NOTE TO USERS

Promotion of Cortical Neuronal Differentiation by Groucho-Related Gene 6

Nathalie Marçal

Center for Neuronal Survival

Montreal Neurological Institute

McGill University

Montreal, Quebec, Canada

Submitted August, 2004

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science (Neurological Sciences)

Your file Votre référence
ISBN: 0-494-12499-7
Our file Notre référence
ISBN: 0-494-12499-7

ACKNOWLEDGMENTS

I want to first thank my parents without whom this achievement would not have been possible. Merci pour votre support, votre amour et vos encouragements qui n'ont jamais cessés. I am grateful to my supervisor Dr Stefano Stifani for his advice, patience, guidance and knowledge he provided to me during my work. Thank you to Stéphanie Bélanger-Jasmin, and Yeman Tang for their help in this work. I want to thank also Dr Philip Barker, Dr David Ragsdale and Dr Timothy Kennedy, members of my committee, for encouragement and advice.

Thank you Francesca, Hugh, Michel-Olivier and Manuel for your great support. Thank you Francesca and Michel-Olivier for all the good moments and laughs we had! I'll never forget this!

A big thanks to all members of the lab for their help!

A special thank to François for your love, support and patience. You have been always there for me!

Thank you to Dr Michael Greenwood and Dr Rosemary Panetta for their encouragement and their great philosophical and scientifical conversations! They have always believed in me!

CONTRIBUTIONS OF AUTHORS

I performed experiments shown in Figs 1, 2A, 2C-J, 3-9. Yeman Tang performed study shown in Figure 2B and Stéphanie Bélanger-Jasmin performed studies shown in Figure 10. Dr. Jinjun Dang kindly provided the Grg6 serum.

ABSTRACT

The Groucho/Transducin-like Enhancer of split (Gro/TLE) proteins are a family of transcriptional corepressors involved in a variety of cell differentiation mechanisms in both invertebrates and vertebrates. In particular, they act as negative regulators of neuronal development. Gro/TLEs can be recruited to DNA by forming complexes with a number of DNA-binding transcription factors and are thus involved in the regulation of numerous genes. The aim of this study was to characterize a new member of the Gro/TLE family named Groucho-related gene 6 (Grg6). It is reported here that Grg6 is expressed in selected regions of the murine embryonic nervous system in both mitotic progenitor cells and postmitotic neurons. Exogenous expression of Grg6 in cortical neural progenitor cells does not significantly affect neuronal differentiation. However, when co-expressed with Gro/TLE1 and the anti-neurogenic Gro/TLE-binding protein brain factor 1 (BF-1; also called Foxg1), Grg6 causes an increase in the number of differentiated neurons. In agreement with these findings, Grg6 interacts with BF-1 and decreases transcriptional repression mediated by BF1:Gro/TLE complexes. In addition, Grg6 disrupts the interaction between BF-1 and Gro/TLE1. Together, these results suggest that Grg6 acts as a negative regulator of BF1 activity and as a positive regulator of cortical neuronal differentiation.

RÉSUMÉ

Les protéines Groucho/Transducin-like Enhancer of split (Gro/TLE) forment une famille de co-répresseur transcriptionnel impliqués dans une multitude de mécanismes de différentiation cellulaire chez les vertébrés et les invertébrés. En particulier, ils régulent de façon négative le développement neuronal. Structurellement, les protéines Gro/TLE n'ont pas la capacité de se lier à l'ADN. Cependant, elles peuvent y être recrutées en formant des complexes avec un certain nombre de facteurs de transcription liants l'ADN. Ainsi, elles sont impliquées dans la régulation de plusieurs gènes. Le but de cette étude était de caractériser un nouveau membre de la famille de Gro/TLE appelé Groucho-related gene 6 (Grg6). Nous avons découvert que Grg6 est exprimé dans des régions particulières du système nerveux embryonnaire de la souris, et ce aussi bien dans les cellules mitotiques progénitrices que dans les neurones post-mitotiques. L'expression exogène de Grg6 dans les cellules neuronales progénitrices du cortex n'a pas d'effet significatif sur la différentiation neuronale. Cependant, lorsque co-exprimé avec Gro/TLE1 et le facteur anti-neurogénique Brain Factor 1 (BF-1), pouvant lui-même se lier aux protéines Gro/TLE, Grg6 cause une augmentation dans le nombre de neurones différenciés. En accord avec ces résultats, Grg6 se lie à BF-1 et diminue la répression transcriptionnelle médiée par les complexes BF-1:Gro/TLE. De plus, Grg6 empêche l'interaction entre BF-1 et Gro/TLE1. Ensemble, ces résultats suggèrent que Grg6 agit en tant que régulateur négatif de l'activité de BF1 et régule de façon positive la différentiation neuronale du cortex.

TABLE OF CONTENTS

1. INTRODUCTION

The development of higher species is a complex and fascinating process. From the egg to the fully developed organism, the events are multiple and must be well controlled. Cell division is a fundamental process at the basis of organ growth, while cell differentiation is responsible for the incredible variety of cell types that compose the mature organism. This thesis review will focus on the mechanisms underlying mammalian central nervous system development and, in particular, those regulating neurogenesis.

The developing mammalian central nervous system shows both symmetric and asymmetric division of the neural progenitor cell population. Symmetric divisions give rise to two identical daughter cells that expand the progenitor cell population (proliferative divisions). On the other hand, asymmetric divisions produce two different types of siblings that later will differentiate into multiple types of neurons and glia (differentiative divisions) (reviewed by Doe et al., 1998). Neurogenesis depends on cell proliferation and thus cell cycle regulation. A number of key regulators of the cell cycle have been well conserved during evolution. Among these, cyclin-dependant kinases (cdks) are central components of the cell-cycle control system in eukaryotic cells. For example, it has been shown that the size of the brain is regulated by the cell-cycle inhibitor p27Kip1, because mice lacking this component exhibit enlarged brains (Nakayama et al., 1996). A tight regulation of the cell cycle will not only determine the proliferation of the neural precursor cells but also will influence the fraction of progenitors that exit the cell cycle and thus differentiate.

During mammalian neurogenesis, neural precursor cells will undergo different stages in the process of differentiation during which they will express characteristic proteins that will make them unique. This will result in the generation of a tremendous variety of cell types that populate the central nervous system. This complex process of cell fate specification is under the control of two general sets of factors: secreted or transmembrane (extrinsic) signals and intrinsic signals. Extrinsic signals, present in a cell's local environment, act on receptors located on the cell surface whose activation initiate signal transduction cascades. Ultimately, these cascades of events will affect gene transcription in the cell nucleus, which in turn will affect structural and physiological changes in differentiating cell. Intrinsic signals operate in a cell autonomous manner and can determine cell fate. Both extrinsic and intrinsic signals cooperate and will gradually establish the final identity of precursor cells (reviewed by Edlund and Jessel 1999).

1.1 Mechanisms of cell differentiation

1.1.1 Drosophila neural cell specification and lateral inhibition

The specification of neural cell identity during the development of the Drosophila nervous system is initiated by the selection of a cluster of cells ("proneural clusters") within the neuroectoderm. These clusters are composed of cells that can become either neural or epidermal, but initially they are all competent to give rise to neural precursors. In fact, the initial rough pattern of these proneural clusters is established by the limitation of "proneural genes" expression to proneural clusters at specific location in the neuroepithelium. These genes encode a number of basic helix-loop-helix (bHLH)

transcription factors that regulate the expression of genes that endow cells with the potential to become neural precursors. Initially, the cells in these groups have equal potential to become neural precursors. But not all the cells that express proneural genes will become neurons. In fact, only one cell within the proneural cluster will predominantly express proneural genes and further differentiate into a neuron. Through the process of lateral inhibition, which is mediated by the Notch signalling pathway, the neighbouring cells are prevented from adopting the neural fate (reviewed by Jan and Jan, 1994). In the absence of Notch expression in the embryo, proneural gene expression continues and all cells develop into neural precursors (Artavanis-Tsakonas, 1983; Hartenstein et al., 1990; reviewed by Ghysen et al, 1993). Such phenotype is characterized by a gross hypertrophy of the nervous system (Lehmann et al, 1981).

1.1.2 Molecular mechanisms controlling Drosophila neurogenesis

The molecular mechanisms involved in lateral inhibition during *Drosophila* neurogenesis are controlled by two classes of bHLH transcription factors. The first class defines the proneural genes, such as the *acheate-scute complex (as-c)*, composed of four tightly linked genes (*acheate*, *scute*, *lethal of scute* and *asense*), *atonal (ato)* and *Daugtherless (Da)* (reviewed by Jan and Jan, 1994). The other class encodes anti-neurogenic components of the Notch signalling pathway like the gene products of the *Enhancer of split complex (E (spl)-c), groucho*, *Suppressor of Hairless* and others (reviewed by Fisher and Caudy, 1998; Artanavis-Tsakonas et al., 1999).

These two families of bHLH proteins act as transcriptional activators or repressors and have been shown to be important in the regulation of *Drosophila* neurogenesis

(reviewed by Jan and Jan, 1994; reviewed by Fisher and Caudy, 1998). The proneural proteins act as transcriptional activators to promote neurogenesis and the Enhancer of split complex proteins act as transcriptional repressors to inhibit neurogenesis (reviewed by Artavanis-Tsakonas et al., 1999).

1.1.3 The role of proneural proteins in Drosophila neurogenesis

Proneural protein expression endows cells with the potential to become neural and thus has a very important role in neural specification during *Drosophila* neurogenesis. Extensive genetic and molecular analysis showed that proneural proteins are in fact signals which promote neural identity and its various specification events during *Drosophila* neural development. It has been shown that proneural proteins are initially expressed in all cells of the proneural cluster. Subsequently, one of these cells accumulates the highest amount and adopts the neural fate. Later, the cells surrounding the specified neural precursor within the proneural cluster reduce or cease to express proneural proteins and adopt a non-neural fate, through the so-called lateral inhibition process (Skeath et al., 1991; Cubas et al., 1991). Mutation analysis of *ac-c* members demonstrated the crucial role of these genes during the development of neural structures. Flies lacking one *as-c* gene showed reduced or complete absence of neural elements, while overexpression resulted in the overproduction of neural structures (Campuzano and Modelell, 1992; reviewed by Jan and Jan, 1994; Jarman et al., 1995).

Molecularly, proneural bHLH proteins can homo- or heterodimerize through the HLH motif and can interact with the ubiquitously expressed Daugtherless protein, forming complexes that can activate transcription when bound to DNA through the basic

region (Cabrera and Alonso, 1991; Van Doren et al, 1994). Proneural proteins bind to the consensus DNA sequence (CANNTG) designated as "E box" and through interaction at this site are thought to positively regulate neurogenesis. The activity of *as-c* members is inhibited by Extramacrochaetae (Emc), an HLH protein lacking the basic domain and thus unable to bind DNA (Van Doren et al., 1992). Emc antagonize as-c proteins by forming non-functional heterodimers leading to the inhibition of neurogenesis.

1.1.4 Drosophila proneural proteins are conserved in mammals

The identification and characterization of homologues of Drosophila proneural proteins in rodents and humans has led to a better understanding of the mechanisms that regulate mammalian neurogenesis. Many mammalian homologues of *Drosophila* proneural proteins have been identified and shown to play similar functions in mammalian neurogenesis (reviewed by Lee et al., 1997; Hassan and Bellen, 2000; Ben Arie et al., 2000). The best characterized is the *Mammalian achaete-scute homologue-1* or *Mash-1* (Johnson et al., 1990; Guillemot and Joyner, 1993).

Mash-1 expression is mainly observed in cells of the developing central nervous system (CNS) and peripheral nervous system (PNS). More specifically, *Mash-1* expression is detected in regions containing undifferentiated neural precursor cells of the ventricular zone (VZ) and also differentiating post-mitotic cells outside the VZ (Guillemot et al., 1993). This expression pattern of *Mash-1* has been revealed to be similar to that of *as-c* genes in *Drosophila* (reviewed by Guillemot, 1999). In addition, *Mash-1* has been shown to be expressed in the developing olfactory epithelium and neural retina (Guillemot et al., 1993; Ahmad, 1995; Jasoni et al., 1996).

Functionally, *Mash-1* is implicated in the regulation of determination and differentiation steps of many types of neurons in both the CNS and PNS. For example, *Mash-1* is required for the generation of autonomic ganglia, olfactory neurons and noradrenergic neurons of the hindbrain (Guillemot et al., 1993; Hirsch et al., 1998).

In agreement with the high *Mash-1* expression in the ventral telencephalon, *Mash-1* mutant mouse embryos show severe loss of neural progenitors in the sub-ventricular zone of the medial ganglionic eminence. Discrete neuronal populations of the basal ganglia and cerebral cortex are also lost (Casarosa et al., 1999). Molecular analysis of *Mash-1* function revealed that *Mash-1* is required to activate the mammalian Notch signalling pathway in the ventral telencephalon, since *Mash-1* mutant embryos exhibited a loss of the expression of Notch ligands *Dll1* and *Dll3*, and the mammalian homologue of *Drosophila* Hairy and Enhancer of split gene, Hes5, a target of Notch signalling (Casarosa et al., 1999). In *Drosophila*, transcription of the *Delta* gene is directly regulated by proneural proteins (Künisch et al., 1994). These results suggest that *Mash-1* is a key component in the regulation of neurogenesis in the ventral telencephalon, where it is required both to specify neuronal precursors and to control the timing of their production (Casarosa et al., 1999).

Molecularly, the mechanism by which Mash-1 activates transcription is very similar to that of *as-c* in *Drosophila*. Mash-1 dimerizes with E47, the mammalian homologue of *Drosophila* proneural protein Daughterless, and together these proteins bind to the E box where they promote transcription of target genes (reviewed by Jan and Jan, 1994).

In vertebrates, other proneural bHLH proteins have been identified with similar molecular and functional properties to their *Drosophila* counterparts. These include neurogenins, NeuroD, Math proteins, which are related to *Drosophila atonal*, and Xash3 or Cash4, which are related to *Drosophila acheate* and *scute* (Guillemot, 1999; Bertrand et al., 2002). It has been previously shown that proneural bHLH proteins are under negative regulation by another family of bHLH factors named Hes (Hairy and Enhancer of split) during both Drosophila and vertebrates neurogenesis. The balance between these factors assures the proper neural development of the brain.

1.1.5 Drosophila Hes proteins

Drosophila Hes proteins define a family of transcription factors characterized by a four amino acids WRPW motif located at their extreme C-terminus which mediates interaction with the general transcriptional corepressor Groucho (Fisher et al., 1996). They possess also a proline-rich region and a region called the "orange" domain or Helix3-Helix4 domain, rich in hydrophobic residues and important for the specificity of interaction (Bae et al., 2000). In addition, they are characterized by a bHLH domain at their N-terminus. The HLH motif mediates homo- or heterodimerization between Hes factors and the basic motif mediates specific interaction with DNA (Ohsako et al., 1994). In contrast to proneural proteins which interact with the DNA sequence CANNTG or E box, Hes factors bind preferentially with the DNA sequence CACNAG, referred to as N box (although they were shown to bind with a lower affinity to the E box as well) (Sasai et al., 1992; Ohsako et al., 1994; Iso et al., 2003). It has been demonstrated that binding

to the N box sequence is required for the *Drosophila* Hairy/Hes proteins to repress *ac* gene expression, both *in vitro* and *in vivo* (Ohsako et al., 1994).

1.1.6 Mammalian and Drosophila Hes proteins share conserved properties

So far, seven mammalian *Hes* genes have been identified, *Hes1* through *Hes7*, and shown to have very important functions during mammalian development. Among all, Hes1 is the best characterized both biologically and molecularly. During the development of the nervous system, Hes1 is specifically expressed by mitotic neural progenitor cells present in the VZ, and its expression decreases when cells start to differentiate (Sasai et al., 1992) Ectopic expression of Hes1 was shown to inhibit neuronal differentiation and the cells expressing Hes1 remained as progenitor cells (Ishibashi et al., 1994; Tomita et al., 1996). This negative effect of Hes1 on differentiation is thought to be in part due to inhibition of the positive bHLH factors like Mash1 (Sasai et al., 1992). On the other hand, Hes1-deficient mice exhibited premature differentiation of neural precursor cells into neurons and expression of bHLH genes such as *Mash1* is up-regulated suggesting that up-regulation of *Mash1* may account for the premature neuronal differentiation and that *Mash1* is a likely target for direct repression by Hes1 (Ishibashi et al., 1995; Tomita et al., 1996). In fact, it was shown that Hes1 binds directly to the *Mash1* promoter and represses its activity (Chen et al., 1997). Together, these results show that Hes proteins are negative regulators of neurogenesis and that their functions have been conserved in both *Drosophila* and mammals. Molecularly, the transcription repression activity of Hes proteins was demonstrated to require the participation of a family of transcriptional co-repressors termed Groucho/TLE proteins.

1.1.7 Groucho/TLE proteins as positive regulators of Hes activity during neurogenesis

In an effort to better understand how Hes proteins function as repressors, a number of studies suggested that the mechanisms underlying Hes activity are mediated by the transcriptional co-repressor protein Groucho (Gro).

In *Drosophila*, genetic studies revealed that loss of *groucho* function resulted in a phenotype similar to that of loss of *Hes* function (Delidakis et al., 1991; Schrons et al., 1992; Paroush et al., 1994; Heitzler et al., 1996). In both cases, supernumerary neurons are produced. These results first suggested a genetic interaction between *groucho* and *Hes* genes (Delidakis et al., 1991; Schrons et al., 1992; Paroush et al., 1994; Heitzler et al., 1996). Groucho and Hes proteins were shown to physically interact with each other through a motif present at the carboxy-terminal domain of Hes proteins called the WRPW motif (Paroush et al., 1994; Fisher et al., 1996). This interaction was shown to be required for Hes mediated transcriptional activity and mutation in the WRPW motif abolished Hes activity (Fisher et al., 1996).

Similar results were obtained in vertebrates, where the mammalian Groucho homologues are referred to as Transducin-like Enhancer of split (TLE) proteins. Studies have demonstrated that Hes1 and Gro/TLEs are coexpressed in a number of tissues during development including the brain (Dehni et al., 1995; Grbavec et al., 1996; Yao et al., 2001) and that they physically interact through the WRPW motif (Fisher et al., 1996; Grbavec et al., 1996). Functionally, Hes1 and Gro/TLE proteins form transcription repression complexes where Hes1 provides a specific DNA-binding activity and Gro/TLE acts as transcriptional corepressor (McLarren et al., 2001; Yao et al., 2001). Consistent with this model, mutations that render Hes1 unable to interact with Gro/TLEs

impair the transcriptional repression activity of Hes1 (McLarren et al., 2000; McLarren et al., 2001; Gratton et al., 2003). All together, these findings strongly suggest that Hes and Gro/TLEs work together, forming transcriptional repression complexes to prevent or delay neuronal differentiation during neurogenesis.

1.1.8 Molecular features of Gro/TLE proteins have been conserved throughout evolution

Drosophila Groucho, a maternally contributed nuclear factor (Hartley et al., 1998; Delidakis et al., 1991), is the founding member of a family of evolutionary conserved transcription corepressor proteins. It was identified in 1968 by a viable mutation that resulted in clumps of extra bristles above the fly adult eyes, resembling the bushy eyebrows of Groucho Marx (Lindsley and Grell, 1968). This family includes the Groucho mammalian homologues Groucho-related-genes (Grgs), the rat enhancer of split proteins (ESPs) and the human Transducin-like Enhancer of split (TLE) proteins 1 through 4 (Stifani et al., 1992; Koop et al., 1996; Leon and Lobe, 1997). Gro/TLE proteins are characterized by two highly conserved domains: the N-terminal glutamine-rich domain (Q domain) and the C-terminal WD-repeat domain (WD domain). They possess also a less well conserved core region composed of a Glycine/Proline-rich domain (GP domain), a region called the CcN domain containing phosphorylation sites for caseine kinase II (CKII) and possible phosphorylation sites for p34cdc2 kinase proximal to a putative nuclear localization sequence (NLS), and finally a Serine/Proline-rich domain (SP domain) (Stifani et al., 1992; Nuthall et al., 2002; Nuthall et al., 2004). The Q domain contains two putative leucine zipper motifs involved in mediating oligomerization between Gro/TLE proteins (Pinto and Lobe, 1996; Chen et al., 1998). It

has been also demonstrated that both the Q and SP domains are important in mediating repression when targeted to a DNA template via fusion to the GAL4 DNA binding domain (Grbavec et al., 1998; Chen et al., 1999). Furthermore, together with the GP domain, the Q domain provides binding sites for histone H3 and histone deacetylases (HDACs), suggesting a mechanism for the transcription repression function mediated by Gro/TLE proteins (Palaparti et al., 1997; Choi and Kim, 1999; Chen et al., 1999; Flores-Saaid et al., 2000; Yao et al., 2001; Brantjes et al., 2001).

The most highly conserved region of the Gro/TLE proteins is the WD domain at the C-terminal end. This region displays a very complex structure made up of seven repeats or blades of about 40 residues, each composed of four-stranded antiparallel β-sheets-the so-called β-blades. Almost each repeat contains the conserved Trp-Asp (WD) motif (Pickles et al., 2002). The WD domain of human TLE1 can functionally replace the WD domain of UNC-37, a Groucho homologue of *C. elegans*, suggesting a high degree of functional conservation (Pflugrad et al., 1997). Several studies have shown that the Gro/TLE WD domain functions as a multifunctional protein-protein interaction site (reviewed in Chen et al., 2000 and Pickles et al., 2002) for a number of transcription factors such as bHLH Hes proteins (Paroush et al., 1994; Fisher et al., 1996; Grbavec et al., 1998; McLarren et al., 2001), winged-helix factors (Wang et al., 200; Yao et al., 2001) and homeodomain proteins containing the engrailed-homology region 1 motifs (Eberhard et al., 2000; Jimenez et al., 1997; Muhr et al., 2001; Tolkunova et al., 1998; Yao et al., 2001).

Mutation analysis has provided a powerful tool to investigate the functional specificity of the WD domain. In *C. elegans*, several UNC-37 mutants have been

characterized and have provided a better understanding of UNC-37 function. UNC-37 is involved in regulating VA motor neuron wiring via interaction with the UNC-4 homeodomain transcription factor. Mutation involving the substitution of a conserved His residue (His539Tyr) in the fifth WD repeat resulted in a neuronal identity switch from a VA motor neuron to a VB motor neuron, translating into movement defects in the worm. Substitution of the WD domain of UNC-37 with that of TLE1 was shown to restore the VA motor neuron identity. These results suggest that the highly conserved WD domain is involved in cell fate decisions and that mutation of that domain may interfere with the interaction of Gro/TLE proteins with specific DNA binding factors (Pflugrad et al., 1997). In agreement with this, another UNC-37 mutation, Glu394Lys, identifies a putative key residue that could mediate specific protein-protein interaction. In the human TLE1 protein, the equivalent residue Glu550 is localized to the second WD repeat. Mutation of this residue for an Ala has been shown to disrupt the interaction between TLE1 and Hes1, but not with Brain-factor 1 (BF-1) or RUNX 1 (M. Buscarlet and S. Stifani, unpublished data). These results suggest that specific residues within the WD domain of Gro/TLE proteins are involved in mediating interaction with specific transcription factors.

As corepressors, Gro/TLE proteins cannot bind to DNA directly but can be targeted to a variety of specific gene regulatory sequences through interaction with numerous DNA-binding transcription factors. In addition to the proteins mentioned previously, Gro/TLEs interact with the paired-like homeodomain repressor Hesx1, a regulator of pituitary development (Dasen et al., 2001). The Q, GP and WD domains of TLE1 are required for the interaction with Hesx1 (Dasen et al., 2001), whereas the

interaction between Gro/TLEs and RUNX proteins is mediated by both the Q and WD domains (McLarren et al., 2000). These results suggest that different DNA binding proteins may interact with different domain of Gro/TLEs. As a result of these interactions with various transcriptional regulators, Gro/TLEs are involved in many developmental processes. For example, *Drosophila* Groucho is required for sex determination, segmentation, eye development, dorsoventral patterning and neurogenesis (Chen et al., 2000) Vertebrate Gro/TLEs have been implicated in events such as neuronal differentiation (Yao et al., 2000), dorsoventral patterning of the neural tube (Muhr et al., 2001), skeletogenesis (Dehni et al., 1995; Thirunavukkarasu et al., 1998; Javed et al., 2000) and hematopoiesis (Levanon et al., 1998; Ren et al., 1999; Eberhard et al., 2000).

1.1.9 Implication of mammalian Gro/TLEs in cell-fate determination and differentiation

Vertebrate Gro/TLE proteins are involved in the regulation of a variety of developmental processes. Mammalian Groucho homologues like the human TLEs, the mouse Grgs and the rat ESPs are expressed during embryogenesis (Dehni et al., 1995; Koop et al., 1996; Leon and Lobe, 1997). In addition and similar to *Drosophila* Groucho, mammalian Groucho proteins have been implicated in the Notch signalling pathway. Both Notch and Gro/TLE genes are coexpressed in epithelial cells during epithelial differentiation (Liu et al., 1996). Also, Gro/TLE2 and Gro/TLE3 are expressed in the developing mouse placenta, where they are coexpressed with Mash2, Notch2, Hes2 and Hes3 (Nakayama et al., 1997). These findings suggest that the function of Gro/TLE proteins in Notch signalling is conserved.

During neurogenesis, Gro/TLEs are widely expressed throughout the developing CNS and PNS. Their expression profile has suggested non-redundant functions during the regulation of neuronal determination and differentiation in mammals. Individual Gro/TLE proteins have distinct expression patterns during *in vitro* and *in vivo* cell differentiation of mouse P19 embryonic carcinoma cells. In response to neural induction, Gro/TLE1 expression was shown to be up-regulated, whereas Gro/TLE2 was down-regulated (Husain et al., 1996; Yao et al., 1998). In contrast, Gro/TLE3 (Husain et al., 1996) and Gro/TLE4 levels remained constant during the same period (Yao et al., 1998). Individual Gro/TLE proteins are also expressed in combinatorial as well as complementary patterns during in vivo development of the cerebral cortex and spinal cord of mouse embryos (Yao et al., 1998). In particular, Gro/TLE1 and Gro/TLE3 proteins are robustly expressed in undifferentiated neural progenitor cells located in the VZ, whereas Gro/TLE2 and Gro/TLE4 are preferentially expressed in differentiating/ed postmitotic neurons outside the VZ (Dehni et al., 1995; Liu et al., 1996; Grbavec et al., 1998; Yao et al., 1998). Taken together, these results suggest that Gro/TLE genes are involved in cell proliferation and differentiation processes during mammalian neurogenesis.

1.1.10 Gro/TLE proteins are negative regulators of neurogenesis

Loss-of-function of *Drosophila groucho* results in the production of supernumerary sensory bristles, similar to the phenotype observed in loss-of-function mutations of components of the Notch signalling pathway (Lindsley and Grell, 1968). This suggests that, in vivo, Groucho is a negative regulator of *Drosophila* neurogenesis. A number of

findings suggested that Gro/TLE proteins may perform a similar function during mammalian neurogenesis. Gro/TLE1 is highly expressed in undifferentiated neural progenitor cells located in the VZ. Its expression decreases when newly specified postmitotic neurons are generated, and remains low during early stages of neuronal differentiation (Dehni et al., 1995; Yao et al., 1998). Hes1 is also robustly expressed in undifferentiated neural progenitor cells, where it is co-expressed with Gro/TLE1 (Akazawa et al.,1992; Sasai et al., 1992; Dehni et al., 1995; Yao et al., 1998). These results suggested that Hes1 may work together with Gro/TLE1 to form transcription complexes that act as negative regulators of neuronal differentiation. An involvement of Gro/TLE1 in the development of the nervous system has been also suggested by the finding that the *C. elegans groucho*-related gene, *unc-37*, controls the specification of motor neuron identity during worm development (Pflugrad et al., 1997). The C-terminal domains of UNC-37 and Gro/TLE1 are functionnally interchangeable during motor neuron differentiation, suggesting that Gro/TLE proteins may perform evolutionarily conserved roles during neuronal development. Furthermore, studies with transgenic mice showed that constitutive expression of Gro/TLE1 in postmitotic neurons inhibits neuronal differentiation in the forebrain, leading to increased apoptosis and loss of cortical and striatal neurons in the telencephalon (Yao et al., 2000). All together, these findings strongly suggest that Gro/TLE family members are important negative regulators of mammalian neurogenesis.

1.1.11 Molecular mechanisms underlying the transcriptional repression function of Gro/TLE proteins in development

The molecular mechanisms by which Gro/TLE proteins mediate transcriptional repression are still poorly understood. Nonetheless, a few studies have yielded initial insights on these mechanisms.

Multiple regions of Gro/TLE proteins contribute to their repressive function. The N-terminal Q domain mediates tetramerization, protein-protein interaction, and transcriptional repression (Grbavec et al., 1998; Chen et al.,1998; Song et al., 2004). This domain is characterized by the presence of putative coiled-coils structures, likely involving a pair of amphipathic α-helical motifs, AH1 and AH2, and involved in mediating oligomerization between Gro/TLE proteins (Pinto and Lobe, 1996; Chen et al., 1998; Song et al., 2004). The Q domain of Gro/TLEs was shown to repress transcription in cultured cells when recruited to DNA via fusion to the GAL4 DNA-binding domain (Fisher et al., 1996), and point mutations in the AH1 and AH2 motifs were found to interfere with the ability of Gro/TLEs to mediate transcriptional repression both in vitro and in vivo (Chen et al., 1998; Song et al., 2004), suggesting that tetramerization of Gro/TLE proteins is required for their repressive function. In addition to the Q domain, the GP and SP regions of Gro/TLEs were also found to play direct roles in transcriptional repression. Both regions are able to direct repression when targeted to DNA (Grbavec et al., 1998; Chen et al., 2000).

Gro/TLE proteins have been proposed to be involved in chromatin remodelling. They were shown to associate with chromatin and to specifically interact with the amino-terminal domain of histone H3 (Palaparti et al., 1997). In addition, Gro/TLE1 was shown to associate with the nuclear matrix (Javed et al.,2000). These findings suggested that Gro/TLE-mediated repression may involve chromatin-dependent mechanisms.

Furthermore, *Drosophila* Groucho interacts with the histone deacetylase Rpd3. In particular, these proteins form a complex *in vivo* and Rpd3 interacts directly via the Groucho GP domain (Chen et al., 1999). Genetic analysis of embryos doubly heterozygous for groucho and Rpd3 revealed high levels of embryonic lethality as well as bicaudal patterning defects not seen in single heterozygous embryos, suggesting a functional interaction between Groucho and Rpd3 (Chen et al., 1999). In addition, mutation in Rpd3 prevented the potentiation of Groucho-mediated repression of Rpd3 in cultured cells. Consistent with these findings, Groucho primarily interacts with hypoacetylated histone H3 (Flores-Saaib and Courey, 2000). All together, these observations suggested that, when targeted to DNA, oligomers of Gro/TLE proteins may interact with histones where recruitment of histone deacetylases would cause deacetylation of histones leading to the generation of transcriptionally silenced chromatin structure (Flores-Saaib and Courey, 2000). In agreement with this model, the transcriptional repression activity of complexes of Gro/TLE proteins and the DNA binding transcription factor Brain Factor-1 were shown to utilize histone deacetylase activity (Yao et al., 2001).

Recent studies have also suggested that the recruitment of histone deacetylases may not be the only mechanism by which Gro/TLEs mediate transcriptional repression. In fact, the Q domain of Gro/TLE was shown to interact with the basal transcription factor TFIIE in HeLa nuclear extracts, consistent with the finding that unc-37 genetically interacts with component of the basal transcriptional machinery in *C. elegans* (Yu et al., 2001; Zhang et al., 2002). This result suggests that Gro/TLE proteins may also repress

transcription by establishing inhibitory interactions with the basal transcriptional machinery.

1.1.12 Functional diversity of Gro/TLE proteins as corepressors during development

Drosophila groucho mutant embryos display multiple defects in many developmental processes including neurogenesis, segmentation, sex determination, dorsal/ventral and terminal pattern formation, as well as patterning of the compound eye, suggesting a broad involvement of Groucho during embryogenesis (Paroush et al., 1994, Paroush et al., 199, Dubnicoff et al., 1997, Aronson et al., 1997, reviewed by Parkhurst, 1998 and Chen et al., 2000). In agreement with these findings, Groucho was shown to interact with numerous sequence-specific DNA-binding transcription factors implicated in diverse cell differentiation events (Fisher and Caudy, 1998, Parkhurst, 1998, Chen et al., 2000). Interestingly, a number of these transcription factors appear to recruit Gro/TLE proteins to DNA via short peptide motifs. For example, homeodomain-containing proteins like Engrailed, Goosecoid and Nkx associate with Groucho through the engrailed homology domain 1 (eh1) motif (F-S-I-X-X-I/L-I/L) (Jimenez et al., 1997; Choi et al., 1999), bHLH proteins like Hes, utilize the WRPW motif (Paroush et al., 1994; Fisher et al., 1996, Grbavec et al., 1996; McLarren et al., 2000), and zinc-finger proteins such as Huckebein use the FRPW motif (Goldstein et al., 1999).

Gro/TLE proteins not only associate with factors acting as dedicated transcriptional repressors but also with factors that can act as transcriptional activators. In these cases, Gro/TLEs often function to convert transcriptional activators into repressors. These factors include Rel domain containing proteins like Dorsal (Dubnicoff et al., 1997;

Valentine et al., 1998; Tetsuka et al., 2000), as well as HMGbox proteins such as TCF/LEF family members (Roose et al., 1998; Brantje et al., 2001). In addition, Gro/TLEs interact with proteins that can act as both transcriptional activators and repressors. Such proteins are for example Runt domain proteins like Runt and AML (Aronson et al., 1997; Levanon et al., 1998; McLarren et al., 2000), and Paired domain-containing proteins like Pax5 (Eberhard et al., 2000).

Recent studies have demonstrated that modulation of the phosphorylation state of Gro/TLE may be a mechanism by which the activities of these proteins may be regulated. In particular, the interaction of Gro/TLE with certain DNA binding proteins leads to hyperphosphorylation of Gro/TLE (Eberhard et al., 2000; Nuthall et al., 2002). In particular, the interaction with Hes1 leads to Gro/TLE hyperphosphorylation, increased transcription repression activity and nuclear association (Nuthall et al., 2002). This process is thought to require protein kinase CK2, which can phosphorylate Gro/TLE1 at serine239 in vivo. Mutation of this conserved residue into alanine decreases Hes1-induces hyperphosphorylation of Gro/TLE1 and also reduces both the nuclear association and the transcription repression activity of Gro/TLE1 (Nuthall et al., 2004). Moreover, phosphorylation of S239 was shown to be required for the antineurogenic function of Gro/TLE1 (Nuthall et al., 2004). These results suggest that the transcription repression activity of Gro/TLE proteins is regulated by phosphorylation events induced by interaction with DNA binding partners and provide additional insights into the regulation of Gro/TLE activity during neuronal differentiation.

1.1.13 BF-1 is essential for the development of the telencephalon

During the development of the telencephalon, Gro/TLE proteins were shown to work together with an important regulator of cortical neuronal development named Brain Factor 1 (BF-1, also known as Foxg1) (Kaestner et al., 2000). BF-1 is a member of the Winged-Helix (WH) family of transcription factors (Tao and Lai, 1992). Members of this family are characterized by the presence of a highly conserved 110-aa DNA binding domain, the winged-helix domain, first identified in the HNF-3 proteins (Lai et al., 1990; Lai et al., 1991; Clark et al., 1993). BF-1 plays crucial roles in the development of specific forebrain structure such as telencephalic vesicles, olfactory bulbs and optic vesicles (Tao and Lai, 1992; Hatani et al., 1994; Xuan et al., 1995; Bourguignon et al., 1998).

BF-1 expression in the developing brain is restricted to the telencephalic neuroepithelium, the nasal half of the retina and the optic stalk. In the developing telencephalic neuroepithelium, BF-1 expression is high in the proliferating neural progenitor cells located in the VZ, and continues to be detected in postmitotic cells (Tao and Lai, 1992; Hatani et al., 1994; Dou et al., 1999). In the adult brain, BF-1 persists in neurons and glia of the cerebral hemisphere. A number of studies have demonstrated that BF-1 is an essential regulator of forebrain development. Analysis of BF-1 null mouse embryos revealed that they are normal at E9.5 but by E10.5 they exhibit severe hypoplasia of the cerebral hemispheres. This phenotype appears to be caused by a drastic reduction in the proliferation of the neuroepithelial cells in the VZ of the developing telencephalon as well as a premature onset of neuronal differentiation (Xuan et al., 1995; Huh et al., 1999).

Studies in *Xenopus* also suggested an important role for BF-1 in neuronal development. Ectopic expression of *Xenopus* BF-1 (XBF-1) seems to have a dual effect. High doses of XBF-1 suppressed endogenous neuronal differentiation resulting in an expansion of the neural plate (Bourguignon et al., 1998). Further observations led to the proposal of two mechanisms by which high doses of XBF-1 cause neural plate expansion. First, XBF-1 was shown to convert ectoderm to neural fate, such that more neural ectoderm formed at the expense of epidermis. Second, XBF-1 promoted the proliferation of neuroectodermal cells. Both of these effect are thought to contribute to the expansion of the neural plate (Bourguignon et al., 1998; Hardcastle and Papalopulu, 2000).

Interestingly, neuronal differentiation was ectopically induced in cells adjacent to high XBF-1-expressing cells. In agreement with this and in contrast to XBF-1 high dosage, a low concentration of XBF-1 did not suppress neuronal differentiation but instead induced ectopic neuronal differentiation (Bourguignon et al., 1998). These results suggest that XBF-1 has a dual activity dependent on its concentration, acting as a suppressor or an activator of neurogenesis (Bourguignon et al., 1998).

1.1.14 BF-1 regulates dorsal-ventral and anterior-posterior patterning of the telencephalon

BF-1 null mutant embryos show severe dorsal-ventral patterning defects. Ventral markers such as Dlx-2 and Nkx2.1 are absent whereas dorsal markers such as Emx2 and Pax6 are expanded to more ventral domains (Dou et al., 1999). The dorsal-ventral patterning of the forebrain is regulated by ventral patterning signals such as Sonic Hedgehog (Shh) and dorsal signals such as Bone Morphogenic Proteins (BMP). BMPs derive from the non-

neural ectoderm flanking the neural plate (Shimamura et al., 1997). In BF-1 knockout mice, loss of Shh expression was observed specifically within the developing ventral telencephalon (Huh et al., 1999; Dou et al., 1999). In contrast, BMP2, 4, 6 and 7 were ectopically expressed in the dorsal telencephalon (Dou et al., 1999; Hanashima et al., 2002). These results suggest that BF-1 contribute to dorsal-ventral patterning both through inducing Shh expression and repressing BMP expression (Dou et al., 1999).

Other studies have also suggested a role for BF-1 in anterior-posterior patterning of the forebrain. Cells at the junction between the anterior neural plate and the anterior ectoderm define a structure called the anterior neural ridge (ANR). Fibroblast growth factor 8 (FGF8) is present in the ANR at the four somite stage (Crossley and Martin, 1995; Shimamura et al., 1997) and in neural plate explant studies, it has been demonstrated that FGF8 is able to substitute for the activity of the ANR and to induce BF-1 expression in neural tissue. These observations suggest that FGF8 may be important in the initiation of BF-1 expression in the anterior neural ridge and that a patterning role is attributed to BF-1 for anterior-posterior neural plate axis formation in response to FGF8 (Shimamura et al., 1997).

1.1.15 BF-1 is an important regulator of cell proliferation, differentiation and cell fate

A number of studies have demonstrated that winged-helix transcription factors are important for cell proliferation (Li et al., 1993, 1997; Xuan et al., 1995, Bourguignon et al., 1998; reviewed by Kaufmann and Knorchel, 1996). In addition to its role in neural patterning, BF-1 is a multifunctional molecule involved in other processes during forebrain development. BF-1 loss-of-function studies in mice showed a reduction of

progenitor cell proliferation and their premature differentiation, which results in reduced cerebral hemisphere size (Xuan et al., 1995). In addition, ectopic expression of XBF-1 in *Xenopus* caused a suppression of neuronal differentiation and an expansion of the neural tube, possibly due to both an increase in neural progenitor cell proliferation and a switch of tissue fated to become ectoderm to a neural fate (Bourguignon et al., 1998; Hardcastle and Papalopulu, 2000). These results suggest that BF-1 might regulate neural progenitor cell proliferation and the timing of neuronal differentiation in the developing telencephalon (Xuan et al., 1995; Bourguignon et al., 1998; Dou et al., 1999; Hardcastle and Papalopulu, 2000).

More recent studies have suggested that BF-1 controls the proliferation and differentiation of progenitor cells through different mechanisms. Genetic analysis in mice where endogenous BF-1 protein have been replaced with a DNA binding defective form of BF-1 (BF-1^{NHAA}) revealed that BF-1 regulates progenitor cell proliferation and differentiation in the neocortex through distinct DNA binding-independent and binding-dependent mechanisms, respectively (Dou et al., 2000; Hanashima et al., 2002). Compared with wild-type embryos, the proliferation of progenitor cells was maintained in BF-1^{NHAA} mutant embryos, indicating that BF-1^{NHAA} protein can substitute for the wild-type BF-1 protein (Hanashima et al., 2002). This result suggested that BF-1 promotes progenitor cell proliferation through a DNA binding-independent mechanism. Consistent with this finding, previous *in vitro* studies demonstrated that BF-1 antagonizes the antiproliferative activity of Transforming-Growth-Factor β (TGF-β) through a DNA binding-independent mechanism (Dou et al., 2000; Rodriguez et al., 2001). TGF-β and related peptides are expressed in the developing brain during neurogenesis (reviewed by

Böttner et al., 2000). They inhibit the proliferation of many types of epithelial cells in the embryos (Feijen et al., 1994; Furuta et al., 1997; Pelton et al., 1991; Moustakas et al., 2002). TGF-β signalling cascade is initiated when TGF-β ligands binds to the TGF-β type II receptor, which in turn trans-phosphorylates and activates the TGF-β type I receptor. This leads to the phosphorylation of cytoplasmic Smad3 proteins, which associate with Smad4. Together, they are translocated to the nucleus where they form transcriptional complexes with different DNA binding partners. Ultimately, the TGF-β cascade leads to the transcriptional activation of cyclin-dependent inhibitors such as p21Cip1 and p15nk4b (Alexandrow and Moses, 1995). BF-1 was shown to be involved in the negative regulation of the TGF-β signalling cascade by, in part, interacting with the transcriptional coactivator partner of Smad2, FAST-2 (Dou et al., 2000) and by interacting with Smad proteins themselves, preventing them from binding DNA in the nucleus (Rodriguez et al., 2001). As an indirect effect of these interactions, BF-1 was shown to repress expression of the cyclin-dependent kinase inhibitor p21Cip1 (Rodriguez et al., 2001; Seoane et al., 2004).

In contrast to the mechanism by which BF-1 regulates progenitor cell proliferation, BF-1 was shown to negatively regulate neuronal differentiation through a DNA binding-dependent mechanism. In fact, compared with wild-type embryos, embryos carrying the DNA-binding defective form of BF-1 exhibited an increase in the number of cells stained for the neuronal marker MAP2 in the mantle zone, suggesting that the regulation of neuronal differentiation requires the DNA binding activity of BF-1 (Hanashima et al., 2002). It was previously shown that BMP genes such as BMP2, 4, 6 and 7 are ectopically expressed in the telencephalic neuroepithelium of BF-1 (-/-) mutant

(Dou et al., 1999; Hanashima et al., 2002). The BMPs belong to the TGF-β superfamily and they have been demonstrated to play a role during neurogenesis. For example, BMP4 inhibits proliferation of neural progenitor cells and promotes their differentiation into neurons. Mutant embryos for the DNA-binding defective form of BF-1 also exhibited ectopic expression of BMPs like the null mutant suggesting the requirement of a DNA binding activity by BF-1 to repress BMPs expression and, consequently, to inhibit neuronal differentiation.

Studies in *Xenopus* have reported that BF-1 affects directly the expression of the cdk inhibitor p27Xic1, without prior protein synthesis (Hardcastle and Papalopulu, 2000). p27Xic1 expression is restricted to the mesoderm where it coincides with non-dividing cells. It has been reported that p27Xic1 is highly expressed in cells destined to become primary neurons and is crucial at early stages of neurogenesis (Vernon et al., 2003). Overexpression of p27Xic1 promotes ectopic neurons formation while its ablation prevents differentiation of primary neurons (Vernon et al., 2003). XBF-1 was shown to regulate p27Xic1 expression in a dose-dependent manner where high or low doses of XBF-1 suppressed or ectopically induced p27Xic1 expression (Hardcastle and Papalopulu, 2000), suggesting that regulation of p27Xic1 may be a mechanism by which XBF-1 affects the differentiation of neural progenitor cells.

Recent studies have provided additional insight into the functions of BF-1 in cell fate regulation during mammalian cerebral corticogenesis. The adult mammalian cerebral cortex comprises six layers of neurons. The neurons in each layer are generated in an orderly sequence during development and exhibit unique traits. Transplantation studies have suggested that early-born neurons can adopt later cell fate but not the converse

(Jessell, 2000; McConnell and Kaznowski, 1991; Frantz and McConnell, 1996; Desai and McConnell, 2000). Cajal-Retzius (CR) cells, which reside in layer 1, are the earliest born neurons and they have been demonstrated to be very important for the development of a well organized cerebral cortex (D'Arcangelo et al., 1995; Frotscher, 1995; Hirotsune et al., 1995; Marin-Padilla, 1998). In the cortex of mice lacking BF-1, CR neurons were overproduced at the expense of ER81 neurons of layers VI and V, which are usually generated later (Hanashima et al., 2004). BF-1 is normally expressed by neuronal progenitor cells and postmitotic neurons but is completely absent in mature CR neurons in the cortex suggesting that early neuronal fate is actively suppressed by BF-1 in later progenitors (Hanashima et al., 2004). Conditional knock-out of BF-1 in later progenitor cells, after CR neurons were generated, demonstrated that in mice where BF-1 expression was rescued, the correct laminar organization was restored (Hanashima et al., 2004). Active removal of BF-1 in these mice reinitiated CR cell production. Interestingly, cell-labelling with bromodeoxyuridine showed that the progenitor cells that are normally destined to generate deeper layer neurons (ER81) switched fated to produce CR neurons instead (Hanashima et al., 2004). Together, these results suggested that BF-1 is required to suppress CR cell fate and implicate BF-1 in the regulation of early cortical cell fate during mammalian corticogenesis.

1.1.16 BF-1 may act as both as a transcriptional repressor and activator

BF-1 acts primarily as a transcriptional repressor (Li et al., 1995; Bourguignon et al., 1998; Yao et al., 2001). This was first suggested based on the analysis of the avian BF-1 homologue Qin. Qin shares 98% sequence homology with BF-1 in the DNA binding

domain and the N-terminus (Li et al., 1995) and it can bind DNA and mediate repression of a basally active promoter in transiently transfected mammalian cell lines (Li et al., 1995; Li et al., 1997). The first suggestion that BF-1 mediates transcriptional repression came from a study in *Xenopus* where ectopic expression of fusion proteins of the DNA binding domain of XBF-1 and the strong repressor domain of Drosophila Engrailed mimicked the effects of full-length XBF-1 overexpression (Bourguignon et al., 1998). The repression domain of Engrailed was shown to repress transcription through interaction with Groucho (Jimenez et al., 1997; Tolkunova et al., 1998). Thus, these findings suggested that BF-1 may also utilize Groucho to mediate its repressive function. This possibility was supported by the fact that the winged-helix protein HNF-3β was shown to interact with Gro/TLE proteins (Wang et al., 2000) and that BF-1 and Gro/TLE proteins are coexpressed in neural progenitor cells of the mammalian telencephalon (Dehni et al., 1995; Xuan et al., 1995; Yao et al., 1998; Yao et al., 2000). Following these observations, BF-1 was shown to directly interact with TLEs *in vivo* (Yao et al., 2001). Furthermore, the ability of BF-1 to mediate transcriptional repression was promoted by TLEs. This activity may require the participation of histone deacetylase since BF-1 was shown to associate with HDAC1 in mammalian cells and BF-1 repressive function was reduced by an inhibitor of histone deacetylase activity (Yao et al., 2001). These results led to the proposal of a mechanism by which BF-1 recruits TLEs and histone deacetylases to repress transcription and, consequently, to negatively regulate neuronal differentiation in the developing telencephalon (Yao et al., 2001).

Other evidence suggested that BF-1 may also work as a transcriptional activator. This comes from a study in Xenopus where overexpression of fusion proteins of XBF-1

to the transcription activation domain of the adenoviral E1A protein specifically suppressed neuronal differentiation, similar to the phenotype caused by ectopic expression of wild-type XBF-1 protein (Bourguignon et al., 1998). The molecular mechanisms by which BF-1 might act as a transcriptional activator are still unclear and under investigation.

1.1.17 Groucho-related-gene 6 (Grg6)

Recently, a new member of the Gro/TLE family of proteins has been identified and designated Groucho-related-gene 6 or Grg6 (Dang et al., 2001). Very little is known about Grg6. It has been discovered based on studies designed to identify downstream targets regulated by the oncogenic fusion protein E2A-HLF in mice. E2A-HLF is a chimeric transcription factor formed by the fusion of the transactivation domain of E2A and the bZIP DNA-binding and protein dimerization domain of Hepatic Leukemia Factor (HLF) (Hunger et al., 1992; Inaba et al., 1992). E2A-HLF is involved in pro-B cell development where it can block apoptosis of murine pro-B lymphocytes (Inaba et al., 1996). Several Grg proteins were shown to be up-regulated in response to enforced expression of E2A-HLF: Grg6 was one of them.

DNA sequence analysis revealed that Grg6 is related to Gro/TLE proteins with the main region of homology being the WD domain (Dang et al., 2001). However, Grg6 encodes a shorter protein of 65kDa compared to the average 95kDa for Gro/TLE proteins (Stifani et al., 1992; Dang et al., 2001). Like other Gro/TLE proteins, Grg6 is widely express both in the developing embryo and adult mice (Stifani et al., 1992; Dang et al., 2001). Based on this structural similarity, our objective was to learn more about the

molecular functions of Grg6 and, ultimately, its possible involvement in neuronal differentiation. To address this, we have tested whether Grg6, like other Gro/TLEs, is expressed in the developing nervous system. We have also examined whether Grg6 is able to physically and functionally interact with DNA-binding proteins known to form transcriptional complexes with other Gro/TLE proteins during neuronal development. We have further investigated whether Grg6 is able to negatively regulate transcription like other Gro/TLEs. Finally, to determine whether Grg6 is able to antagonize neuronal differentiation like other Gro/TLE proteins, we have examined the effect of exogenous Grg6 expression on the neuronal differentiation of cortical progenitor cells. The results of these studies are presented in this thesis and are the first characterization of the functions of Grg6.

2. MATERIALS AND METHODS

2.1 Plasmids. PCR was used to amplify the sequence encoding full-length Ggr6 (oligonucleotide primers Grg6-1 [5'-GATGACTTCCCACAGACAGAGC-3'] and Grg6-2 [5'-GTGTACCACATCAAGTACTGA-3']) using pMT-CB6-Ggr6 plasmid as template (Dang et al., 2001). The Grg6 primers map the start and stop codon of the Grg6 sequence. The PCR product with blunt ends was subcloned into pCMV2-FLAG (Sigma) digested with *Eco*RV. Construct peGFP-Grg6 was generated by first digesting pCMV2-FLAG-Grg6 with *Eco*RI and *Kpn*1 followed by subcloning into peGFP-C1 (Clonetech) digested with *Eco*RI and *Kpn*1. Plamid pCMV2-HA-Grg6 was obtained by digesting pCMV2-FLAG-Grg6 with *Hind*III and *Kpn*1 followed by subcloning into pCMV2-HA digested with *Hind*III and *Kpn*1. pcDNA3-GAL4-Grg6 was generatd by subcloning the Grg6 PCR product obtained with primers Grg6-1 and Grg6-2 into the filled-in BamH1 site of pcDNA3-GAL4bd. Plasmids pMYC-Gro/TLE4, pCMV2-FLAG-Gro/TLE1, pCMV2-FLAG-BF-1, pCMV2-FLAG-Hes1, pCMV2-FLAG-Hes1ΔWRPW, pcDNA3-GAL4bd-Gro/TLE1, pEBG-Gro/TLE1, pEBG-Gro/TLE1 (1-135), p6B-CMV-Luc, (luciferase gene under the control of the cytomegalovirus [CMV] promoter linked to six BF-1 binding sites), p6N-βactin-Luc (luciferase gene under the control of the β-actin promoter linked to six Hes1 binding sites), p5X-UAS GAL4-SV40-Luc (luciferase gene under the control of the simian virus 40 promoter linked to five GAL4 upstream activation sequence (UAS_G) sites) were described previously (Sasai et al., 1992; Li et al., 1995; Grbavec et al., 1998; Yao et al., 1998; Grbavec et al., 1999; Eberhard et al., 2000; McLarren et al., 2000; McLarren et al., 2001; Yao et al., 2001; Swingler et al., 2004).

2.2 Transient transfection, protein-protein interaction assays and Western blotting analysis. Human HeLa or HEK 293 cells were grown and, when appropriate, transfected with the SuperFect reagent (Qiagen) as described previously (McLarren et al., 2001, Nuthall et al., 2002). Coprecipitation assays using plasmids pEBG-Gro/TLE1 and pEBG-Gro/TLE1 (1-135) (or pEBG as control), immunoprecipitation experiments with anti-FLAG-epitope (Sigma) or anti-Gro/TLE1 antibodies, and Western blotting studies were performed as described previously (Dehni et al., 1995, Husain et al., 1996, Palaparti et al., 1997, McLarren et al., 2000, McLarren et al., 2001, Yao et al., 2001, Nuthall et al., 2002). The antibodies used for Western blotting analysis were anti-FLAG (Sigma), anti-GFP (Molecular Probes), anti-hemagglutinin (anti-HA) (Roche), anti-panTLE (Stifani et al., 1992, Dehni et al., 1995, Palaparti et al., 1997), anti-GST and anti-GAL4bd (Santa Cruz Biotechnology), anti-MYC (BD Pharmingen) or anti-Gro/TLE1 (Husain et al., 1996, Yao et al., 1998, Yao et al., 2000). Rabbit polyclonal antibodies against Grg6 (Dang et al., 2001) were kindly provided by Dr. Jinjun Dang and used for Western blotting in a 1:200 dilution after affinity purification.

2.3 Affinity purification of Grg6 antibodies. Kidneys from adult mouse were dissected and prepared in a fine tissue suspension in saline (0.9% NaCl) (1g of tissue/ml of saline). 8 ml of acetone were added per 2ml of tissue suspension and incubated on ice for 30 min, followed by centrifugation at 10 000xg for 10 min. The pellet was resuspended with fresh acetone, vigorously mixed and incubated on ice for 10 min, followed by centrifugation at 10 000xg for 10 min. The pellet was transferred to a piece of filter paper and allowed to air-dry at room temperature. The powder was kept at 4°C. Fifty µl of Grg6 serum was

then mixed with 1 ml of PBS and kidney acetone powder was added to a final concentration of 1%, followed by incubation at 4°C for 30 min and centrifugation at 10 000xg for 10 min. The supernatant was recovered and considered as acetone treated Grg6 serum. 293A cells were transfected with FLAG-Grg6 (total of 8 μg DNA), cell lysates were prepared and subjected to SDS-PAGE on a 8% gel followed by tranfer to a nitrocellulose membrane. A vertical strip was cut and subjected to Western blot analysis with anti-FLAG antibodies to visualize the position of migration of FLAG-Grg6. Based on that position, a horizontal strip was cut and incubated for 2 hrs in incubation buffer [I.B., 25 mM Tris/HCl, pH 7.6, 150 mM NaCl, 0.05% Triton X-100, 5% milk powder], followed by incubation with acetone treated Grg6 serum. The nitrocellulose strip was washed extensively with I.B. and PBS. The bound Grg6 IgGs were eluted by incubation for 5 min in 1M acetic acid and 100mM glycine, pH 3.0. The supernatant was collect and mixed immediately with an equal volume of 1M KH_2PO_4. The affinity purified Grg6 antibodies were stored at -20°C.

2.4 Indirect immunofluorescence. COS-7 and cortical neural progenitor cells were cultured on four well chamber slides (Nalgene Nunc Int.) as described (Gratton et al., 2003), fixed and permeabilized in 0.1% IGEPAL in Hepes-buffered saline containing 4% paraformaldehyde. Cos-7 cells were transfected with either peGFP-Grg6 or pCMV2-FLAG-BF-1. Primary antibodies for immunofluorescence were either mouse anti-FLAG, rabbit anti-Grg6, mouse anti-Ki67 (1:25; BD Pharmingen) (a marker of undifferentiated neural progenitor cells) or mouse anti-NeuN (1:50; Chemicon) (a marker of differentiated neurons). Detection was as described in Gratton et al., (2003).

2.5 Transcription assays. HeLa or HEK 293 cells were transiently transfected using the SuperFect reagent. The total amount of tranfected DNA was adjusted in each case to 3μg per well using pEFBos. Transcription studies were performed with reporter plasmids p6B-CMV-Luc (500 ng/transfection), p6N-βactin-Luc (1μg/transfection) or p5X-UAS GAL4-SV40-Luc (2 μg/transfection). Effector plasmids included pCMV2-FLAG-BF-1 (15 ng/transfection), pCMV2-FLAG-Grg6 (25 and 50 ng/transfection), pcDNA3-Gro/TLE1 (100 ng/transfection), pCMV2-FLAG-Hes1 (50 ng/transfection), pcDNA3-GAL4bd (500 ng/transfection), pcDNA3-GAL4bd-Grg6 (50, 200 and 500 ng/transfection), or pcDNA3-GAL4bd-Gro/TLE1 (50, 200 and 500 ng/transfection). In each case, 250 ng of pCMV-βgalactosidase plasmid DNA was cotransfected to provide a means of normalizing the assays for transfection efficiency. Luciferase activity values were expressed as mean values ± SD. For analysis of FLAG-BF-1, FLAG-Grg6, FLAG-Hes1, GAL4bd-Grg6 and GAL4bd-Gro/TLE1 expression, one-tenth of total cell lysates was loaded on SDS-PAGE gels, followed by Western blotting analysis with anti-FLAG or anti-GAL4bd antibodies.

2.6 Cortical neural progenitor cell culture, transfection, and analysis of neuronal differentiation. Primary neural progenitor cell cultures were established from dorsal telencephalic cortices dissected from embryonic day (E) 13.5 mouse embryos as described previously (Gratton et al., 2003). Cells were seeded into 4-well chamber slides (Nalgene Nunc Int.) ($4x10^5$ cells/well) coated with 0.1% poly-D lysine and 0.2% Laminin (BD Biosciences). Cells were cultured in Neurobasal medium supplemented with 1% N2, 2% B27, 0.5 mM glutamine, 1% pennicilin-streptomicin (Invitrogen) and 40 ng/ml FGF2

(Collaborative Res.). After 48h in vitro, cells were transfected as described in Gratton et al., (2003) using plasmids encoding either enhanced GFP alone (0.2 μg/well) or combination of GFP and Gro/TLE1 (0.3 μg/well), Grg6 (0.3μg/well) or BF-1 (0.3 μg/well). The total amount of DNA was adjusted to 1.0 μg/transfection. Cells were allowed to differentiate until day 3 in vitro, when they were fixed and subjected to double-label immunocytochemical analysis of the expression of GFP, Ki67, or NeuN. Digital image acquisition and analysis were performed with Northern Eclipse software (Empix Inc.). Results were expressed as mean values ± SD.

2.7 RT-PCR. Total mRNA was extracted from cortical progenitor cells taken from the dorsal telencephalon of E15.5 mice embryos and subjected to reverse transcription with M-MuLV reverse transcriptase (NEB) as described in the manufacturer's instructions. As negative control, reactions were also performed without reverse transcriptase (RT). cDNA samples were stored at -20˚C. The thermocycling parameters and the sequences of the specific primers for PCR are as follows. 94˚C for 4 min, 25 cycles at 94˚C for 1 min, 50˚C for 45 s and 72˚C for 45 s, and 72˚C for 6 min using sense primer 1 (5'-CCTAGCACAGCACTCTG-3'), antisense primer 2 (5'-TGGATACAACTTACCTG-3'), or antisense primer 3 (5'-GTGTACCACATCAAGTACTGA-3'). These primers amplify a ~1000 bp product (primers 1 and 3) or a ~300 bp product (primers 1 and 2). Twenty microliters of the PCR products was electrophoresed on a 1.0% agarose gel.

2.8 Preparation of whole cell lysates from neural tube tissues. Forebrain, midbrain and spinal cord tissues were dissected from E16.0 mouse embryos and lysed for 30 min at

4ºC in lysis buffer containing 0.25 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100 and proteinase inhibitor cocktail "Complete". Each lysate were loaded on a 8% SDS-PAGE gel and transferred to a nitrocellulose membrane followed by Western blotting analysis with affinity purified anti-Grg6 antibody (1:200).

3. RESULTS

3.1 Characterization of Grg6 antibodies. To begin to examine the properties of Grg6, we characterized a previously described anti-Grg6 antibody, following affinity purification as described in Materials and Methods (Dang et al., 2001) (Fig. 1). Lysates from human 293A cells non-transfected or transfected with FLAG epitope-tagged forms of Gro/TLE1 or Grg6, were subjected to Western blot analysis with the affinity purified anti-Grg6 antibody (Fig. 1A, lanes 1-3) or anti-FLAG antibody (Fig. 1A, lanes 4-6). The anti-FLAG antibody recognized FLAG-Gro/TLE1 and FLAG-Grg6 as proteins of roughly 95 and 67 kDa, respectively (Fig. 1A. lanes 5-6). The affinity purified anti-Grg6 antibody decorated only the FLAG-Grg6 protein but not FLAG-Gro/TLE1. This antibody reacted with a slower component of roughly 67 kDa and a species of slightly faster mobility (Fig. 1A, lane3). The slower 67 kDa component comigrated with the FLAG-Grg6 protein recognized by the anti-FLAG antibody, suggesting that it may correspond to full-length Grg6. The faster form likely corresponds to a break-down product of the protein lacking the N-terminal FLAG epitope, since it was not detected using the anti-FLAG antibody (Fig. 1A, cf. lane 3 and 6). No immunoreactive bands were decorated by the anti-Grg6 antibody in non-transfected cells or in cells transfected with FLAG-Gro/TLE1 (Fig. 1A, cf. lane 3 and lanes 1-2). To further characterize the anti-Grg6 antibody, immunocytochemical studies were performed in COS-7 cells transfected with a fusion protein of GFP and Grg6. We found that the GFP fluorescence and Grg6 immunoreactivity overlapped (Fig. 1, cf. panel D and panels B-C), suggesting further that the anti-Grg6 antibody reacts with Grg6. Interestingly, Grg6 exhibited a predominatly

cytoplasmic localization in these cells. This is in contrast to the nuclear localization of canonical Gro/TLE proteins (Stifani et al., 1992). Together, these results suggest that the anti-Grg6 antibody is specific to Grg6 and that this protein is localized to the cytosol in transfected COS-7 cells.

3.2 Grg6 expression in neural cells. Gro/TLE proteins are expressed in both the CNS and PNS where their expression is correlated with both neural progenitors and differentiated neurons (Stifani et al., 1992; Dehni et al., 1995; Liu et al., 1996; Grbavec et al., 1998; Yao et al., 1998). Because previous studies have shown the presence of *Grg6* transcripts in adult brain tissues (Dang et al., 2001), we investigated whether Grg6 is also expressed in the developing mammalian nervous system. Lysates from forebrain or midbrain tissues from E16.0 mouse embryos, together with lysates from 293A cells either non-tranfected or transfected with FLAG-Grg6, were subjected to Western blot analysis using the anti-Grg6 antibody (Fig. 2A). This antibody detected FLAG-Grg6 in transfected cells but not in non-transfected cells (Fig. 2A, cf. lanes 1 and 2). A band of about 65 kDa was detected in both forebrain and midbrain lysates (Fig. 2A, cf. lanes 2-4), suggesting the presence of endogenous Grg6 in these tissues. A similar immunoreactive band was also observed in spinal cord tissues (data not shown). These results suggest that Grg6 is expressed in brain tissues.

Based on these findings, we focused our study of Grg6 on the forebrain, where the role of Gro/TLE proteins has been characterized previously. In particular, Gro/TLE1 was shown to be expressed in the forebrain and to act as a negative regulator of neurogenesis in that region (Dehni et al., 1995; Yao et al., 1998; Yao et al., 2000). To examine the

expression of Grg6 transcripts in neural cells, we performed RT PCR analysis on mRNA isolated from primary cultures of cortical neural progenitor cells obtained from the telencephalon of E15.5 mouse embryos using primers specific to regions of the WD domain of Grg6 (Fig. 2B). Using two different combinations of oligonucleotide primers, we detected specific PCR products of about 300 and 1000 bp (Fig. 2B, lanes 1 and 3). These products were not observed if the mRNA was not treated with reverse transcriptase (Fig. 2B, lanes 2 and 4). These results suggest that Grg6 is expressed in cortical neural progenitor cells of the dorsal telencephalon. To further test this possibility, we subjected cultures of cortical progenitor cells to immnunocytochemical analysis using anti-Grg6 antibodies and antibody against the mitotic cell marker Ki67 (Fig. 2C-F), or the neuronal marker NeuN (Fig. 2G-J). As shown in panels E and I, Grg6 immunoreactivity overlapped with both Ki67 and NeuN expression, suggesting that Grg6 is express in both mitotic cells and neurons. Importantly, endogenous Grg6 expression was mainly nuclear in these primary cell cultures. Together, these findings suggest that, like other Gro/TLE proteins, Grg6 is expressed in differentiating neural cells. Moreover, they suggest that Grg6 may be involved in nuclear functions in these cells.

3.3 Interaction of Grg6 with Brain-Factor 1 but not with Hes1. The expression of Grg6 in the telencephalon suggests that, like other Gro/TLE proteins, Grg6 may be implicated in cortical neurogenesis. To begin to address this question, we tested whether Grg6 might interact with DNA-binding proteins that are known to form transcription complexes with other Gro/TLE proteins during neuronal development in the forebrain. The winged-helix protein BF-1 was previously shown to be expressed in the

telencephalon (Tao and Lai, 1992; Hatani et al., 1994; Dehni et al., 1995; Xuan et al., 1995; Yao et al., 1998; Yao et al., 2000). BF-1 and Gro/TLE interact with each other and can repress transcription together (Yao et al., 2001). The binding of Gro/TLE proteins to BF-1 involves their WD40 domain (Yao et al., 2001). Since the WD40 domains of Grg6 and Gro/TLE are highly related (Dang et al., 2001), Grg6 might also be able to interact with BF-1. To test this possibility, we performed coimmunoprecipitation assays in 293A cells transfected with FLAG-BF-1 and GFP-Grg6. We found that GFP-Ggr6 coimmunoprecipitated with FLAG-BF-1 (Fig. 3A, lane 4) but not when cells were transcfected with the FLAG vector alone (Fig. 3A, lane 5). GFP alone (Fig. 3A, lane 3) did not interact with FLAG-BF-1 (Fig. 3A, lane 6). These results suggest that Grg6 shares with Gro/TLE proteins the ability to interact with BF-1. To further investigate the interaction of Grg6 with BF-1, we tested if the intracellular localization of Grg6 changed in the absence or presence of BF-1. COS-7 cells were transfected with GFP-Grg6 alone (Fig. 4A-C) or in combination with FLAG-BF-1 (Fig. 4G-I), followed by double-label analysis of GFP and BF-1 expression. As shown above in Fig. 1, Grg6 displayed non-nuclear staining when expressed in the absence of BF-1 (Fig. 4C in green). BF-1 alone was localized to nuclei (Fig. 4F in red). In contrast, when cells were cotransfected with both Grg6 and BF-1, a significant amount of Grg6 immunoreactivity was detected in the nucleus, where BF-1 was also localized (Fig. 4G-I). A quantification of three separate experiments is shown in Fig. 4J. Together with the results of coimmunoprecipitation studies, these findings strongly suggest that Grg6 can interact with BF-1 and that this interaction results in a recruitment of Grg6 to nuclei.

To test the specificity of the interaction of Grg6 with BF-1, we asked if Grg6 would interact with Hes1, which is another transcription factor known to negatively regulate neuronal differentiation and to interact with Gro/TLEs. 293A cells, which express endogenous Gro/TLEs (Fig .3G, lanes 1-2), were transfected with HA-Grg6 (Fig. 3F, lanes 1-2) together with FLAG-Hes1 (Fig. 3E, lane 1) or with a truncated form of Hes1 lacking the WRPW motif necessary for Gro/TLE binding (FLAG-Hes1ΔWRPW) (Fisher et al., 1996; McLarren et al., 2001) (Fig. 3E, lane 2). Immunoprecipitation with anti-FLAG antibodies resulted in the coimmunoprecipitation of endogenous Gro/TLEs with FLAG-Hes1 (Fig. 3J, lane 3) but not FLAG-Hes1ΔWRPW (Fig. 3J, lane 4). HA-Grg6 did not coimmunoprecipitate with either Hes1 or Hes1ΔWPRW (Fig. 3I, lanes 3-4). Taken together, these findings demonstrate that Grg6 can interact specifically with BF-1, suggesting that Grg6 is involved in BF-1 functions.

3.4 Inhibition of BF-1-mediated transcriptional repression by Grg6. Previous studies have demonstrated that BF-1 acts primarily as a transcriptional repressor. Futhermore, it was shown that BF-1 can interact with Gro/TLEs and that the latter promote BF-1-mediated repression (Yao et al., 2001). To determine if BF-1 and Grg6 might also functionally interact, we monitored BF-1-mediated transcriptional repression in the absence or presence of Grg6. 293A cells were transfected with a previously described (Li et al., 1995) reporter construct containing the luciferase gene under the control of a CMV promoter linked to six tandem copies of a BF-1 binding site. Transfection of this reporter plasmid (p6B-CMV-Luc) alone resulted in a strong basal expression of the luciferase gene, which was designated as 100% (Fig. 5, lane 1). Cotransfection of a BF-1 expression

plasmid led to a repression of basal transcription (Fig. 5, lane 2). However, when BF-1 was cotransfected with Grg6, its repressive effect was significantly reduced (Fig .5, cf. lanes 2 and 3). In contrast, Gro/TLE1 potentiated BF-1-mediated repression (Fig. 5, cf. lanes 2 and 5). Cotransfection of Gro/TLE1 with BF-1 and Grg6 antagonized the negative effect of Grg6 on BF-1 (Fig. 5, cf. lanes 3 and 4). Grg6 alone had no significant effect on the activity of the p6B-CMV-Luc reporter (Fig. 5, cf. lanes 1 and 6) and neither Grg6 not Gro/TLE1 had a significant effect on the activity of the CMV promoter alone (Fig. 5, lanes 7-9). Moreover, Grg6 expression did not appear to affect BF-1 expression (data not shown). These results strongly suggest that, in contrast to Gro/TLE proteins, Grg6 is a negative regulator of BF-1 transcription repression activity.

3.5 Lack of a functional interaction between Grg6 and Hes1. Based on our previous results suggesting that Grg6 does not interact with Hes1, we hypothesized that Grg6 would not affect Hes1-mediated repression. To examine this possibility 293A cells were transfected with the previously described (Sasai et al., 1992) p6N-βactin-Luc reporter construct, containing the luciferase gene under the control of a basal β-actin promoter liked to six tandem copies of a Hes1 binding site. Cotransfection of Hes1 resulted in a repression of the reporter gene expression (Fig. 6A, cf lanes 2-4). Cotransfection of increasing amounts of Grg6 together with Hes1 had no significant effect on the repressor ability of Hes1 (Fig. 6A, cf. lanes 3-4 and 2). The presence of Grg6 alone had no significant effect on the reporter gene expression (Fig. 6A, cf. lanes 5-6 and 1). Both Hes1 and Grg6 proteins were expressed at similar levels (Fig. 6B). These results, together with the fact that Grg6 does not interact with Hes1, suggest further that Grg6 is not

involved in the regulation of Hes1 and further suggest the specificity of Grg6 for BF-1. These results also suggest that Grg6 does not sequester transcriptional cofactors away from Hes1.

3.6 Transcriptional repression by Gro/TLE1 but not Grg6. Gro/TLEs are non-DNA binding proteins. However, previous studies have demonstrated that Gro/TLEs can repress transcription in cultured cells when directly recruited to DNA by fusion to the GAL4 DNA binding domain (Fisher et al., 1996; Grbavec et al., 1998; Chen et al., 2000). The negative effect of Grg6 on BF-1 suggested that, in contrast to other Gro/TLEs, Grg6 may not be able to mediate transcriptional repression if recruited to DNA. To test this possibility, HeLa cells were transfected with a previously described (Catron et al., 1995; Fisher et al., 1996) reporter plasmid containing the luciferase reporter gene downstream from a simian virus 40 promoter liked to five tandem copies of the GAL4 upstream activation sequence (UAS), either alone or in combination with plasmids encoding GAL4bd or fusion proteins between GAL4bd and Grg6 or Gro/TLE1 (Fig. 7A). In agreement with previous studies (Fisher et al., 1996), the expression of GAL4bd alone resulted in a moderate (approx. 1.5 to 2.0-fold) activation of the reporter gene above basal levels (Fig. 7A, cf. lanes 1 and 2). Cotransfection of increasing amount of GAL4bd-Gro/TLE1 led to a repression of basal transcription from the UAS-simian virus40 promoter (Fig. 7A, cf. lanes 1 and 6-8). In contrast, cotransfection of increasing amounts of GAL4bd-Grg6 did not repress nor activate basal transcription (Fig. 7A, cf. lanes 1 and 3-5). Both proteins were expressed at equal levels (Fig. 7B). These results suggest that, in

contrast to other Gro/TLE proteins, Grg6 does not act as transcriptional repressor when targeted to DNA.

3.7 Reduced interaction of BF-1 with Gro/TLE1 in the presence of Grg6. Both Grg6 and Gro/TLE1 bind to BF-1. All three proteins are expressed in the telencephalon (Tao and Lai, 1992; Hatani et al., 1994; Dehni et al., 1995; Xuan et al., 1995; Yao et al., 1998; Yao et al., 2000; Fig. 2). To elucidate the mechanisms underlying the inhibitory effect of Grg6 on BF-1, we tested whether Grg6 might compete with Gro/TLE1 for binding to BF-1, thus titrating away the corepressor function that Gro/TLE provides to BF-1. 293A cells were transfected with FLAG-BF-1 alone or together with GFP-Grg6, followed by immunoprecipitation with anti-Gro/TLE1 antibodies to precipitate endogenous Gro/TLE1 proteins (Fig. 8). In the absence of GFP-Grg6 (Fig. 8, lane 1), FLAG-BF-1 coimmunoprecipitated efficiently with endogenous Gro/TLE1 (Fig. 8D, lane 5). However, when Grg6 was cotransfected with BF-1 (Fig. 8, lane 3), we failed to detect coimmunoprecipitation of BF-1 with Gro/TLE1 (Fig. 8, lane 7). Grg6 also failed to coprecipitate with endogenous Gro/TLE1, both in the absence or presence of BF-1 (Fig. 8B and E, cf. lanes 2-3 and 6-7). Under these conditions, Grg6 expression did not affect significantly the levels of expression of BF-1 or Gro/TLE1 (Fig. 8A and C, cf. lanes 1 and 3), suggesting that the absence of BF-1 in the immunoprecipitate was not simply due to a decrease in the levels of expression of these proteins. Together, these results suggest that Grg6 can antagonize the interaction of BF-1 with Gro/TLE1. They also suggest that Grg6 does not interact with Gro/TLE.

Previous studies have shown that Gro/TLEs can homo- or heterodimerize (Pinto and Lobe, 1996; Chen et al., 1998). We therefore directly tested whether Grg6 might interact with Gro/TLE1 (Fig. 9). 293A cells were transfected with plasmids encoding either MYC-Gro/TLE4, FLAG-Grg6, GST-Gro/TLE1 or GST-Gro/TLE1(1-135), a form of Gro/TLE1 containing only the first 135 amino acids required for dimerization. Gro/TLE4 (Fig. 9C, lane 4) coprecipitated with GST-Gro/TLE1 (Fig. 9D and F, lane 8). In contrast, Grg6 (Fig. 9A, lanes 1-3) failed to coprecipitate with GST-Gro/TLE1, GST-Gro/TLE1(1-135) or GST alone (Fig. 9B and F, lanes 5-7). Similar results were obtained in immunoprecipitation studies preformed with FLAG-Grg6 and MYC-Gro/TLE4 using FLAG antibodies, where Gro/TLE4 failed to coimmunoprecipitate with FLAG-Grg6 (data not shown). Taken together, these findings suggest that Grg6 can disrupt the interaction between BF-1 and Gro/TLE. This effect may be the result of a competition by Grg6 with Gro/TLE for BF-1 binding.

3.8 Promotion of cortical neurogenesis by Grg6. BF-1 and Gro/TLE1 can negatively regulate neuronal differentiation in the developing forebrain (Xuan et al., 1995; Yao et al., 2000). To determine whether Grg6 might also participate in the regulation of the development of cortical neurons, we transfected exogenous Grg6 in primary cultures of neural progenitor cells isolated from the dorsal telencephalon of E13.5 mouse embryos. These cortical progenitor cells endogenously express Grg6, Gro/TLE1, as well as BF-1 (Tao and Lai, 1992; Hatani et al., 1994; Dehni et al., 1995; Yao et al., 2001; Fig. 2). Enhanced GFP was coexpressed to identify the transfected cells. Exogenous Gro/TLE1 expression inhibited neuronal differentiation and led to an increase in the number of

undifferentiated neural progenitor cells expressing the mitotic cell marker protein Ki67 (Fig. 10, cf. bars 1 and 2) and a decrease in the number of differentiated neurons expressing the neuronal marker protein NeuN (Fig. 10, cf. bars 7 and 8). In contrast to Gro/TLE1, exogenous expression of Grg6 alone had no antineurogenic effect (Fig. 10, cf. bars 1 and 3, and 7 and 9). However, exogenous coexpression of Grg6 together with BF-1 led to a significant increase in the number of differentiated neurons (Fig. 10, cf. bars 7 and 10). When Grg6 was coexpressed with Gro/TLE1 and BF-1, Grg6 blocked the antineurogenic effect of Gro/TLE1 and led to a decrease in the number of progenitor cells and an increase in the number of differentiated neurons (Fig. 10, cf. bars 2 and 5, bars 8 and 11, and 11 and 12). Taken together, these results suggest that Grg6 antagonizes the antineurogenic activity of BF-1:Gro/TLE1 complexes and promotes neuronal differentiation

4. DISCUSSION

4.1 Grg6 and Gro/TLE have both similar and different functional properties. Gro/TLE proteins lack DNA-binding activity of their own but become recruited to specific gene regulatory sequences in context-dependent manners by forming complexes with a number of different DNA-binding transcription factors. Most if not all of these interactions require the C-terminal WDR domain conserved in all Gro/TLE family members. Grg6 shares with Gro/TLEs a conserved WDR domain but otherwise shows little similarity. This situation suggested that Grg6 might share at least certain protein-protein interaction properties with Gro/TLEs but that these might be correlated with different functional effects. The present studies have shown that similar to Gro/TLEs, Grg6 forms homodimers. However, it does not heterodimerize with either Gro/TLE1 (this study) or Gro/TLE4 (N.M and S.S., unpublished data), in contrast to the ability of Gro/TLEs to homo- and heterodimerize with each other (Palaparti and Stifani, 1997; Grbavec et al., 1998; Chen and Courey, 2000;). The inability of Grg6 to bind to Gro/TLEs may be due to the lack of the two conserved N-terminal leucine zipper-like motifs that mediate Gro/TLE oligomerization (Song et al., 2004). Although the structural elements that underlie Grg6 homodimerization remain to be fully elucidated, it is possible that a putative leucine zipper-like motif at its N-terminus may be involved in this ability. Another similarity between Grg6 and Gro/TLEs is the fact that both are expressed in neural progenitor cells and differentiated neurons. In particular, Grg6 is expressed in cortical progenitor cells that also express Gro/TLEs, as well as BF-1 and Hes1 (Sasai et al., 1992; Xuan et al., 1995; Yao et al., 1998, 2000, 2001). This coexpression is correlated

with a specific interaction of Grg6 with BF-1 but not Hes1. These observations suggest that Grg6 and Gro/TLEs share the ability to bind to certain common transcription factors through the conserved WDR domain. The sequence dissimilarities within the WDR domain of Grg6 and Gro/TLEs may be responsible for the different protein-protein interaction properties of these molecules. This situation may facilitate our understanding of how specific regions of the WDR domain contribute to the binding of different cofactors.

In contrast to Gro/TLEs, Grg6 does not mediate transcriptional repression when recruited to DNA. This conclusion is suggested by the finding that Grg6 does not repress transcription when fused to the DNA-binding domain of GAL4 and does not promote, but instead suppresses, transcriptional repression mediated by BF-1. It is likely that Grg6 can not repress transcription due to the lack of a significant relatedness to the N-terminal Q and GP domains of Gro/TLEs. Both of these regions are involved in functions that are believed to be important for Gro/TLE-mediated transcriptional repression, namely protein oligomerization and interactions with histone deacetylases and components of the basal transcriptional machinery (Palaparti and Stifani, 1997; Chen and Courey, 2000; Zhang and Emmons, 2002; Song et al., 2004). It is likely that Grg6 is unable to form complexes with histone deacetylases and/or other general transcriptional regulators that associate with Gro/TLEs. This possibility is suggested by our observation that Grg6 suppresses neither Gro/TLE- nor Hes1-mediated transcriptional repression and thus does not seem able to interact with, and titrate away, cofactors required by Gro/TLE and/or Hes1. It is also entirely possible that Grg6 may not participate in other, yet to be characterized, mechanisms underlying transcriptional repression by canonical Gro/TLEs.

Also in contrast to Gro/TLEs, Grg6 does not appear to be generally localized to nuclei and its intracellular localization is cell type-dependent. The nuclear association of Gro/TLEs is mediated by their CcN motif, which includes a nuclear localization sequence and phosphorylation sites for the protein kinases CK2 and cdc2 (Nuthall et al., 2002b, 2004). Grg6 harbors a domain that exhibits a limited similarity to the CcN domain of Gro/TLEs but does not contain a defined nuclear localization sequence. These observations suggest that Grg6 may not have an intrinsic ability to translocate to nuclei and may depend on interactions with other factors to become localized to the nucleus. In that regard, we have observed that Grg6 is localized to the cytosol of COS7 cells when transfected alone but can be detected in the nucleus when cotransfected together with BF-1. More importantly, endogenous Grg6 immunoreactivity was observed in the nuclei of cortical progenitor cells, where BF-1 is also endogenously expressed. These observations strongly suggest that Grg6 intracellular localization is dependent on the cellular environment and is influenced by its association with other proteins.

4.2 Grg6 suppresses BF-1 and promotes neuronal differentiation. Previous studies have demonstrated that BF-1 is a critical regulator of the progenitor-to-neuron transition in the developing mammalian telencephalon. BF-1 knock-out animals exhibit premature differentiation of the neural progenitor cells, leading to early depletion of the progenitor population and reduced cerebral hemisphere size (Xuan et al., 1995). These findings suggested that BF-1 promotes cell proliferation and/or inhibits cellular differentiation in the telencephalon. The antineurogenic activity of BF-1 appears to be mediated in conjunction with Gro/TLE corepressors. More specifically, BF-1 and Gro/TLE genes are

coexpressed in neural progenitor cells of the mammalian telencephalon (Dehni et al., 1995; Yao et al., 1998; Yao et al., 2001), and Gro/TLE1 was shown to negatively regulate neuronal differentiation in the forebrain (Yao et al., 2001). Moreover, BF-1 physically interacts with Gro/TLEs and the latter promote BF-1-mediated transcriptional repression (Yao et al., 2001). To determine if Grg6 might also be involved in the regulation of neuronal differentiation in the mammalian forebrain, we investigated if Grg6 is expressed in neural progenitor cells of the telencephalon and if it can interact with BF-1. Previous studies have demonstrated that Grg6 transcripts are present in the embryonic and adult mouse brain (Dang et al., 2001). In agreement with these observations, we have found that Grg6 is expressed in regions of the neural tube such as the forebrain, midbrain and spinal cord. More specifically, we have shown that Grg6 is expressed in both neural progenitors and differentiated neurons of the telencephalon, like other Gro/TLE proteins. Based on these observations, we have examined the effect of exogenous Grg6 expression in primary cultures of cortical neural progenitor cells. These investigations have revealed that in contrast to Gro/TLE1, exogenous expression of Grg6 alone has no detectable effect on *in vitro* cortical neurogenesis. This finding strongly suggests that Grg6 is not an anti-neurogenic factor like Gro/TLE1. However, Grg6 promoted cortical neurogenesis when it was cotransfected with BF-1 and the coexpression of Gro/TLE1 did not antagonize this effect to a significant extent. The Grg6-mediated promotion of neuronal differentiation appears to be the result of a recruitment of supernumerary progenitors into the neuronal lineage. Taken together, these observations suggest that Grg6 promotes cortical neuronal differentiation by inhibiting the repressive function of BF-1. This possibility is consistent with our demonstration that

Grg6 interacts with BF-1 in mammalian cells and that this interaction results in a recruitment of Grg6 to nuclei. This interaction is likely to involve the WD domain of Grg6. This interaction seems specific for BF-1, since we have shown that Grg6 does not interact with Hes1. Our studies have also demonstrated that Grg6 antagonizes BF-1-mediated transcriptional repression and reduces the interaction between BF-1 and Gro/TLE1 in mammalian cells. These findings lead us to propose that the inhibition of BF-1 activity may represent at least one mechanism utilized by Grg6 to promote neuronal differentiation. Interestingly, exogenous expression of Grg6 alone had no neurogenic effect in cortical progenitor cells, where BF-1 and Gro/TLE proteins are coexpressed. These observations suggest that Grg6 may act on BF-1 in a dosage dependant manner.

It has been shown previously that BF-1 inhibits neuronal differentiation through a DNA-dependent mechanism. Embryos expressing a DNA-binding defective form of BF-1 exhibit ectopic expression of BMP genes, such as BMP4, that were shown to inhibit proliferation of neural progenitor cells and to promote their differentiation into mature neurons (Dou et al., 1999; Hanashima et al., 2002). It may be possible that by antagonizing the binding of BF-1 to Gro/TLE1, Grg6 may prevent BF-1 from repressing BMP gene expression and, consequently, may promote neuronal differentiation. It will be important to test these possibilities in the future.

An additional mechanism by which Grg6 may promote neurogenesis together with BF-1 is suggested by the finding that BF-1 may not only act as transcriptional repressor but also as a transcriptional activator (Bourguignon et al., 1998). It is tempting to speculate that by binding to BF-1 and inhibiting the interaction with Gro/TLE1, Grg6 may be involved in converting BF-1 from a transcriptional repressor to a transcriptional

activator. As a result, Grg6:BF-1 complexes may activate, rather then suppress the expression of genes that promote neurogenesis. In that regard, we found that Grg6 promoted neuronal differentiation together with BF-1 in cortical progenitor cells but failed to convert BF-1 to an activator in transcription assays in transfected 293A cells. This apparent discrepancy may reflect the fact that Grg6 may have a weaker effect on BF-1 in mammalian cell lines compared to neural progenitor cells. This may be caused, among other things, by different levels of expression of Gro/TLEs. Moreover, based on transcription assays performed in 293A or HeLa cells, it is unlikely that Grg6 acts by itself as a transcriptional activator. Thus, cofactors that may collaborate with Grg6 in neural progenitor cells may either not be present in non-neural cells or may be not able to influence Grg6 activity. In the future, it may be informative to perform transcription assays in cortical progenitor cells with Grg6 alone and together with BF-1 to begin to answer these questions.

4.3 Possible mechanisms of suppression of BF-1 transcription repression activity by Grg6. Our studies have shown that Grg6, in contrast to other Gro/TLE proteins, antagonizes BF-1-mediated repression in mammalian cells. This effect is unlikely to be due to a decrease in the stability of BF-1 expression since the presence of Grg6 did not affect BF-1 levels of expression. To begin to elucidate the mechanisms by which Ggr6 suppresses BF-1 activity, we have tested whether Gr6 may act as a transcriptional activator. Our results suggested that, in contrast to other Gro/TLE proteins, Grg6 can neither repress nor activate transcription in transfected cell lines. This suggests in turn that Grg6 is unlikely to influence BF-1 through its own transcriptional activity. However,

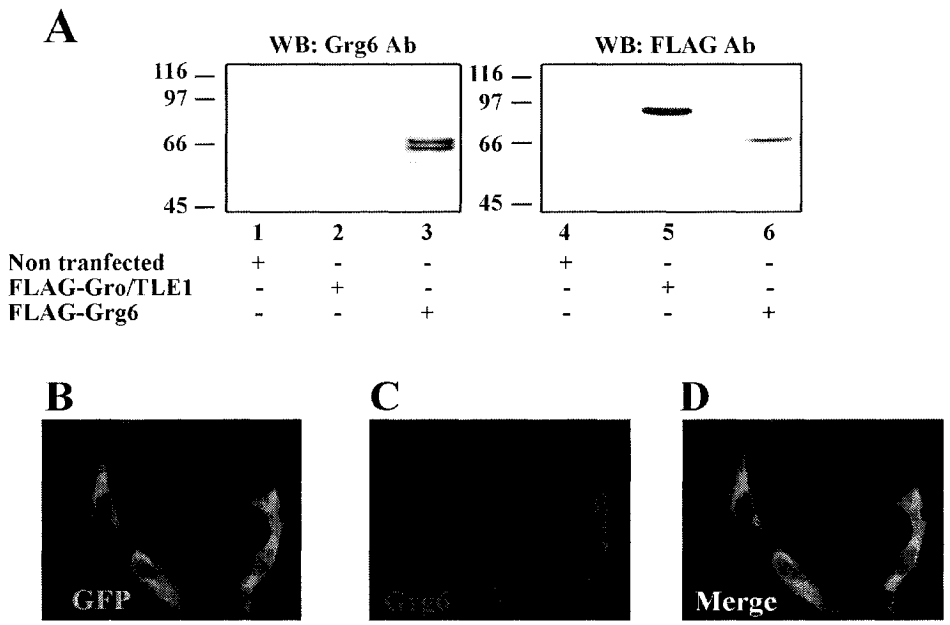
we cannot exclude the possibility that Grg6 may have a transcriptional activity in a more physiologically relevant environment. Transcription assays with Grg6 in cortical progenitor cells will provide more insight into whether Grg6 acts as a transcriptional activator.

Another possibility is that Grg6 may prevent Gro/TLE from associating with BF-1 thus depriving BF-1 of an important transcriptional corepressor. This possibility is consistent with our finding that the coimunoprecipitation of BF-1 with Gro/TLE1 is significantly reduced in the presence of Grg6. This effect does not appear to be due to the formation of Grg6:Gro/TLE hetero-oligomers because we failed to observe an interaction between Grg6 and Gro/TLE. In agreement with this, a computer program that predicts the presence of putative coiled-coil motifs (Lupas A et al., 1996) does not suggest that Grg6 contains an N-terminal structure similar to the two coiled-coils that are thought to mediate dimerization of Gro/TLEs (Pinto and Lobe, 1996; Chen et al., 1998). Alternatively, ternary complexes of BF-1, Grg6 and Gro/TLE may form but may not be competent to mediate transcriptional repression due to the presence of Grg6. Our findings also argue against this possibility since immunoprecipitation assays have shown no formation of Grg6:BF-1:Gro/TLE complexes. Thus, it appears more likely that Grg6 competes with Gro/TLE for BF-1 binding. This situation may result in the formation of complexes that can bind to DNA but are unable to repress transcription efficiently. Alternatively, Grg6:BF-1 complexes may not be able to bind to DNA. In the future, a combination of chromatin immunoprecipitations, EMSA experiments, and transcription assays will be required to test these possibilities further.

4.4 Biological relevance of Grg6 activity. The expression of Grg6, as well as that of BF-1, continues in differentiated cells. Adult tissues, including the brain, still express Grg6 (Dang et al., 2001). This expression pattern suggests that Grg6 may function in mature cells in addition to undifferentiated cells. An interesting role for Grg6 in the differentiation and maturation of neuronal cells may come from the recent finding that BF-1 plays an important role in early cortical cell fate. Mice lacking BF-1 exhibited overproduction of Cajal-Retzius neurons at the expense of ER81 neurons suggesting that BF-1 is required to suppress Cajal-Retzius cell fate (Hanashima et al., 2004). However, the molecular mechanisms underlying this process are still unknown. Based on our present findings, where Grg6 antagonizes BF-1 activities and promotes neuronal differentiation, it is tempting to speculate that Grg6 may represent a possible player that positively regulate CR cell fate. Further elucidation of the mechanisms underlying Grg6 activity will clarify important processes regulating vertebrate neurogenesis.

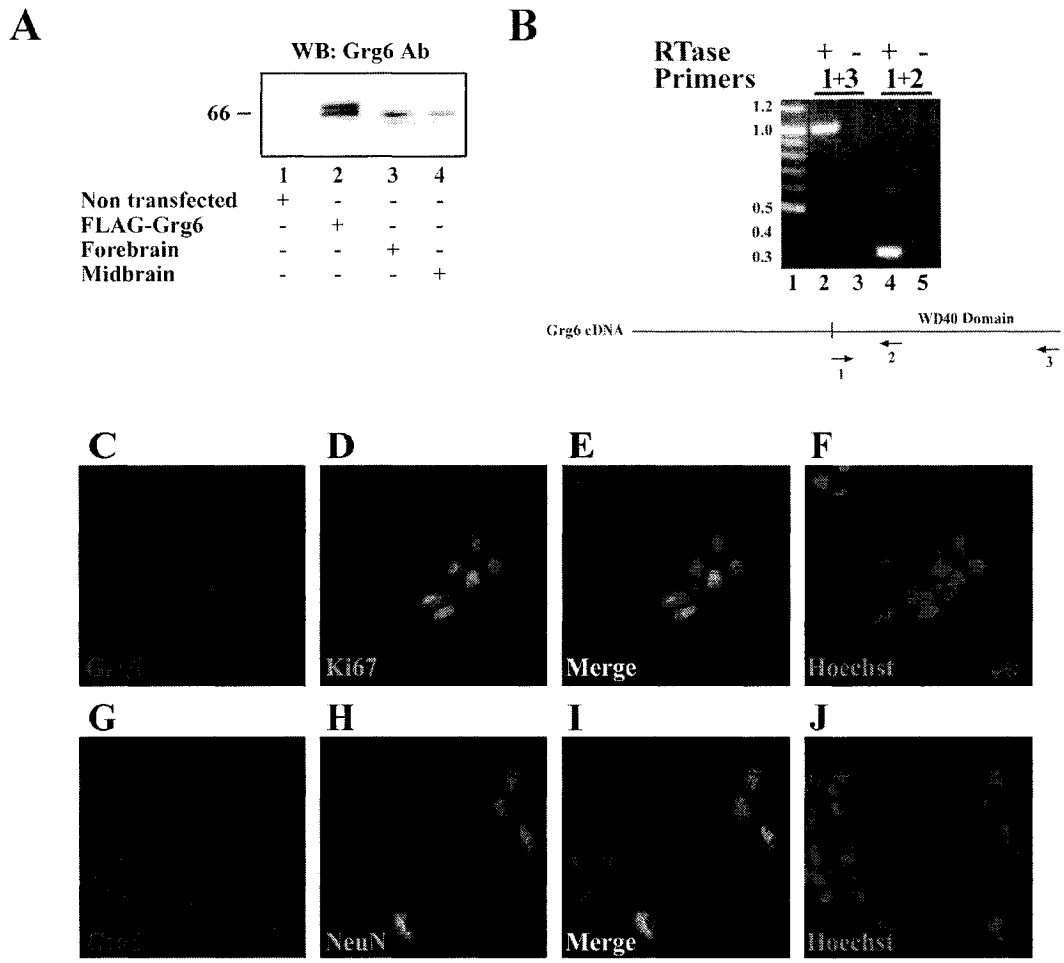
5. FIGURES AND LEGENDS

FIGURE 1



Characterization of Grg6 antibodies. (A) 293A cells were either non-transfected (lanes 1 and 4) or transfected with FLAG-Gro/TLE1 (lanes 2 and 5) or FLAG-Grg6 (lanes 3 and 6), followed by Western blotting (WB) with either anti-Grg6 (lanes 1 to 3) or anti-FLAG (lanes 4 to 6) antibodies (Ab). Positions of size standards are indicated in kilodaltons. (B-D) Immunocytochemical analysis. COS-7 cells were transfected with GFP-Grg6, fixed and subjected to double-labeling analysis of the expression of GFP (panel B) or Ggr6 (panel C). The combined GFP and Grg6 staining is shown in panel D.

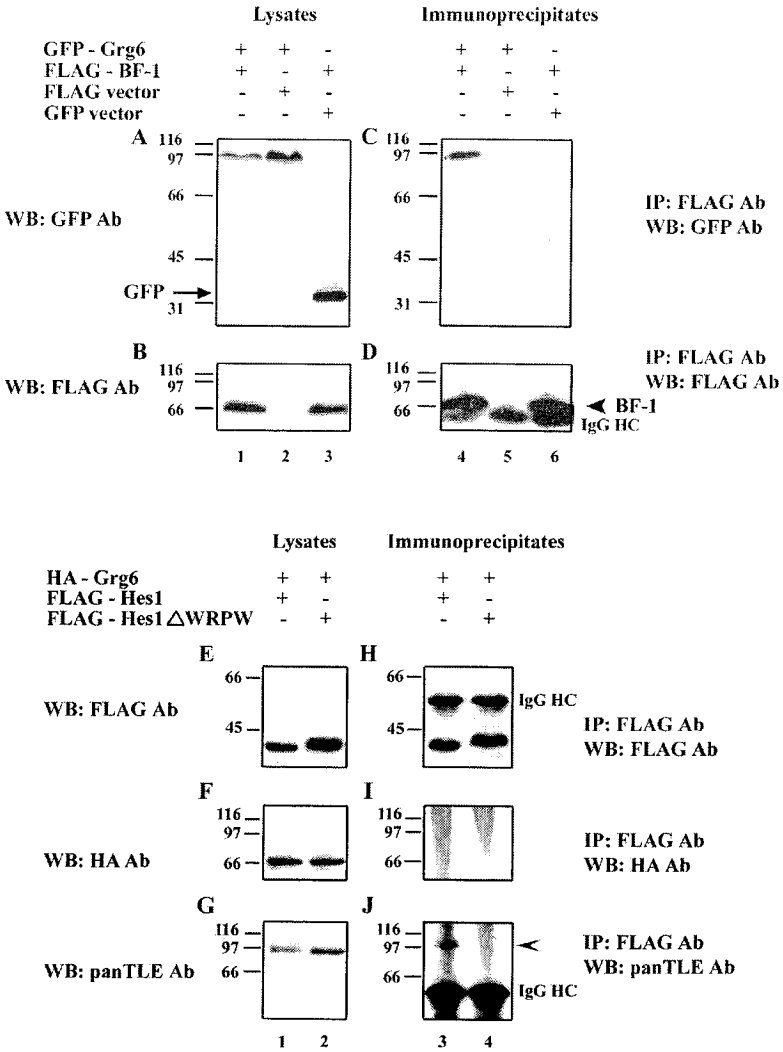
FIGURE 2



Grg6 expression in neural tissues. (A) Protein extracts from 293A cells either non-transfected (lane 1) or transfected with FLAG-Grg6 (lane 2), together with lysates from forebrain (lane 3) or midbrain (lane 4) tissues from E16 mouse embryos were subjected to SDS-PAGE on an 8% gel. Western blotting (WB) was performed with the affinity purified anti-Grg6 antibodies (Ab). Molecular size standard is indicated in kilodaltons. (B) RT-PCR analysis. mRNA was extracted from cortical progenitor cells taken from the dorsal telencephalon of E15.5 mouse embryos. mRNA was then incubated in the absence

(-) or presence (+) of reverse transcriptase (RT). The ensuing cDNA was subjected to PCR using either primers 1 and 3 (lanes 2 and 3) or primers 1 and 2 (lanes 4 and 5). Lane 1 was loaded with the indicated DNA size standards in kb. A schematic of the Grg6 cDNA is represented with the positions of the different primers indicated underneath. (C-J). Primary cultures of E13.5 mouse embryonic cortical neural progenitor cells were grown in chamber slides, fixed and subjected to double-labeling analysis of the expression of Grg6 (panels C and G), Ki67 (panel D) or NeuN (panel H). The combined Grg6/Ki67 (panel E) and Grg6/NeuN (panel I) stainings are shown. Hoechst staining was used to label nuclei (F and J).

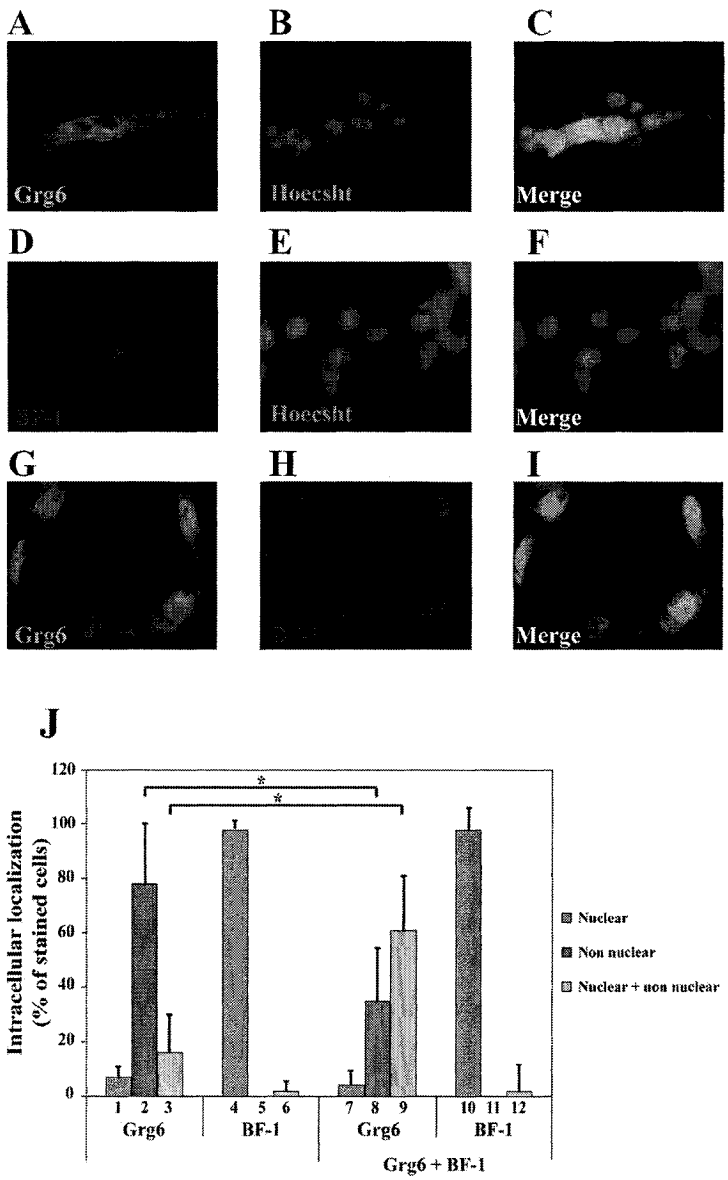
FIGURE 3



Grg6 interacts with BF-1 but not Hes1. (A-D) 293A cells were transfected with a plasmid encoding FLAG-BF-1 in the absence or presence of GFP-Grg6, or GFP alone. One-tenth of each cell lysate was subjected to SDS-PAGE (A and B) and the remaining lysates were subjected to immunoprecipitation (IP) with anti-FLAG antibodies (Ab) (C and D). Samples were analysed by Western blotting (WB) with anti-GFP (A and C) or anti-FLAG (B and D) antibodies. The arrow in A points to the position of migration of

the GFP protein (lane 3). The arrowhead in D points to the position of migration of BF-1 (lanes 4 and 6). IgG HC, immunoglobulin G heavy chain. (E-J). 293A cells were cotransfected with plasmids encoding either FLAG-Hes1 or FLAG-Hes1ΔWRPW together with HA-Grg6. Cell lysates were collected and one-tenth was subjected to Western blotting with anti-FLAG (E) or anti-HA (F) antibodies, or antibodies that recognize all Gro/TLE proteins (panTLE) (G). The remaining lysates were immunoprecipitated with anti-FLAG antibodies (H-J) followed by Western blotting with anti-FLAG (H), anti-HA (I) or panTLE (J) antibodies. The arrowhead in panel J points to the position of migration of endogenous Gro/TLE proteins. IgG HC, immunoglobulin G heavy chain. Position of size standards are indicated in kilodaltons.

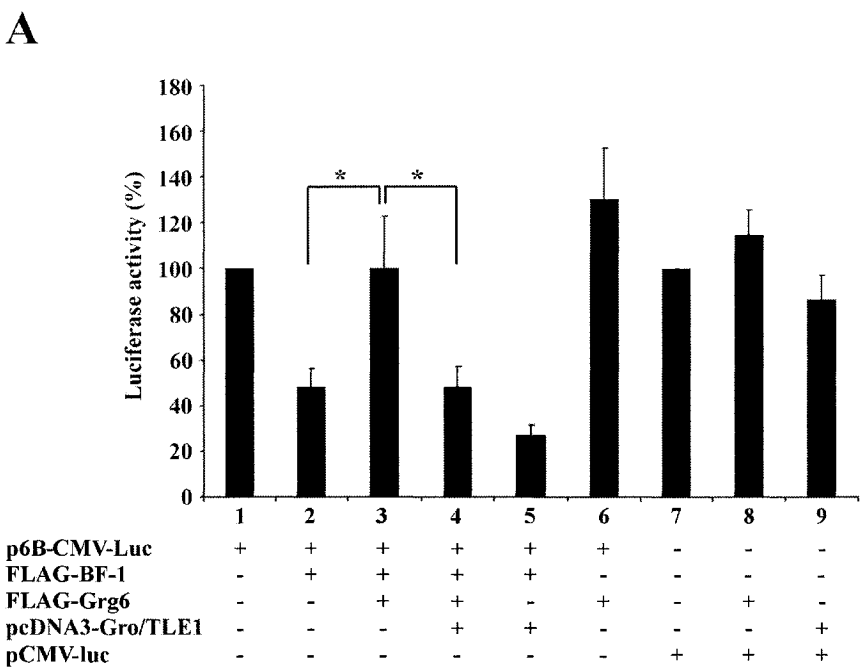
FIGURE 4



Intracellular distribution of Grg6 in the absence or presence of BF-1. (A-I) COS-7 cells were transfected with GFP-Grg6 (A-C), FLAG-BF-1 (D to F) or GFP-Grg6 together with FLAG-BF-1 (G-I). Twenty-four hours later, cells were fixed and subjected to double-labeling analysis of the expression of GFP (Grg6, A and G) or BF-1 (D and H).

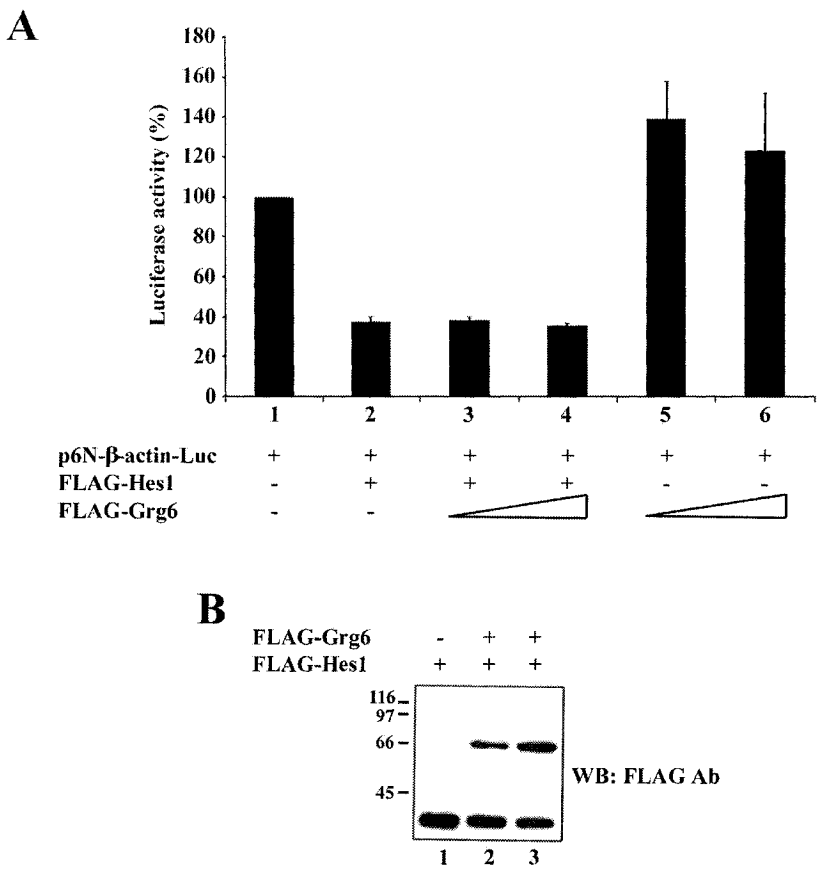
Hoechst staining is shown in B and E. The combined stainings are shown in C, F and I. (J) Quantification of the intracellular distribution of Grg6 (bars 1-3), BF-1 (bars 4-6), or Grg6 in the presence of BF-1 (bars 7-12), defined as the percentage of stained cells showing either a nuclear (green columns), non-nuclear (pink columns), or both nuclear and non-nuclear (blue columns) staining. Results are shown as means ± SD (n=7). *, $P<$ 0.01.

FIGURE 5



Inhibition of BF-1-mediated transcriptional repression by Grg6. 293A cells were transfected with the reporter construct p6B-CMV-Luc (500 ng/transfection; bars 1 to 6) and the expression vectors pCMV2-FLAG-BF-1 (15 ng/transfection; bars 2 to 5), pCMV2-FLAG-Grg6 (50 ng/transfection; bars 3 and 4) or pcDNA3-Gro/TLE1 (100 ng/transfection; bars 4 and 5). The basal activity of the reporter construct in the absence of any expression plamids was considered as 100%. Luciferase activity was expressed as the mean ± SD of at least three independent experiments performed in duplicate. Neither Grg6 (bar 8) nor Gro/TLE1 (bar 9) had a significant effect on a control reporter (pCMV-Luc; 500 ng/transfection) containing the CMV promoter but no BF-1 binding sites. *, $P< 0.05$.

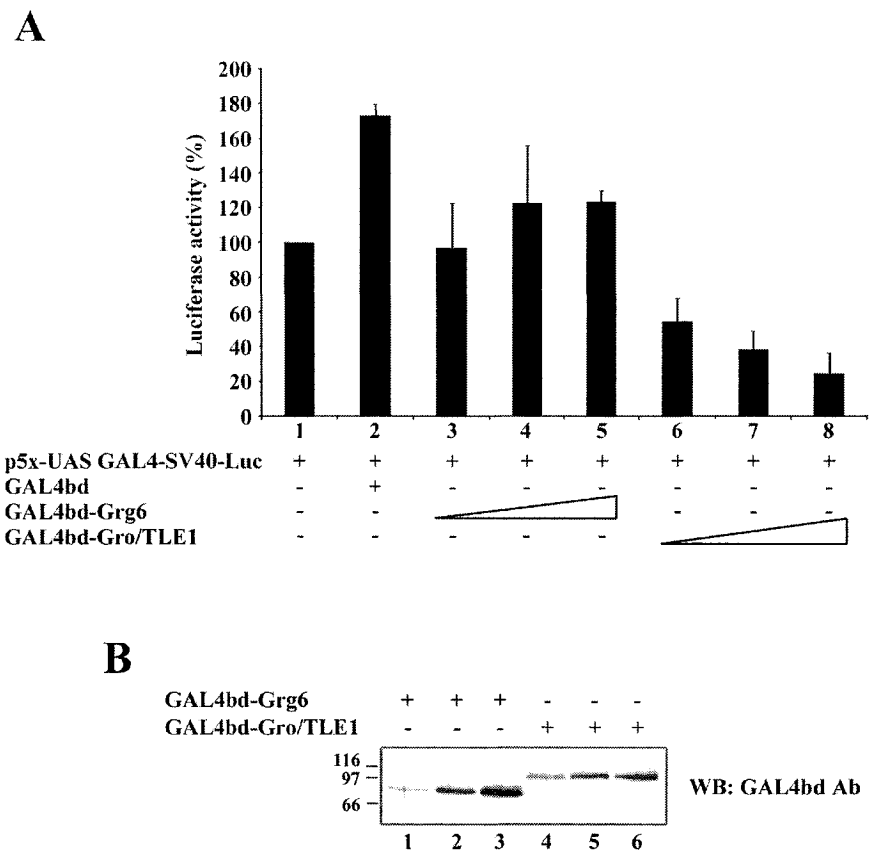
FIGURE 6



Transcriptional repression by Hes1 in the absence or presence of Grg6. (A) 293A cells were transfected with the reporter construct p6N-β actin-Luc (1 μg/transfection; bars 1 to 6) and the expression vectors pCMV2-FLAG-Hes1 (50 ng/transfection; bars 2 to 4) and increasing amounts of pCMV2-FLAG-Grg6 (25 and 50 ng/transfection; bars 3 and 4). The activity of the reporter gene in the absence of any expression plasmids was considered as 100%. Luciferase activity was expressed as the average ± SD of at least three independent experiments performed in duplicate. Grg6 had no effect on the activity of the reporter construct (bars 5 and 6). (B) Western blotting analysis of Grg6 and Hes1.

Lysates from 293A cells transfected with pCMV2-FLAG-Hes1 (50 ng/transfection) in the absence (lane 1) or presence of increasing amount of pCMV2-FLAG-Grg6 (lane 2; 25 ng/transfection and 3; 50 ng/transfection) were subjected to Western blotting with anti-FLAG antibodies.

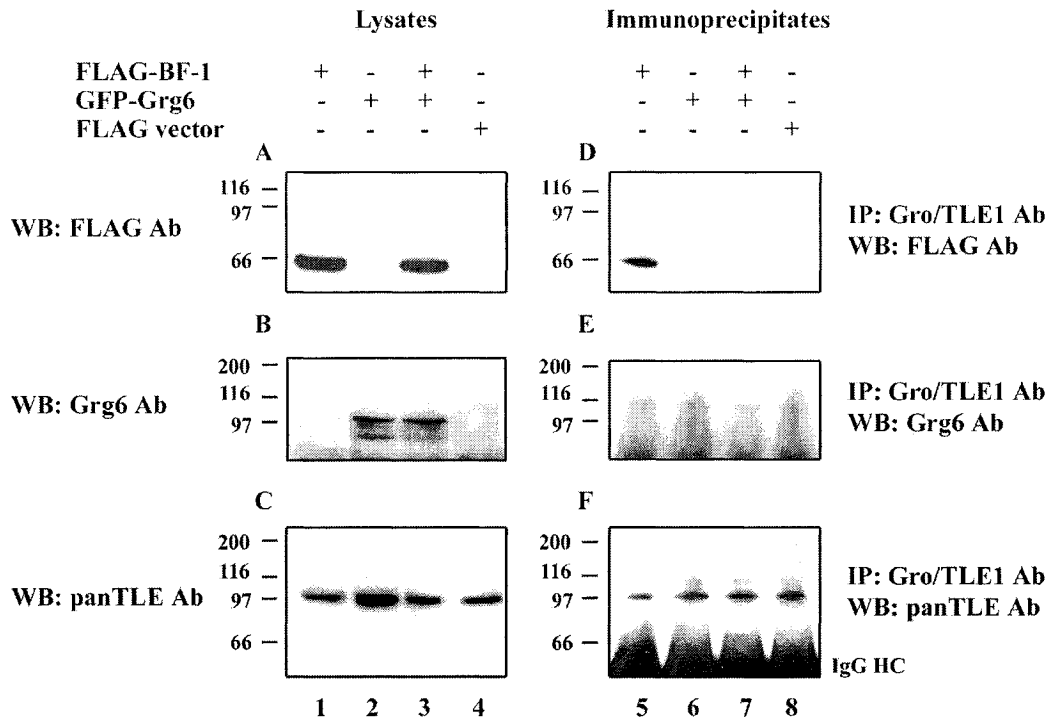
FIGURE 7



Repression of basal transcription by Gro/TLE1 but not Grg6. HeLa cells were transfected with the reporter plasmid p5X-UAS GAL4-SV40-Luc (500 ng/transfection; bars 1 to 8) alone or in combination with GAL4bd (500 ng/transfection; bar 2) or increasing amounts of either GAL4bd-Grg6 (50, 200, 500 ng/transfection, bars 3 to 5) or GAL4bd-Gro/TLE1 (50, 200, 500 ng/transfection, bars 6 to 7). The basal activity of the reporter construct in the absence of any expression plasmids was considered as 100%. Values represent means ± SD of at least three independent experiments performed in duplicate. (B) Western blotting analysis of Grg6 and Gro/TLE1. Lysates from HeLa cells

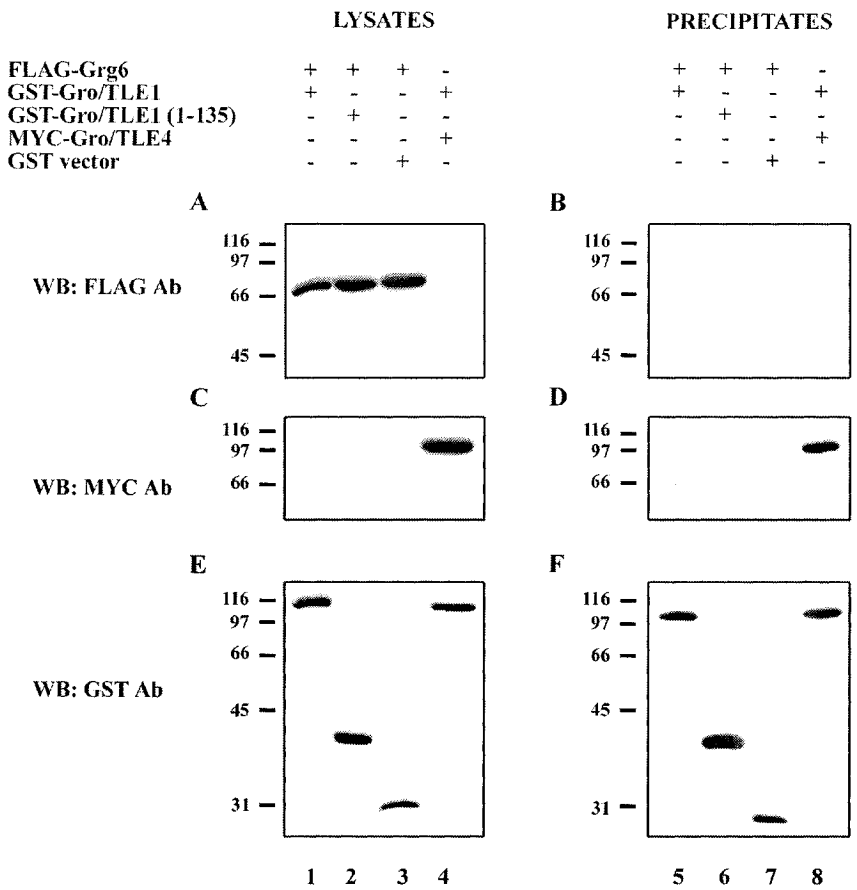
transfected with increasing amounts of either GAL4bd-Grg6 (50, 200, 500 ng/transfection, bars 1 to 3) or GAL4bd-Gro/TLE1 (50, 200, 500 ng/transfection, bars 4 to 6) were subjected to Western blotting with anti-GAL4bd antibodies.

FIGURE 8



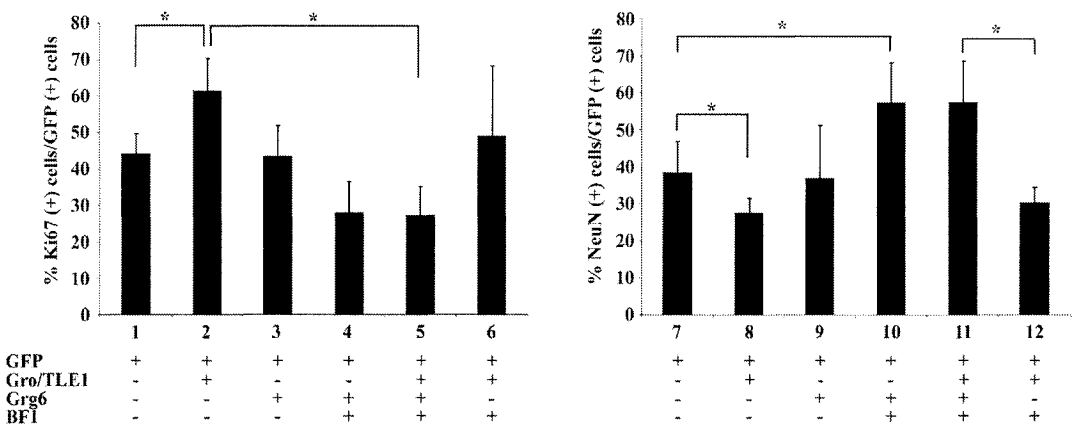
Effect of Grg6 on the interaction between BF-1 and Gro/TLE1. 293A cells, which express endogenous Gro/TLE, were transfected with FLAG-BF-1 (lane 1), GFP-Grg6 (lane 2), FLAG-BF-1 together with GFP-Grg6 (lane 3) or FLAG vector alone (lane 4). One-tenth of each cell lysate was subjected to Western blotting (WB) with anti-FLAG (A), anti-Grg6 (B) or panTLE (C) antibodies (Ab). The remaining lysates were subjected to immunoprecipitation (IP) with anti-Gro/TLE1 antibodies (D to F) followed by Western blotting analysis with anti-FLAG (D), anti-Grg6 (E), or panTLE (F) antibodies. IgG HC., immunoglobulin heavy chains. Positions of size standards are indicated in kilodaltons.

FIGURE 9



Grg6 does not heterodimerize with Gro/TLE1. 293A cells were transfected with plasmids encoding the indicated combinations of proteins. Cells lysates were collected, and the fusion proteins were isolated on glutathione-Sepharose beads. The precipitated material was subjected to SDS-PAGE (lanes 5 to 8) on a 12% gel, together with one-tenth of each input lysate collected prior to incubation with glutathione-Sepharose beads (lanes 1 to 4). This was followed by Western blotting (WB) with anti-FLAG (A and B), anti-MYC (C and D) or anti-GST (E and F) antibodies. Positions of size standards are indicated in kilodaltons.

FIGURE 10



Promotion of cortical neurogenesis by Grg6. E13.5 mouse embryonic cortical progenitor cells were transfected with plasmids encoding either GFP alone or in combination with Gro/TLE1, Grg6, or BF-1, as indicated. Forty-eight hours later, cells were fixed and subjected to double-labeling analysis of the expression of GFP, NeuN, or Ki67. Shown is the quantitation of the percentage of GFP-Ki67 (bars 1 to 7) of GFP-NeuN (bars 7 to 12). Results are shown as the mean ± SD (n=5), *, $P< 0.05$, **, $P< 0.001$.

6. REFERENCES

Ahmad I. (1995) Mash-1 is expressed during ROD photoreceptor differentiation and binds an E-box, E(opsin)-1 in the rat opsin gene. Brain Res Dev Brain Res. 90, 184-189.

Akazawa C, Sasai Y, Nakanishi S, Kageyama R. (1992) Molecular characterization of a rat negative regulator with a basic helix-loop-helix structure predominantly expressed in the developing nervous system. J Biol Chem. 267, 21879-21885.

Alexandrow MG, Moses HL. (1995) Transforming growth factor beta and cell cycle regulation. Cancer Res. 55, 1452-1457.

Aronson BD, Fisher AL, Blechman K, Caudy M, Gergen JP. (1997) Groucho-dependent and -independent repression activities of Runt domain proteins. Mol Cell Biol. 17, 5581-5587.

Artavanis-Tsakonas S, Muskavitch MA, Yedvobnick B. (1983) Molecular cloning of Notch, a locus affecting neurogenesis in Drosophila melanogaster. Proc Natl Acad Sci. 80, 1977-1981.

Artavanis-Tsakonas S, Rand MD, Lake RJ. (1999) Notch signaling: cell fate control and signal integration in development. Science. 284, 770-776.

Bae S, Bessho Y, Hojo M, Kageyama R. (2000) The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation. Development. 127, 2933-2943.

Ben-Arie N, Hassan BA, Bermingham NA, Malicki DM, Armstrong D, Matzuk M, Bellen HJ, Zoghbi HY. (2000) Functional conservation of atonal and Math1 in the CNS and PNS. Development. 127, 1039-1048.

Bertrand N, Castro DS, Guillemot F. (2002) Proneural genes and the specification of neural cell types. Nat Rev Neurosci. 3, 517-530.

Bottner M, Krieglstein K, Unsicker K. (2000) The transforming growth factor-betas: structure, signaling, and roles in nervous system development and functions. J Neurochem. 75, 2227-2240.

Bourguignon C, Li J, Papalopulu N. (1998) XBF-1, a winged helix transcription factor with dual activity, has a role in positioning neurogenesis in Xenopus competent ectoderm. Development. 125, 4889-4900.

Brantjes H, Roose J, van De Wetering M, Clevers H. (2001) All Tcf HMG box transcription factors interact with Groucho-related co-repressors. Nucleic Acids Res. 29, 1410-1419.

Cabrera CV, Alonso MC. (1991) Transcriptional activation by heterodimers of the achaete-scute and daughterless gene products of Drosophila. EMBO J. 10, 2965-2973.

Campuzano S, Modolell J. (1992) Patterning of the Drosophila nervous system: the achaete-scute gene complex. Trends Genet. 8, 202-208.

Casarosa S, Fode C, Guillemot F. (1999) Mash1 regulates neurogenesis in the ventral telencephalon. Development. 126, 525-534.

Catron KM, Zhang H, Marshall SC, Inostroza JA, Wilson JM, Abate C. (1995) Transcriptional repression by Msx-1 does not require homeodomain DNA-binding sites. Mol Cell Biol. 15, 861-871.

Cau, E., Gradwohl, G., Fode, C. and Guillemot, F. (1997) *Mash1* activates a cascade of bHLH regulators in olfactory neuron progenitors. *Development* 124, 1611-1621.

Chen G, Nguyen PH, Courey AJ. (1998) A role for Groucho tetramerization in transcriptional repression. Mol Cell Biol. 18, 7259-7568.

Chen G, Fernandez J, Mische S, Courey AJ. (1999) A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in Drosophila development. Genes Dev. 13, 2218-2230.

Chen G, Courey AJ. (2000) Groucho/TLE family proteins and transcriptional repression. Gene. 249, 1-16.

Choi CY, Kim YH, Kwon HJ, Kim Y. (1999) The homeodomain protein NK-3 recruits Groucho and a histone deacetylase complex to repress transcription. J Biol Chem. 274, 33194-33197.

Clark KL, Halay ED, Lai E, Burley SK. (1993) Co-crystal structure of the HNF-3/fork head DNA-recognition motif resembles histone H5. Nature. 364, 412-420.

Crossley PH, Martin GR. (1995) The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. Development. 121, 439-451.

Cubas P, de Celis JF, Campuzano S, Modolell J. (1991) Proneural clusters of achaete-scute expression and the generation of sensory organs in the Drosophila imaginal wing disc. Genes Dev. 5, 996-1008.

Dang J, Inukai T, Kurosawa H, Goi K, Inaba T, Lenny NT, Downing JR, Stifani S, Look AT. (2001) The E2A-HLF oncoprotein activates Groucho-related genes and suppresses Runx1. Mol Cell Biol. 21, 5935-5945.

D'Arcangelo G, Miao GG, Chen SC, Soares HD, Morgan JI, Curran T. (19950 A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. Nature. 374, 719-723.

Dasen JS, Barbera JP, Herman TS, Connell SO, Olson L, Ju B, Tollkuhn J, Baek SH, Rose DW, Rosenfeld MG. (2001) Temporal regulation of a paired-like homeodomain repressor/TLE corepressor complex and a related activator is required for pituitary organogenesis. Genes Dev. 15, 3193-3207.

Dehni G, Liu Y, Husain J, Stifani S. (1995) TLE expression correlates with mouse embryonic segmentation, neurogenesis, and epithelial determination. Mech Dev. 53, 369-381.

Delidakis, C., A. Preiss, D.A. Hartley, and S. Artavanis-Tsakonas. (1991) Two genetically and molecularly distinct functions involved in early neurogenesis reside within the *Enhancer-of-split* locus of *Drosophila melanogaster. Genetics* 129, 803-823.

Desai AR, McConnell SK. (2000) Progressive restriction in fate potential by neural progenitors during cerebral cortical development. Development. 127, 2863-2872.

Doe CQ, Fuerstenberg S, Peng CY. (1998) Neural stem cells: from fly to vertebrates. J Neurobiol. 36, 111-127.

Dou CL, Li S, Lai E. (1999) Dual role of brain factor-1 in regulating growth and patterning of the cerebral hemispheres. Cereb Cortex. 9, 543-550.

Dubnicoff T, Valentine SA, Chen G, Shi T, Lengyel JA, Paroush Z, Courey AJ. (1997) Conversion of dorsal from an activator to a repressor by the global corepressor Groucho. Genes Dev. 11, 2952-2957.

Eberhard, D., G. Jimenez, B. Heavy, and M. Busslinger. (2000) Transcriptional repression by Pax5 (BSAP) through interaction with corepressors of the Groucho family. EMBO J. 19, 2292–2303.

Edlund T, Jessell TM. (1999) Progression from extrinsic to intrinsic signaling in cell fate specification: a view from the nervous system. Cell. 96, 211-224.

Feijen A, Goumans MJ, van den Eijnden-van Raaij AJ. (1994) Expression of activin subunits, activin receptors and follistatin in postimplantation mouse embryos suggests specific developmental functions for different activins. Development. 120, 3621-3637.

Fisher AL, Ohsako S, Caudy M. (1996) The WRPW motif of the hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain. Mol Cell Biol. 16, 2670-2677.

Fisher A, Caudy M. (1998) The function of hairy-related bHLH repressor proteins in cell fate decisions. Bioessays. 20, 298-306.

Flores-Saaib RD, Courey AJ. (2000) Analysis of Groucho-histone interactions suggests mechanistic similarities between Groucho- and Tup1-mediated repression. Nucleic Acids Res. 28, 4189-4196.

Frantz GD, McConnell SK. (1996) Restriction of late cerebral cortical progenitors to an upper-layer fate. Neuron. 17, 55-61.

Frotscher M. (1998) Cajal-Retzius cells, Reelin, and the formation of layers. Curr Opin Neurobiol. 8, 570-575.

Furuta Y, Piston DW, Hogan BL. (1997) Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. Development. 124, 2203-2212.

Ghysen A, Dambly-Chaudiere C. (1993) The specification of sensory neuron identity in Drosophila. Bioessays. 5, 293-298.

Goldstein RE, Jimenez G, Cook O, Gur D, Paroush Z. (1999) Huckebein repressor activity in Drosophila terminal patterning is mediated by Groucho. Development. 1999 Sep;126(17):3747-55.

Gratton MO, Torban E, Jasmin SB, Theriault FM, German MS, Stifani S. (2003) Hes6 promotes cortical neurogenesis and inhibits Hes1 transcription repression activity by multiple mechanisms. Mol Cell Biol. 23, 6922-6935.

Grbavec D, Stifani S. (1996) Molecular interaction between TLE1 and the carboxyl-terminal domain of HES-1 containing the WRPW motif. Biochem Biophys Res Commun. 223, 701-705.

Grbavec D, Lo R, Liu Y, Stifani S. (1998) Transducin-like Enhancer of split 2, a mammalian homologue of Drosophila Groucho, acts as a transcriptional repressor, interacts with Hairy/Enhancer of split proteins, and is expressed during neuronal development. Eur J Biochem. 258, 339-349.

Guillemot F, Joyner AL. (1993) Dynamic expression of the murine Achaete-Scute homologue Mash-1 in the developing nervous system. Mech Dev. 42, 171-185.

Guillemot F, Lo LC, Johnson JE, Auerbach A, Anderson DJ, Joyner AL. (1993) Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell. 75, 463-476.

Guillemot F. (1999) Vertebrate bHLH genes and the determination of neuronal fates. Exp Cell Res. 253, 357-364.

Hanashima C, Shen L, Li SC, Lai E. (2002) Brain factor-1 controls the proliferation and differentiation of neocortical progenitor cells through independent mechanisms. J Neurosci. 22, 6526-6536.

Hanashima C, Li SC, Shen L, Lai E, Fishell G. (2004) Foxg1 suppresses early cortical cell fate. Science. 303, 56-59.

Hardcastle Z, Papalopulu N. (2000) Distinct effects of XBF-1 in regulating the cell cycle inhibitor p27(XIC1) and imparting a neural fate. Development. 127, 1303-1314.

Hartenstein V, Posakony JW. (1990) A dual function of the Notch gene in Drosophila sensillum development. Dev Biol. 142, 13-30.

Hartley, D.A., A. Preiss, and S. Artavanis-Tsakonas. (1988) A deduced gene product from the *Drosophila* neurogenic locus, *Enhancer-of-split,* shows homology to mammalian G-protein beta subunit. *Cell* 55, 785-795.

Hassan BA, Bellen HJ. (2000) Doing the MATH: is the mouse a good model for fly development? Genes Dev. 14, 1852-1865.

Hatini V, Tao W, Lai E. (1994) Expression of winged helix genes, BF-1 and BF-2, define adjacent domains within the developing forebrain and retina. J Neurobiol. 25, 1293-12309.

Heitzler P, Bourouis M, Ruel L, Carteret C, Simpson P. (1996) Genes of the Enhancer of split and achaete-scute complexes are required for a regulatory loop between Notch and Delta during lateral signalling in Drosophila. Development. 122, 161-171.

Hirotsune S, Takahara T, Sasaki N, Hirose K, Yoshiki A, Ohashi T, Kusakabe M, Murakami Y, Muramatsu M, Watanabe S, et al. (1995) The reeler gene encodes a protein with an EGF-like motif expressed by pioneer neurons. Nat Genet. 10, 77-83.

Hirsch MR, Tiveron MC, Guillemot F, Brunet JF, Goridis C. (1998) Control of noradrenergic differentiation and Phox2a expression by MASH1 in the central and peripheral nervous system. Development. 125, 599-608.

Huh S, Hatini V, Marcus RC, Li SC, Lai E. (1999) Dorsal-ventral patterning defects in the eye of BF-1-deficient mice associated with a restricted loss of shh expression. Dev Biol. 211, 53-63.

Hunger SP, Ohyashiki K, Toyama K, Cleary ML. (1992) Hlf, a novel hepatic bZIP protein, shows altered DNA-binding properties following fusion to E2A in t(17;19) acute lymphoblastic leukemia. Genes Dev. 6, 1608-1620.

Husain J, Lo R, Grbavec D, Stifani S. (1996) Affinity for the nuclear compartment and expression during cell differentiation implicate phosphorylated Groucho/TLE1 forms of higher molecular mass in nuclear functions. Biochem J. 317, 523-531.

Inaba T, Roberts WM, Shapiro LH, Jolly KW, Raimondi SC, Smith SD, Look AT. (1992) Fusion of the leucine zipper gene HLF to the E2A gene in human acute B-lineage leukemia. Science. 257, 531-534.

Inaba T, Inukai T, Yoshihara T, Seyschab H, Ashmun RA, Canman CE, Laken SJ, Kastan MB, Look AT. (1996) Reversal of apoptosis by the leukaemia-associated E2A-HLF chimaeric transcription factor. Nature. 382, 541-544.

Ishibashi M, Moriyoshi K, Sasai Y, Shiota K, Nakanishi S, Kageyama R. (1994) Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system. EMBO J. 13, 1799-1805.

Ishibashi M, Ang SL, Shiota K, Nakanishi S, Kageyama R, Guillemot F. (1995) Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. Genes Dev. 9, 3136-3148.

Iso T, Kedes L, Hamamori Y. (2003) HES and HERP families: multiple effectors of the Notch signaling pathway. J Cell Physiol. 194, 237-255.

Jan YN, Jan LY. (1994) Neuronal cell fate specification in Drosophila. Curr Opin Neurobiol. 4, 8-13.

Jarman AP, Sun Y, Jan LY, Jan YN. (1995) Role of the proneural gene, atonal, in formation of Drosophila chordotonal organs and photoreceptors. Development. 121, 2019-2030.

Jasoni CL, Reh TA. (1996) Temporal and spatial pattern of MASH-1 expression in the developing rat retina demonstrates progenitor cell heterogeneity. J Comp Neurol. 369, 319-327.

Javed A, Guo B, Hiebert S, Choi JY, Green J, Zhao SC, Osborne MA, Stifani S, Stein JL, Lian JB, van Wijnen AJ, Stein GS. (2000) Groucho/TLE/R-esp proteins associate with the nuclear matrix and repress RUNX (CBF(alpha)/AML/PEBP2(alpha)) dependent activation of tissue-specific gene transcription. J Cell Sci. 113, 2221-2231.

Jimenez, G., Z. Paroush, and D. Ish-Horowicz. (1997) Groucho acts as a corepressor for a subset of negative regulators, including Hairy and Engrailed. Genes Dev. 11, 3072–3082.

Johnson JE, Birren SJ, Anderson DJ. (1990) Two rat homologues of Drosophila achaete-scute specifically expressed in neuronal precursors. Nature. 346, 858-861.

Kaestner KH, Knochel W, Martinez DE. (2000) Unified nomenclature for the winged helix/forkhead transcription factors. Genes Dev. 14, 142-146.

Kaufmann E, Knöchel W (1996) Five years on the wings of *forkhead.* Mech Dev 57: 3–20.

Koop KE, MacDonald LM, Lobe CG. (1996) Transcripts of Grg4, a murine groucho-related gene, are detected in adjacent tissues to other murine neurogenic gene homologues during embryonic development. Mech Dev. 59, 73-87.

Kunisch M, Haenlin M, Campos-Ortega JA. (1994) Lateral inhibition mediated by the Drosophila neurogenic gene delta is enhanced by proneural proteins. Proc Natl Acad Sci U S A. 91, 139-143.

Lai E, Prezioso VR, Smith E, Litvin O, Costa RH, Darnell JE Jr. (1990) HNF-3A, a hepatocyte-enriched transcription factor of novel structure is regulated transcriptionally. Genes Dev. 4, 1427-1436.

Lai E, Prezioso VR, Tao WF, Chen WS, Darnell JE Jr. (1991) Hepatocyte nuclear factor 3 alpha belongs to a gene family in mammals that is homologous to the Drosophila homeotic gene fork head. Genes Dev. 5, 416-427.

Lee JE. (1997) Basic helix-loop-helix genes in neural development. Curr Opin Neurobiol. 7, 13-20.

Leon C, Lobe CG. (1997) Grg3, a murine Groucho-related gene, is expressed in the developing nervous system and in mesenchyme-induced epithelial structures. Dev Dyn. 208, 11-24.

Levanon, D., R. E. Goldstein, Y. Bernstein, H. Tang, D. Goldenberg, S. Stifani, Z. Paroush, and Y. Groner. (1998) Transcriptional repression by AML1 and LEF-1 is mediated by the TLE/Groucho corepressors. Proc. Natl. Acad. Sci. USA 95, 11590–11595.

Li J, Vogt PK. (1993) The retroviral oncogene qin belongs to the transcription factor family that includes the homeotic gene fork head. Proc Natl Acad Sci U S A. 90, 4490-44494.

Li J, Chang HW, Lai E, Parker EJ, Vogt PK. (1995) The oncogene qin codes for a transcriptional repressor. Cancer Res. 55, 5540-5544.

Li J, Thurm H, Chang HW, Iacovoni JS, Vogt PK. (1997) Oncogenic transformation induced by the Qin protein is correlated with transcriptional repression. Proc Natl Acad Sci U S A. 94, 10885-10888.

Lindsley EB, and Grell EH (1968) Genetic Varaition of Drosophila malanogaster, Publ. No 627. Carnegie Institute, Washington, DC.

Liu Y, Dehni G, Purcell KJ, Sokolow J, Carcangiu ML, Artavanis-Tsakonas S, Stifani S. (1996) Epithelial expression and chromosomal location of human TLE genes: implications for notch signaling and neoplasia. Genomics. 31, 58-64.

Lupas A. (1996) Prediction and analysis of coiled-coil structures. Methods Enzymol. 266, 513-525.

Marin-Padilla M. (1998) Cajal-Retzius cells and the development of the neocortex. Trends Neurosci. 21, 64-71.

McConnell SK, Kaznowski CE. (1991) Cell cycle dependence of laminar determination in developing neocortex. Science. 254, 282-285.

McLarren KW, Lo R, Grbavec D, Thirunavukkarasu K, Karsenty G, Stifani S. (2000) The mammalian basic helix loop helix protein HES-1 binds to and modulates the transactivating function of the runt-related factor Cbfa1. J Biol Chem. 275, 530-538.

McLarren, K. W., F. Theriault, and S. Stifani. (2001) Association with the nuclear matrix and interaction with Groucho and RUNX proteins regulate the transcription repression ability of the basic helix loop helix factor Hes1. J. Biol. Chem. 276, 1578–1584.

Muhr J, Andersson E, Persson M, Jessell TM, Ericson J. (2001) Groucho-mediated transcriptional repression establishes progenitor cell pattern and neuronal fate in the ventral neural tube. Cell. 104, 861-873.

Nakayama K, Ishida N, Shirane M, Inomata A, Inoue T, Shishido N, Horii I, Loh DY, Nakayama K. (1996) Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. Cell. 85, 707-720.

Nakayama H, Liu Y, Stifani S, Cross JC. (1997) Developmental restriction of Mash-2 expression in trophoblast correlates with potential activation of the notch-2 pathway. Dev Genet. 21, 21-30.

Nuthall HN, Husain J, McLarren KW, Stifani S. (2002a) Role for Hes1-induced phosphorylation in Groucho-mediated transcriptional repression. Mol Cell Biol. 22, 389-399.

Nuthall HN, Joachim K, Palaparti A, Stifani S. (2002b) A role for cell cycle-regulated phosphorylation in Groucho-mediated transcriptional repression. J Biol Chem. 277, 51049-51057.

Nuthall HN, Joachim K, Stifani S, (2004) Phosphorylation of Serine 239 of Groucho/TLE1 by Protein Kinase CK2 is important for inhibition of neuronal differentiation. Mol. Cell. Biol. 24, 8395-8407.

Ohsako S, Hyer J, Panganiban G, Oliver I, Caudy M. (1994) Hairy function as a DNA-binding helix-loop-helix repressor of Drosophila sensory organ formation. Genes Dev. 8, 2743-2755.

Palaparti A, Baratz A, Stifani S. (1997) The Groucho/transducin-like enhancer of split transcriptional repressors interact with the genetically defined amino-terminal silencing domain of histone H3. J Biol Chem. 272, 26604-26610.

Parkhurst SM. (1998) Groucho: making its Marx as a transcriptional co-repressor. Trends Genet. 14, 130-132.

Paroush, Z., R. L. Finley, T. Kidd, S. M. Wainwright, P. W. Ingham, R. Brent, and D. Ish-Horowicz. (1994) Groucho is required for *Drosophila* neurogenesis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins. Cell 79, 805–815.

Paroush Z, Wainwright SM, Ish-Horowicz D. (1997) Torso signalling regulates terminal patterning in Drosophila by antagonising Groucho-mediated repression. Development. 124, 3827-3834.

Pelton RW, Johnson MD, Perkett EA, Gold LI, Moses HL. (1991) Expression of transforming growth factor-beta 1, -beta 2, and -beta 3 mRNA and protein in the murine lung. Am J Respir Cell Mol Biol. 5, 522-530.

Pflugrad A, Meir JY, Barnes TM, Miller DM. (1997) The Groucho-like transcription factor UNC-37 functions with the neural specificity gene unc-4 to govern motor neuron identity in C. elegans. Development. 124, 1699-1709.

Pickles LM, Roe SM, Hemingway EJ, Stifani S, Pearl LH. (2002) Crystal structure of the C-terminal WD40 repeat domain of the human Groucho/TLE1 transcriptional corepressor. Structure (Camb). 10, 751-761.

Pinto M, Lobe CG. (1996) Products of the grg (Groucho-related gene) family can dimerize through the amino-terminal Q domain. J Biol Chem. 271, 33026-33031.

Rodriguez C, Huang LJ, Son JK, McKee A, Xiao Z, Lodish HF. (2001) Functional cloning of the proto-oncogene brain factor-1 (BF-1) as a Smad-binding antagonist of transforming growth factor-beta signaling. J Biol Chem. 276, 30224-30230.

Roose J, Molenaar M, Peterson J, Hurenkamp J, Brantjes H, Moerer P, van de Wetering M, Destree O, Clevers H. (1998) The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. Nature. 395, 608-612.

Sasai Y, Kageyama R, Tagawa Y, Shigemoto R, Nakanishi S. (1992) Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. Genes Dev. 6, 2620-2634.

Seoane J, Le HV, Shen L, Anderson SA, Massague J. (2004) Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell. 117, 211-223.

Shimamura K, Rubenstein JL. (1997) Inductive interactions direct early regionalization of the mouse forebrain. Development. 124, 2709-218.

Schrons H, Knust E, Campos-Ortega JA. (1992) The Enhancer of split complex and adjacent genes in the 96F region of Drosophila melanogaster are required for segregation of neural and epidermal progenitor cells. Genetics. 132, 481-503.

Song H, Hasson P, Paroush Z, Courey AJ. (2004) Groucho oligomerization is required for repression in vivo. Mol Cell Biol. 24, 4341-4350.

Stifani S, Blaumueller CM, Redhead NJ, Hill RE, Artavanis-Tsakonas S. (1992) Human homologs of a Drosophila Enhancer of split gene product define a novel family of nuclear proteins. Nat Genet. 2, 119-127.

Skeath JB, Carroll SB. (1991) Regulation of achaete-scute gene expression and sensory organ pattern formation in the Drosophila wing. Genes Dev. 5, 984-995.

Swingler TE, Bess KL, Yao J, Stifani S, Jayaraman PS. (2004) The proline-rich homeodomain protein recruits members of the Groucho/Transducin-like enhancer of split protein family to co-repress transcription in hematopoietic cells. J Biol Chem. 279, 34938-34947.

Tao W, Lai E. (1992) Telencephalon-restricted expression of BF-1, a new member of the HNF-3/fork head gene family, in the developing rat brain. Neuron. 8, 957-966.

Tetsuka T, Uranishi H, Imai H, Ono T, Sonta S, Takahashi N, Asamitsu K, Okamoto T. (2000) Inhibition of nuclear factor-kappaB-mediated transcription by association with the amino-terminal enhancer of split, a Groucho-related protein lacking WD40 repeats. J Biol Chem. 275, 4383-4390.

Thirunavukkarasu, K., M. Mahajan, K. W. McLarren, S. Stifani, and G. Karsenty. (1998) Two domains unique to osteoblast-specific transcription factor Osf2/Cbfl contribute to its transactivating function and its inability to heterodimerize with Cbfß. Mol. Cell. Biol. 18, 4197–4208.

Tolkunova, E. N., M. Fujioka, M. Kobayashi, D. Deka, and J. B. Jaynes. (1998) Two distinct types of repression domain in Engrailed: one interacts with the Groucho corepressor and is preferentially active on integrated target genes. Mol. Cell. Biol. 18, 2804–2814.

Tomita K, Ishibashi M, Nakahara K, Ang SL, Nakanishi S, Guillemot F, Kageyama R. (1996) Mammalian hairy and Enhancer of split homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. Neuron. 16, 723-734.

Valentine SA, Chen G, Shandala T, Fernandez J, Mische S, Saint R, Courey AJ. (1998) Dorsal-mediated repression requires the formation of a multiprotein repression complex at the ventral silencer. Mol Cell Biol. 18, 6584-6594.

Van Doren M, Powell PA, Pasternak D, Singson A, Posakony JW. (1992) Spatial regulation of proneural gene activity: auto- and cross-activation of achaete is antagonized by extramacrochaetae. Genes Dev. 6, 2592-2605.

Van Doren M, Bailey AM, Esnayra J, Ede K, Posakony JW. (1994) Negative regulation of proneural gene activity: hairy is a direct transcriptional repressor of achaete. Genes Dev. 8, 2729-2742.

Vernon AE, Devine C, Philpott A. (2003) The cdk inhibitor p27Xic1 is required for differentiation of primary neurones in Xenopus. Development. 130, 85-92.

Wang, J. C., M. Waltner-Law, K. Yamada, H. Osawa, S. Stifani, and D. K. Granner. (2000) Transducin-like Enhancer of split proteins, the human homologs of *Drosophila* Groucho, interact with hepatic nuclear factor 3ß. J. Biol. Chem. 275, 18418–18423.

Xuan S, Baptista CA, Balas G, Tao W, Soares VC, Lai E. (1995) Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres. Neuron. 14, 1141-1152.

Yao J, Liu Y, Husain J, Lo R, Palaparti A, Henderson J, Stifani S. (1998) Combinatorial expression patterns of individual TLE proteins during cell determination and differentiation suggest non-redundant functions for mammalian homologs of Drosophila Groucho. Dev Growth Differ. 40, 133-146.

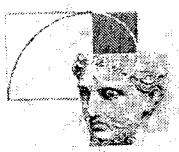
Yao J, Liu Y, Lo R, Tretjakoff I, Peterson A, Stifani S. (2000) Disrupted development of the cerebral hemispheres in transgenic mice expressing the mammalian Groucho homologue transducin-like-enhancer of split 1 in postmitotic neurons. Mech Dev. 93, 105-115.

Yao J, Lai E, Stifani S. (2001) The winged-helix protein brain factor 1 interacts with groucho and hes proteins to repress transcription. Mol Cell Biol. 21, 1962-1972.

Yu X, Li P, Roeder RG, Wang Z. (2001) Inhibition of androgen receptor-mediated transcription by amino-terminal enhancer of split. Mol Cell Biol. 21, 4614-4625.

Zhang H, Emmons SW. (2002) Caenorhabditis elegans unc-37/groucho interacts genetically with components of the transcriptional mediator complex. Genetics. 160, 799-803.

7. APPENDICES



Montreal Neurological Institute and Hospital

A Teaching and Research Institute at McGill University

Internal Radioisotope User Permit

Issued by:

The Radiation Safety Committee of the
Montreal Neurological Institute and Hospital
Authorized by the Canadian Nuclear Safety Commission
CNSC Radioisotope Licence Number: 01187-2-08.1

1. Radioisotope User Permit Number : MNI_022
   Classification : Basic
   Date of Issue : May 1, 2003
   Revision Date : N/A
   Expiry Date : April 30, 2008

   RSO Office: 398-8927
   RSO Pager: 406-3069
   Locating: 8888-53333
   Home: 525-0220

2. Name of Principal Investigator : Stifani, Stefano

3. Department : CNS

4. Location(s) approved by this permit : F102, BT-218, F104

5. Radioisotopes approved by this permit : See non-shaded cells in table below.

Note: The permit holder needs written authorization by the CNSC for projects requiring more than 10,000 exemption quantities (E. Q.) of a radioactive substance.

approved for use of:	^{3}H	^{14}C	^{18}F	^{32}P	^{35}S	^{45}Ca	^{51}Cr	^{125}I
10'000 exemption quantities (E. Q)				100 MBq or 2.7 mCi	1 TBq or 27 Ci			
your possession limit is:				370 MBq or 10 mCi	370 MBq or 10 mCi			

6. Personal Dosimeters Required : Yes

7. Method of Disposal : All radioactive waste (solid and liquid) must be disposed of through the containers provided by the McIntyre Waste Management Facility (McGill) and brought to the central waste storage cage in the basement of the MNH/I (room 045 B).

8. Special Conditions:

Gloves and lab coats mandatory.
Weekly wipe tests required in areas where radioisotopes are used.
Ring badges required for staff using > 50MBq (~1.5mCi) of ^{32}P.
Monitoring of all work surfaces where ^{32}P is used at the end of the work day.
Radio-iodinations: Must be carried out in a working fume hood. Schedule thyroid monitoring. Use of proper survey equipment during radio-iodine manipulations.

The Radiation Safety Officer, MNH/I:___ _ (E.Meyer, RSO, ext. 8927)

Guidelines for completing the form are available at www.mcgill.ca/rgo/animal

McGill University
Animal Use Protocol – Research

For office use only
Protocol #: 4381
Investigator #: 303
Approval End Date: April 30, 2005
Facility Committee: M N I

Title: Coordinated regulation of progenitor cell proliferation and neuronal differentiation in the mammalian telencephalon.
(must match the title of the funding source application)

New Application: **Renewal of Protocol: # 4381** **Pilot**: **Category** *(see section 11)*: **D**

1. Investigator Data:

Principal Investigator: Dr. Stefano Stifani **Phone #:** 398-3946
Department: Neurology and Neurosurgery **Fax#:** 398-1319
Address: 3804 rue University, Montreal, QC, H3A 2B4 **Email:** stefano.stifani@mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: Stefano Stifani **Work #:** 514-398-3946 **Emergency #:** 514-830-0334
Name: Francesca Theriault **Work #:** 514-398-6255 **Emergency #:** 514-270-8595

3. Funding Source:

External: X	**Internal**:
Source (s) : CIHR	**Source (s)** :
Peer Reviewed: X YES NO**	**Peer Reviewed**: YES NO**
Status : X Awarded Pending	**Status**: Awarded Pending
Funding period: 01/04/2001–31/03/2006	**Funding period**:

For Office Use Only:

ACTION	BY	DATE
CCC		
DB	L/	16/04
APPROVED		

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/rgo/animal

Proposed Start Date of Animal Use (d/m/y):	01/05/2004	**or ongoing**:
Expected Date of Completion of Animal Use (d/m/y):	30/04/2005	**or ongoing**:

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature: **Date:** 11 March 2004

Chair, Facility Animal Care Committee:		**Date:** March 15, 2004
University Veterinarian:		**Date:** April 22, 2004
Chair, Ethics Subcommittee (as per UACC policy):		**Date:** 4/21/04
Approved Animal Use	**Beginning:** MAY 1, 2004	**Ending:** April 30, 2005

This protocol has been approved with the modifications noted in Section 13.

18 MAR. 2004

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: Dr. Stefano Stifani TELEPHONE: 398-3946

ADDRESS: Montreal Neurological Institute FAX NUMBER: 398-1319

3801 rue University E-MAIL: mdst@musica.mcgill.ca

DEPARTMENT: Neurology and Neurosurgery

PROJECT TITLE: Coordinated regulation of progenitor cell proliferation and neuronal differentiation in the mammalian telencephalon.

2. FUNDING SOURCE: CIHR XX NSERC NIH FCAR FRSQ

INTERNAL OTHER (specify)

Grant No.: Awarded Beginning date 01/04/2001 End date 31/03/2006

3. Indicate if this is

Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
Approval End Date

New funding source: project previously reviewed and approved under an application to another agency.

Agency Approval End Date

XX New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafty Manual". Containment Level (c

Principal Investigator (Course director

e: 05 03 01
day month year

DR. DALIUS J. GRIGERIS
Dept. of Microbiology & Immunology
McGill University
3775 University Street
Montreal, Quebec H3A 2B4
Canada

Chairperson, Biohazards Committee:

e: 20-03-01
day month year

Approved period: be

y 31-03-2006
month year

* as defined in the "McGill Laboratory Bi

2