely separated in the preceding chapters, this was done more for reasons of explanatory clarity, rather than for reasons of scientific proof. Indeed, there is a great need for molecules that link the processes of regional fate specification to the processes of cell type specification and telencephalic growth. With its expression throughout the majority of the telencephalic tissue, Foxg1 is in a particularly good position to function as an integrator of multiple developmental events, which are regulated by multiple molecular pathways.

Whilst it may seem far-fetched that one protein could provide such a central role in telencephalic development, new evidence may support such a claim. It has recently been shown that multiple splicing isoforms of *Foxg1* are present in mice and humans (Shoichet et al., 2005) and there is also evidence that various phosphorylated forms of Foxg1 exist (N. Papalopulu, unpublished observations). Consequently, rather than being expressed as one protein, there may be several isoforms and phosphoforms of Foxg1, each with important roles in telencephalic development. It will be very interesting to determine how important these new complexities of Foxg1 expression and regulation

turn out to be. In any case, there is an increasing perception in the literature that developmental signalling pathways, which have long been studied as linear pathways, actually interact and combine in complex, non-linear ways. It is therefore important that nodes through which these pathways interact are identified. An interesting example from the literature of such a node is the convergence of BMP, Fgf and insulin-like growth factor (IGF) signalling pathways on the transcription factor Smad1 (Pera et al., 2003). These different pathways have distinct effects on Smad1 phosphorylation and direct different downstream activities of the Smad1 protein, which, therefore, integrates the activities of several signalling inputs. It would be very interesting to determine whether Foxg1 has any analogous functions in the developing telencephalon.

APPENDIX A. Materials and Methods for Molecular Biology

DNA EXTRACTION

Mouse Genomic DNA

DNA was extracted from adult mouse ear biopsy samples and embryonic tissue samples for PCR genotyping by the Hotshot method (Truett et al 2000). Tissues were lysed by heating to 96°C for 30 minutes in 50-200µl alkaline lysis buffer (25mM NaOH, 0.2mM disodium EDTA, pH12). Samples were then cooled to 4°C and neutralised by adding an equal volume of neutralisation buffer (40mM Tris-HCl, pH5). 1µl of the lysate was then used for each PCR reaction.

Plasmid DNA

Plasmid DNA was extracted from bacteria using Qiagen mini or midi-prep kits. DNA was then analysed by restriction enzyme digest to confirm the nature of the plasmid and the yield was quantified by running on an agarose gel against DNA standards of known concentration (Roche).

DNA / RNA PRECIPITATION

DNA and RNA were precipitated using ammonium acetate. The following general protocol was used for a given volume *n* of nucleic acid:

1. Add 0.4 x *n* 5M NH_4.OAc, pH5.5
2. Add 2.5 x *n* 100% ethanol
3. Vortex
4. Spin at 13,000 rpm for 30 minutes at 4 °C
5. Discard supernatant
6. Wash in 75% ethanol
7. Spin at 13,000 rpm for 10 minutes at 4 °C
8. Air dry pellet
9. Re-suspend in appropriate volume of ddH_2O

AGAROSE ELECTROPHORESIS

DNA and RNA were routinely separated using agarose electrophoresis. Low meting point agarose was dissolved by boiling in 1X TBE. Ethidium bromide (at 10μg/ml) was added to 0.1μl/ml TBE-Agarose. Gels were left to set (~30 minutes) at RT before being covered in 1X TBE, samples loaded, and electrophoresed at 45mA/cm for varying amounts of time.

RESTRICTION DIGESTS

Restriction enzymes were typically obtained from New England Biolabs (NEB), Promega or Fermentas, dependent on availability. Digests were conducted at 37 °C (unless the enzyme was active at a different temperature) for one to two hours. A general protocol for a single digest was as follows:

Digest of 20 µl	2 µl 10X Reaction Buffer 2 µl 10X BSA 1 µl Enzyme (10 units) n µl DNA (required µg quantity) (20 - (5 + n)) µl ddH_2O

Reaction buffer at 10X was supplied by the manufacturer, 100X BSA was supplied by the manufacturer and made to 10X fresh for each digest with ddH_2O. Whilst digest volumes and quantities of DNA were altered as required, all digests were scaled according to the above and the final concentration of total enzyme never exceeded 10% of the reaction volume.

PCR (POLYMERASE CHAIN REACTION)

PCR was used widely throughout this thesis, mainly for genotyping genomic DNA extracted from adult and embryonic mice. The following describes the general conditions used for every reaction, methods of optimisation typically used where this was required, followed by the notation used to define experimental conditions in each specific reaction.

Primers were designed using the web application Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The sequences of all primers can be found in table A1.

PCR Reaction Conditions

All PCR reactions to genotype Foxg1 mutant mice and embryos with genomic DNA as a template used the following basic set of conditions, reagents were generally supplied by Promega and primers synthesised to order by MWG-Biotech, and their sequences are shown below.

PCR Reaction Mix (Foxg1 PCRs)	2.0 µl 10X Reaction Buffer 0.6 µl 25mM $MgCl_2$ 1.0 µl 5' Primer (10µM) 1.0 µl 3' Primer (10µM) 0.4 µl dNTP mix (Promega) 1 µl DNA Template (see above) 0.2 µl Taq Polymerase (1 unit) 13.8 µl ddH_2O
PCR Program	Step 1 : 2 Minutes at 96 °C Step 2 : 30 Seconds at 96 °C Step 3 : 30 Seconds at 58°C Step 4 : 30 Seconds at 72 °C

	Step 5 : Go To Step 2 34 Times Step 6 : 2 Minutes at 72 °C

Genotyping of Gli3 mutant mice utilised a multiplex PCR reaction, with the conditions as shown below.

PCR Reaction Mix (Gli3 PCR)	2.0 µl 10X Reaction Buffer 0.6 µl 25mM $MgCl_2$ 0.5 µl 5' *Gli3* Primer (10µM) 0.5 µl 3' *Gli3* Primer (10µM) 0.5 µl 5' *Gli* ^{Xt} Primer (10µM) 0.5 µl 3' *Gli* ^{Xt} Primer (10µM) 0.4 µl dNTP mix (Promega) 1 µl DNA Template (see above) 0.2 µl Taq Polymerase (1 unit) 13.8 µl ddH_2O
PCR Program	Step 1 : 2 Minutes at 95 °C Step 2 : 20 Seconds at 94 °C Step 3 : 20 Seconds at 63°C Step 4 : 50 Seconds at 72 °C Step 5 : Go To Step 2 34 Times

Primers used:

Allele	Forward Primer	Reverse Primer	Product (bp)
Foxg1	CTGACGCTCAATGGCATCTA	TTTGAGTCAACACGGAGCTG	438
Foxg1^{lacZ}	GCTGGACATGGGAGATAGGA	GACAGTATCGGCCTCAGGAA	550
Foxg1^{cre}	CATTTGGGCCAGCTAAACAT	ATTCTCCCACCGTCAGTACG	308
Gli3	GGCCCAAACATCTACCAACACATAG	GTTGGCTGCTGCATGAAGACTGAC	193
Gli^{Xtj}	TACCCCAGCAGGAGACTCAGATTAG	AAACCCGTGGCTCAGGACAAG	580

REVERSE TRANSCRIPTASE PCR (RT-PCR)

cDNA Synthesis

Routinely M-MLV reverse transcriptase (Promega) was used with 1μg of RNA. Where starting material was more limited a Sensiscript reverse transcriptase kit was used (Qiagen).

cDNA Synthesis Reaction Mix (M-MLV) (RNA and Hexamers were mixed with ddH_20 and incubated at 70°C for 5 Minutes prior to reaction)	5 μl 5X Reaction Buffer 2.5 μl dNTP mix (10mM) 1 μl M-MLV Enzyme (10 units) 1 μl Random hexamers (0.5μg) 0.6 μl RNasin Inhibitor (40U/μl) n μl RNA (n μl =1μg) (25 - (10.1 + n)) μl ddH_2O
cDNA Synthesis Reaction Mix (Sensiscript)	2 μl 10X Reaction Buffer 2 μl dNTP mix

	1 μl Sensiscript Enzyme (10 units) 1 μl Random hexamers (0.5μg) 0.25 μl RNasin Inhibitor (40U/μl) n μl RNA (n μl ≤ 50ng) (20 - (6.25 + n)) μl ddH_2O
cDNA synthesis reaction conditions	Step 1 : 60 Minutes at 42°C Step 2 : 5 Minutes at 93°C

RT-PCR Reaction conditions

For RT-PCR with a cDNA template, reaction mix and standard PCR conditions were as follows:

RT-PCR Reaction Mix	2.0 μl 10X Reaction Buffer 1.2 μl 25mM $MgCl_2$ 0.7 μl 5' Primer (10μM) 0.7 μl 3' Primer (10μM) 0.4 μl dNTP mix (Promega) 1 μl cDNA Template 0.2 μl Taq Polymerase (1 unit) 13.8 μl ddH_2O
PCR Program	Step 1 : 1 Minute at 95 °C Step 2 : 30 Seconds at 95 °C Step 3 : 30 Seconds at 58°C

	Step 4 : 30 Seconds at 72 °C Step 5 : Go To Step 2 25-32 Times Step 6 : 5 Minutes at 72 °C Step 7 : 4 °C Forever

For RT-PCR the number of amplificatory PCR cycles was adjusted depending on the abundance of the transcript to try and keep product levels within the linear range, thus allowing a limited degree of comparison of amplification efficiency between samples when analysed by agarose gel electrophoresis.

CULTURE OF *E. coli* TO CLONE DNA PLASMIDS

Heat-Shock Transformation

Plasmids (10 to 100ng) were transformed into chemically competent *E. coli* (strain DH5α, prepared in house) by heat shock at 42°C for 40 seconds following incubation on ice for 30 minutes. Bacteria were allowed to recover in 300µl SOC medium, and then were plated out on LBA containing ampicillin and incubated overnight at 37 °C.

Culture of E. coli *for mini-prep and midi-prep extraction of plasmid DNA*

Small cultures of 2ml LB medium were inoculated with *E. coli* from a freshly streaked plate and cultured overnight at 37°C with agitation for mini-preps.

50ml cultures were inoculated and cultured in the same fashion for midi-preps.

BACTERIAL GROWTH MEDIA

SOC Recovery Medium

0.5% Yeast extract, 2.0% tryptone, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 20mM $MgSO_4$, 20mM glucose dissolved in water and autoclaved to sterilize.

LB Broth (LB) Medium

1 tablet (Sigma L-7275) per 50ml dissolved in water and autoclaved to sterilize.

LB Agar Plating Medium

35g (Sigma L-2897) dissolved in 1L of water, autoclaved and poured into plastic Petri dishes under sterile conditions.

Antibiotics

Ampicillin (Roche: 835 242):

Stock dissolved in 50% ethanol stored at –20°C and diluted to working concentration of 100 μg/ml in LB or LB-Agar at temperatures not exceeding 50°C.

Appendix B. Media for primary culture of telencephalic cells and pre-implantation embryos.

PRIMARY NEURAL CELL CULTURE

*Stock solutions for serum free culture medium**

Antibiotics: Gentamycin (100mg) Kanamycin (200mg) added to 20ml sterile double deionised water, filter sterilised and stored in 1ml aliquots at –20°C.

Putrescene: 100μM stock (161.1mg/100ml in sterile double deionised water, filter sterilised and stored in 4ml aliquots at –70°C).

Progesterone: 20μM stock (6.29mg/100ml ethanol stored in 1ml and then 50μl aliquots at –70°C).

Na_2SeO_3: 30μM stock (5.2mg/100ml in sterile double deionised water, filter sterilised and stored in 50μl aliquots at –70°C).

L-glutamine: 0.2M stock (6.344g/100ml in sterile double deionised water, filter sterilised and store in 2ml and then 50μl aliquots at –70°C).

Preparation of serum free culture medium

Prepared by mixing the following reagents together under sterile conditions and stored for not more than two weeks at 4°C until use. Medium was warmed and equilibrated in a 37°C humidified incubator containing 5% CO_2 for at least one hour prior to use.

100ml F12 (Hams) (Sigma: N4888)

100ml Dulbecco's modified Eagles' medium (DMEM) (Sigma: D5671)

1mg Insulin (Sigma: I6634. Final concentration 5μg/ml)

2mg apo-transferrin (Sigma: T1147. Final concentration 10μg/ml)

3ml HEPES buffer (Sigma: H0887)

0.24g Na_2HCO_3 (Sigma: S5761. Final concentration 0.12mg/ml)

3ml antibiotics stock* (Gentamycin + Kanamycin) (Sigma: G1264 + K1377)

2ml putrescene stock* (Sigma: P5780. Final concentration 16.11μg/ml)

20μl progesterone stock* (Sigma: P8783. Final concentration 6.29ng/ml)

20μl Na_2SeO_3 stock* (Sigma: S5261. Final concentration 5.2ng/ml)

2ml $_L$-glutamine stock* (Sigma: G2128. Final concentration 25μg/ml)

'EBSS' medium used during embryonic brain dissection

Prepared by mixing the following reagents together under sterile conditions and stored for not more than two weeks at 4°C until use.

100ml Earle's balanced salt solution 10x (EBSS) (Sigma: E-7510)

0.22g Na_2HCO_3 (Sigma: S5761. Final concentration 22mg/ml)

0.065g Glucose (Sigma: G-7021. Final concentration 6.5mg/ml)

900ml double deionised water.

EBSS was oxygenated by bubbling with 95% O_2 and chilled on ice prior to use.

COLLECTION AND CULTURE OF PRE-IMPLANTATION EMBRYOS

Stock solutions for KSOM medium for culturing and handling embryos

Media were first made as stock solutions, which were then combined prior to usage, as shown below. Embryos were flushed from the reproductive tracts in KSOMH and then aggregated in KSOM. All reagents were purchased from Sigma.

Stock	Component	Mass (g)	Concentration (mM)
Stock A	NaCl	5.550	95.0
(100ml)	KCl	0.186	2.50
	KH_2PO_4	0.048	0.35
	$MgSO_4.7H_2O$	0.049	0.20
	Sodium Lactate (60%)	1.869	10.00
	Glucose	0.036	0.20
	EDTA	0.004	0.01
	Penicillin	0.060	97.5u/ml
	Streptomycin	0.050	37.5u/ml
Stock B	$NaHCO_3$	2.101	25.00
(100ml)	Phenol Red	0.01	0.20
Stock C	Sodium Pyruvate	0.022	0.2
(10ml)		(2ml of 100mM)	
Stock D	$CaCl_2.2H_2O$	0.252	1.71
(10ml)			

Stock E (10ml)	L-Glutamine	0.146	1.00
Stock F (100ml)	HEPES Adjust to pH7.2	4.764	20

All stocks were stored at 4°C. Stocks A,D,E &F were replaced at least every 3 months. Stocks B&C were replaced every 2 weeks.

To make Culture Media

Stock (ml)	Embryo Culture (KSOM)	Embryo Handling (KSOMH)
A	1.0	5.0
B	1.0	1.0
C	0.1	0.5
D	0.1	0.5
E	0.1	0.5
F	-	5.0
ddH_2O	7.7	37.5
BSA	10mg	50mg
Total Volume	10.0ml	50.0ml

Solutions were filter sterilized through 0.2μm filters (Millipore) prior to use.

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Foxg1 is required for specification of ventral telencephalon and region-specific regulation of dorsal telencephalic precursor proliferation and apoptosis

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Abstract

Null mutation of the *Foxg1* gene causes hypoplasia of the mouse telencephalon and loss of ventral telencephalic structures. We show that a crucial early requirement for Foxg1 is in the induction of ventral cell fate in the telencephalon. To study later proliferative defects, we have adapted an iododeoxyuridine and bromodeoxyuridine double labeling protocol for use in the developing embryo, which allows estimation of cell cycle kinetics in a single specimen. This technique is used to demonstrate that the cell cycle is prematurely lengthened in the *Foxg1*-null telencephalon. These defects are first apparent at embryonic day 10.5 (E10.5) and are most severe in the rostral telencephalon. We show that apoptosis is also reduced in the same rostral domain. These defects correspond temporally and spatially with a dramatic reduction in expression of the potent signaling molecule *Fgf 8*. We also show that in the absence of *Foxg1* an excess of neurons is produced from E11.5, depleting the progenitor pool and limiting the growth of the $Foxg1^{-/-}$ telencephalon. The increase in neurogenic division coincides with an increase in BMP signaling, as detected by immunohistochemistry for phosphorylated smad-1, -5, and -8. This study reinforces *Foxg1*'s position as a major regulator of telencephalic neurogenesis and supports the idea that *Foxg1* controls precursor proliferation via regulation of Fgf signaling and differentiation via regulation of Bmp signaling.
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Introduction

The embryonic telencephalon arises from the most rostral region of the neural tube, later giving rise to the adult cerebral cortex, hippocampus, olfactory bulbs, and basal ganglia. Soon after its specification, the telencephalon undergoes a period of rapid expansion where the progeny of every cell division re-enters the cell cycle (Bhide, 1996; Caviness et al., 1995). Following this period, there is a gradual slowing of the rate of growth as the progenitor cell cycle lengthens and an increasing proportion of newly born cells exit the cell cycle to differentiate into neurons (Sheth and Bhide, 1997; Takahashi et al., 1996). During this period of telencephalic neurogenesis, the vast majority of neurons that will populate the adult brain for the lifetime of the animal are produced. In order to ensure that the correct types of neurons are produced in the right numbers at the right time and place, the behavior of the telencephalic progenitor cells must be accurately choreographed. Three factors have a major influence on the cellular output from the proliferative zones: the rate of cell proliferation, the rate of cell differentiation, and the rate of cell death. Any failure to correctly regulate any of these crucial processes may have a profound effect on the number of cells produced and the structure of the brain region concerned. Understanding how these processes are modulated at a genetic level remains a major challenge.

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between the number of cells in the L_{cells} and the S_{cells} fractions is equal to the ratio between T_i (which equals 1.5 h) and T_s (Shibui et al., 1989):

$$T_i/T_s = L_{cells}/S_{cells} \quad \therefore T_s = T_i/(L_{cells}/S_{cells})$$

(where L_{cells} = IddU$^+$/BrdU$^-$ and S_{cells} = IddU$^+$/BrdU$^+$)

By exactly the same logic, the calculated T_s can be used to estimate T_c:

$$T_s/T_c = S_{cells}/P_{cells} \quad \therefore T_c = T_s/(S_{cells}/P_{cells})$$

P_{cells} is the total number of proliferating cells in the sampling area. In this case, P_{cells} is estimated by counting the total number of cells in the ventricular zone (VZ) in the sampling area. This equation, therefore, rests on the assumption that all cells in the ventricular zone are proliferating. Previous authors have shown that 98–100% of cells in the VZ of the wild-type telencephalon are indeed actively proliferating (Caviness et al., 1995; Estivill-Torrus et al., 2002; Takahashi et al., 1993, 1995). Here, 8 h cumulative BrdU labeling at E10.5 (Figs. 1D and E) and proliferating cell nuclear antigen (PCNA) staining at each age (Supplementary Fig. 1 and data not shown) in *Foxg1*$^{-/-}$ mutant and wild-type brains labeled all VZ cells show that they are all proliferating. This technique also assumes that the precursor cells consist of a single proliferating population with the same cycling kinetics. Evidence suggests this is indeed the case during early telencephalic development. However, around E13.5 in the dorsal telencephalon (Takahashi et al., 1995b) and E11.5 in the ventral telencephalon (Sheth and Bhide, 1997), a second proliferative population arises within the germinal zone. Since the cell cycle kinetics of these populations are very similar to those of the primary proliferative population (Sheth and Bhide, 1997; Takahashi et al., 1995b), this aspect of telencephalic development should not affect results generated by this technique.

For the calculation of BrdU labeling index in the ventralmost telencephalon, the number of BrdU-labeled cells in two 100-μm sampling bins spaced 100 μm apart in the ventralmost region of the telencephalon was counted and expressed as a percentage of all VZ cells. A total of 14 counts were made in each of three control and *Foxg1*$^{-/-}$ embryos in sections spaced evenly through the rostrocaudal axis of the telencephalon.

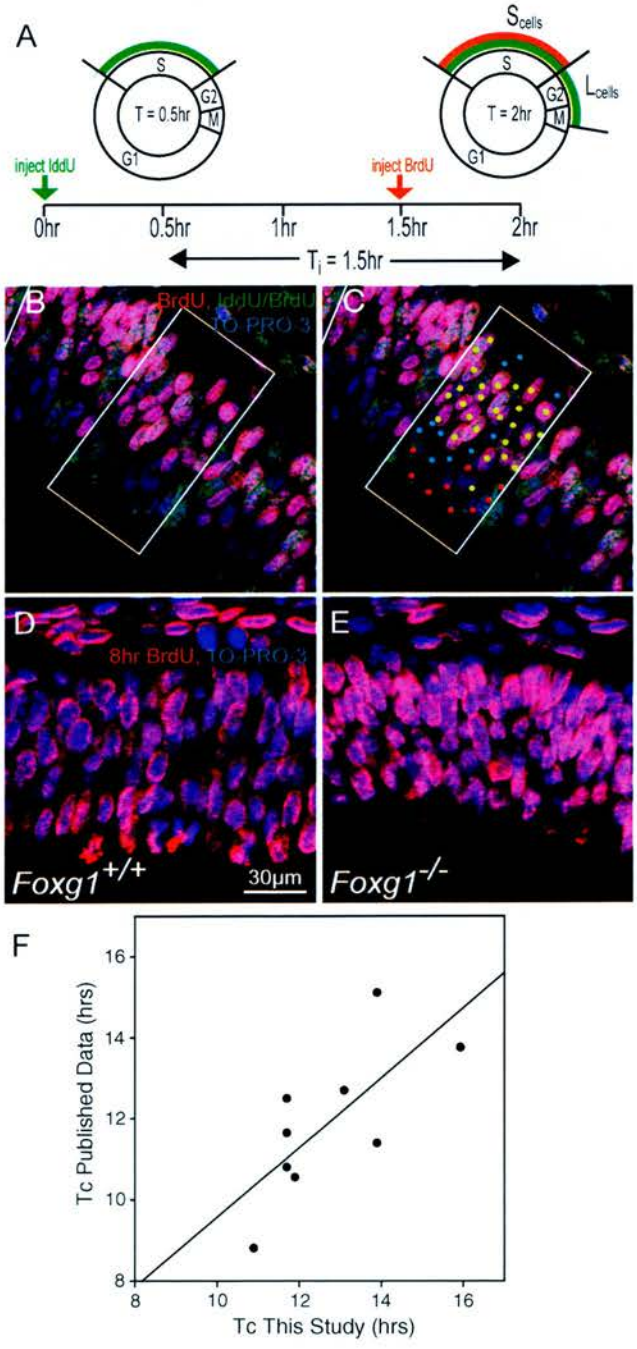


Fig. 1. Calculation of cell cycle kinetics by IddU/BrdU double labeling. (A) To estimate cell cycle kinetic parameters, the pregnant mouse is injected with IddU at $T = 0$ h to label all cells in S-phase at the beginning of the experiment. At $T = 1.5$ h, an injection of BrdU is given and the embryos are fixed after a short survival period of 0.5 h, sufficient to label the S-fraction (S_{cells}) at the end of the labeling period (Nowakowski et al., 1989). During the 1.5 h interval when cells are exposed to IddU but not BrdU (T_i), some of the initial S-phase cohort will leave S-phase and consequently will be labeled just with IddU; this is the leaving fraction (L_{cells}). (B and C) Monoclonal antibodies specific for both BrdU and IddU (green), and BrdU alone (red), can then be used to identify cells in the S-fraction (red and green double-labeled cells in B marked with yellow spots in C) and cells in the L-fraction (green only in B and C, marked with red spots in C); blue spots in C mark unlabelled cells. (D and E) At the beginning of telencephalic neurogenesis (~E10.5), essentially all VZ cells are actively proliferating in both wild-type and *Foxg1*$^{-/-}$ telencephalon, since they all become labeled with BrdU following 8 h cumulative labeling at E10.5. Therefore, a nuclear counterstain can be used to count all VZ cells which represent the P_{cells} fraction (all stained nuclei in B and yellow, red, and light blue marked nuclei in C). The L_{cells}, S_{cells}, and P_{cells} fractions can then be used to calculate T_c and T_s (see Materials and methods). (F) When T_c estimates obtained in wild-types with this technique at a range of ages are plotted against those obtained using BrdU cumulative labeling for matched ages in the same tissues (Bhide, 1996; Estivill-Torrus et al., 2002; Takahashi et al., 1996; Yuasa et al., 2002), there is a significant correlation ($P = 0.022$, Pearson correlation test, $n = 9$), as shown by the linear regression (gradient = 0.97, $r = 0.73$).

This approach to measuring cell cycle kinetics is fast, reliable, and can be used to estimate parameters in defined groups of cycling cells in individual embryos. Estimates of cell cycle parameters in wild-type embryos generated in this study are very similar to those obtained at a range of ages by various authors using the more traditional technique of BrdU cumulative labeling (Bhide, 1996; Estivill-Torrus et al., 2002; Takahashi et al., 1995a,b; Yuasa et al., 2002) (Fig. 1F). The correlation between the wild-type values of T_c obtained here and those previously published at comparable ages is statistically significant ($P = 0.022$, Pearson correlation test, $n = 9$).

Cell cycle time increases precociously in the Foxg1$^{-/-}$ telencephalon

Cell cycle kinetics of telencephalic progenitor cells were estimated at several ages during early and mid-neurogenesis in control (wild-type and *Foxg1*$^{+/-}$ heterozygotes, see Materials and methods) and *Foxg1*$^{-/-}$ embryos (Fig. 2). Numerous counts were made in sampling bins throughout the telencephalon at E9.5, E10.5, and E11.5, and in the dorsal telencephalon at E14.5, to give an average estimate of T_c for each embryo at each age. Patterns of IddU and BrdU labeling were very similar in control (Fig. 2A) and mutant sections (Fig. 2B). Cells in S-phase are BrdU labeled (red) and, as expected, were found in the outer half of the ventricular zone. Cells which had left S-phase during the labeling period (IddU-labeled only, green) had moved towards the ventricular surface. The presence of unlabelled mitotic figures (asterisks in Figs. 2A and B) shows that T_i is shorter than the length of G2 and M-phases (T_{G2+M}) so the number of cells in the L_{cells} fraction is not artificially inflated by the counting of recently divided cells. These findings suggest that interkinetic nuclear migration and the general mode of telencephalic precursor proliferation are not severely affected by the loss of *Foxg1*. Fig. 2C shows that at E9.5 mean T_c is approximately 7 h in control and *Foxg1*$^{-/-}$ telencephalon. Around E10.5, neurogenesis commences and the first post-mitotic neurons appear at the pial surface of the telencephalic neuroepithelium. As neurogenesis proceeds, there is a gradual lengthening of T_c with age in both control and *Foxg1*$^{-/-}$ telencephalon (Fig. 2C). This slowing of progenitor proliferation rate is more severe and occurs earlier in the *Foxg1*$^{-/-}$ telencephalon. At E14.5, the mid-point of wild-type neurogenesis, when cell output should be at its peak (Caviness et al., 1995; Takahashi et al., 1996), the mean cell cycle time of *Foxg1*$^{-/-}$ dorsal telencephalic precursors is more than twice that of their wild-type counterparts (29 h and 14 h, respectively). The difference in T_c between control and mutant observed over time is statistically significant ($P = 0.002$, univariate general linear model, $n = 12$).

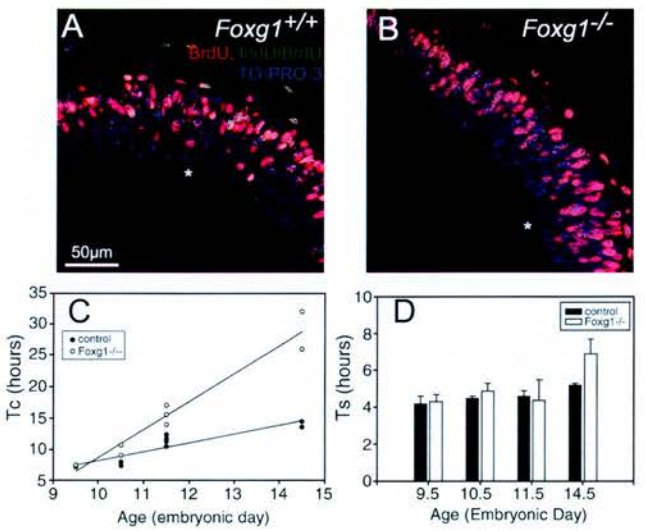


Fig. 2. Cell cycle times increase precociously in the *Foxg1*$^{-/-}$ telencephalon. Patterns of IddU and BrdU labeling within the ventricular zone are very similar in (A) wild-type and (B) *Foxg1*$^{-/-}$ telencephalon at E10.5, suggesting that inter-kinetic nuclear migration and the general mechanics of precursor cell division are not severely perturbed in the mutant. The presence of non-IddU-labeled mitotic figures (asterisks) shows that $T_i <$ the length of G2 and M-phases (T_{G2+M}). These facts suggest that it is appropriate to apply the IddU/BrdU labeling technique to control and experimental embryos. (C) Mean T_c increases with developmental age in both control and *Foxg1*$^{-/-}$ telencephalon, but the trend is accelerated in mutant embryos, as shown by linear regressions. The difference in T_c between control and mutant telencephalon over developmental time is significant ($P = 0.002$, analysis of variance by general linear model, $n = 12$ embryos). (D) Histogram showing mean $T_s \pm$ SD at a range of ages in *Foxg1*$^{-/-}$ embryos (open bars) and control embryos (filled bars). T_s remains relatively constant at 4–5 h in both wild-type and mutant at all ages analyzed, except at E14.5 when T_s is longer in the *Foxg1*$^{-/-}$ dorsal telencephalon.

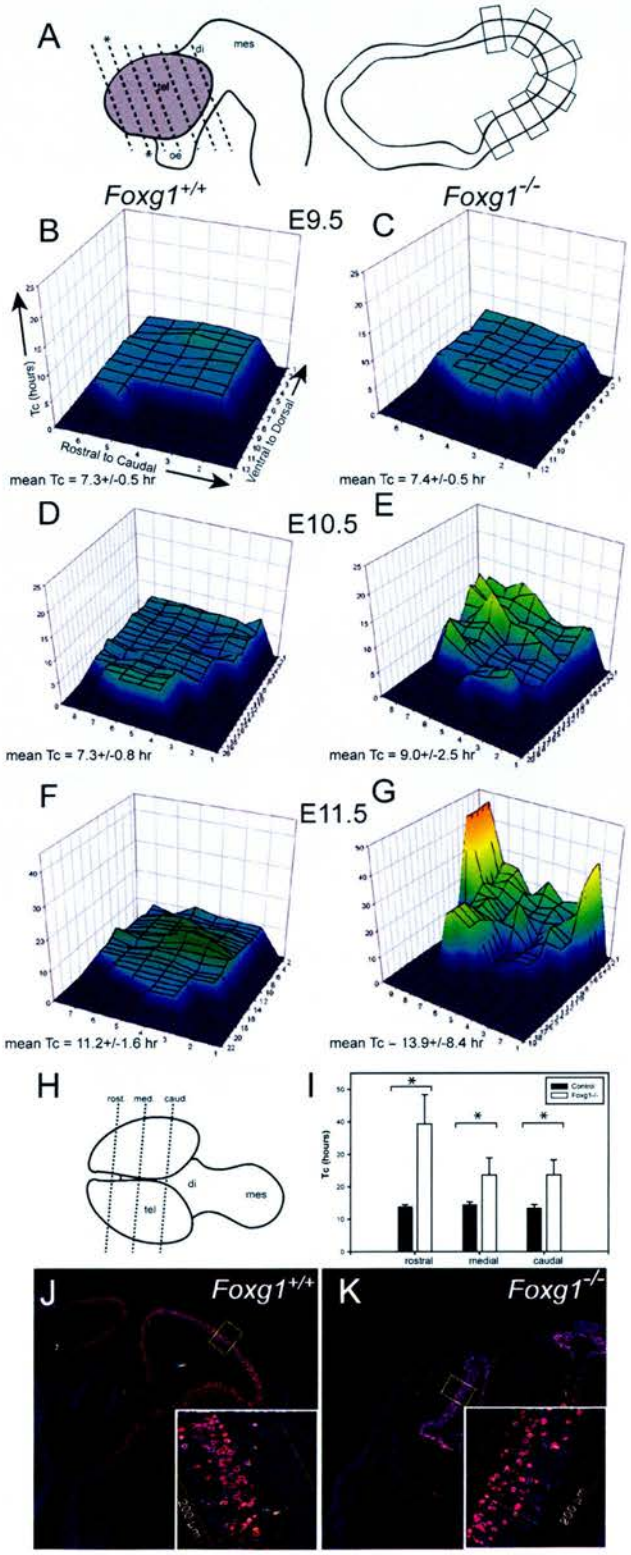
Interestingly, the length of S-phase is very similar in wild-type and mutant precursors, averaging between 4 and 5 h (very similar to wild-type T_s measured by other authors; Bhide, 1996; Estivill-Torrus et al., 2002; Takahashi et al., 1995a,b) at all ages except E14.5, where it is lengthened as the mutant cell cycle becomes more disregulated (Fig. 2D).

These results suggest that while the mode of progenitor cell division is not grossly altered in the *Foxg1*$^{-/-}$ telencephalon, there is an early and significant reduction in proliferation rate from E10.5, co-incident with the start of neurogenesis. From this age onwards, the mutant telencephalon is notably reduced in size (Xuan et al., 1995; and this study). This reduction in proliferation rate is very likely to contribute to the observed hypoplasia.

Cell cycle time is altered in a region-specific fashion

At E9.5, the *Foxg1*$^{-/-}$ telencephalon is similar in size to wild-type controls. However, as development proceeds, the mutant telencephalon appears to grow more slowly than wild-type telencephalon. At E12.5, the telencephalon of *Foxg1*$^{-/-}$ embryos is clearly hypoplastic relative to that of controls and this is more apparent rostrally than caudally (Fig. 3, compare A and C with B and D, and E and G with F and H). Morphologically there is also a loss of ventral telencephalic structures. The ganglionic eminences, which

medial (ventral) and lateral (dorsal) domains. Nkx2.1 expression marks prospective ventral diencephalon (caudally) and ventral telencephalon (rostrally). In *Foxg1*$^{-/-}$ embryos at this age, the expression of Nkx2.1 does not extend rostrally into the prospective telencephalon (arrows



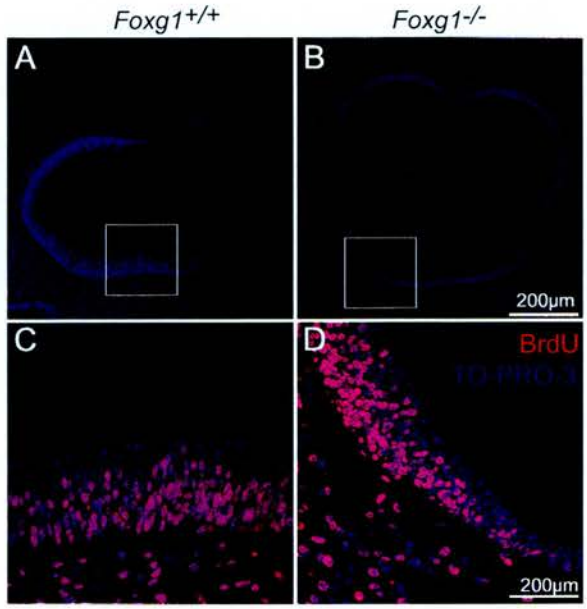


Fig. 5. BrdU labeling in ventralmost telencephalon. (A and B) Low power images of comparable coronal telencephalic sections from wt and *Foxg1*$^{-/-}$ embryos. (C and D) Higher power images of boxed areas in A and B showing cells labeled with BrdU following a 30-min pulse of BrdU (red). Nuclei are counterstained with TO-PRO-3.

in Figs. 6A and B), consistent with a failure to induce the ventral telencephalon. At E9.5, there is a total absence of Nkx2.1 expression in the ventral region of the *Foxg1*$^{-/-}$ telencephalon (Fig. 6D), but not in the ventral diencephalon (Fig. 6H). At E9.5, Mash1, which appears in the ventralmost telencephalon of normal embryos at this age (Fig. 6E), is also absent from the *Foxg1*$^{-/-}$ telencephalon (Fig. 6F) but is still present in the dorsal and ventral diencephalon (Fig. 6G). By E10.5, Gsh2 gene expression becomes detectable in

Fig. 4. Cell cycle time is increased in a region-specific fashion in *Foxg1*$^{-/-}$ mutants. T_c and T_s were estimated in sampling bins covering the whole of the developing telencephalon at E9.5, E10.5, and E11.5 as schematized in A. Approximately every 6–10th section was imaged. The section was divided into 100-μm-wide sectors and cell counts were made through the depth of the telencephalic wall from the dorsal to ventralmost extent of the telencephalon in alternate sectors (the coronal section shown in A is taken from level marked by asterisks in the lateral view). This allowed the mapping of T_c values measured at evenly spaced interval across the surface of the telencephalon. (B–G) 3D plots from representative embryos at the three ages analyzed. (B and C) At E9.5, T_c was constant across the telencephalon in control and mutant telencephalon. (D and E) By E10.5, populations of cells in rostral and dorsal portions of the *Foxg1*$^{-/-}$ had inflated T_c's relative to control. (F and G) At E11.5, lengthening of T_c was more severe and was observed through much of the mutant telencephalon. (H) At E14.5, groups of three non-adjacent sections in caudal, rostral, and medial positions were analyzed. (J and K) Cell cycle analysis was conducted in a 200-μm counting bin placed in a dorso-lateral position of the neocortex in mutant and control sections. (I) At all three rostrocaudal levels, T_c was significantly higher in the *Foxg1*$^{-/-}$ dorsal telencephalon (open bars) than in controls (filled bars) ($P < 0.05$, Student's t test, $n = 3$). Histogram shows mean $T_c \pm$ SD. tel, telencephalon; di, diencephalon; oe, optic eminence; mes, mesencephalon.

markers Nkx2.1, Gsh2, and Dlx in all rostrocaudal levels of the *Foxg1*$^{-/-}$ telencephalon later in development (at E12.5), demonstrating that ventral specification is not simply delayed (data not shown). Concurrent with the loss of ventral marker gene expression, dorsal cell types are over-represented when *Foxg1* is absent. Pax3, which marks the dorsalmost of telencephalic cells (Fig. 6K), is expressed in a slightly expanded dorsal zone in *Foxg1*$^{-/-}$ telencephalon and appears ectopically in a number of laterally and ventrally positioned cells at E9.5 (Fig. 6L). At E10.5, the roofplate Pax3 domain is clearly expanded in *Foxg1* mutants relative to control, while the ectopic lateral and ventral expression seen at E9.5 is no longer apparent (Figs. 6M and N). Pax6, whose expression is normally restricted to the dorsal telencephalon (Fig. 6O), is expanded throughout the whole of the *Foxg1*$^{-/-}$ telencephalon at E9.5, E10.5, and E11.5 (Fig. 6P and data not shown).

The gene expression studies described here provide good support for the idea that *Foxg1* is required for the induction of the ventral telencephalon, and in the absence of this transcription factor, dorsal telencephalic fates are expanded.

Apoptosis is reduced in the rostral telencephalon of Foxg1$^{-/-}$ *mutants*

As well as the reduced precursor proliferation rate measured here, it is possible that an increase in the level of programmed cell death contributes to the morphological abnormalities and hypoplasia of the *Foxg1*$^{-/-}$ telencephalon. To examine levels of apoptosis in the developing telencephalon, whole-mount TUNEL was performed on *Foxg1*$^{-/-}$ embryos and wild-type littermates. At E9.5, TUNEL-labeled cells in the telencephalon are restricted to the dorso-rostral midline (Aoto et al., 2002; Furuta et al., 1997; Ohkubo et al., 2002) (Figs. 7A and B). At this age, although it appears that there may be slightly fewer labeled cells in *Foxg1*$^{-/-}$ telencephalon, this was not consistent (n = 3 per genotype). A day later, at E10.5, there is a clear and consistent reduction in the number of TUNEL labeled cells in this rostral domain in the *Foxg1* mutant telencephalon at E10.5 in all embryos analyzed (n = 3) (compare Figs. 7C and E with D and F and Supplementary movie 1 with movie 2). Scanning TUNEL-labeled embryos with optical projection tomography (OPT) (Sharpe et al., 2002) showed that apoptotic cells are in the telencephalic neuroepithelium, rather than the overlying ectoderm. Few apoptotic cells were observed in ventral regions of *Foxg1* mutant or control embryos, and as such, increased cell death does not seem to contribute to the impaired development of the *Foxg1*$^{-/-}$ ventral telencephalon. Patterns of apoptosis in non-telencephalic parts of *Foxg1*$^{-/-}$ embryos are otherwise indistinguishable from those in wild-types, with labeled cells in the midline of the diencephalon and mesencephalon, in the facial ectoderm, somites, and limb buds (Supplementary movies 1 and 2), suggesting that the observed decrease in TUNEL-labeled cells in the telencephalon results specifically from the loss of *Foxg1*.

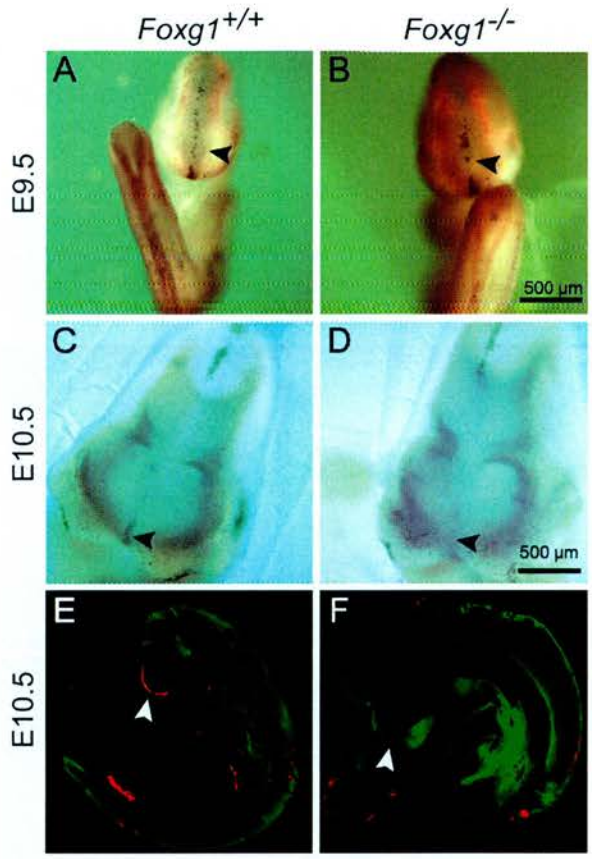


Fig. 7. Apoptosis is reduced in the rostral telencephalic midline of *Foxg1*$^{-/-}$ mutants. (A and B) Whole mount TUNEL shows that apoptotic cells are present in the rostral midline of the wild-type (arrowhead in A) and *Foxg1*$^{-/-}$ telencephalon (arrowhead in B) at E9.5. At E10.5, TUNEL-labeled cells are still observed in the rostral telencephalon of controls (arrows in C, E) but almost completely absent in the *Foxg1* mutant (arrows in D and F). Some TUNEL-labeled embryos were scanned with optical projection tomography (OPT) allowing for the digital reconstruction of the complete set of optical sections for each embryo, which provided verification that this rostral apoptotic population is in the neuroepithelium, rather than the overlying surface epithelium. Examples of pseudo-colored optical sections are shown in E and F with regions of TUNEL-labeling in red.

Neurogenesis is accelerated in the Foxg1$^{-/-}$ *telencephalon*

In addition to the regulation of proliferation rate and the rate of cell death, the rate at which the progeny of neurogenic cell divisions withdraws from the cell cycle to differentiate must be tightly controlled to ensure neurons are produced in correct numbers at the correct time. Previous authors have observed an early accumulation of cells expressing markers of post-mitotic neurons in the mantle zone of the *Foxg1*$^{-/-}$ telencephalon from E12.5 onwards (Hanashima et al., 2002; Xuan et al., 1995). Here, these observations are confirmed and extended to show that an excess of neurons is produced from E11.5 onwards.

Since apoptosis is actually reduced in the same territory at this age, the reduction in *Fgf8* expression is not due to programmed cell death of expressing cells. The reduced proliferation rate described above, although it is first observed in the same rostrodorsal domain that expresses *Fgf 8*, also seems an unlikely cause since it coincides with, rather than precedes, the reduction in expression. Previous researchers have shown that BMP4, 6, and 7, which are normally restricted to the dorsomedial wall of the telencephalon, are expanded in the *Foxg1*$^{-/-}$ telencephalon after E11.5 (Dou et al., 1999; Hanashima et al., 2002). Furthermore, it has been shown that enhanced BMP signaling can repress Fgf8 expression in the chick telencephalon (Ohkubo et al., 2002). To examine whether excess BMP signaling might contribute to the loss of Fgf 8 expression described above, we examined expression of direct effectors of BMP signaling: tyrosine phosphorylated forms of Smad-1,5,8 (P-Smad). At E10.5, immunohistochemistry for P-smad revealed positive nuclei around the ventricular surface of the telencephalic neuroepithelium and a slightly increased level of staining in the roofplate region of the telencephalon, where BMP expression has been documented (Furuta et al., 1997). There is no clear difference in P-smad staining in control and *Foxg1*$^{-/-}$ telencephalon at E10.5 (Figs. 9E and F). A day later, however, at E11.5 when BMP expansions have been demonstrated (Dou et al., 1999, Hanashima et al., 2002), increased P-smad staining is observed in the *Foxg1*$^{-/-}$ telencephalon, especially in dorsomedial regions (Figs. 9G and H).

It is also possible that changes in other dorsally restricted signaling molecules, such as the Wnt proteins, may alter *Fgf 8* expression in *Foxg1*$^{-/-}$ mutants. We therefore examined the expression of *Wnt8b* at E10.5. In both control and mutant embryos, *Wnt8b* mRNA was restricted to the dorsomedial telencephalic wall (Figs. 9I and J).

These data suggest that BMP and Wnt signalling activity are present and correctly localized in the *Foxg1*$^{-/-}$ telencephalon at E10.5. However, since they are not expanded at the time that *Fgf 8* expression is reduced (E10.5), it seems unlikely that increases in these dorsally derived signals cause this repression directly.

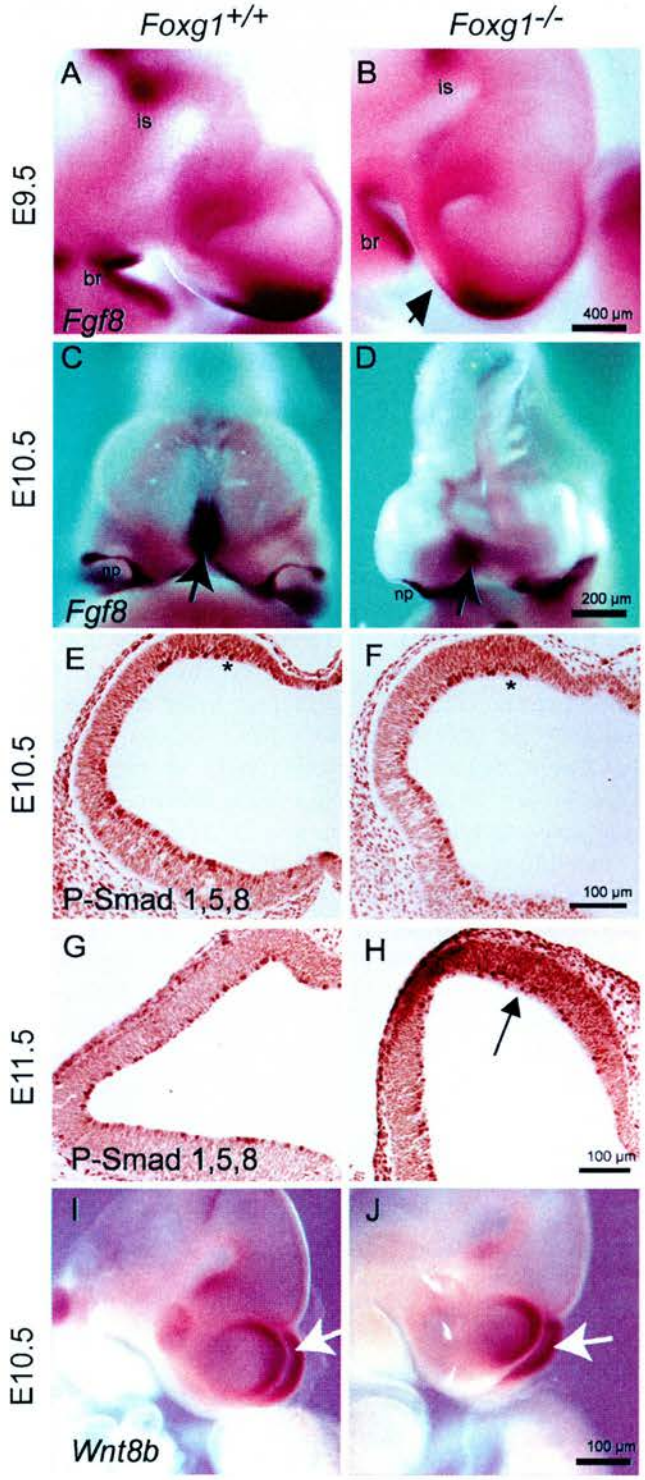


Fig. 9. *Fgf8*, BMP, and Wnt signalling pathways in control and *Foxg1*$^{-/-}$ telencephalon. (A and B) Whole mount in situ hybridization at E9.5 for *Fgf8* shows this signalling molecule is expressed in the rostral telencephalon of both *Foxg1*$^{-/-}$ and wild-type embryos, although it may be slightly reduced and does not extend so far ventrally in the mutant (arrow in B). (C and D) At E10.5, there is dramatic reduction in the level of *Fgf8* expression in the same rostral telencephalic domain in *Foxg1*$^{-/-}$ embryos (arrows). At both ages, *Fgf8* expression in other structures, notably the branchial arches (br), nasal pits (np), and isthmus (is), is not affected by the loss of *Foxg1*. (E, F, G, and H) Immunohistochemistry on coronal sections for phosphorylated Smad-1,5,8 (P-smad), a readout of BMP signalling. At E10.5, there is no clear difference in P-smad staining between mutant and control, with positive cells around the ventricular surface and increased staining in the medial telencephalon (asterisks in E and F). By E11.5, there is increased P-smad staining in the null telencephalon, particularly in the medial telencephalon (arrow in H). (I and J) Whole mount in situ hybridization for *Wnt8b* shows that this Wnt family member is expressed appropriately in a restricted dorsomedial band of control and mutant telencephalon (white arrows).

Discussion

During the neurogenetic interval of telencephalic development (~E10.5–E17.5 in mouse, Caviness et al., 1995;

observations suggest that FGF8 is capable of inducing cellular proliferation in a variety of tissues in the developing embryo. It is expressed in several major outgrowths of the embryo during their rapid expansion: the rostral telencephalon; the apical ectodermal ridge of the limb bud; and the developing tailbud (Crossley and Martin, 1995). More direct experimental evidence that FGF8 is pro-proliferative in the developing central nervous system comes from experiments involving ectopic application of the protein to the midbrain and diencephalon (Martinez et al., 1999) and its transgenic misexpression in the mesencephalon (Lee et al., 1997). Both of these manipulations caused dramatic overgrowth of the midbrain and caudal forebrain. We know of no previous studies which have directly related telencephalic FGF8 to proliferation rate. However, correlative evidence presented here suggests that FGF8 may have a similar pro-proliferative role in the most anterior brain structure. Reduced FGF8 levels may contribute to the reduced precursor proliferation rate observed in the rostral telencephalon of the *Foxg1* mutant.

While FGF8 level may have a direct relationship with proliferation rate, the relationship to cell survival does not seem to be so straightforward. High levels of apoptosis are seen in the same terminal outgrowths listed above, which express high levels of FGF8 and are proliferating rapidly, perhaps suggesting that very high exposure to FGF8 can promote programmed cell death. A recent paper suggested that such a paradoxical relationship is observed between FGF8 dosage and cell survival (Storm et al., 2003). Increasing or eliminating FGF8 caused increased apoptosis in telencephalic explants, while a hypomorphic allele that produces lower levels of FGF8 led to enhanced survival. The result presented here is directly comparable to this finding. At E10.5, when *Fgf8* expression is greatly reduced, but not totally eliminated in the *Foxg1* mutant, an enhancement of cell survival is seen.

It is very interesting to consider why *Fgf8* expression is lost from the $Foxg1^{-/-}$ telencephalon. One possibility is that it is a consequence, rather than a cause, of the reduced rostrodorsal proliferative defects described here. However, this seems an unlikely explanation for two reasons. Firstly, while the reduction in *Fgf8* is spatially correlated with reduced proliferation rate, it is also temporally coincident. Reduced proliferation would have to precede the *Fgf8* reduction to explain it. Secondly, the reduced cell death observed in the same area that expresses *Fgf8* would be predicted to cause an expansion, rather than a contraction of the number of cells expressing this factor. A more likely reason for the loss of *Fgf8* may be the absence of the potent ventrally derived signalling molecule Sonic Hedgehog (Shh) from the $Foxg1^{-/-}$ telencephalon (Huh et al., 1999). In $Shh^{-/-}$ embryos, just like in the $Foxg1^{-/-}$ embryos, *Fgf8* is induced but not maintained, although in the former *Fgf8* expression is lost somewhat earlier, at E9 (Ohkubo et al., 2002). It is also possible that *Foxg1* may have a more direct effect on the *Fgf8* promoter itself and is perhaps required for the maintenance, if not the induction, of telencephalic *Fgf8* transcription after E9.5.

In this paper, we also provide evidence that cell fate choice is misregulated during neurogenesis in $Foxg1^{-/-}$ embryos. While neuron production at E10.5 appears similar to wild-type (Hanashima et al., 2004; and data not shown), at E11.5 the number of post-mitotic neurons in the mantle zone of the mutant is significantly greater than that of controls. This finding suggests that either the neurogenic program is ectopically activated in precursors without cell division or that there is an increase rate of neurogenic divisions (either symmetrical or asymmetrical) in the $Foxg1^{-/-}$ telencephalon at this early stage of neurogenesis. This early depletion of the proliferative population combines with decreased proliferation rates of the remaining precursors to drastically limit the growth of the $Foxg1^{-/-}$ telencephalon.

While the slowing in proliferation rate and reduction in apoptosis are focussed specifically in rostrodorsal areas, the defects in the early differentiation rate do not share this spatial pattern. Instead, the early appearance of post-mitotic neurons occurs at all levels of the telencephalon. Given that FGF8 dosage has not been linked to neuronal differentiation rate and that the observed cell fate defect is more diffusely located, we find it unlikely that the reduction in *Fgf8* expression described here contributes to this aspect of the mutant phenotype. One possibility here is that the increased rate of neuronal differentiation is caused by the increased expression of Bmps 2, 4, 6, and 7 whose expression expands beyond their normal dorsal midline domain (Furuta et al., 1997) to fill much of the telencephalic neuroepithelium of $Foxg1^{-/-}$ embryos at E11.5 (Dou et al., 1999; Hanashima et al., 2002). Various studies in vivo and in vitro suggest that Bmps can increase the differentiation rate of telencephalic neuronal precursors (Li et al., 1998; Mehler et al., 2000). Consistent with this hypothesis, when *Foxg1* is replaced by a DNA-binding defective form of the gene, the proliferative defects in the dorsal telencephalon seem to be rescued, while the early differentiation and, importantly, expanded Bmp expression are not (Hanashima et al., 2002). This does suggest that excess Bmp can induce neuronal differentiation, but may not influence precursor proliferation kinetics, at least at the developmental stage studied. Foxg1 may therefore be required to repress BMP expression outside of the cortical hem and protect the telencephalon from this powerful differentiative signal. It has also been suggested that Foxg1 is required cell-autonomously to repress production of the earliest born telencephalic neurons, Cajal–Retzius cells, which are produced in excess in $Foxg1^{-/-}$ mutant mice (Hanashima et al., 2004). Another recent study suggested that Foxg1 is required within the telencephalon to suppress the cytostatic action of Transforming Growth Factor β (TGFβ) (Seoane et al., 2004).

In summary, these findings emphasize the position of Foxg1 as a key regulator of several aspects of the

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