Tcf4 is a target gene of the imprinted gene *Zac1* during mouse neurogenesis

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List of Abbreviations

°C	Celsius
AG	Androgenetic
Ara-C	Arabinofuranosyl Cytidine
AS	Angelman Syndrome
bHLH	basic Helix-Loop-Helix
BMP	Bone Morphogenic Protein
BP	Basal Progenitor
Bp / Kb	Base pairs / Kilo base pairs
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic
ChIP	Chromatin Immuno-Precipitation
CIAP	Calf Intestinal Alkaline Phosphatase
CNS	Central Nervous System
CP	Cortical Plate
C-terminal	Carboxy-terminal
DAPI	4′,6-Diamidin-2-phenylindol
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMR	Differentially Methylated Region
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTPs	Desoxyribonucleosidtriphosphate
DOX	Doxycycline
E	Embryonic day
EB	Embryoid body
EGF	Epidermal Growth Factor
eGFP	enhanced Green Fluorescent Protein
EGL	External Granular cell Layer
EP	Electroporation buffer
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
FGF2	basic Fibroblast Growth Factor
Fig.	Figure
GABA	γ-Aminobutyric acid
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GMEM	Glasgow Minimum Essential Medium
GnRH	Gonadotropin-Releasing Hormone
GST	Glutathione S-transferase
HAT	Histone Acetyltransferase
HRP	Horseradish Peroxidase
Hrs/Min/Sec	Hours/Minutes/Seconds
ICC	Immunocytochemistry
ICR	Imprinted Control Region
IG	Imprinted Gene
IgG	Immunoglobulin G
IGL	Internal Granular cell Layer
IGN	Imprinted Gene Network
IHC	Immunohistochemistry
INM	Interkinetic Nuclear Migration
IPC	Intermediate Progenitor Cell
KO	Knock-Out
KSR	Knockout Serum
Lif	Leukemia inhibitory factor
LPR	Linker Proline-rich Region
mES	mouse Embryonic Stem cell
N/D	Not Determined
NEP	Neuroepithelial cells
NS	Neurosphere
N-Terminal	Amino-terminal
OD	Optical Density
P	Postnatal day
PACAP	Pituitary adenylate cyclase-activating peptide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDL	Poly-D-lysine
PFA	Paraformaldehyde
PG	Parthenogenetic
PIC	Protease Inhibitors cocktail
poly I:C	Polyinosinic:polycytidylic acid
PWS	Prader-Willi Syndrome
qRT-PCR	Real time Quantitative Polymerase Chain Reaction
RA	Retinoic Acid
RGC	Radial Glial Cell
RNA	Ribonucleic Acid
RNase	Ribonuclease
RPM	Rotation Per Minute
RT	Room Temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SCN	Suprachiasmatic Nucleus
SD	Standard Deviation
SGZ	Sub-Granular Zone
shRNA	Small hairpin RNA
SNP	Single Nucleotide Polymorphism
SVZ	Sub-Ventricular Zone
TET	Tetracycline
TNDM	Transient Neonatal Diabetes Mellitus
U	Unit
UV	Ultraviolet
VZ	Ventricular Zone
WCE	Whole Cell Extract
WT	Wild Type
ZF	Zinc Finger
ZF7	Zinc Finger seven mutated

Abstract

During neurogenesis, the balance between factors promoting stem cell maintenance and those favoring cell differentiation, controls the transition from proliferative cell division to neurogenic cell division. Transcriptional and cell cycle regulators are key players of progenitor cell fate decision, and hence control the establishment of the neuronal networks which mediate cognitive functions later in life. Recent studies showed that imprinted genes regulate neurodevelopment and contribute to mental disorders. Here we show that the paternally expressed gene *Zac1* might promote neuronal differentiation by functioning as a transactivator of the *Tcf4* gene during neurogenesis.

The *Tcf4* gene encodes a basic helix–loop–helix (bHLH) transcription factor which belongs to the E-protein family. Tcf4 has been shown to mediate cell proliferation and migration. In addition, it is required for the neuronal differentiation of *Math1*-expressing populations in the hindbrain. In human, *TCF4* plays a critical role during human brain development and in cognitive functions. Haploinsufficiency of *TCF4* causes the Pitt Hopkins syndrome, and common variations in *TCF4* intragenic region were recently associated with schizophrenia and bipolar disorder. We showed that Zac1 coordinately binds to the proximal promoter and first intron of *Tcf4 in vitro* and *in vivo* and induces specifically its expression in several mouse neural cell types (embryonic stem cells, neural stem cells and mature neuronal populations) and adult neuronal population. We could also show that *ZAC1*, the human *Zac1* orthologue activates *TCF4* in vitro, suggesting conservation across species. Zac1 also regulates the expression of the *Tcf4* target gene $p57^{kip2}$ and hence, might control the cell cycle arrest and the migration of neuronal precursors in the developing brain.

Altogether we could identify (to our knowledge) the first direct regulator of *Tcf4* gene expression in the developing and adult brain. This suggests that the imprinted gene *Zac1* might contribute to the brain development by promoting neuronal differentiation of specific progenitor populations.

Introduction

I. Formation of the central nervous system (CNS)

The CNS arises from the neural plate, a specialized region of the ectoderm that is the outermost of the three primitive germ layers. During embryonic development, the neural plate folds and forms the neural tube composed of neuroepithelial cells (NEP). Initially, the whole neural tube will differentiate into major subdivisions along an anterior-posterior axis giving rise to the brain and spinal cord. At first, the most anterior part of the neural tube differentiates into the three primary brain vesicles: the prosencephalon, the mesencephalon and the rhombencephalon. These vesicles are later subdivided into secondary vesicles, respectively: the telencephalon and diencephalon; the mesencephalon; the metencephalon and the myencephalon. The telencephalon gives rise to the neocortex dorsally and the basal ganglia ventrally. The resulting cavity forms the lateral ventricles. The diencephalon gives rise to the retina, the thalamus and the hypothalamus. The tectum originates from the mesencephalon. Finally, the metencephalon gives rise to the pons and the cerebellum, while the myencephalon differentiates into the medulla oblongata *(Fig. 1)*.

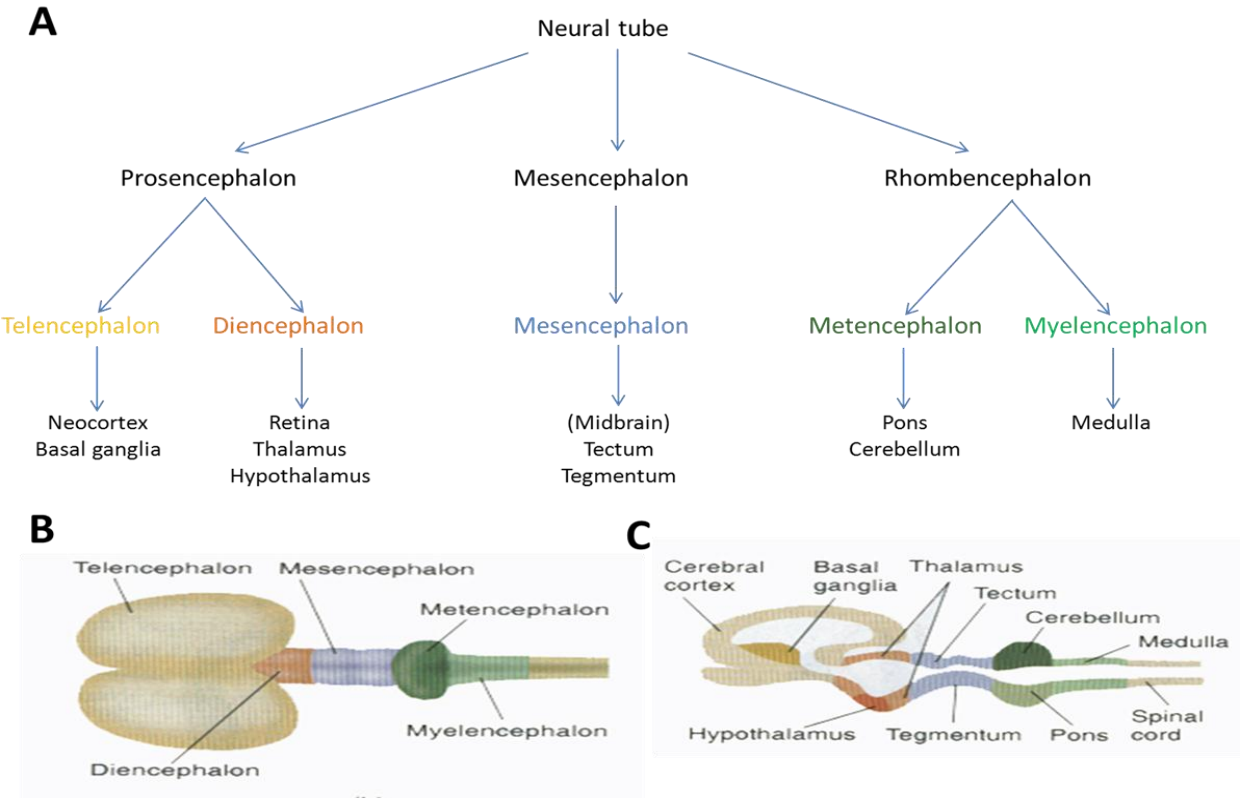


Figure 1: **Formation of the brain.** A) During development, the neural tube differentiates first into second, then third vesicles, which ultimately generate the different brains regions. The telencephalon and myelencephalon are the most rostral and caudal third vesicles, respectively. B) Rostrocaudal organization of the third vesicles. C) Scheme of the brain regions derived from the third vesicles, in a sagittal view. (Figure adapted from Carlson, 2004)

The central nervous system is composed of diverse cell subtypes whose spatio-temporal patterning is highly controlled and conserved during evolution. During brain development, all cell subtypes are generated from heterogeneous progenitors cells or neural stem cells which undergo sequential differentiation steps under the control of signaling molecules such as fibroblast growth factors (Fgfs), Wingless proteins (Wnts), sonic hedgehog (Shh), retinoic acid (RA), nodals, and bone morphogenic proteins (Bmps). In addition, cell differentiation is also under the control of intrinsic factors, as for instance specific combination of transcription factors in concert with epigenetic gene regulation which predispose the cells towards certain fates.

II. Neural stem cells and neural progenitors

The formation of the neural tube polarizes the cells with their apical side facing the inward zone which later becomes the ventricular zone (VZ) and their basal side facing towards the basal lamina and the pial surface (Götz and Huttner, 2005). NEP are characterized by the expression of different markers such as the intermediate filament *Nestin* (Lendahl et al., 1990), the SoxB1 transcription factor family members *Sox1*, *Sox2*, and *Sox3* (Wood and Episkopou, 1999). During mitosis, the nuclei of NEP migrate in a cell cycle dependent movement called Interkinetic Nuclear Migration - INM (apical to basal in G1, and basal-to-apical in G2), leading to the pseudo-stratification of the cell layers lining the ventricle (Taverna and Huttner, 2010). Before the onset of neurogenesis, all NEP expand via symmetric divisions and with the onset of neurogenesis, they switch to asymmetric mode of division to generate radial glial cells (RGC), basal progenitors (BP) also called intermediate progenitor cells (IPC) and post-mitotic neurons (preplate neurons) *(Fig. 2)* (Miyata et al., 2001, Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004).

a) Radial glial cells

RGC differentiate from NEP with the onset of neurogenesis, around embryonic day 10.5 (E.10.5) and acquire typical astrocytic features such as the presence of glycogen granules and the expression of astrocytic markers: the astrocytic specific glutamate and aspartate transporter (*GLAST*) (Shibata et al., 1997), the brain lipid-binding protein (*BLBP*) (Feng et al., 1994), *Tenascin-C* (Bartsch et al., 1992), the Ca2+ binding protein (*S100ß*) , *Vimentin* (Schnitzer et al.,

1981) and in the dorsal telencephalon *PAX6* (Götz et al., 1998; Heins et al., 2002). RGC exhibit apical–basal polarity and span the entire cortical wall. Their cell bodies are located at the most apical part of the cortical wall, with an apical process at the ventricular surface and a basal process at the pial surface. Their nuclei undergo INM and therefore RGC are, like NEP, layered in a pseudostratified epithelium manner (Kriegstein and Götz, 2003). In the telencephalon, RGC go through several rounds of mitosis and can therefore either increase the pool of multipotent proliferating cells upon clonal division or give rise to another RGC and to neuronal or glial progenitor after asymmetric cell division. These progenitors then migrate along their parental cell´s´ radial fibers (Malatesta et al., 2008) *(Fig. 2)*. During development, the differentiation potential of the RGC is regulated in a temporal manner, as neurogenesis precedes gliogenesis and oligodendrogenesis. In later stages of brain development, the vast majority of RGC fully differentiate into astrocytes, except small populations located in specific niches, namely the subventricular zone (SVZ) of the lateral ventricules and the subgranular zone (SGZ) of the dentate gyrus (Lois and Alvarez-Buylla, 1993; Johansson et al., 1999; Alvarez-Buylla et al., 2001; Merkle et al., 2004; Kempermann et al., 2004). These cells do not undergo terminal differentiation and maintain the ability to generate adult new born neurons and glia (Morrens et al., 2012).

b) Intermediate Progenitor cells

In the developing neocortex, ventral telencephalon and thalamus, cells are mainly born from RGC in the ventricular zone (VZ) but delaminate from that, and migrate in the basal direction to form a second layer of basal or intermediate progenitors cells (IPC), the sub-ventricular zone (SVZ) (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Wang et al., 2011). In the developing mammalian cortex, the SVZ is located between the VZ and the preplate *(Fig. 2)*. IPC are characterized by the expression of the T-domain transcription factor *Tbr2/Eomes* (Englund et al., 2005). They loose their basal process and can undergo asymmetric mitosis only a few times, mainly generating a pair of neuronal precursors, and rarely a pair of IPC (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). Upon asymmetric division, their daughter cells are restricted to neuronal lineage and have a limited mitotic cycle of 1-3 cycles (Noctor et al., 2004). Hence, the SVZ contains progenitor cells and migrating neurons derived from the VZ and IPC *(Fig. 1)*. In contrast to the progenitors derived from the RGC which can differentiate into neurons of all cortical layers, IPC are believed to generate mainly neuronal precursors fated to migrate and occupy the upper layers of the cortex (Arnold et al., 2008). This view is supported by the

expression in IPC of markers characteristic of upper layers neurons, such as the non-coding *RNASvet1* (Tarabykin et al., 2001) and the homeodomain transcription factors *Cux-1* and *Cux-2* (Nieto et al., 2004). In addition, phylogenic studies report that the enlargement of the SVZ during mammalian evolution parallels the expansion of the upper cortical layers (Cheung et al., 2010; Martínez-Cerdeño et al., 2006).

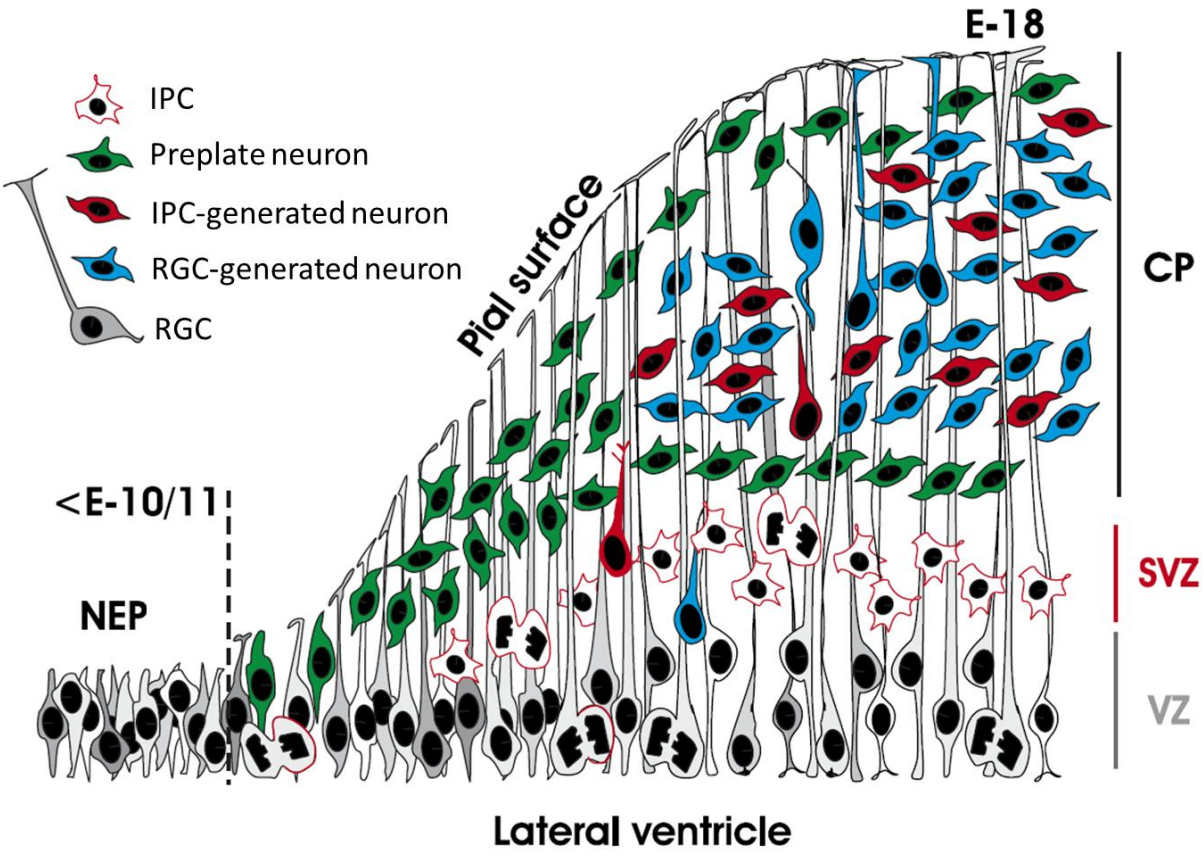


Figure 2: **The different cell populations during embryonic corticogenesis.** With the onset of neurogenesis, NEP cells progressively convert to RGC that elongate following the thickening of the neural tube wall. IPC (outlined in red) are generated at early stages by NEP, and at later stages by RGC. They accumulate in the SVZ. Preplate neurons (green) are the first post-mitotic neurons generated during corticogenesis and delineate apically the SVZ. At later stages, neurons derive from both RGC (blue) and IPC, migrate towards the cortical plate (red) to form the 6 cortical layers. CP cortical plate; SVZ subventricular zone;VZ ventricular zone (Figure adapted from Malatesta et al, 2008).

c) Proneural factors and E-proteins

During brain development, many factors and signaling pathways have been shown to play a role in the differentiation of neural progenitors. Among others, the basic Helix-Loop-Helix (bHLH) factors are key controllers of the proliferation, specification, differentiation and migration of progenitors during neurogenesis (Cai et al., 2000; Ge, 2006). The bHHL factors are characterized

by the presence of a basic helix loop helix domain that allows them, upon homo- or hetero-dimerization to bind to specific DNA sequences, the so-called E-Box (CANNTG) (Ephrussi et al., 1985; Murre et al., 1989). I will discuss here the role of the bHLH that play an important role in cell fate decision during corticogenesis, which includes the E-protein family and the proneural factors - Neurogenin, Mash, Math and NeuroD family members.

The proneural factors are bHLH proteins whose expression promotes the differentiation of the neural stem cells. These factors are directly or indirectly capable of modulating cell cycle progression and initiate transcriptional cascades controlling the fate of the committed precursors (Farah et al., 2000; Bertrand et al., 2002; Ross et al., 2003; Castro et al., 2011). During brain development, there are 2 classes of factors promoting neurogenesis, the "specification" factors such as Neurogenins, Maths, Mashs and NeuroD factors, whose expression is spatiotemporally controlled, and their ubiquitously expressed dimerization partners, the E-proteins (Bertrand et al., 2002; Bhattacharya and Baker, 2011). The specification factors control migration, specific neuronal fate acquisition (e.g glutamatergic vs GABAergic differentiation) and gain/loss of function studies highlighted their importance in the timing and in the control of cell fate (differentiation into neurons, astrocytes or oligodendrocytes (Lee et al., 1995; Cai et al., 2000; Fode et al., 2000; Tomita et al., 2000; Nieto et al., 2001; Parras et al., 2002; Parras et al., 2007).

During development, proneural factors are expressed at low levels in proliferating undifferentiated progenitors. With the onset of neurogenesis the expression of specification factors (*Neurogenin 1/2*, *Math1* and *Mash1*) and the E-protein family members (the two splice variants of *E2A*: *E12* and *E47*, *HEB* and *E2-2* also named *Tcf4*) increases. This results in the inhibition of the proliferation of progenitors, inhibition of astrogenesis (Sun et al., 2001) and in the induction of target genes, such as NeuroD genes which are required for terminal neuronal differentiation (Sun et al., 2001; Ross et al., 2003; Roybon et al., 2010).

Many studies have tried to address how cellular diversity of the brain can be achieved when only few specification factors are expressed. The number of dimerization partners and their specific association during fate decision could provide a way to modulate proneural bHLH functions.

The E-proteins were previously considered as non-specific cofactors of the specification factors. Indeed, in contrast to *Neurogenin2* and *Mash1* null mutants, *HEB* and *E2A* knockout mice did not display any gross morphological changes in brain anatomy, and these mutant mice exhibit higher expression of the other members of the E-proteins family. This suggests compensatory

mechanisms, where the dosage of E-protein and not the member identity is the key criteria to promote neuronal differentiation (Ravanpay and Olson, 2008). This view is supported by the fact that the 3 E-proteins are derived from a common ancestor (*Daughterless*) whose sequence was highly conserved throughout evolution and by the fact that their pattern of expression is overlapping during hematopoiesis and neurogenesis. In the hematopoietic lineage, where E-proteins play an important role in B and T-Lymphocytes development (Murre, 2005), *HEB* driven by the endogenous *E2A* promoter can functionally replace *E2A* in supporting B-cell commitment and differentiation (Zhuang et al., 1998). In addition, *in vitro* studies in the embryo-derived teratocarcinoma P19 cell line, showed that co-transfection of *NeuroD2* in combination with each E-protein, promoted neuronal differentiation with the same efficiency (Ravanpay and Olson, 2008).

The model of non-specific coactivating function of E-proteins was recently challenged by the study of Flora et al., which provided the first *in vivo* evidence for an exclusive function of dimers formed between a proneural bHLH factor and a specific E-protein (Flora et al., 2007). In this study, the authors identified a critical role for the heterodimer Tcf4/Math1 in the development of the mouse hindbrain. Interestingly in *Tcf4* knockout mice, they observed deficits in migration and differentiation of rhombic lip-derived $Math1^{+}$ progenitors in the pontine nucleus, that could not be compensated by the expression of the other E-proteins. Thus, Tcf4/Math1 heterodimers can exclusively activate specific differentiation programs required for pontine nucleus neurons. Altogether, the authors suggest the existence of two classes of neural progenitors in the developing hindbrain. The first class can differentiate when any one of the three E-proteins genes is deleted, because of functional compensation by the remaining E-proteins; and the second class requires the presence of Math1/Tcf4 heterodimers to activate the correct differentiation program. The authors propose that similar regulation occurs in specific neural progenitors in different regions of the developing neural tube, and that Tcf4 might additionally interact with various proneural bHLH such as the Mash1 (Persson et al., 2000b), or the NeuroD family members (Brzózka et al., 2010) to control brain development.

This hypothesis is supported by the fact that *TCF4* is critical for human nervous system development and cognitive function. Indeed, haploinsufficiency of *TCF4* causes mental retardation and the Pitt-Hopkins syndrome, a neurodevelopmental disorder characterized by mental retardation, seizures and hyperventilation (Amiel et al., 2007; Brockschmidt et al., 2007; Zweier et al., 2007; Greenblatt et al., 2008; de Pontual et al., 2009; Rosenfeld et al., 2009; Marangi et al., 2011; Whalen et al., 2012). Moreover, a trinucleotide repeat in intron 3 and a

single nucleotide polymorphism (SNP) in intron 4 were associated with increased risk of developing bipolar disorder or schizophrenia in adulthood respectively (Breschel et al., 1997; Cai et al., 2000; Li et al., 2010; Steinberg et al., 2011). Thus, whilst severe dysfunction of *TCF4* causes developmental defects, subtle changes at its transcription level might predispose to psychiatric diseases.

Altogether this suggests that E-proteins are partly redundant during development and that the control of their expression is of particular relevance to coordinate the transcription mechanisms controlling neuronal differentiation.

d) Repressors of neurogenesis and maintenance of pluripotency by Hes and Id proteins

At the onset of neurogenesis, the expression of proneural bHLH factors transiently increases to induce differentiation of specific progenitors. To counteract the neurogenic effect of proneural factors, and prevent the simultaneous differentiation of all progenitors in the VZ, the differentiating cells maintain the adjacent cells in undifferentiated state by a process called lateral inhibition (Artavanis-Tsakonas et al., 1999; Lai, 2004). Neuronal precursors express the Notch ligand at their surface which activates the Notch signaling pathway in the adjacent cells, inducing the expression of the repressor of the proneural genes, the *Hes* genes. The *Hes* genes are the mammalian homologues of *Drosophila hairy* and *Enhancer of split*. Upon activation of the transmembrane receptor Notch by its ligand Delta-like protein 1 (Dll1), the Notch intracellular domain (NICD) is released from the membrane and translocated to the nucleus where it forms a complex with RBP-J. The complex RBP-J/NICD acts as a transcriptional activator; whilst in absence of NICD, RBP-J functions as a repressor of *Hes1* and *Hes5* genes (Jarriault et al., 1995; Kopan and Ilagan, 2009; Imayoshi et al., 2010).

The lateral inhibition model is considered to explain the opposite pattern of *Hes1* and the Notch ligand *Dll1* expression in the nervous system during early ontogeny (Bettenhausen et al., 1995; Hatakeyama and Kageyama, 2006). This model, also called “salt-and-pepper” model states that at early stages, neural progenitors express similar levels of Dll1 and proneural genes. However, some cells express higher levels of Dll1, due to stochastic events, and activate more efficiently the notch signaling in the adjacent cells. The latter cells express then higher levels of Hes1 and Hes5, which lead to the lower expression of the proneural genes and Notch ligands, and

maintenance of stemness. Consequently, the former cells are less activated by Notch ligands, which induces the expression of proneural genes and Notch ligand, ultimately promoting neuronal differentiation.

However, recent observations that *Hes1* expression oscillates with a period of about 2-3 hours in neural precursors, which in turn induces the inverse oscillation of *Dll1* and *Neurogenin2* expression, have added another layer of complexity to the lateral inhibition model (Kageyama et al., 2008; Shimojo et al., 2008). By contrast, *Hes5* expression does not seem to oscillate, and is independent in NEP on Notch canonical pathway, but is first induced by active DNA demethylation of its promoter (Hitoshi et al., 2011).

Hes1 and *Hes5* genes are the direct effectors of Notch signaling during brain development and play key roles in the development of the telencephalon, where they can sustain progenitors in an undifferentiated, proliferative state and inhibit their neuronal differentiation (Ishibashi et al., 1995; Nakamura et al., 2000; Ohtsuka, 2001; Hatakeyama, 2004; Mizutani et al., 2007). They act as repressor of gene expression by recruiting repressor complexes such as Groucho/TLE to their target gene promoters (Yao et al., 2001), where they can either directly bind to DNA elements called N boxes (CACNAG) (Takebayashi et al., 1994) or can be tethered by other transcription factors. Their targets include, among others, key regulators of differentiation of glutamatergic and GABAergic neurons, namely *Neurogenin2* and *Mash1* (Baek, 2006; Holmberg et al., 2008; Imayoshi et al., 2008). Hes5 was also showed to heterodimerize *in vitro* with the E-protein E47, sequestrating it from its interacting proneural factor and hence, inhibits the transcriptional activity of the complex E-protein/proneural factor (Akazawa et al., 1992). However, recent experiments *in vivo* argue against this mechanism and rather favor the view that Hes proteins act as transcriptional repressors of proneural genes during neurogenesis (Holmberg et al., 2008).

Like *Hes1* and *Hes5*, *Id* genes can inhibit neurogenesis via two mechanisms: either by sustaining *Hes1* expression in neural stem cells (NSC) (Bai et al., 2007), or by competiting with the E-proteins for binding to the proneural factors (Jung et al., 2010). Id proteins possess a helix-loop-helix domain but lack the basic domain required for DNA binding. Hence, they can form heterodimers with other bHLH factors or their dimerization partners, the E-proteins; and prevent the binding of proneural/E-protein complexes to the promoter of their target genes (Jen et al., 1992; Kreider et al., 1992). During brain development, *Id1*, *Id2* and *Id3* are abundantly expressed in proliferating NEP (Tzeng, 2003) and facilitate self-renewal and proliferation of NSC by

inhibiting the transcriptional cascade controlled by proneural factors (Lyden et al., 1999; Jung et al., 2010).

During brain development, the cross-regulation of the proneural factors and pluripotency factors and their relative proportion determine both the cell fate and the timing of differentiation. Before the onset of neurogenesis, Hes and Id activity promotes proliferation of neural progenitors and inhibits the expression of neurogenic factors. Upon onset of neurogenesis, progenitors exit the cell cycle and concomitantly a transient increase in the expression of proneural bHLH factors and their dimerization partners, the E-proteins triggers the sequential expression of specification bHLH factors, ultimately leading to the generation of distinct neuronal populations.

In the last decade, several studies revealed the importance of transcription factors during neurogenesis; however it has now become clear that many gene families control brain development. Among them, the imprinted genes were identified as important regulators of cognitive function and brain development.

III. Brain development and imprinted genes

In 1984, Barton et al. in the UK (Barton et al. 1984) and Mcgrath and Solter in the USA (McGrath and Solter, 1984) discovered that parthenogenetic (PG) and androgenetic (AG) embryos (having two maternal or paternal genome copies respectively) do not complete embryogenesis. AG embryos died at the 8-somite stage and displayed retarded embryonic growth and extra-embryonic tissue overgrowth. PG embryos died at the 25-somite stage and exhibited reduced extra-embryonic tissue growth. The authors concluded that the maternal and paternal contributions to the embryonic genome in mammals are not equivalent, and that a diploid genome derived from only one of the two parental sexes is incapable of supporting complete embryogenesis. This discovery suggested that genes expressed from the paternal allele might have different functions than those expressed by the maternal allele, hinting for a differential contribution of the parental genomes to the embryonic development. These genes were identified later as expressed in a parent-of-origin-specific manner and named imprinted genes (IG) (Barlow et al. 1991). The molecular and cellular mechanisms underlying imprinting have been extensively studied, and it was shown that allele-specific expression is mediated by different epigenetic modifications on the two parental chromosomes (DNA methylation and Histone tail modifications). These modifications are established during gametogenesis and are maintained throughout life (Bartolomei and Ferguson-Smith, 2011; Kaneda, 2011). Furthermore, IG

expression is thought to be highly susceptible to environmental conditions (e.g dietary, stress) that could modify the epigenetic mark controlling the allele specific expression (Jaenisch and Bird, 2003).

Since the discovery of the paternally expressed *Igf2* gene and maternally expressed *Igf2r* and *H19* genes in 1991 (Barlow et al., 1991; DeChiara et al., 1991), approximately 100 *IG* have been identified. Recently, using new-generation sequencing methods for high-resolution of mouse brain transcriptome, Gregg et al. identified over 1300 RNAs (protein-coding and putative non coding) showing a parental bias in expression; suggesting that the number of IG is much higher than expected (Gregg et al., 2010a). However to accept all these genes as *bona fide* IG, the precise epigenetic mechanisms governing their biased expression need to be identified. The rationale of imprinting is still under debate with the most developed theory being the conflict theory (or kinship theory) (Haig and Westoby, 1989). According to this concept, imprinting developed from a genetic conflict between paternal and maternal genome interests (Haig and Westoby, 1989; Haig and Graham, 1991; Moore and Haig, 1991). In this model, genomic imprinting has evolved in species where females carry the offspring of more than one male during their life span and provide most of the post-fertilization nutrition. The father´s interests are to promote the survival and the reproductive success of his progeny over other´s, whereas mother´s interests are to equally provide resources between the different offsprings whilst maintaining enough resources available for further pregnancies. Therefore paternal genes are expected to maximize resources received from the mother and to promote maternal care to an individual offspring; whereas maternal genes restrain resource acquisition and maternal provisioning (Haig, 1997; Haig, 2004). The antagonistic function of IG in resource acquisition is further supported by the number of imprinted genes regulating placental function, *in utero* growth and the suckling behavior (Itier et al., 1998; Lefebvre et al., 1998; Li, 1999; Plagge et al., 2004; Isles and Holland, 2005).

IV. Genomic imprinting plays key roles in brain function

Imprinting has been clearly linked to social behavior and human cognitive function through studies of Prader-Willi syndrome (PWS) and Angelman syndrome (AS), which result from a paternally or maternally inherited deletion of an imprinted gene cluster (including *Snrp, UBE3A* and *Necdin* genes) on the same chromosomal region 15q11-13 (Cassidy et al., 2000). PWS is

associated with hypotonia, hyperphagia, hypogonadotrophic phenotype, stubbornness and compulsive traits (Cassidy et al., 2011), whereas AS is associated with absence of speech, ataxia, happy demeanor and inappropriate laughter (Williams et al., 2006). Interestingly, individuals with Angelman syndrome have anatomical brain abnormalities including cortical atrophy (Dörries et al., 1988), Purkinje cell loss and ventricular enlargement (Jay et al., 1991), indicating that IG play a role during brain development and brain structure organization.

The specific effect of IG on brain size and organization was previously demonstrated using chimeric mice (Allen et al., 1995) generated by aggregating wild type (WT) cells with PG or AG cells. Both types of chimera survived but they had different phenotypes: The brain, especially the forebrain of PG mice, was larger than WT brains, in contrast to the AG mice brains that were smaller. This study also demonstrated that during neurogenesis, AG and PG cells distributed in the brain in distinct patterns: AG cells preferentially localized to the hypothalamus (especially in the preoptic area), septum and the bed nucleus of stria terminalis whereas PG cells were mainly found in the striatum, hippocampus and neocortex. This study indicated a role of the imprinted genes in neurodevelopment and further suggested that the paternal and the maternal genomes may impact differentially on distinct brain systems.

Comprehensive studies of the expression pattern of IG in the brain revealed that these genes show a substantial variability in their spatio-temporal expression but also in some case, in their imprinting status (Gregg et al., 2010a; Gregg et al., 2010b); which might be an additional level of fine-tuning of the IG function. The dynamic nature of IG gene expression in the developing brain also suggests that these genes might be required for differentiation or proliferation of neural progenitors. However, although the expression of IG in the brain has been extensively studied, for most of them their functions and mechanisms of action during neurogenesis remain unclear.

For few genes, the mechanism of action has been identified by loss of function approaches which revealed that the paternally expressed genes *Peg3* and *Necdin* and the maternally expressed gene Cdkn1c/$p57^{kip2}$ have profound impacts on brain development. For instance, the paternally expressed putative transcription factor *Peg3* is required for the generation of oxytocin neurons in the hypothalamus. *Necdin* enhances the differentiation of GABAergic neurons in the forebrain (Kuwajima et al., 2006) and is required for the development of gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus (Miller et al., 2009). The cyclin kinase inhibitor $p57^{kip2}$ coordinates multiple stages of corticogenesis by controlling the proliferation, differentiation and

migration of neural precursors (Tury et al., 2011a). These examples indicate that, like proneural genes, IG are important factor controlling brain development.

V. The paternally expressed gene *Zac1* as regulator of embryonic development

In 1997, Spengler et al, using a functional expression cloning technique (Spengler et al., 1993), isolated two factors capable of activating the type I PACAP receptor in a kidney epithelial cell line: *p53* and a new zinc finger protein (Spengler et al., 1997). Like *p53*, this factor can concomitantly induce apoptosis and regulates cell cycle progression in a p53-dependent (Huang et al., 2001) and independent manner (Kamikihara et al., 2005), and was named after its function: Zinc finger protein regulator of Apoptosis and cell Cycle arrest 1 (*Zac1*). The rat orthologue of *Zac1*, *Lot1* (for Lost-On-Transformation) was identified in malignantly transformed rat ovarian surface epithelial cells (Abdollahi et al., 1997a) whilst the human orthologue *ZAC1* was discovered in 1998 and mapped to the chromosome 6q24-25, a region frequently deleted in solid tumors of different tissues (Varrault et al., 1998). Interestingly, loss of *ZAC1* expression has been observed in numerous tumor types, including breast tumors, ovary tumors and pituitary adenomas (Abdollahi et al., 1997b; Bilanges et al., 1999; Pagotto et al., 2000; Cvetkovic et al., 2004; Basyuk et al., 2005). Finally, *Zac1* inhibits tumor formation in nude mice (Spengler, 1997), suggesting a function for *Zac1* as tumor suppressor.

ZAC1 is a member of the Pleiomorphic Adenoma Gene (PLAG) family (Kas, 1998), and is also named *PLAGL1* (Pleiomorphic Adenoma Gene-Like 1) due to its structure analogy with the member of the PLAG family, *PLAG1* and *PLAGL2*. These three members are structurally similar but appear to have different functions. Indeed, *Zac1/Lot1/ZAC1* is a candidate tumor suppressor gene, whereas *PLAG1* and *PLAGL2* are proto-oncogenes (Kas et al., 1997; Zheng et al., 2010). In this dissertation, *Zac1* will refer to the mouse gene while *ZAC1/PLAGL1* and *Lot1* refers to the human and the rat orthologues, respectively.

a) *Zac1*/Lot1/PLAGL1 protein structure

Zac1 is located on chromosome 10 and its transcripts are spliced from 10 exons (Piras et al., 2000), where the last two are coding for a 693 or 704 amino acids long protein (Spengler, 1997; Warzée et al., 2010). *Zac1* contains seven N-terminal C2H2 zinc-fingers required for DNA

binding to palindromic elements or direct repeats elements *(Fig. 3)*. The linker Proline-Rich region (LPR) confers transactivational activity to Zac1. The C-terminal domain, together with the zinc finger domain, can recruit the general coactivator p300 and modify its HAT activity (Hoffmann et al., 2003; Hoffmann et al., 2006).

Lot1 encodes a 583 amino acid long protein and present the same structure as *Zac1*, however it does not contain the proline rich tripeptides motifs (PLE, PMQ or PML) (Abdollahi et al., 1997a). *ZAC1* codes for a 463 amino-acid protein that also shares a strong homology with *Zac1*, yet it lacks the proline rich tripeptide motifs, and the C-terminal regions rich in P,Q,L residues and PE,E repeats *(Fig. 3)*. Despite these structural differences, *Zac1* and *ZAC1* seem to have similar functions in apoptosis and growth inhibition (Bilanges et al., 1999).

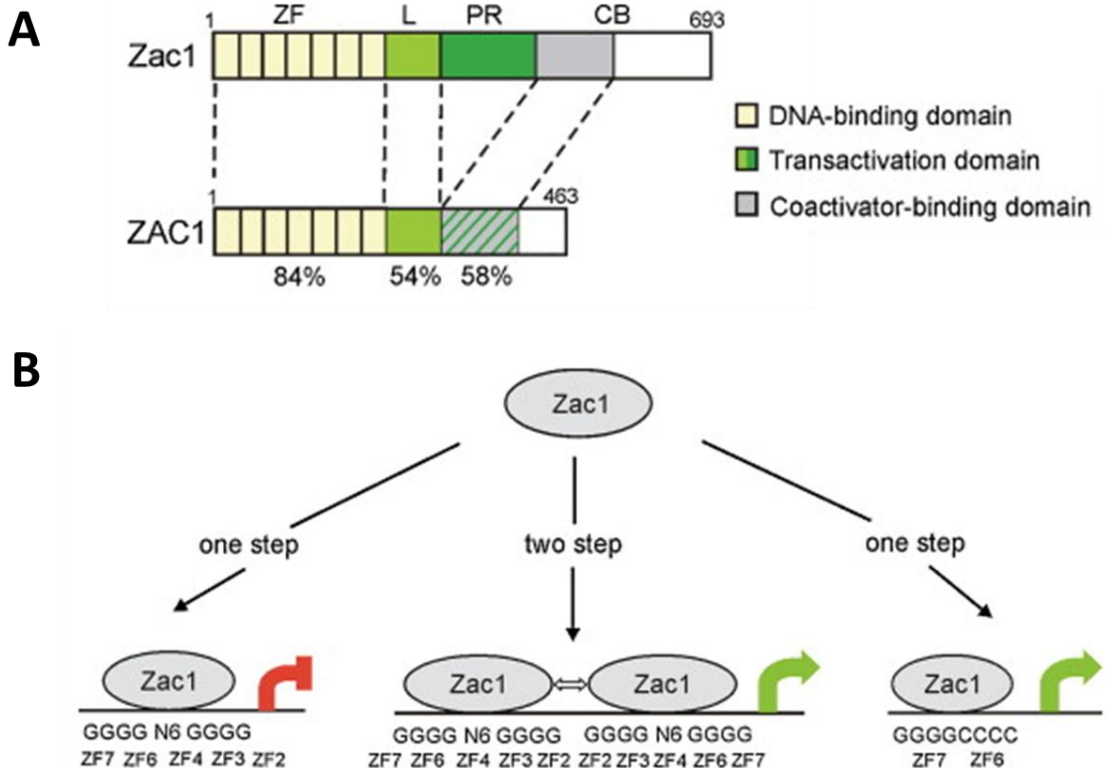


Figure 3**: Structure and transcriptional activities of Zac1. A) Scheme of Zac1 proteins.** Numbers denote amino acids, and domains are boxed. The homology (%) between mouse (Zac1) and human (ZAC1) proteins is indicated. Mouse Zac1 and human ZAC1 protein possess identical zinc-finger domains (ZF) that are involved in DNA-binding. The linker (L) confers transactivation in conjunction with the proline-repeat (PR) domain which exists solely in mouse. The coactivator-binding domain (CB) can recruit the general coactivators p300/CBP. B) Model of Zac1 transcriptional activities based on differential DNA-binding. Zac1 binding as a monomer to the palindrome G_4C_4, via zinc finger 6 and 7 confers transactivation. DNA binding occurs by multiple zinc finger contacts, including zinc fingers 2 to 4, 6, and 7. Zac1 binding to the direct repeat element $(G_4N_6G_4)_2$ promotes dimerization through zinc

finger 2 and transactivation. Multiple zinc fingers, including 2 to 4, 6, and 7, contribute to DNA binding. Zac1 binding to one half-site of the direct repeat element confers repression instead of transactivation (Figures modified from Theodoropoulou et al. 2010).

b) Expression pattern of *Zac1*

i. During development

Zac1 is abundantly expressed in the developing nervous system as well as in non-neural tissues including the myocardium, skeletal muscle, chondrogenic tissue, body wall of the umbilical region, limb buds, branchial arches, liver primordium, pancreas and somites (Valente and Auladell, 2001; Tsuda et al., 2004; Alam et al., 2005; Valente et al., 2005; Yuasa et al., 2010; Du et al., 2011) *(Table 1).* In the developing brain, between E9.5 and E15, *Zac1* is expressed in regions with active cellular proliferation such as the ventricular zone of the third and fourth ventricles (Alam et al., 2005; Chung et al., 2011) and the developing retina (Ma et al., 2007a). In the telencephalon, the expression of *Zac1* is mainly restricted to dividing progenitors and is patterned into a dorsal-ventral gradient with a highest expression in the dorsal cortical domains (Alam et al., 2005) indicating a role for *Zac1* in corticogenesis (Mattar et al., 2008). *Zac1* is also expressed in differentiating areas such as the cortical preplate, deep layer of the cortex (III-IV), the arcuate nucleus and the amygdaloid region (Valente and Auladell, 2001). In the cerebellum, *Zac1* is expressed in the ventricular zone overlying the fourth ventricle, in the external granular cell layer (EGL), in differentiating GABAergic interneurons and a subset of Golgi cells (Chung et al., 2011).

From E15 until postnatal day 0 (P0), *Zac1* expression remains strong in the differentiating areas, where it was expressed earlier and in the different germinative layers of the brain: VZ and EGL of the cerebellum (Valente and Auladell, 2001; Chung et al., 2011). In the cortex, *Zac1* is expressed at much reduced levels in the SVZ, and at intermediate levels in a band of cells in the developing cortical plate (Alam et al., 2005).

ii. At postnatal stages

In adult mice, *Zac1* is highly expressed in the anterior pituitary, in chondrogenic sites, at diverse levels in the brain, and faintly in peripheral tissue, such as stomach, kidney, adrenal gland, heart and lung (Spengler, 1997; Valente and Auladell, 2001).

After birth *Zac1* expression decreases in the VZ and the SVZ but increases in certain neuronal populations, as for instance in the pyramidal neurons of the CA3 of the hippocampus and in the

migrating neurons in the rostral migratory stream. Moreover its expression increases in differentiated neurons of the amydgaloid area, in the arcuate nucleus and other hypothalamic nuclei (Valente and Auladell, 2001). In the cerebellum, *Zac1* is transiently expressed in the EGL and its derivatives (in a subset of Purkinje cells, and in GABAergic interneurons) from P0 until P20; suggesting a role for *Zac1* in the differentiation/maturation of specific GABAergic population of the cerebellum (Valente and Auladell, 2001; Chung et al., 2011). Interestingly, Lot1 is transiently expressed in the developing suprachiasmatic nucleus from P1 until P20, where its expression shows a circadian rhythm peaking at day time, suggesting a role for Lot1 in maturation of suprachiasmatic nucleus (SCN) neurons (Maebayashi et al., 1999).

Hence the pattern of expression in progenitors and differentiated cells suggests that *Zac1* plays a role in both control of proliferation, consistent with its role as cell cycle regulator, but also in differentiation of specific neuronal subpopulations. However, the precise molecular mechanism by which *Zac1* regulates neurogenesis is still unknown.

c) Imprinting of *Zac1*

Transient neonatal diabetes mellitus (TNDM) is a rare condition characterized by intrauterine growth retardation, dehydration and hyperglycemia due to low levels of insulin secreted during the first months after birth (Shield et al., 1997). Despite an apparent remission by 3 months, there is a tendency for children to develop diabetes in later life (Temple, 2002). In 1995, Temple et al. (Temple et al., 1995) reported that TNDM is associated with paternal uniparental disomy of the human chromosome 6, thus suggesting a pathogenetic role for imprinted genes. This was further supported by the discovery that patients with paternal disomy of the chromosome 6, duplication of paternal 6q (Temple, 1996) and methylation defect on the maternal allele (Gardner, 2000) present the same phenotype. The genomic region associated with TNDM was further defined (Temple, 1996) and two paternally-expressed candidate genes were identified: *ZAC1* and the non coding RNA *HYMAI* (Arima et al., 2000; Kamiya, 2000) whose exclusive paternal expression relies on DNA methylation in the promoter region of the maternal allele (Arima, 2001; Mackay et al., 2002; Arima et al., 2006). This differentially methylated region (DMR) is a so called imprinting control region (ICR) that mediates the parent-of-origin specific expression. Zac1 ICR harbors a CpG island where the maternal allele is methylated (preventing its expression) and where the paternal is devoid of methylation and can be expressed *(Fig. 4)*. Therefore, duplication of the paternal allele, or defects in methylation of the maternal allele on chromosome 6, result in overexpression of the *ZAC1/HYMAI* genes which causes TNDM. The role of *ZAC1/HYMAI* in

TNDM was also evidenced by the study of Ma et al, (Ma et al., 2004) in which transgenic mice harboring the human TNDM locus upon paternal transmission display, hyperglycemia in neonates which resolves in juvenile mice and impaired glucose tolerance in adult. Overexpression of *ZAC1*/*HYMAI* in these transgenic mice recapitulated the key features of TNDM and implicated a role for *ZAC1*/*HYMAI* in pancreatic beta-cells development and function (Ma et al., 2004).

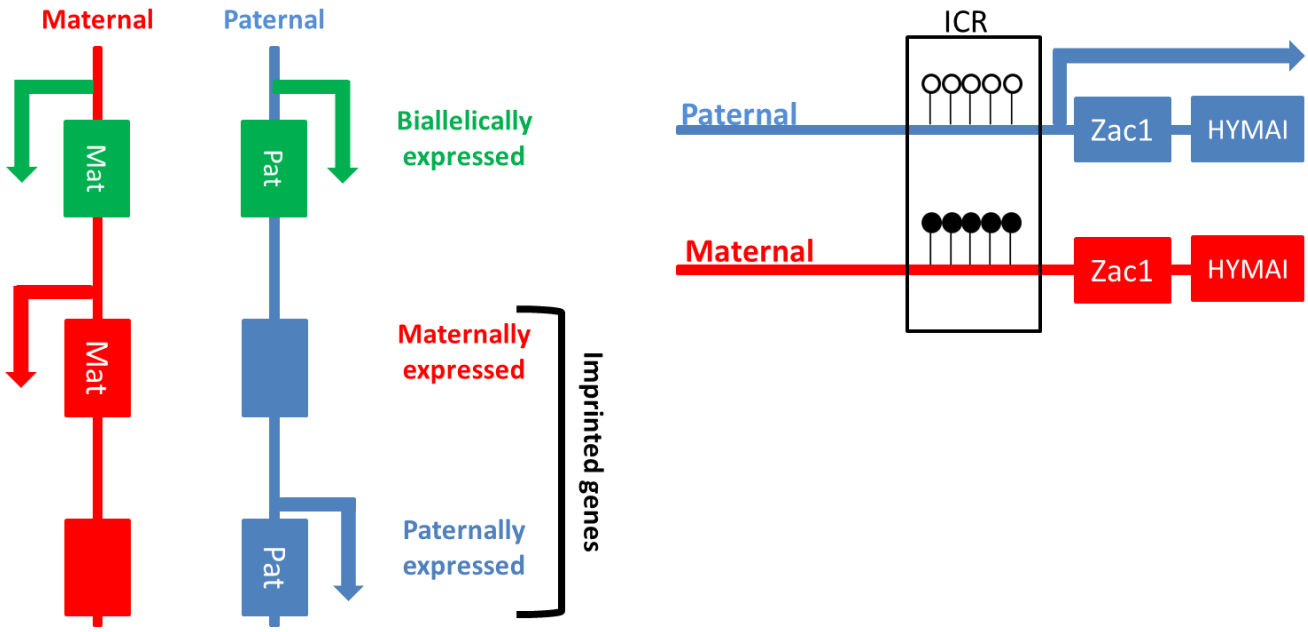


Figure 4: **Paternal expression of the imprinted gene Zac1.** A) Imprinted genes are expressed in a parent-of origin manner. Most of the genes are biallelically expressed (green boxes). Only a small subset of genes is exclusively expressed either from the maternal (Mat) or paternal (Pat) allele. These genes are named imprinted genes. B) The paternal expression of the *Zac1* and *HYMAI* genes is mediated by a differential cytosine methylation in the imprinted control region (ICR). The ICR of Zac1/HYMAI is a CpG island which is located in the promoter of the Zac1/HYMAI genes. The ICR is highly methylated on the maternal allele (●●●●●), which prevents the Zac1/HYMAI gene expression; whereas it is devoid of methylation on the paternal allele (○○○○○), which enables gene transcription.

d) Biological function of *Zac1*

The mechanism of imprinting is conserved between mouse and human (Arima et al., 2006) and therefore, knockout mice (KO) can be obtained by inactivating the *Zac1* paternal allele. Varrault et al (Varrault et al., 2006) used this approach to create *Zac1* KO ($Zac1^{+/-pat}$). *Zac1* mutant mice show intrauterine growth retardation, impaired bone formation, lung defects and neonatal lethality. Interestingly, in contrary to what is expected from a tumor suppressor gene with proapoptotic and cell-cycle-blocking activities, KO of *Zac1* resulted in embryonic growth restriction. This is however consistent with the kinship theory of imprinting, where paternally

expressed genes are growth-promoting. Hence, the authors suggest that number of genes have been evolutionally selected to be imprinted according to their physiological properties rather than their molecular functions (Varrault et al., 2006).

To gain insight into the function of *Zac1*, Varrault et al. (Varrault et al., 2006) compared microarray data sets to identify genes that were co-expressed with *Zac1*. They identified an imprinted gene network (IGN) which controls embryonic growth and differentiation, and where the downregulation of *Zac1* alters the expression of several other imprinted genes such as *p57^{kip2}*/*Cdkn1c*, *IGF2*, *H19* and *Dlk1 (Fig. 5)*. They further showed that *Zac1* directly controls the expression of *IGF2* and *H19* by binding to their shared enhancer E2 on chromosome 7; however they could not identify by which mechanism *Zac1* acts on the other imprinted genes.

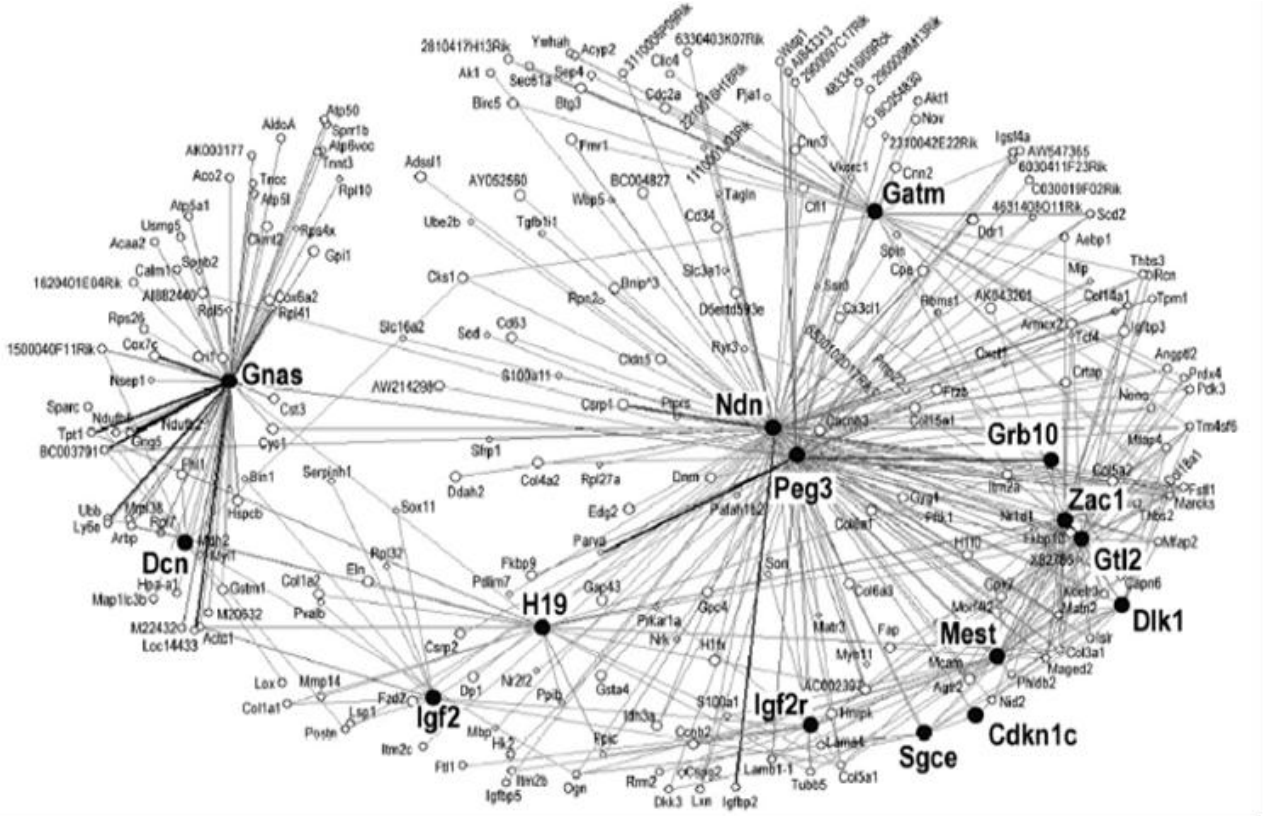


Figure 5: ***Zac1 b*elongs to an imprinted gene network.** Genes co-expressed with imprinted genes, including *Zac1*, were deduced from a body of 116 microarray data sets. 246 genes linked to at least three imprinted genes were then selected. The links between genes have different gray densities according to the number of data sets in which the genes are coregulated (light gray = 4 to black = 10). (Varrault et al. 2006) (Varrault et al., 2006).

Anatomic structure	E9.5	E10.5	E12	E14	E16	E18	P0−P9	>P12
	Whole embryos				Brain slices			
Body wall of the umbilical region	++	++	+++ +					
Costal cartilage (cc)	−	−	++					
Forelimb (fl)	−	+/−	+++ +					
Hindlimb (hl)	−	+/−	+++ +					
Liver primordium (l)	+++ +	+++ +	++					
Midbrain (fm)	++	+	+					
Neural tube (nt)	+++	+++	++					
Optic eminence (oe) or neural retina (nr)	−	+/−	+++					
Pericardial region or Heart (h)	+++	+++ +	+					
Second branchial arch	−	+	++					
Somite (s)	+/−	+	++					
Telencephalic vesicle (tv)	++	++	++					
Dorsal diencephalic sulcus				+++	++	++		
Dorsal thalamus				+	−	−		
Entopeduncular nucleus				+	−	−		
Epithalamus area				+++	−	−		
Geniculate body, dorsal lateral and medial (g)				−	+/−	+		
Interventricular foramen of Monroe (IVF)				+	+/−	+/−		
Mammillothalamic tract (MTa)				++	−	−		
Medial forebrain bundle, posterior				+/−	+/−	+/−		
Medial vestibular nucleus				+/−	+/−	+/−		
Neuroepithelium of aqueduct of Sylvius (nAQ)				+++	+++	+++		
Neuroepithelium of fourth ventricle				+/−	+/−	+/−		
Neuroepithelium of infundibulum n(IF)				+++ +	+++	+++		
Neuroepithelium of pineal recess				++	++	−		
Neuroepitheium of periventricular hypothalamic nucleus				+	+	+		
Neuroepithelium of preoptic recess				+/−	+	++		
Neuroepithelium of third ventricle				+++	+++	+++		
Neuroepithelium of ventral hypothalamic sulcus (nvhs)				+++	+++	+++		
Reticular thalamic nucleus				+/−	+	+		
Subcommissural organ (SCO)				−	+/−	+		
Tegmentum (Tg)				++	++	++		
Ventricular zone (VZ)				+++ +	+++ +	+++ +	++	+/−
Subventricular zone (SVZ)				+/−	+	++	+	+/−
Marginal zone (MZ)				+	+/−	+/−	−	−
Cortical plate (CP)				−	+/−	+	+/−	−
Arcuate nucleus (arc)				++	++	++	++	+++ +
Dorsomedial hypothalamic nucleus (dmh)				++	++	++	+++	++
Ventromedial hypothalamic nucleus (vmh)				++	++	++	++	++
Ventral posterior thalamic nucleus (vpm)				+	++	++	+/−	−
Dorsomedial thalamic nucleus (dmt)				−	+/−	+	+	+
Amygdaloid area (AA)				+	+	+	+	++
CA3 field of the hippocampus, posterior levels (Py)				−	−	−	+++	+++ +
Pyramidal cells of the hippocampal formation				−	−	−	+/−	+/−
Granular cells of the dentate gyrus				−	−	−	+/−	+/−
Septal area				−	−	−	−	+/−
Piriform area				−	−	−	−	+/−
Choroid plexus (ChPl)				+++	+++	+++	++	+
Medulla (Me)				−	−	+/−	+/−	+
Dorsal cochlear nucleus (cod)				−	−	+	+/−	−
External granular layer of the cerebellum (EGL)						+++	++	−
Internal granular layer of the cerebellum (IGL)						−	+	−
Differentiating field of the olfactory bulb				++	++	++	+	+
Accessory olfactory bulb (differentiating field)							++	++
Nerve of olfactory bulb							+	+/−
Mitral cell layer of the olfactory bulb (Mi)							+/−	+
Anterior hypothalamic area (AH)							+	++
Posterior hypothalamic nucleus (PH)							+	++
Lateral hypothalamic nucleus (LH)							+++	++
Purkinje cells (Pj)							++	+

[a] +++ +, intense; +++, strong; ++, moderate; +, weak; +/−, very weak.

Table 1: **Expression pattern of *Zac1* during mouse embryonic development and postnatally**. The intensity of the signal detected by *in situ* hybridization (ISH) using a mouse *Zac1* cDNA riboprobe is indicated. (Valente and Auladell, 2001).

e) *Zac1* as regulator of progenitor cell differentiation

Accruing evidence describes a role for *Zac1* function in the control of progenitor cell fate during development. Indeed, it was recently identified as an essential transcription factor for cardiac morphogenesis (Yuasa et al., 2010) and for neurogenesis in the retina (Ma et al., 2007a; Ma et al., 2007b). *Zac1* also regulates, upon PACAP activation, the proliferation of neuronal precursors in the developing cerebellum (Fila et al., 2009); and is necessary for the differentiation of distinct GABAergic neuronal populations in the cerebellum (Chung et al., 2011).

An unpublished work of Tony Valente describes in more details the effects of *Zac1* KO on murine brain development. Mutant mice had smaller brains, displayed in some cases hydrocephalia and increased mitotic activity in the VZ and SVZ of the lateral and third ventricles. Changes in neuronal and glial cell population ratio (especially GABAergic interneurons, catecholaminergic neurons and oligodendrocytes) were observed, supporting a role for *Zac1* in the proliferation and differentiation of specific neural progenitors during brain development (http://www.tesisenred.net/bitstream/handle/10803/840/2.RESULTADOS.pdf?sequence=3).

Aim of the thesis

The paternally expressed gene *Zac1*/*ZAC1* has been previously identified as a cell cycle regulator. Interestingly, it acts as a transcription factor, which, like proneural genes, shows a dynamic expression in the neuroepithelia of the developing brain. *Zac1* is part of an imprinted gene network that regulates embryonic growth and was recently shown to control the proliferation and/or the differentiation of distinct neural progenitors. However, the molecular mechanism by which *Zac1* might regulate neurogenesis has not been addressed yet. As *Zac1* functions as a transcription factor, we hypothesized that identifying its transcriptional targets would help to better understand how *Zac1* participates in progenitor cell fate decision. To identify *Zac1* target genes, we used the same approach as described in the study of Barz et al. (Barz et al., 2001). We applied genome-wide expression analysis and identified, among others, the E-protein *Tcf4* as putative *Zac1* target gene. The aim of the work presented here was to validate the dimerization partner of proneural factors, *Tcf4*, as direct *Zac1* target gene and to investigate how this regulation could contribute to brain development.

Materials and Methods

I. Materials

a) Molecular biology

Chemicals - DNA/ RNA analysis

Reagent	Company
1kb DNA ladder	Life technologies
Agar	Life technologies
Agarose – electrophoresis grade	Life technologies
Agarose – universal	Peqlab
Ampicillin	Roth
Chloroform	Roth
Chloroform	Roth
Phenol	Roth
dNTPs	Thermo scientific
DMSO	Roth
EDTA	Sigma
Ethanol	Roth
Ethidium bromide	Biomol
Glycogen	Thermo scientific
Glycerol	Roth
Isoamylalcohol	Merck
IPTG	Thermo scientific
β-mercaptoethanol	Merck
Oligonucleotides	Metabion
2-propanol	Merck
2-propanol - RNAse free	Merck
Tris	Sigma
Tryptone	Roth
Sodium acetate	Merck
Sodium hydroxyde	Merck
X-Gal	Sigma
Yeast extract	Life technologies

Chemicals – Protein analysis

Reagent	Company
3mm membrane	Whatman
Bradford assay	Biorad
Bovine serum albumin	Sigma
Coomasie blue	Biorad
DAPI	Life technologies
Developing solution	Kodak
Fixing solution	Kodak
Formaldehyde	Merck
Glycine	Riedel-deHaen
L-Glutathione reduced	Sigma
Glutathionine	Amersham
Methanol	Roth
Mowiol	Roth
Milk powder	
NaCl	Merck
Non fat dried milk	
Normal Goat Serum	Abcam
Ponceau	Roth
Protease inhibitor cocktail	Sigma Aldrich
Protein marker	Thermo scientific
SDS	Biomol
Slim Fast Chocolate powder	
TEMED	Sigma
Tris	Sigma
Triton X-100	Roth
Tween 20	Roth

DNA/RNA modifying enzymes

Enzyme	Company
Accuprime GC-rich	Life technologies
CIAP	Thermo scientific
DNA polymerase (*Taq*.)	Thermo scientific
DNA polymerase (Pfu)	Thermo scientific
Proteinase K	Boehringer Manheim
Restriction enzymes	Thermo scientific
Revertaid Reverse transcriptase	Thermo scientific
RNase	Sigma
T4-DNA-Ligase	Thermo scientific

Molecular biology kits

Kit	Company
96-PCR purification plate	Macherey-Nagel
BigDyeTerminator v3.1	ABI
Lipofectamine 2000	Life technologies
Magna ChIP G kit	Merck
Montage SEQ96 cleanup kit	Millipore
NucleoSpin plasmid quick-prep	Macherey Nagel
NucleoSpin PCR purification	Macherey-Nagel
Nucleospin RNA II	Macherey-Nagel
pGEM-T vector system	Promega

b) Cell culture materials

media/solutions	Company
DMEM	Life technologies
DMEM-F12	Life technologies
EBBS	Life technologies
ESGRO medium	Biozol
FCS	Life technologies
Kanamycin	Life technologies
Knock Out Serum	Life technologies
Neurobasal	Life technologies
Neurobasal A	Life technologies
OPTIMEM	Life technologies
PANSera ES4	Pan Biotech
PBS	Life technologies
Pen/Strep	Life technologies
Trypsin	Life technologies

reagents	Company
All-trans Retinoic acid	Sigma Aldrich
Accutase	Merck
B-mercaptoethanol	Sigma Aldrich
Dexamethasone (Fortecortin)	Merck
DnaseI	Worthington
Doxycycline	Sigma Aldrich
Epidermal growth factor (Human)	Peprotech
Fibroblast growth factor basic (Human)	Peprotech
Gelatine	Sigma
Leukemia inhibitory Factor (LIF)	Merck
N2 supplement	Life technologies
Poly-D-Lysine	Life technologies
Poly (I-C) (Cat # Tlrl-pic)	Invivogen
Soy bean trypsin inhibitor	Sigma Aldrich
Tetracycline	Sigma Aldrich

c) Antibodies

I. Primary antibodies

Epitope	Type	Company / source
ß-Actin	Mouse monoclonal (8H10D10)	Cell signaling
Flag	Mouse monoclonal (M2)	Sigma aldrich
GFAP	Mouse polyclonal	Dako
Hemagglutinin (HA)	Mouse monoclonal (HA-7)	Sigma aldrich
ITF2	Mouse monoclonal Ri3b9	Kind gift from Dr. Herbst
Nestin	Mouse monoclonal (2Q178)	Abcam
NeuN	mouse monoclonal (A60)	Millipore
Tcf4	Rabbit polyclonal	Pineda
Tcf4-B	Rabbit polyclonal	Pineda
TuJ-1	Mouse monoclonal (TU-20)	Abcam
Zac1-LPR	Rabbit polyclonal	Pineda
Zac1-LPR	Guinea Pig polyclonal	Pineda
Zac1- C	Guinea Pig polyclonal	Pineda

II. Secondary antibodies

Immunocytochemistry (all from Dianova)	Immunoblot (all from Sigma Aldrich)
Dylight 488 conjugated™ Donkey anti-guinea pig IgG	**Sheep anti-mouse IgG peroxidase conjugated**
Dylight 594 conjugated™ Donkey anti-guinea pig IgG	**Goat anti-rabbit IgG peroxidase conjugated**
Dylight 488 conjugated™ Donkey anti-mouse IgG	
Dylight 594 conjugated™ Donkey anti-mouse IgG	
Dylight 488 conjugated™ Donkey anti-rabbit IgG	
Dylight 594 conjugated™ Donkey anti-rabbit IgG	

II. Methods

a) Animals

All animals used in these experiments (C57BL/6N and Cd1 mice) were obtained from Charles River Laboratory (Charles River, Sulzfeld, Germany), upon arrival they were housed, at the animal facility of Max-Planck Institute of Psychiatry, under standard conditions [temperature controlled (21°C) environment and 12h light:12h dark cycle (lights on at 06:00)]. For neurospheres and primary embryonic cultures, female Cd1 mice were checked daily at 7:00 am and those that had mated, as evidenced by the existence of a vaginal plug, were then housed with other pregnant mice. The positive plug date was termed E1. For postnatal cerebellar neuronal cultures, male and female Cd1 juvenile mice were housed with the mother; the date of birth was termed P1.

b) DNA analysis

I. PCR reaction

Standard PCR were performed in Biometra T-Gradient thermocyclers (Biometra, Germany) using Fermentas Taq polymerase (Thermo scientific). PCR reaction mix was prepared as followed : 2 µl template, 2.5 µl 10×reaction buffer, 1.5 µl dNTPs (10 mM each), 0.5 µl forward primer (10 pmol/µl), 0.5 µl reverse primer (10 pmol/µl), 4 µl $MgCl_2$ (25 mM), 1U Taq polymerase, distilled water to 25 µl. If not indicated otherwise, the following PCR conditions were used: Initial denaturation (95 °C, 3 min); then 35 cycles [denaturation (95 °C; 1 min) – annealing (see table 3 for annealing temperatures; 30s) – elongation (72 °C; 1 min)]; then final elongation (72 °C, 5 min).

II. Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyse the quality of DNA and to separate fragments by size. Depending on the size of the DNA molecules, agarose solutions were prepared ranging from 0.8 to 2% (w/v) in 1 x TBE buffer (90 mM Tris, 90 mM Boric acid, 2 mM EDTA). Ethidium bromide was added to a final concentration of 0.25 µg/ml. The samples to be analysed were mixed with 1x loading dye (6x loading dye: 40% v/v sucrose, 0.25% xylene cyanol, and 0.25% bromophenol blue). The voltage applied depended on the distance between the electrodes. In

general, the voltage applied was between 4 and 6 V/cm. As size standard, a 1kb DNA ladder (Invitrogen) was used. DNA or RNA was visualized under UV light.

III. Recovery of DNA from agarose gels

The desired DNA band was cut out from an electrophoresis-grade agarose gel under UV light and transferred into a 1.5 ml tube. The Macherey-Nagel PCR purification kit was used to recover the DNA fragment from the gel. NT buffer was added to the tube which was incubated at 65 °C on a thermomixer (Eppendorf, Germany) with gentle shaking until gel melting. The mixture was transferred to a PCR purification column. After purification, DNA was eluted in 15 µl TE buffer.

c) RNA analysis

I. RNA isolation

Total RNAs from brain tissues or cell lines were isolated using the Nucleospin RNA II kit (Macherey Nagel). All plastic and glassware and the pestle used were autoclaved before use to inactivate RNases. Brain tissues were first crushed using a pestle and resuspended in lysis buffer. The homogenate was then further passed several times through a hypodermic syringe (29G), and processed following manufacturer´s protocol. RNA was eluted in 30 µl DEPC-treated water and RNA concentrations were measured spectrophotometrically using the Nanodrop.

II. cDNA synthesis and subsequent gene-specific PCR

A total volume of 20 µl was used for cDNA synthesis reaction using the RevertAid™ Premium Reverse Transcriptase (Thermo scientific). Total RNA (200 ng) was subjected to a reverse transcription reaction in the presence of 1 µl oligo $(dT)_{18}$ (100 pm), 1 µl dNTPs (10 mM each) and adding sterile water to 12 µl. Then the mixture was heated to 65 °C for 5 minutes and quick chilled on ice. The contents of the tube were collected by brief centrifugation before adding 5× RT buffer, 1 µl Ribolock^{Tm} RNase inhibitor (Thermo scientific) and 1 µl RevertAid™ Premium Reverse to the reaction. The samples were incubated first 10 minutes at 25 °C, then 40 minutes at 50 °C. Finally, the reaction was inactivated by heating at 85 °C for 5 minutes. The cDNA was used as template for amplification in PCR reactions.

III. Quantitative PCR analysis

mRNA expression levels and DNA amounts precipitated during Chromatin immunoprecipitation (ChIP) were analysed by quantitative Real Time PCR, using the Absolute Blue QPCR Sybr green mix (ABgene) and the MJ Mini Opticon light cycler (Bio-Rad). All experiments were performed according to manufacturer's instructions. The primers used for qRT-PCR were designed across exons to avoid genomic DNA amplification. The different primers used are listed (Table 2) and experiments were performed in triplicates. Fluorescence was assessed each cycle after elongation phase. At the end of each run, a melting curve (50-95 °C with 0.05 °C/sec) was generated to evaluate the quality of the PCR product. Cycle threshold values (Ct values) were determined using the Opticon monitor 3 software (Biorad). Threshold and noise band were set in all compared runs to the same level. Relative gene expression was determined by the $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001) using the real PCR efficiency calculated from an external standard curve and normalized to the expression of the house keeping gene Mas.

RT-PCR/qRT-PCR

	Gene investigated	Forward	Reverse	Annealing T°
Mouse	*Gapdh*	5′- ccatcaccatcttccaggagcgag –3′	5′- gatggcatggactgtggtcatgag-3′	58
	Mas (ATPJ5)	5′- tattggcccagagtatcagca – 3′	5′- ggggtttgtcgatgacttcaaat-3′	61
	N-cadherin	5′-cggtttcacttgagagcaca-3′	5′-gctacaatgacgtccaccct-3′	58
	Nestin	5′-aggaaccaaaagagacaggtg-3′	5′-ttcctcagatgagaggtcaga-3′	57
	Oct4	5′- ggcgttctctttggaaaggtgttc – 3′	5′- ctcgaaccacatccttctct-3′	60
	$p57^{Kip2}$	5′-caggacgagaatcaagag-3′	5′-ggttcctgctacatgaac-3	57
	Tcf4 /Tcf4-B	5′-aactatggagatgggact-3′	5′-tttggtgggcgaaagggtt-3′	55
	Tcf4-A	5′-atgggcggcaactctttgat	5′-ctggcttggaggaaggatag-3′	61
	TuJ-1	5′- tagtggagaacacagacgaga –3′	5′- ctgctgttcttactctggatg-3′	57
	Zac1	5′- gccttcgtctccaagtataagctg –3′	5′- atcgccattgctcgccgag –3′	64
Human	*TCF4-B*	5′-tccaggtttgccatcttcagt-3′	5′-gcctgggcagtccctattg-3′	56
	ZAC1	5′-gtgctactggaccacctcaa-3′	5′-tcatcagctcctgtgagtgg-3′	56
	GAPDH	5′- cctgcaccaccaactgcttagc –3′	5′- caggtccaccactgacacgttgg-3′	62

ChIP

	Distance to TSS (bp)	Forward	Reverse	Annealing T°
Tcf4 locus	-3387	5′-gataggcaaaatggtgcagt-3′	5′-ttctgataccaggcacggtt-3′	56
	-485	5′-gcactaaccaagttctcacactcc-3′	5′-tgaccctgagctggaagagtagga-3′	61
	-89	5′-gagccacgacctgagtgagt-3′	5′-ggcactgcggcttataaaga-3′	61
	1933	5′-aggaggtggtggtggcggag-3′	5′-aaagtgcgctgtgcccgg-3′	61
	2648	5′-aatcccatccgccccta-3′	5′-agtcccaatccttggaga-3′	65
	Isoform A promoter	5′-ttgagcgtctgacagcagc-3′	5′-ccttcagttgcattttccca-3′	56
$p57^{Kip2}$ locus	-2374	5′-ccctcattctgttcctc-3′	5′-ttcacctcttgcctctc-3′	55
	-1220	5′-cttccaaaccccgtctct-3′	5′-caggacttccggacatactt-3′	55

Table 2: **Primers used for RT-PCR, real time qRT-PCR and ChIP analysis.**

d) Plasmids

I. Reporter constructs

pGL3-*Tcf4*prom: The promoter region of the mouse *Tcf4* gene (NM_013685.2) (-887 bp until +46 bp) was amplified by PCR from Bl6 mouse kidney genomic DNA. The primers used for this PCR reaction contained a MluI site in the forward primer (5´-ATT ACG CGT GGT GTT AAG GAT GTG AA-3´) and a BglII site in the reverse primer (5´-CTT AGA TCT GTA ATC CAT TCA CAT CCG GGC-3´). The PCR product was first cloned into pGEM-T (Promega) vector and verified by sequencing. The mouse *Tcf4* promoter fragment was released from the pGEM-T-*Tcf4*prom vector by MluI and BglII double digestion. Meanwhile, PGL-3 basic vector (Promega) was also double digested with MluI and BglII (all restriction enzymes were obtained from Thermo scientific), and dephosphorylated by adding 1U of calf intestinal alkaline phosphatase (CIAP) to avoid recircularization of the vector. After purification on electrophoresis grade agarose gel, the *Tcf4* promoter fragment and pGL3 basic vector were ligated at a ratio 2:1 overnight at 4 °C. After ligation, plasmid DNA was precipitated by adding 1 µl glycogen, 1 µl NaAc and 2 volumes of pure ethanol to the ligation mix and placing it in liquid nitrogen for 1 min. Samples were then centrifuged at 4 °C (12000 g, 20 min), and the pellet was washed by 70% ethanol prior to another centrifugation step (4 °C, 12000 g, 10 min) to pellet desalted plasmid DNA. Plasmid DNA was resuspended in 10 µl TE buffer and 5 µl were used for transformation. DH5 bacteria were transformed with ligation product and plated on agar plate overnight at 37°C. Plasmid DNA was amplified by miniprep preparation and the presence of the insert upstream of the luciferase reporter gene in the MluI/BglII sites of the PGL3 basic vector was assessed by enzymatic restriction mapping. Positive clones were amplified and DNA isolated from maxiprep was used in transfection assay. **pGL3-*Tcf4*intr1** – The DNA fragment coding for a part of intron 1 of the mouse *Tcf4* gene (+1087/+2754 bp) was amplified by PCR from Bl6 mouse kidney genomic DNA using the accuprime GC-rich polymerase (Invitrogen). The primers used for this PCR reaction included MluI site in the forward primer (5´-TTC ACG CGT ATG TGC ATG GGT TTC TGT ATG G-3´) and a BglII site in the reverse primer (5´-CAA AGA TCT CCT AGA AAC ATG GAA ATA ACC GC-3´). The PCR product was first cloned into pGEM-T vector and verified by sequencing. The fragment coding for the intron 1 of the mouse *Tcf4 gene* was then subcloned upstream of the firefly luciferase in the MluI/BglII sites of the pGL-3 basic vector. **pGL3-*Tcf4*prom-ex1:** The first exon and a part of the first intron of the mouse *Tcf4* gene (+46/+1080) was amplified by PCR from Bl6 mouse kidney genomic DNA. The primers used for

this PCR reaction contained a BglII site in the forward primer (5´-GGA AGA TCT AAT GTA TCT TTC AGG GAA ACC T-3´) and MluI-BglII sites in the reverse primer (5´-TTT AGA TCT ACG CGT CAC CGA GCA CCT CAT TTT C-3´). The PCR product was first cloned into pGEM-T vector and verified by sequencing. The fragment was subcloned downstream of the mouse *Tcf4* promoter gene in the BglII site of the PGL3-*Tcf4*prom vector. The MluI cassette containing *Tcf4* promoter and first intron was further subcloned upstream of the *Tcf4* intron 1, in the MluI site of the pGL-3 *Tcf4*intr vector to create the **pGL3-*Tcf4*reg. pGL3-*Tcf4*prom rev**: The same fragment as described for the pGL3-*Tcf4*prom was amplified by PCR from Bl6 mouse kidney genomic DNA. Both primers used for this PCR reaction were coupled with a BglII site. The PCR product was first cloned into pGEM-T (Promega) vector and subcloned into the BglII site of the pGL3-basic vector. **pGL3-*Tcf4*intr1 rev**: the fragment containing the DNA fragment coding for the intron 1 of *Tcf4* gene was excised from the pGL3-*Tcf4*intr1 vector and subcloned into the MluI/HindIII sites of the pGEMZF(+) vector. The fragment was then subcloned in the KpnI/MluI sites of the PGL-3 basic vector. The reverse orientation of the DNA fragment coding for *Tcf4* promoter and the first intron was confirmed by restriction mapping. **pGL3-TK-*Tcf4*intr1** – The DNA fragment coding for the Thymidine Kinase (TK) promoter was amplified by PCR from Bl6 mouse kidney genomic DNA. Both primers used for this PCR reaction were coupled with KpnI restriction sites. Forward primer (5´- ATT GGT ACC GAG CTC CAC CGC GGT GGC GG-3´). Reverse primer (5´-TTT GGT ACC CTC GAG ATC TGC GGC AC -3´). The PCR product was first cloned into pJET1.2 vector and verified by sequencing. The fragment coding for the TK promoter was then subcloned upstream of the *Tcf4* first intron in the KpNI site of the **pGL3-*Tcf4*intr1** vector.

II. Expression vectors

The **pRK7-FLAG** vector was created by cloning the oligonucleotides AGC TTC TCG AGA TGG ACT ATA AGG ACG ATG ACG ATA AGG and AGA GCT CTA CCT GAT ATT CCT GCT ACT GCT ATT CCC TAG into the HindIII and BamHI digested pRK7 vector. These oligonucleotides encode for the short hydrophilic 8 amino acid (aa) peptide Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys. This epitope is likely to be located on the surface of a fusion protein due to its hydrophilic nature and, therefore, accessible to antibodies. The small size of the peptide limits interference with the fusion protein's function and transportation.

pRK7-GFP: The coding sequence of the enhanced green fluorescent protein (eGFP) was amplified by PCR using pEGFP-C1 vector (Clontech) as template. The primers used for this PCR

reaction included a BamHI site in the forward primer (5´- AAA GGA TCC ATG GTG AGC AAG GGC GAG GAG C-3´) and an EcoRI site in the reverse primer (5´-TTT GAA TTC CTT GTA CAG CTC GTC CAT GCC-3´). The PCR product was first cloned into pGEM-T vector and verified by sequencing. The eGFP was then subcloned in the BamHI/EcoRI sites of the PRK7 vector.

pRK7-Zac-eGFP: The coding sequence of the mouse *Zac1* gene (NM_009538.2) including the start codon but not the stop codon, was amplified by PCR using the pfu DNA polymerase and PRK-Flag *Zac1* as template. The primers used for this PCR reaction were coupled with a BamHI sites in the forward primer (5´-GGA TCC ATG GCT CCA TTC CGC TGT CAA -3´) and in the reverse primer (5´-GGA TCC AAC TGT CCA TTT CTT ATA GAC GAG-3´). The PCR product was first cloned into pJET1.2 vector and verified by sequencing. The coding sequence of *Zac1* was then subcloned in frame upstream of the eGFP in the BamHI site of the PRK7-eGFP vector.

The human *TCF4*-B (**pCDNA3-ITF2B**) and *TCF4*-A (**pCDNA3-ITF2A**) (Herbst et al., 2009a) expression vectors were kind gifts from Dr. Andreas Herbst (Department of Medicine II, University of Munich, Marchioninistrasse 15, 81377, Munich; Germany) and were used for recombinant proteins production and antibodies validation. The BamHI/XhoI (blunted) fragments encoding for the human *TCF4*-B or *TCF4*-A were subcloned into the BamHI/EcoRV sites of the pRK-Flag to generate the **pRK-Flag-*TCF4*-B** and **pRK-Flag-*TCF4*-A** respectively.

The mouse *Tcf4*-B (**PGK-mITF2B**) and *Tcf4*-A (**PGK-mITF2A**) (Skerjanc et al., 1996), expression vectors were kind gifts from Dr. Ilona Skerjanc (Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, Ottawa; Canada). The mouse flag-tagged *Tcf4* expression vector (**pCDEF3-Flag E2-2**) (Tanaka et al., 2009) was a kind gift from Dr. Susumu Itoh (Department of Experimental Pathology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba; Japan). These plasmids were used for antibodies validation.

e) Recombinant protein construct

GST-Tcf4-B (pGex2tk-TCF4-B) and GST-TCF4 total (pGex2tk-TCF4) plasmids were used to produce recombinant GST fused peptides. For prokaryotic expression, the human TCF4-B cDNA coding for the amino acid (aa 32-154) (isoform B specific) or coding for the amino acids (aa 418-536) (present in both Tcf4 isoforms) were amplified by PCR using the pCDNA3-ITF2B expression vector as template. The primers used for this PCR reaction were coupled with a BamHI site in the forward primer (Total *TCF4*: 5´-ATT GGA TCC ATG CAT GGA ATC ATT

GGA CCT T-3´ / TCF4-B: 5´-CAT GGA TCC AGC AGT GGG AAA AAT GGA-3´) and an EcoRI site in the reverse primer (Total *TCF4*: 5´-ACC GAA TTC ATC TAA TTT CTT GTC CTC CGA-3´ / TCF4-B: 5´-TAC GAA TTC ATT ATT GCT AGA ATA CTG ATA-3´). The fragments were first cloned into the pJET1.2 vector (Thermo scientific) and subcloned into the BamHI/EcoRI sites of the pGex2tk (Pharmacia) vectors. All constructs used in this study were entirely sequence verified.

e) Plasmid preparation

I. Plasmid miniprep

In order to screen positive recombinants, plasmid DNA was extracted from *E.coli* DH5α. Colonies were picked from an agar plate and incubated in 1.5 ml growth medium supplemented with ampicillin (200 µg/ml). Cultures were incubated 6-8 hours or overnight at 37 °C with vigorous shaking. 1 ml of each overnight culture was transferred in a 1.5 ml reaction tube. Bacteria were pelleted down using a bench-top centrifuge (1 min, 13000 g, RT). The cell pellet was resuspended in 200 µl TEG (25 mM Tris pH 8.0, 10 mM EDTA, 100 µg/ml RNAse A, 1% glucose) on shaking platform or vortexer, lysed by adding 200 µl alkaline SDS (200 mM NaOH, 1% (w/v) SDS) and mixed immediately by inverting the tube for 6-8 times. After incubation at RT for 5 minutes, the reaction was stopped by adding 200 µl 3M KAc. The tube was inverted again for 6-8 times and then left on ice for 5 minutes and centrifuged (10 min, 13000 g, RT). The supernatant containing plasmid DNA was transferred into a new 1.5 ml reaction tube and mixed with 500 µl 2-propanol. The tube was then centrifuged (10 min, 13000 g, RT) to pellet the plasmid DNA. The pellet was washed with 70% ethanol and centrifuged (5 min, 13000 g, RT). The supernatant was carefully removed; the pellet was air-dried and dissolved in 10 µl TE (10 mM Tris, 1 mM EDTA, pH 8). The plasmid DNA can be used immediately or stored in a -20 °C freezer.

II. Plasmid maxiprep

To obtain more than 100 µg of plasmid DNA, maxi-preparation of plasmid DNA was performed. Two days prior plasmid extraction, DH5α were transformed and selected on ampicillin-supplemented agar plate. A single growing colony was picked up from the agar plate and resuspended in 4 ml of SOB medium (tryptone 20 µg/ml, yeast extract 5 µg/ml, NaCl 10 mM, KCl 2.5 mM) supplemented with ampicillin (200 µg/ml). After incubation overnight at 37 °C

with vigorous shaking, bacterial suspension was transferred in 500 ml flask containing 40 ml of TB_A (tryptone, 12 µg/ml; yeast extract, 24 µg/ml; glycerol, 0.4%) and 10 ml of TB_B (KH_2PO_4, 0.17 M; K_2HPO_4, 0.72 M) supplemented with ampicillin (200 µg/ml). The culture was incubated overnight at 37°C with vigorous shaking. The plasmids were then purified using a NucleoBond® PC 100 kit (Macherey-Nagel).

f) Cell culture and transfection experiments

I. Cell cultures

All cells were kept in a 5% CO_2 humidified atmosphere at 37 °C.

LLC-PK1 cells are an epithelial cell line (ATCC No. CL-101) originally derived from porcine (pig) kidneys. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. In this study, LLC-PK1 cells were used for reporter assay and for electroporation of pRK-Flag *Zac1* and *Tcf4* expression vectors to validate Tcf4 antibodies.

DLD1-ITF2 cell line is a kind gift from Dr. Andreas Herbst (Department of Medicine II, University of Munich, Marchioninistrasse 15, 81377, Munich; Germany) and is a stable clone overexpressing the human *TCF4*-B transgene under the control of a Tetracycline dependent promoter (Tet ON system). It was generated from the human colorectal adenocarcinoma cell line DLD1 (ATCC No. CL-101). Cells were grown in DMEM medium supplemented with 10 % FCS and penicillin/streptomycin. Expression of the transgene *TCF4*-B was induced for 48 hrs by adding doxycycline to the growing medium (1 µg/ml final concentration). This cell line was used for antibody validation.

C17.2 (ECACC No 7062902) is an immortalized mouse neural progenitor cell line capable of differentiation *in vitro* and *in vivo*. The cell line was established by retroviral-mediated transduction of the avian myc oncogene into mitotic progenitor cells of neonatal mouse cerebellum. In this study, C17.2 cells were first used to create stable clones overexpressing *Zac1* under the control of a tetracycline dependent promoter –Tet OFF system- (C23_11 clone), to identify by microarray (24k mouse cDNA arrays - Max-Planck-Institute of Psychiatry, Munich; Germany) (Landgrebe et al., 2002) scanned on a Perkin-Elmer Life Sciences ScanArray 4000 laser scanner genes whose expression was differentially regulated upon *Zac1* overexpression. C17.2 cells were used then to validate *Tcf4* as a direct target gene of *Zac1*. C17.2 cells were grown in DMEM supplemented with 10% FCS and penicillin/streptomycin. C23_11 were grown

in the same medium supplemented with 100 ng/ml tetracycline. To induce expression of *Zac1* transgene, cells were first rinsed once with PBS, before adding fresh growing medium tetracycline free.

46C cell line is a mouse embryonic stem cell line growing as adherent monolayer which was generated by gene targeting of E14Tg2a (Aubert et al., 2003). The open reading frame of the early marker of neuroepithelial cells, Sox1 is replaced with the coding sequence of eGFP linked to a puromycin-resistant gene, that allows monitoring of neural differentiation. Cells were routinely propagated without feeders in Lif-supplemented GMEM medium containing 10% Knockout serum (KSR) and differentiated into neural cells by Lif-withdrawal and medium change as described previously (Ying and Smith, 2003). Differentiated cells were grown in a normal differentiation medium that consists in a mix 1:1 of DMEM-F12 and Neurobasal media to which 0.5 mM Glutamine, 0.05 mg/ml BSA, B27 serum replacement and N2 supplement (containing: human transferrin 1 mM, insulin recombinant full chain 500 mg/L, progesterone 2 µM, putrescine 10 mM, selenite 3 µM) were added. For embryoid bodies formation, 46C cells were resuspended at $2x10^4$ cells/ ml in differentiation in suspension medium containing a mix 1:1 GMEM medium supplemented with 10% Knockout serum (KSR)/ DMEM-F12 supplemented with 0.05 mg/ml BSA, 0,02 mM ß-mercaptoethanol and N2 supplement. Cells were grown in suspension in a 6-cm Petri dish for 4 days, with medium changed every second day. For retinoic acid treatment, cells were differentiated for 4 days in normal differentiation medium complemented with 0.1 µM of All-trans Retinoic Acid (Sigma Aldrich).

Neurosphere (NS) cultures- At E15, pregnant mice were killed by cervical dislocation and uteri were withdrawn. Extra-embryonic tissues were removed and after opening the skull, brains were carefully extracted under a hood in sterile conditions. Each 3 brains were then cut into small pieces with thin sterilized scissors and they were transferred into a 1.5 ml reaction tube containing 1 ml of digestion medium containing a mix 1:1 accutase / 0.05 % trypsin supplemented with 100 U/ml DNase. Tubes were incubated 10 min at 37 °C on a shaking platform. Digestion reaction was stopped by transferring the tube content into a 15 ml tube containing 5 ml of DMEM-F12 supplemented with 10% FCS. Non digested tissue was finally dissociated mechanistically with a 10 ml plastic pipette. After a brief centrifugation at 70 g for 1 min, the supernatant containing cell suspension was collected and transferred into a new tube. This process was repeated until the pellet disappeared. Tubes were then centrifuged at 800 g for 5 min and cell pellets were resuspended into 1 ml of growing medium containing a mix 1:1

DMEM-F12 / Neurobasal to which 0.5 mM Glutamine, 0.05 mg/ml BSA, B27 serum replacement and N2 supplement were added. Cells were counted using a hemacytometer, and were seeded in uncoated T75 Flasks at $1x10^5$ cells/ml in growing medium supplemented with 10 ng/ml EGF and FGF2 (final concentration) to allow cells to grow in suspension and to form aggregates after 1 day in culture. Medium was changed every second day, and NS were passaged after 5 days in culture. After the second passage, cells were seeded in differentiation medium at $1x10^5$ cells/well in 6-well plates. The differentiation medium was renewed every second day. Neuronal differentiation was performed as described (Fath et al., 2009), briefly cells were seeded on PDL-coated plates, in Neurobasal-A medium to which N2 supplement, 0.5 mM glutamine and 2.5 µM arabinofuranosyl cytidine (Ara-C) (Sigma Aldrich) were added. Astrocytic differentiation was induced by plating the cells on uncoated plate in a medium containing a mix 1:1 DMEM-F12 / Neurobasal medium to which 0.5 mM glutamine, 0.05 mg/ml BSA, B27 serum replacement, N2 supplement, 1% of PANSera ES4 and 10^6 U/ml of Lif were added.

Neural precursors, primary hippocampal neurons and primary cerebellar neurons were dissected from brains of E15, P3 and P7 Cd1 mice respectively. Briefly, the region surrounding the lateral ventricle, the hippocampi and cerebelli were carefully dissected and all meninges and blood vessels were removed. Tissue was then sliced using a McIIwair tissue chopper and pieces were digested for 10 min with gentle shaking at 37 °C in 5 ml of EBBS supplemented with 0.05% trypsin, 0.03 mg/ml BSA and 100 U/ml DNase. Digestion was stopped by adding 15 ml of Neurobasal A medium supplemented with 0.4 mg/ml Soy bean trypsin inhibitor, 3 mg/ml BSA, 2% FCS (all final concentrations) and B27 serum replacement. After a brief centrifugation step at 70g, the supernatant containing cells in suspension was filtered through a 30 µm nylon strainer and the undigested tissue was mechanistically dissociated using a BSA pre-coated pipette. This process was repeated until the pellet disappeared. Cells were pelleted at 200 g for 6 min and resuspended in 3 ml of Neurobasal-A medium supplemented with B27 serum replacement. The cell suspension was then layered on a BSA gradient and samples were centrifuged at 70g for 6 min. Cells were resuspended in Neurobasal-A medium supplement with 0.5 mM glutamine, 0.1 mM kanamycin and B27 serum replacement and plated at $5x10^4$ cells/well in 12-well plates and at $5x10^5$ cells/well in 6-well plates.

II. Transfection

LLC-PK1 cells were seeded the day prior to electroporation at $1x10^7$ cells per 15-cm culture dish. Cells were harvested by trypsinization and resuspended in 1 ml electroporation buffer (1x EP buffer) (50 mM K_2HPO_4; 20 mM CH_3KO_2; 20 mM KOH). 50 µl of cell suspension was added to a 100 µl mixture containing different amount of pRK-Flag *Zac1* or pCDEF3-Flag E2-2, pCDNA3-ITF2A, PGK-mITF2B, PGK-mITF2A, pCDNA3-ITF2B, 4 µl $MgSO_4$, 20 µl 5 x EP buffer, 2 µg PAM vector DNA, and water. The mix DNA-cells was transferred into a 4 mm electroporation cuvette (Molecular Bioproducts) and left at room temperature for 10 minutes. DNA was electroporated into the cells by using a BTX 600 electroporator (290V, 500µF, 720Ω). After pulse delivery, the cells were immediately plated in a 10-cm culture dishes, and cultivated for 24 hrs.

C17.2, SK-NM-C, DLD-1 cells were all transfected using Turbofect in vitro transfection reagent (Thermo Scientific) according to manufacturer´s instructions (details of methods and amount of transfected DNA will be detailed in results part). For *Zac1* sh-RNA experiments, a pool of 5 different MISSION® shRNA Plasmid DNA targeting *Zac1* (Sigma Aldrich) was transfected in C17.2 cells using Turbofect in vitro transfection reagent. The parent vector PLKO.1 was used as control.

Clone	Zac1 mRNA region targeted	Sequence (5´-> 3´)
NM_009538.1-2608s1c1	3´UTR	CCGGGACGCTATTGATGTCTCCATTCTCGAGAATGGAGACATCAATAGCGTCTTTTTG
NM_009538.1-1011s1c1	CDS	CCGGCCACTGTGATAGATGCTTCTACTCGAGTAGAAGCATCTATCACAGTGGTTTTTG
NM_009538.1-1197s1c1	CDS	CCGGCCAGAGCAATTTCCAACTCATCTCGAGATGAGTTGGAAATTGCTCTGGTTTTTG
NM_009538.1-1732s1c1	CDS	CCGGCCAATTATTCTTCAGGAGCATCTCGAGATGCTCCTGAAGAATAATTGGTTTTTG
NM_009538.1-1198s1c1	CDS	CCGGCAGAGCAATTTCCAACTCATTCTCGAGAATGAGTTGGAAATTGCTCTGTTTTTG

Table 3: **Clone Id number, and sequences of the sh-RNA used to knock down *Zac1* expression**

Differentiating cells derived from neurospheres and 46C cells were transfected using lipofectamine 2000 reagent (Life technologies) according to manufacturer´s instructions. For luciferase assay, cells were seeded 48 hrs before transfection in a 12-well plate at $3x10^5$ cells/well. In each well, 6 µl of lipofectamine 2000 reagent were added to 50 µl of OPTIMEM medium and mixed with 50 µl of OPTI-MEM medium containing 1 µg of luciferase reporter constructs, 0.5 µg pRK7-β-gal and increasing amounts of pRK-Flag *Zac1* or pRK-Flag *Zac1*-ZF7. After 20 minutes incubat-ion to allow DNA-Lipofectamine complexes to form, the mix was added to the well. 24 hrs after transfection, luciferase activity was measured.

Primary hippocampal and cerebellar neurons were nucleofected with different amounts of pRK7-Zac1-GFP and pRK7-GFP using the Amaxa® Basic Neuron SCN Nucleofector® Kit and the Nucleofector™ technology (Lonza) [program SCN basic neuron 3]. After nucleofection, cells were seeded in 6-well plates at $5x10^5$ cells/well for RNA extraction and qRT-PCR, and in 12-well plates at $1x10^5$ cells/well for immunocytochemistry. Gene expression analysis and immunocytochemistry were performed 3 days after transfection.

g) Luciferase assay

To measure promoter activity, cells were washed twice with PBS and then thoroughly lysed in 100 µl lysis buffer (75mM Tris-HCl, 10mM MgCl2, 1% Triton X-100, 2mM ATP, 1 mM DTT). 50 µl of aliquots were measured in a LKB luminometer for 20 seconds. As an internal control of transfection efficiency, the luciferase readings were normalized on β-galactosidase activity from a cotransfected expression vector (pRK7-β-gal) (Hoffmann et al., 2003). The β-gal activity in the extracts was measured as described previously (Spengler et al., 1993).

h) Protein preparation

I. Protein concentration and purity

Bradford assays were used to determine the concentration of all proteins used in this study. The concentrated assay buffer was first diluted 1:5 and standards were prepared containing a range of 20 to 200 µg protein (BSA) to a standard volume. The samples were diluted (2 µl in 200 µl water) to an estimated concentration of 20 to 200 µg/ml. 800 µl Bradford assay was added to each sample and protein standards, and the absorbance was measured at 590nm. Protein concentrations of samples were deduced from the standard curve. Coomasie blue staining was then further used to assess the purity, the integrity of recombinant protein preparations and the rough quantity. Proteins were separated by electrophoresis on a 10 % polyacrylamide gel. Size markers and BSA standards (50, 250, 1000 ng) were included. Proteins were separated by applying 100 V to the gel. The gel was then soaked in 0.2% Coomassie blue for 1h and destained in 40% methanol, 50% acetic acid solution overnight. The gel was then blotted on to paper and dried.

II. Recombinant proteins

Recombinant GST-*TCF4* fusion proteins were used to produce anti-sera after injection in rabbits. After transformation of DH5α bacteria with pGEX-2TK-*TCF4* vectors, single colonies were

grown at 37 °C in 50 ml 2YT (0.16 % tryptone, 0.1 % yeast extract, 0.1 % NaCl) overnight, to which 450 ml 2YT were added, and bacteria were grown at 37 °C until sufficient cell density was reached, indicated by an OD_{600} of 0.5-1.0. Production of fusion proteins was then induced by adding 1 mM IPTG to the cultures which were incubated for 2 hours at 30 °C. The GST-proteins were purified using glutathione-sepharose beads (Hoffmann et al., 2003) and purity after elution was assessed by Coomasie blue staining.

i) Antibody production

500 µg of GST fusion proteins (GST-*TCF4*-B and GST-*TCF4*) were sent to PINEDA Antikörper-Service (Berlin) as antigens to immunize rabbits against the mouse *TCF4*-B protein and the 2 mouse protein isoforms of *TCF4*. On the first day of immunisation, animals received an intradermal injection of the GST fusion protein completed with Freund´s complete adjuvant to enhance immune response. Later, animals were subcutaneously injected with the antigen solution and Freund´s incomplete adjuvant (day 20, 30, 40, 60). At day 60, immunoreactivity of the antisera was verified by western blot. In case of insufficient immunoreactivity, animals received one more injection of antigen solution and Freund´s incomplete adjuvant at day 75 and serum was extracted at day 90. Once validated by western blot, antisera were tested for further applications (Immunocytochemistry, immunohistochemistry and chromatin immunoprecipitation) and their optimal conservation conditions were determined.

j) Western blots

Cells were seeded in 10 cm or 6-well plate 24 hours before harvesting. The cells were first washed twice with cold PBS, and then scrapped in 0.2 to 0.5 ml TE buffer supplemented with a protease inhibitor cocktail (PIC). Cells were disrupted by passing the homogenate several times through a hypodermic syringe (29G). An aliquot of 2 µl was kept at this step for measuring protein concentration by Bradford assay. Proteins were then denatured by adding 4X Laemmli buffer (200 mM Tris-HCl pH 6.8, 8 % SDS, 40 % glycerol, 0.4 % Bromophenol blue, 0.1 % β-mercaptoethanol) and incubating the mix at 95 °C for 5 minutes. Samples were stored at -20 °C until use. Equivalent amounts of protein (10 to 100 µg) were subjected to electrophoresis on an SDS-PAGE 8 % polyacrylamide gel. The gel was then blotted onto a nitrocellulose membrane, and equal loading of protein in each lane was assessed by brief staining of the blot with Ponceau red. To prevent antibody unspecific binding, membranes were blocked for 1 hr in (10 mM Tris, 0.5 mM NaCl, 0.25% Tween, 4% (w/v) Slim Fast powder, pH 7) and incubated overnight at 4 °C

with primary antibodies diluted in the blocking solution. Membranes were washed 3 X 5 min in (10 mM Tris, 0.5 mM NaCl, 0.1% Tween, 4%, pH 7) and incubated for 1 h at room temperature with HRP conjugated anti-rabbit (1/4000), mouse (1/2000) or guinea pig (1/4000) antibodies (Sigma-Aldrich) , diluted in blocking solution. Specific reactions were revealed with the ECL Western blotting detection reagent (GE healthcare). The first film was exposed for 30 second to check signal appearance and then exposure time was adjusted to the strength of the signal.

k) Immunohistochemistry

I. Fixation of mouse brains and cryosections

Prior to cutting cryosections from embryonic and postnatal mouse brains, the tissue was fixed overnight at 4 °C in 4 % PFA in PBS, rinsed with PBS and placed overnight in 20% sucrose/PBS at 4 °C. Then, the brain or head was embedded in Tissue Tek, frozen and cut into 10 µM cryosections. Sections were collected on SuperFrost microscope slides and stored at -20 °C.

II. Immunocytochemistry (ICC) / Immunohistochemistry (IHC)

Cells were seeded on coverslips in 12-well plates, fixed with pre-warmed 4% paraformaldehyde in PBS containing 2% sucrose for 5 minutes at 37 °C and rinsed with PBS. Frozen brain sections were allowed to warm-up slowly at room temperature for 30 min before washing them twice with PBS. The same protocol was then applied to both coverslips and brain sections.

Coverslips were incubated 2 hours with blocking solution (5 % BSA, 5% normal goat serum, 0.05% Triton X-100, 50 mM Tris-HCl, 50 mM NaCl, pH 7.4). Coverslips were then incubated with the primary antibodies diluted in blocking solution overnight in a humidifying chamber at 4 °C. Coverslips were then washed three times with PBS for 10 minutes and incubated at room temperature for 2 hrs with secondary antibodies diluted in blocking solution (1/1000). Coverslips were washed with PBS three times for 10 minutes prior addition of 4′,6-Diamidin-2-phenylindol (DAPI) diluted in PBS (1/6000) for 5 minutes. Finally, coverslips were washed with PBS three times for 10 minutes before mounting in Mowiol on SuperFrost microscope slides. Confocal images were taken using an Olympus laser scanning confocal microscope. Phase-contrast and fluorescence images were taken using an Olympus BX61 microscope equipped with a F-view II digital camera.

f) Chromatin immunoprecipitation (ChIP)

ChIP experiments were performed *in vitro* and *in vivo* to investigate the binding of *Zac1* and *Tcf4* at several gene loci. For *in vivo* ChIP, frozen tissues were stored at -80 °C in 1.5 ml tubes. First, tissue was crushed using a pestle and resuspended into 300 µl of ice-cold PBS containing a protease inhibitor cocktail (PIC) (Sigma Aldrich). The homogenate was then further passed several times through a hypodermic syringe (29G). Tubes were then incubated 15 min at room temperature on a rotating platform in presence of 1 % formaldehyde to cross-link proteins with DNA. Excessive formaldehyde was quenched by adding glycine (final concentration of 125 mM). Pellets were washed twice in ice-cold PBS containing PIC, by successive centrifugation steps (5 min at 800 g, 4°C). Samples were then processed according to the Upstate Biotechnology ChIP Kit (Magna ChIP G, Millipore) protocol with the following modifications: Samples were sonicated 3 times for 5 minutes using the Bioruptor^{TM} from Diagenode (cycles of: 30 seconds "ON" / 30 seconds "OFF") in a wet ice bath. After sonication, 5 µl of the sheared material was loaded on a 1 % agarose gel to check the fragment sizes. The DNA smear on the gel sould range between 200 bp and 1000 bp. DNA concentration in the sheared chromatin was determined with a SmartSpec Plus spectrophotometer (Bio-Rad). The chromatin samples were either subjected to immunoprecipitation or stored at -80 °C until further use. 1 OD_{260nm} chromatin DNA was then subjected to immunoprecipitation using *Zac1* LPR rabbit antibody (1/100) or *Tcf4* total rabbit antibody (1/50). To reduce unspecific binding during immunoprecipitations, antibodies were pre-incubated with sheared chromatin DNA on a rotating platform at 4 °C overnight, while Dynabeads Protein G (Invitrogen) were added in the following day and incubated for 1 hour at 4°C on a rotating platform. After reverse crosslinking, protein digestion, and DNA isolation using the UltraClean PCR Clean-Up Kit (Mobio), fragments were dissolved in 100 µl TE buffer and subjected to standard or real-time PCR.

For *in vitro* ChIP, samples were processed according to the protocol described here above with the following modifications: samples were sonicated three times for 3 minutes and 5 OD_{260nm} chromatin DNA was subjected to immunoprecipitation.

l) Statistical analysis and computer software

Transcriptional factor binding sites analysis were performed using Genomatix software. Primers were designed according to the general guidelines by the software Oligo 6. Fluorescent images were analyzed using Fluoview FV10-ASW software. Cells were counted manually using Image J software. Graphs and statistical analysis were performed using Microsoft Excel software, pictures and figures were created using Excel, PowerPoint and Corel Draw.

Results

To identify *Zac1* target genes that could underlie its function in neural progenitors, we performed gene expression analysis in the immortalized neural stem cell line C17.2 (Lynch et al., 1996). This cell line can be routinely maintained as neural progenitors in standard cell culture conditions and can be transfected with high efficiency. The C17.2 (ECACC No 7062902) cell line is an immortalized mouse neural progenitor cell line capable of differentiation *in vitro*. The cell line was established by retroviral-mediated transduction of the avian *myc* oncogene into mitotic progenitor cells of neonatal mouse cerebellum. We generated a panel of inducible C17.2 *Zac1* clones using a Tet-off system (Hoffmann et al., 1997). By cultivating cells in absence or presence of tetracycline, ectopic *Zac1* expression was switched on and off respectively. The clone C23_11 was selected because of its maximal *Zac1* induction (approximately 2 fold) and because ectopic *Zac1* expression lead to a strong growth inhibition. As expected from a cell cycle regulator gene, induction of Zac1 lead to a 70 % growth inhibition after 8 days in culture, (*Fig. 6* - by the courtesy of Udo Schmidt-Edelkraut).

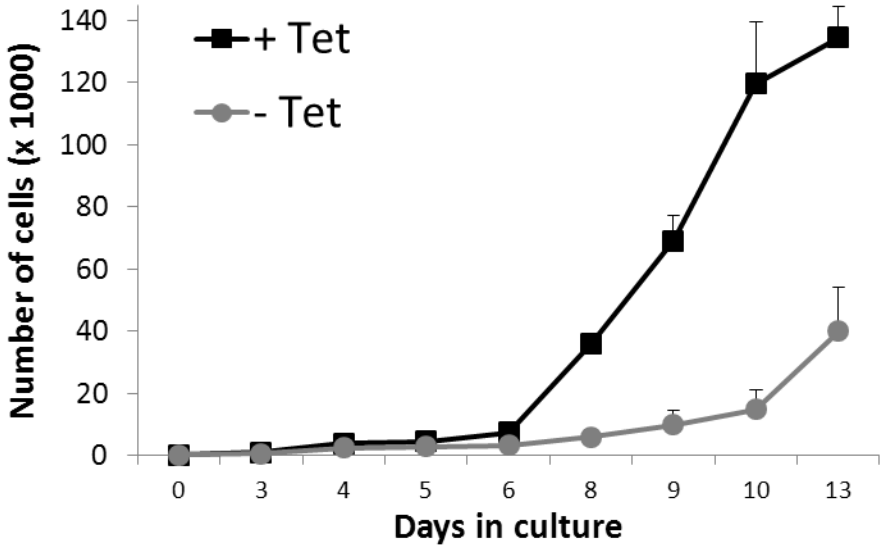


Figure 6: **Induced *Zac1* expression in C23_11 clone inhibits cell proliferation**. Cells were seeded at 2000 cells/well of a 24-well plate, cultivated in the presence or absence of tetracycline (+Tet/-Tet conditions) and cell numbers were measured daily with a particle counter Z1 (Coulter). Medium was renewed every 2nd day. *Points*, mean of three independent experiments; *bars*, standard deviation (SD).

I. *Zac1* induces expression of *Tcf4* in the neural stem cell line C17.2.

Using cDNA microarrays, a comparative genome-wide expression analysis of the inducible *Zac1* clone cultivated for 8, 16, and 24 hours with or without tetracycline was performed. Only genes exhibiting a >1.5-fold difference between tetracycline-positive and tetracycline-negative conditions were considered. Collectively, *Zac1* induction differentially affected 127 common genes (data not shown); belonging to different functional groups, including metabolism, transcription, proliferation, signaling/transport, and cell structure.

Among these differentially regulated with known function during brain development was the isoform B of the *Tcf4 (E2-2/ITF2/SEF-2)* gene, exhibiting a 1.7-fold activation. We validated *Tcf4* up-regulation following *Zac1* induction by quantitative RT-PCR (qRT-PCR) analysis which revealed a 1.5-fold increase in *Tcf4* mRNA. Finally, a time course analysis revealed a transient increase in *Zac1* induction peaking at 9 hrs and normalizing at 24 hrs after tetracycline removal, which was mirrored by *Tcf4* gene expression, thus supporting the idea of direct regulation of *Tcf4* gene expression by *Zac1* (*Fig. 7*).

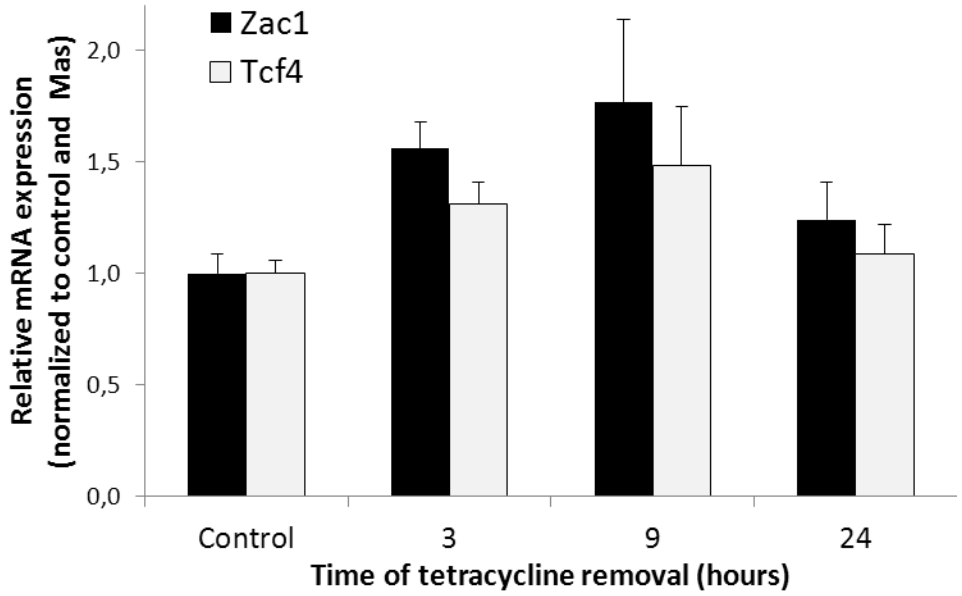


Figure 7: **Induced Zac1 expression in C23_11 clone increases Tcf4 gene expression at different time points after tetracycline removal.** Bars represent the induction in gene expression determined by qRT-PCR, normalized to control conditions in presence of tetracycline, and to the house keeping gene Mas. Bars represent the mean of 3 independent experiments performed in duplicates, SD is shown.

The direct transactivation mechanism was confirmed by transfecting wild type (WT) *Zac1* construct in the parental cell line C17.2, which led to an increase in both *Tcf4-B* gene expression and protein levels.(*Fig. 8.A-B*).

There are 2 known protein isoforms of TCF4, namely TCF4-A and TCF4-B, with both sharing an identical C-terminal part and having a distinct N-terminal part. *TCF4*-B the longest and best characterized isoform, possesses an extra transactivation domain (Herbst and Kolligs, 2008) and can act both as a transactivator (Pscherer et al., 1996; Persson et al., 2000a; Muir et al., 2006; Herbst et al., 2009a) and a repressor (Skerjanc et al., 1996; Chen and Lim, 1997; Lu et al., 2005). *TCF4*-A has weaker transactivation capacity and its function is less well understood (Herbst et al., 2009b; Sobrado et al., 2009). In order to determine if only one or the two isoforms were regulated by *Zac1*, we performed qRT-PCR analysis using isoform-specific primers, which revealed that *Zac1* regulates specifically the expression of the *Tcf4*-B variant (*Fig. 8.A*). Beyond this point in this thesis, if not stated otherwise, *Tcf4* will refer exclusively to *Tcf4*-B gene.

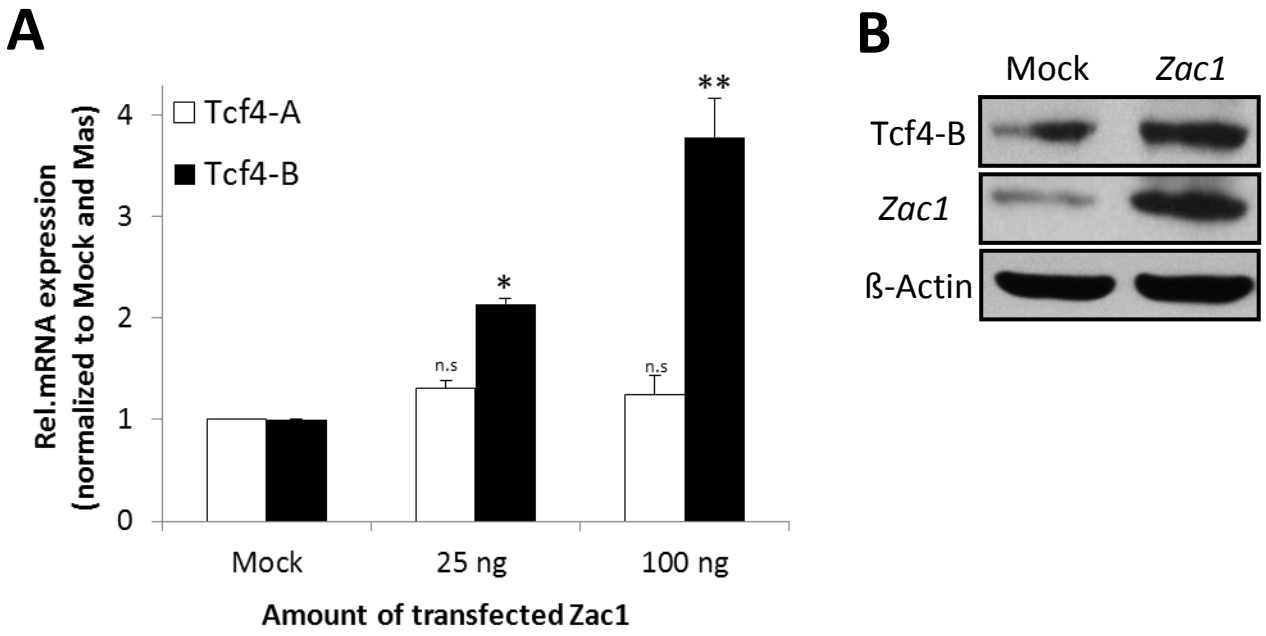


Figure 8: ***Zac1* regulates *Tcf4*-B gene expression.** A) qRT-PCR analysis revealing that *Tcf4-B* gene expression increases in a *Zac1*-dependent manner in C17.2 cells transfected for 24 hrs with increasing amount of pRK-Flag-Zac1. Bars represent the mean of 4 independent experiments; SD is shown.*p<0.05, **p<0.01 (t-test). B) *TCF4*-B protein levels in C17.2 cells are increased after transfection with 100 ng of pRK-Flag-Zac1 as revealed by immunoblot - 50 µg of whole cell extract (WCE) were blotted.

To determine if *Zac1* can act as transactivator or coactivator of *Tcf4* gene expression, we next transfected C17.2 cells with either the DNA-binding defective mutant form (Zac1 ZF7mt) or the

transactivation domain deleted mutant form (Zac1 ΔLPR) (Hoffmann et al., 2003). Quantitative RT-PCR and immunoblot analysis revealed that in contrast to WT, Zac1 ΔLPR overexpression did not affect *Tcf4* expression, while Zac1 ZF7mt induced *Tcf4* at reduced levels (*Fig. 9.A-B*). Since deletion of the LPR domain does not impair the coactivator function of Zac1 (Hoffmann and Spengler, 2008) but abolished completely the induction of *Tcf4* by Zac1, we conclude that Zac1 is not a coactivator of *Tcf4* gene expression. In addition, its induction by Zac1 requires an intact Zac1 DNA-binding domain, indicating that Zac1 needs to bind to DNA to induce the *Tcf4* gene expression. These effects were not due to quantitative differences in expression of the different *Zac1* constructs, as immunoblot analysis showed that all of them were expressed at similar level (*Fig. 9.B*).

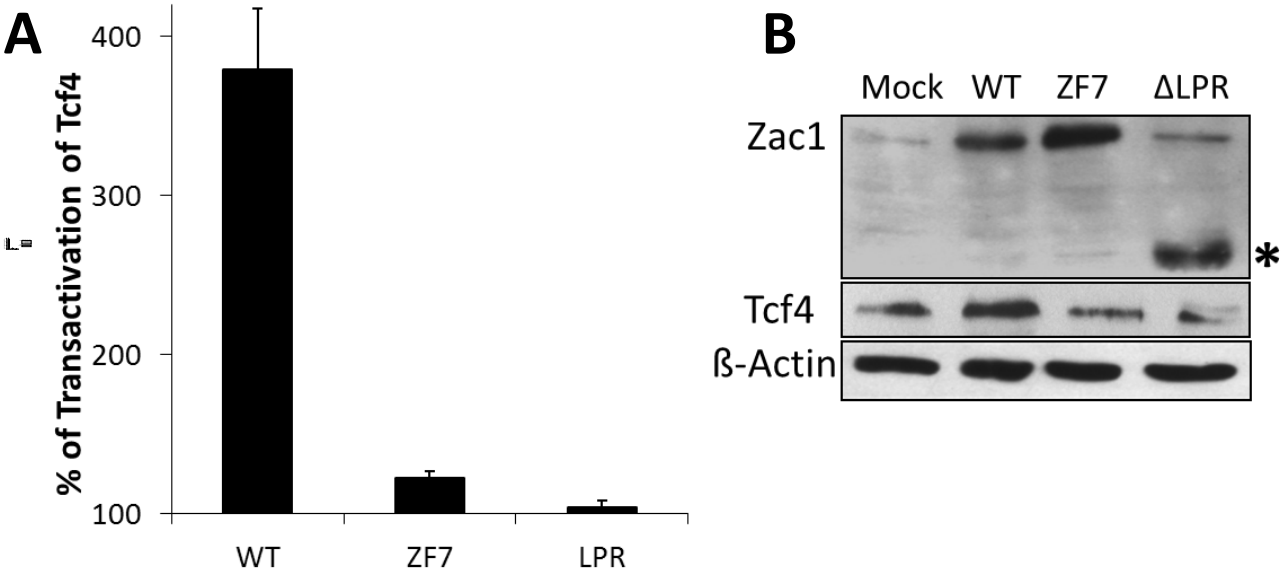


Figure 9: ***Zac1* act as a transactivator of the *Tcf4* gene.** A) C17.2 cells were transfected with 100 ng of different Zac1 constructs (WT, ZF7 and ΔLPR) and *Tcf4* gene expression was measured by qRT-PCR 24 hrs after transfection and normalized to mock transfected conditions (100%) and to the expression of the house keeping gene Mas. DNA binding or transactivation domain deficient mutants cannot induce *Tcf4* expression ruling out coactivation mechanism of *Tcf4* by *Zac1*. Bars represent the mean of 2 independent experiments ran in duplicates; SD is shown B) Protein levels of Tcf4 and Zac1 mutants were assessed by immunoblot – 50 µg of whole cell extract (WCE) were loaded; Zac1 constructs were expressed at similar levels, whereas Tcf4 protein level is increased only when the DNA binding domain and transactivation domains of Zac1 is intact. * Due to the deletion of the linker proline rich domain, the *Zac1* mutant ΔLPR proteins migrate to lower molecular weight (~ 70 kda) compared to WT and ZF7 (~130 kda).

Computational analysis, using the genomatix software, revealed *Zac1* putative potential binding sites at *Tcf4* promoter and first intron whereas no putative binding sites could be identified on the promoter of the isoform A of *TCF4* (*Fig. 10.A*). ChIP analysis showed that *Zac1* occupies

specifically the *Tcf4* proximal promoter and the first intron, supporting a direct transactivation mechanism (*Fig. 10.B*).

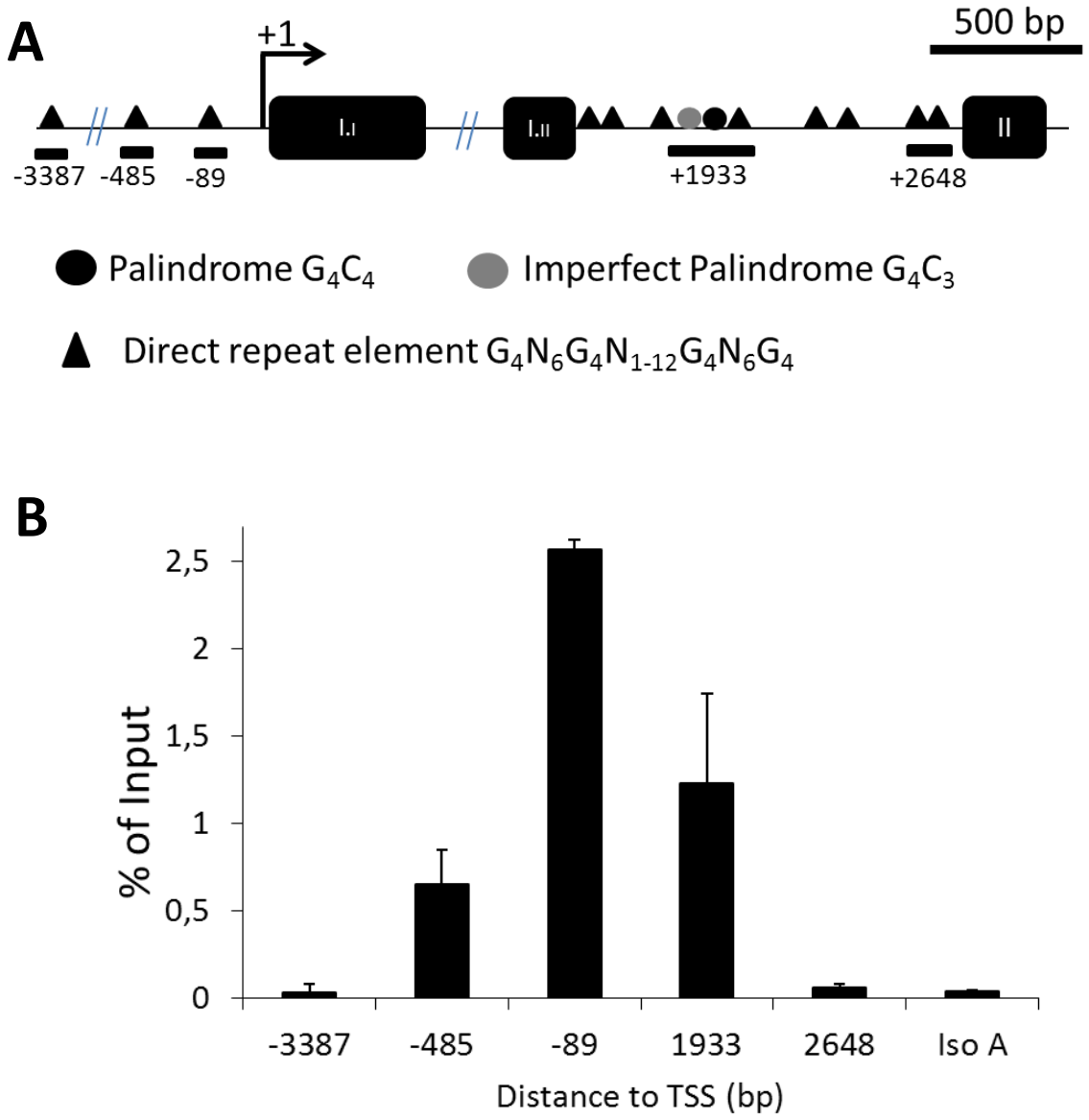


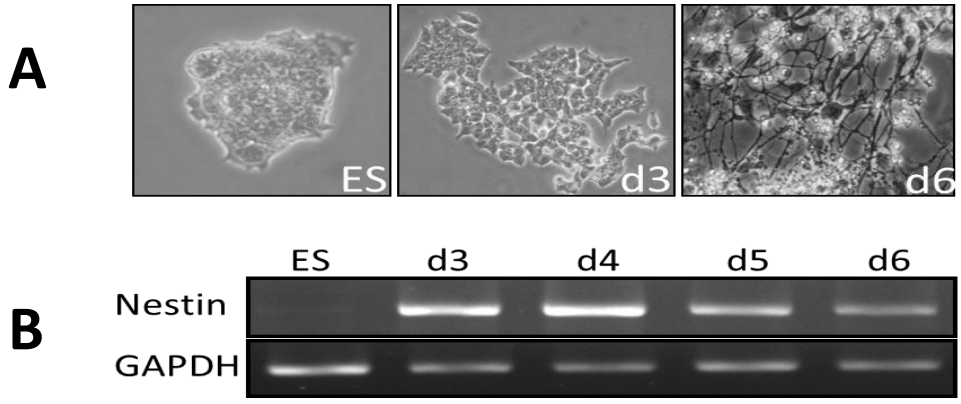
Figure 10: ***Zac1* occupies *Tcf4* promoter and first intron in C17.2 cells.** A) Schematic representation of *Tcf4* promoter and first intron; black boxes represent the 2 first exons (2 alternative exons I and the exon II); putative binding Zac1 sites are pictured as triangles and grey or black circles, numbers indicate their distance in base pair to the transcriptional start site (TSS or +1). Black bars indicate the genomic regions amplified by the primers used for ChIP experiment. B) ChIP experiments revealed Zac1 occupancy at *Tcf4* proximal promoter and at elements in the first intron, but not on the promoter of the isoform A (Iso A). Values were first calculated as % of input, to which negative control (IgG) values were subtracted. Bars represent the mean of 3 independent experiments with duplicates; SD is shown.

Altogether these findings suggest that *Tcf4* is a direct *Zac1* target in the mouse C17.2 cell line and that transactivation might be mediated via the *Zac1* binding sites in the proximal promoter and/or in the first intron.

II. *Zac1* regulates *Tcf4* gene during neuronal differentiation of mouse embryonic stem cells.

To study the precise molecular regulation of *Tcf4* gene by *Zac1* during neurogenesis, we used a well-characterized *in vitro* model of differentiation: the neuronal differentiation of the mouse 46C mES cell line. Hoffmann and Spengler (Hoffmann and Spengler, 2008), previously showed that *Zac1* expression is up-regulated during early neuronal differentiation of the mouse embryonic stem cell line 46C. This is mimicking the transient increase in *Zac1* expression observed in neuroepithelial cells and in neuronal progenitors *in vitro* and *in vivo* (Valente et al., 2005; Elkabetz et al., 2008; Abranches et al., 2009; Koch et al., 2009). We thus assessed next, whether *Zac1* regulates the expression of the proneural factor *Tcf4* during early neurogenesis.

Upon Leukemia inhibitory factor (Lif) withdrawal from the medium, 46C cell colonies flattened and spread to give rise to single cells that acquired progressively neuronal morphology within 3 days. During differentiation, the expression of the neural progenitor cell marker Nestin (*Fig. 11.B*) and late neuronal differentiation marker TuJ-1 (*Fig. 11.C*) increased. In contrast, the expression of the pluripotency marker Oct4 decreased (*Fig. 11.D*). This indicates that the cells lost their pluripotency and acquired a neuronal fate. Interestingly, *Zac1* and *Tcf4* gene expression and protein levels were strongly up-regulated after 6 days of Lif withdrawal, suggesting that both genes might play a role during neuronal differentiation or have specific functions in immature neurons (*Fig. 11.E-F*).



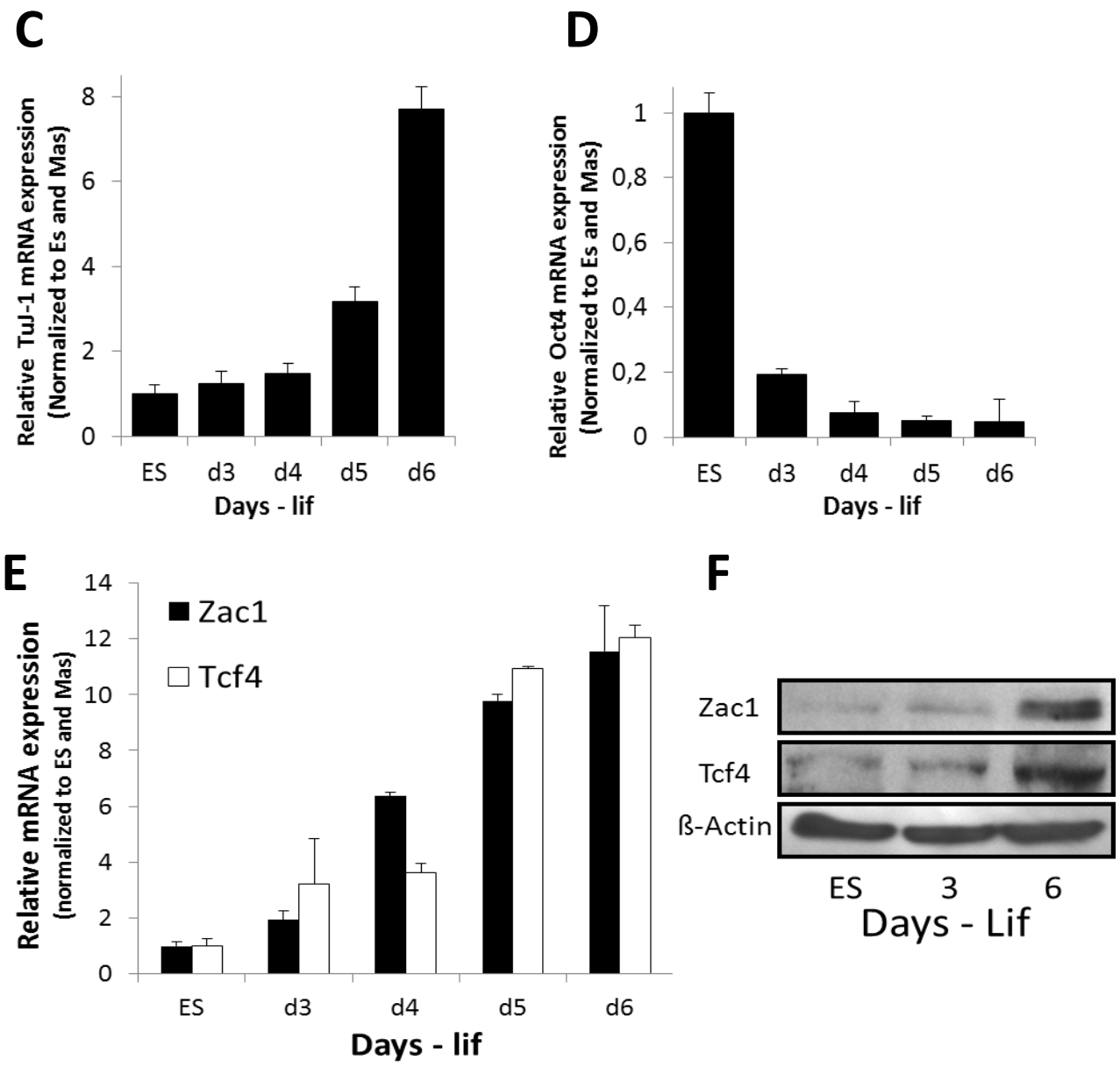


Figure 11: ***Zac1* and *Tcf4* genes are upregulated during neuronal differentiation of the mouse embryonic stem cell line 46C.** A) Light microscopy of mES cells which progressively differentiate into neurons following Lif withdrawal for the indicated number of days. B-C) The expression of the neural stem/progenitor marker Nestin (B) and of the late neuronal marker Tuj1 (C) increase during neuronal differentiation as revealed by RT-PCR and qRT-PCR analysis. D) qRT-PCR analysis of the expression of the pluripotency marker Oct4, which is rapidly downregulated in mES cells upon Lif withdrawal. E) The expression of *Zac1* and *Tcf4* was measured following Lif withdrawal for the indicated number of days. *Zac1* and *Tcf4* are co-induced by Lif withdrawal. (F) Immunoblot analysis of 100 µg of WCE from mES cells grown for the indicated number of days in the absence of Lif. The coinduction of *Tcf4* and *Zac1* mRNAs translates into their increased protein expression. Bars represent the average of 3 independent experiments ran in duplicates; SD is shown.

Two established protocols of neuronal differentiation, namely the embryoid formation (EB) (Watanabe et al., 2005; Kamiya et al., 2011) and treatment with retinoic acid (RA) (Guan et al., 2001) were applied to the mES cells to confirm that *Zac1* and *Tcf4* were co-induced during neuronal differentiation of the 46C cells. Both protocols potently induced neuronal differentiation of the mES as indicated by the loss of Oct4 expression and by the strong up-regulation of the neural progenitor marker Nestin and neuronal markers N-Cadherin and TuJ-1 (*Fig. 12.A*). All treatments were associated with a fast and strong up-regulation of *Zac1* and *Tcf4* gene expression and protein levels during neuronal differentiation (*Fig. 12.B-C*).

We further confirmed that *Tcf4* is a *Zac1* target gene by transfecting *Zac1* in 46C cells, and could show that endogenous *Tcf4* gene expression increased in a *Zac1* dose-dependent manner (*Fig. 13*).

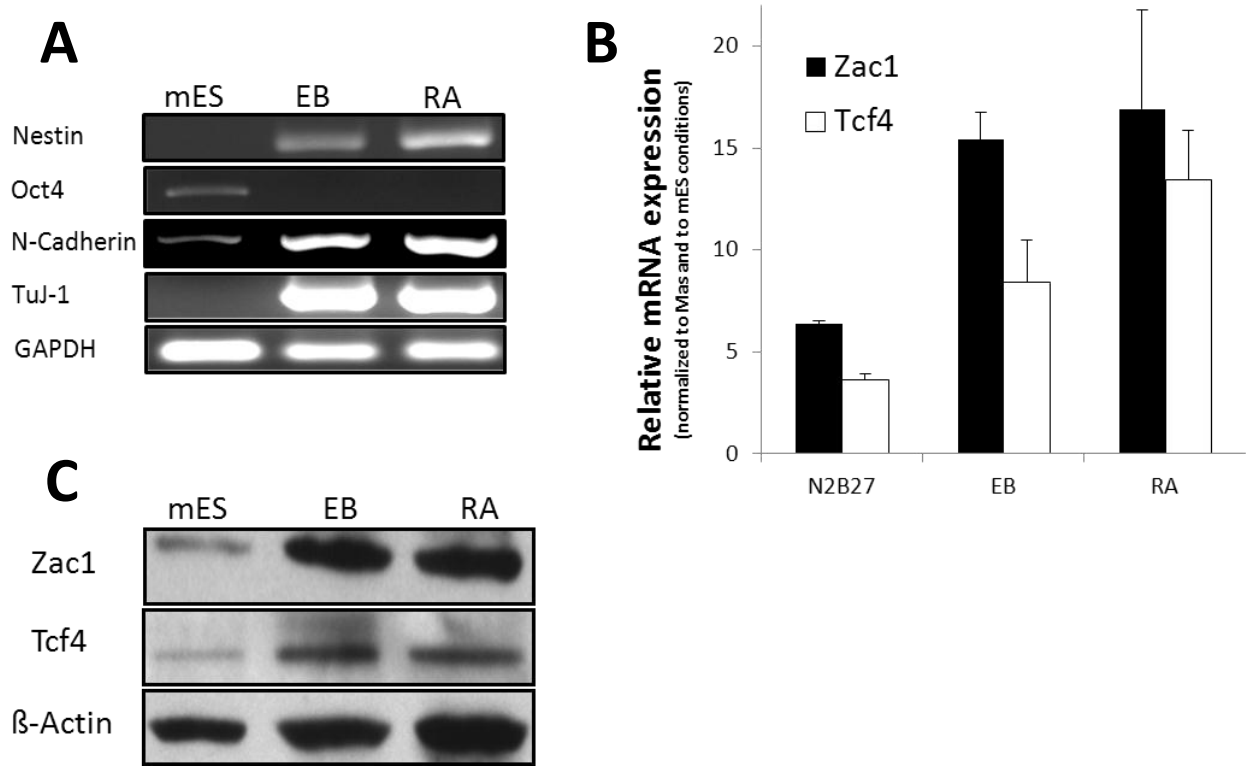


Figure 12: ***Zac1* and *Tcf4* genes are co-induced during neuronal differentiation of mES.** A) RT-PCR analysis showing that embryoid formation (EB) and all-trans retinoic acid (RA) treatment (0.1 µM) for 4 days induced neuronal differentiation of mES. The expression of the neural stem/progenitor marker Nestin and of the neuronal markers N-Cadherin and Tuj1 were increased after treatment in contrast to the expression of the stem cell marker Oct4. B) qRT-PCR analysis showing co-induction of *Zac1* and *Tcf4* expression during neuronal differentiation induced by EB or RA treatments. C) Immunoblot analysis of 100 µg of WCE from mES cells in presence of Lif and

after EB and RA treatments. The co-induction of *Tcf4* and *Zac1* mRNAs translates into their increased protein expression. Bars represent the mean of 2 independent experiments ran in duplicates; SD is shown.

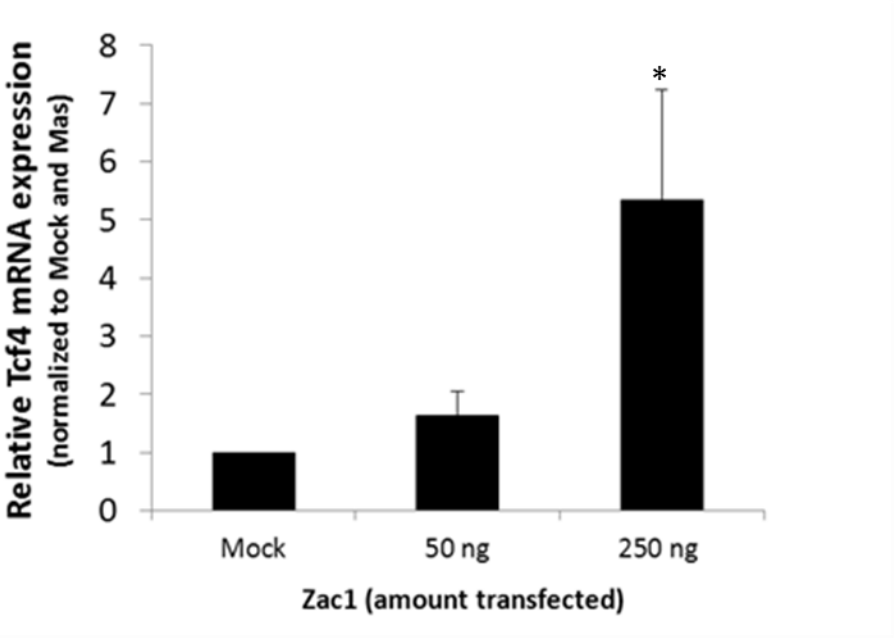


Figure 13: ***Zac1* regulates *Tcf4* gene expression in mES cells (46C).** qRT-PCR analysis revealing that *Tcf4* gene expression increases in a *Zac1*-dependent manner in 46C cells transfected for 24 hrs with increasing amount of pRK-Flag-*Zac1* . Bars represent the average of 3 independent experiments ran in duplicates; SD is shown. The asterisk indicates significant difference ($p<0.05$,*)

To identify *Zac1* binding site(s) mediating *Tcf4* expression, ChIP assays in undifferentiated cells and during neuronal differentiation were carried out and revealed multiple Zac1 binding sites on the *Tcf4* promoter and first intron. Indeed, Zac1 binding was barely detectable in undifferentiated cells, whereas the withdrawal of Lif caused a marked increase in Zac1 occupancy at the *Tcf4* locus. Zac1 binding was restricted to the proximal promoter region (-485 and -89 bp) and to a region containing a 2 palindromic and two direct repeat elements located in the center of the first intron (+1933 bp) of the *Tcf4* gene (*Fig. 14*). The concomitant increase in Zac1 binding to *Tcf4* and the increase in *Zac1* and *Tcf4* gene expression during neuronal differentiation, suggests that *Zac1* might regulate *Tcf4* by associating with its proximal promoter and first intron.

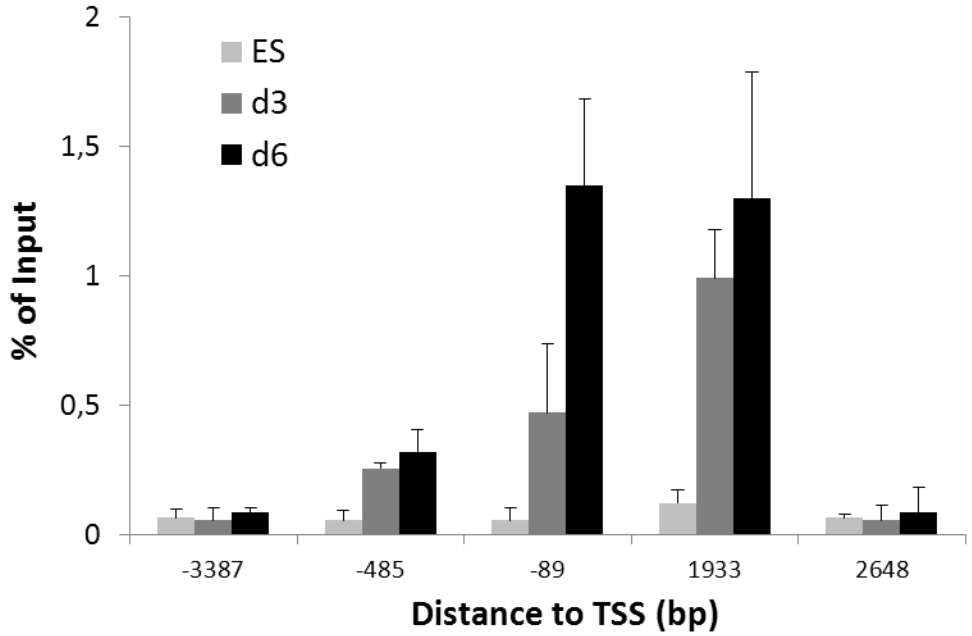


Figure 14: ***Zac1* occupies *Tcf4* proximal promoter and first intron during neuronal differentiation of mES cells.** ChIP assays were conducted in the presence (ES) or absence of Lif for 3 (d3) or 6 (d6) days. In the presence of Lif, *Zac1* binding is barely detectable at the *Tcf4* locus, whereas strong association between *Zac1* and *Tcf4* proximal promoter and first intron (+1933) was detected upon Lif withdrawal. Values were first calculated as % of input, to which negative control (IgG) values were subtracted. Bars represent the average of 3 independent experiments ran in duplicates; SD is shown.

To assess the contribution of each potential binding sites to *Zac1*-mediated *Tcf4* gene regulation, different fragments encoding either the *Tcf4* proximal promoter or *Tcf4* first intron separately, or in conjunction, were cloned upstream of a luciferase reporter gene *(Fig. 15 and Fig. 16.A)*. Luciferase assays were performed in undifferentiated cells. Each fragment met promoter criteria as they exhibit transcriptional activity, with the proximal promoter showing the highest activity. Inverting the orientation of each fragment (3´ to 5´) upstream of the luciferase reporter gene led to a strong decrease (50 to 100 fold) in promoter activity (*Fig. 15*).

Interestingly, while the activity of the promoter or the first intron separately was not affected by *Zac1* overexpression, the activity of the cassette coding for both promoter and first intron of *Tcf4* was induced up to 4 fold. This induction was impaired when the DNA binding defective mutant Zac1ZF7 was transfected (1.4 fold induction) (*Fig. 16.B*), hinting to the specific requirement of Zac1 to bind DNA in order to transactivate *Tcf4*. Altogether we suggest that *Zac1* regulates *Tcf4* gene transcription by a coordinated binding to the proximal promoter and to an element located in the first intron.

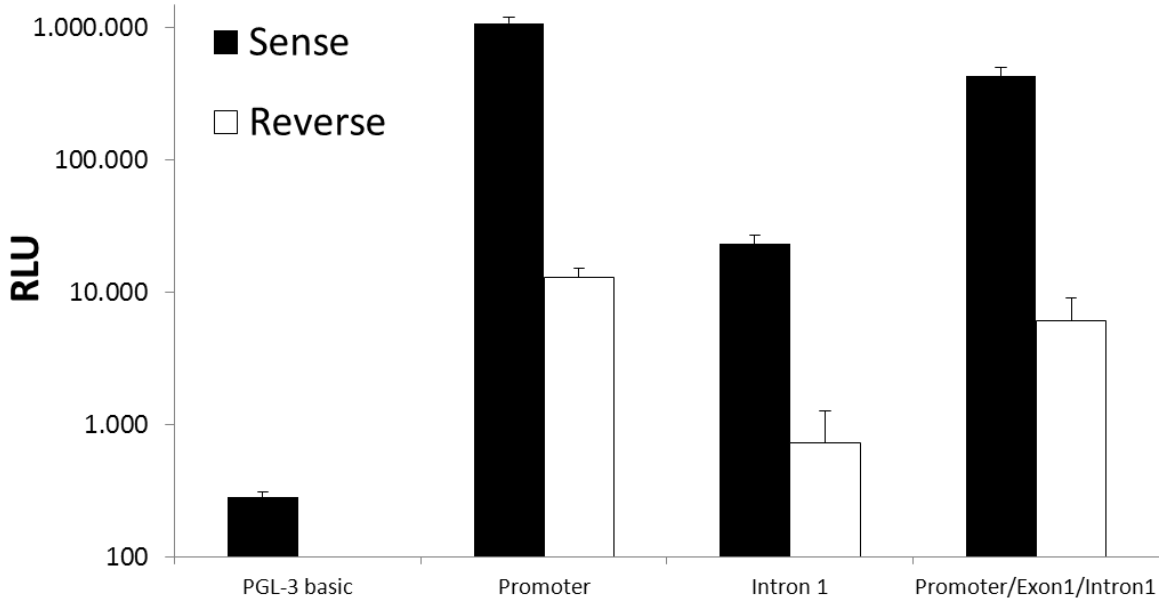


Figure 15: **Transcriptional activities of *Tcf4* promoter, first intron and association of the promoter with the first intron.** 46C cells were transfected with 250 ng of the ß-gal reporter gene, along with 500 ng of pGL3-basic or various PGL-3 *Tcf4* fragments (Promoter: -887/+46 bp; Intron: +1087/+2754 bp; Promoter + Intron: -887/+2754 bp) inserted in the sense orientation (5´-3´; black bars) or in the reverse orientation (3´-5´; white bars), upstream of the luciferase firefly reporter gene. Luciferase activity of the transfected cell extracts were determined 24 hrs after transfection, normalized to ß-gal activity values and plotted on a logarithmic scale. Each fragment exhibits promoter activity which was strongly reduced when they were inserted in the reverse orientation. This suggests that each element can function as promoter. Bars represent the average of 2 independent experiments ran in duplicates; SD is shown.

We asked next if the first intron of *Tcf4* was a *Zac1* responsive element that could enhance transcription in presence of a heterologous promoter containing *Zac1* binding sites. To address this, *Tcf4* first intron was cloned downstream of the TK promoter. Besides an increase in basal promoter activity caused by the promoter activity of the intron, *Zac1* overexpression did not change promoter activity of the heterologous construct (*Fig. 17*). Similar results were obtained when the intron was cloned downstream of the SV40 promoter (data not shown). This suggests that the presence of both *Tcf4* proximal promoter and intron is required for the *Zac1*-mediated regulation. This was further confirmed by inverting the orientation of the intron in association with the promoter, which abolished completely the *Zac1*-induced transcriptional activity.

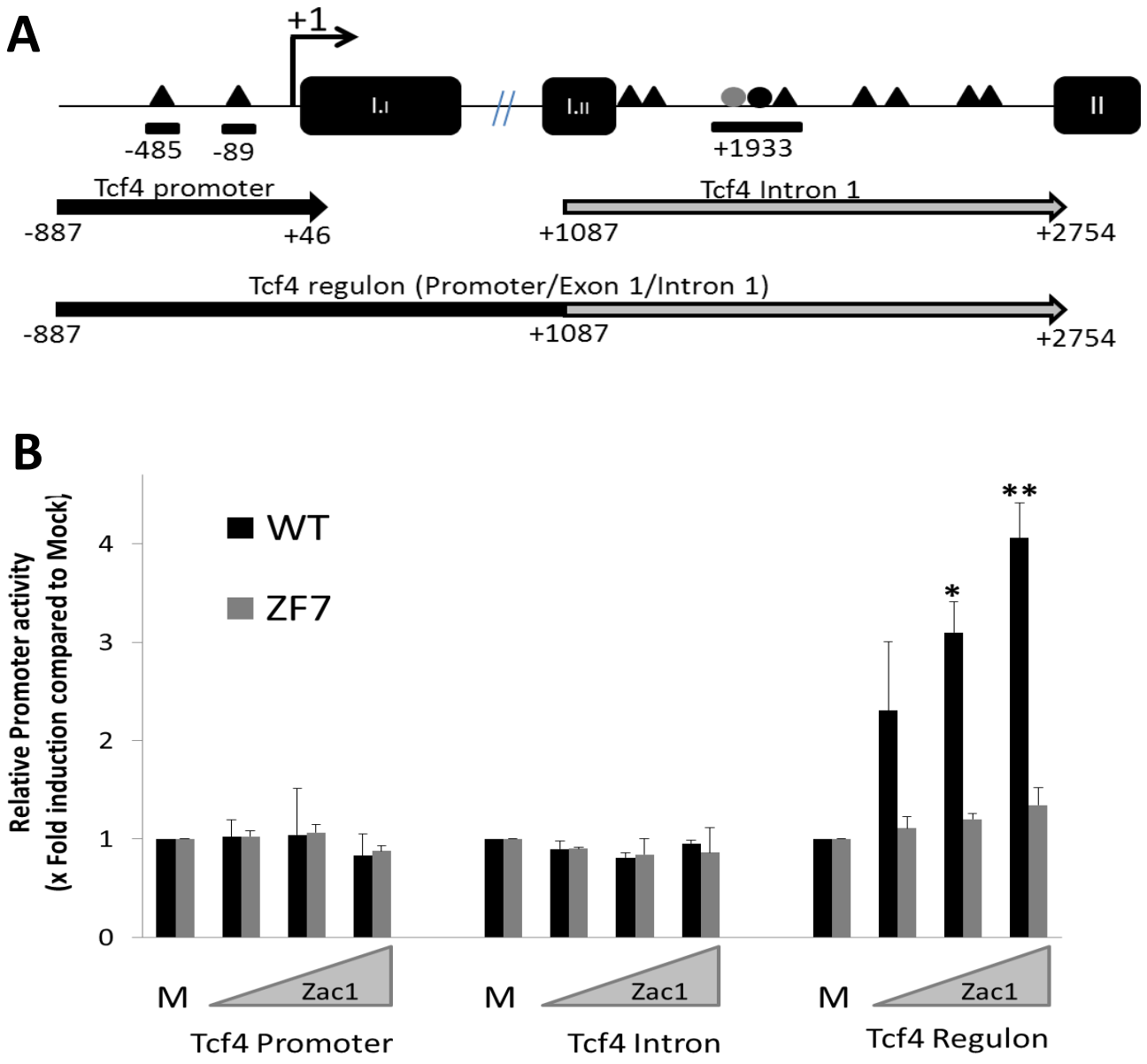


Figure 16: ***Zac1* activates *Tcf4* gene expression by regulating both promoter and intron transcriptional activity**. A) Schematic representation of the *Tcf4* promoter and first intron element cloned upstream of a luciferase reporter gene. Black boxes represent the 2 first exons (2 alternative exon I and the exon II); putative binding Zac1 sites are pictured as triangles and grey or black circles. Numbers indicate the distance in base pair to the TSS (+1). Black bars indicate validated Zac1 binding sites at the *Tcf4* locus, as revealed by ChIP. B) 46C cells were transfected with 250 ng of the ß-gal reporter gene, along with Mock (M) or increasing amount of pRK7-Zac1WT (white bars) or Zac1ZF7 (grey bars) and with 500 ng of PGL-3-Tcf4 fragments. Zac1 transfection increased promoter activity only when *Tcf4* promoter and first intron were together and this activation depends on *Zac1* DNA binding. Luciferase activity of the transfected cell extracts was determined 24 hrs after transfection and normalized to ß-gal activity values and to the mock transfected activity. Bars represent the mean of 4 independent experiments ran in duplicates; SD is shown. (*) $p<0.05$; (**) $p<0.01$ (t-test).

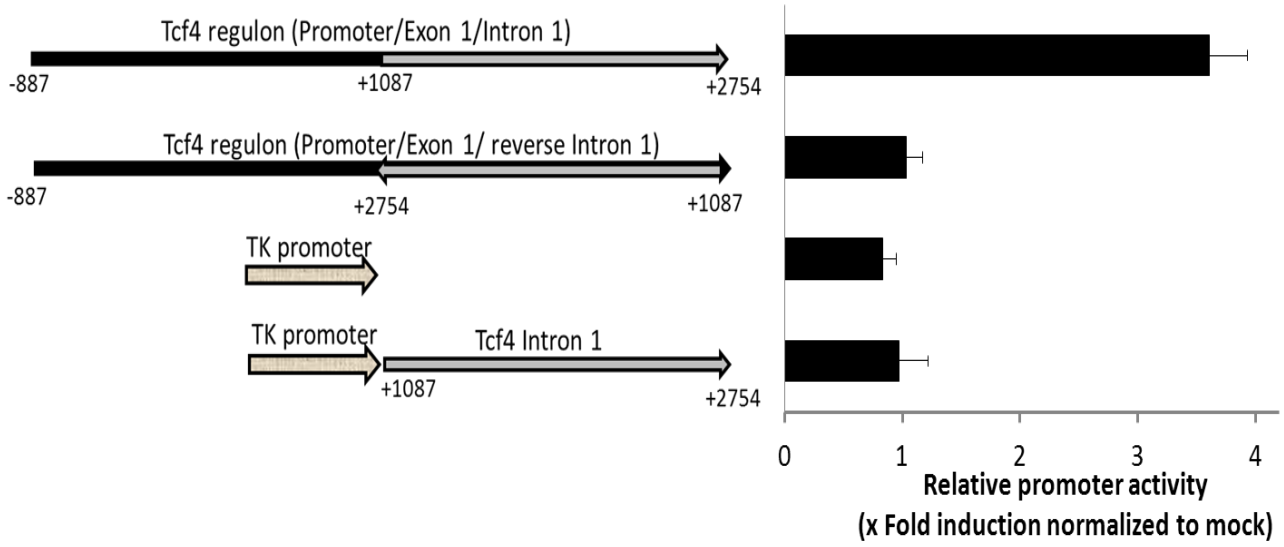


Figure 17: **Tcf4 first intron does not act as enhancer of transcription.** 46C cells were transfected with 250 ng of the ß-gal reporter gene, 100 ng pRK7-Flag Zac1 and with 500 ng of PGL-3-Tcf4 or PGL3-TK fragments. *Tcf4* first intron does not act as a general Zac1-responsive element, and its promoter activity depends on the presence of the naïve *Tcf4* promoter. Luciferase activity of the transfected cell extracts was determined and normalized to ß-gal activity values and to the mock transfected activity Bars represent the average of 3 independent experiments ran in duplicates; SD is shown.

III. *Zac1* regulates *Tcf4* expression during embryonic neurogenesis.

As *Zac1* regulates *Tcf4* expression during early neural differentiation of mES, we asked next whether this regulation also occurs at later developmental stages after the onset of neurogenesis onset. To address this *in vitro*, we performed neurosphere culture from E15 mouse brains. Neurospheres (NS) are spheroid structures grown in suspension and in high concentration of EGF and FGF-2. NS consist of a heterogeneous mix of neural stem cells, neural progenitors and more differentiated cells with a rich extra-cellular matrix surrounding them. Once dissociated and seeded on PDL-coated dish, NS-derived cells show the capacity for self-renewal, Nestin immunoreactivity and the ability to differentiate into neurons, astrocytes and oligodendrocytes (Rao, 2004; Ahmed, 2009). NS were generated from whole embryonic brains and passaged twice every 5 days, with medium change each second day. At the third passage, cells were seeded on PDL-coated plate and grown as monolayer in presence of EGF and FGF-2. After 2 days in culture, the cell population was composed mainly of Nestin positive radial glial-like cells, that co-expressed *Zac1* and *Tcf4* (*Fig. 18.A-C*). Cells were then cultured in different conditions to

enhance neuronal or astroglial differentiation (see material and methods for detailed protocols) (*Fig. 18.A*). As observed during differentiation of mES, *Zac1* expression was induced during differentiation of NSC supporting the idea that *Zac1* is induced during general differentiation of progenitor cells (Alam et al., 2005; Valente et al., 2005). Indeed after 6 days of differentiation, *Zac1* was up-regulated both in neurons and astrocytes; in contrast to the neurogenic factor *Tcf4*, which was restricted to neurons (*Fig. 18.B*) where it was highly expressed (*Fig. 19*).

Reporter assays and ChIP analysis confirmed that *Tcf4* is a *Zac1* target gene in NSC and in differentiated cells with the strongest transactivational effect of Zac1 observed in neurons (*Fig. 20*). Interestingly *Zac1* occupancy on *Tcf4* promoter and first intron increased exclusively during neuronal differentiation, and this, despite an increase in *Zac1* expression during both neuronal and astroglial differentiation. This indicates that Zac1 binding to *Tcf4* locus might be cell-type specific and does not depend on endogenous *Zac1* expression level. Hence, *Zac1* might regulate *Tcf4* gene expression specifically in NSC and during their differentiation into neurons but not into glial cells.

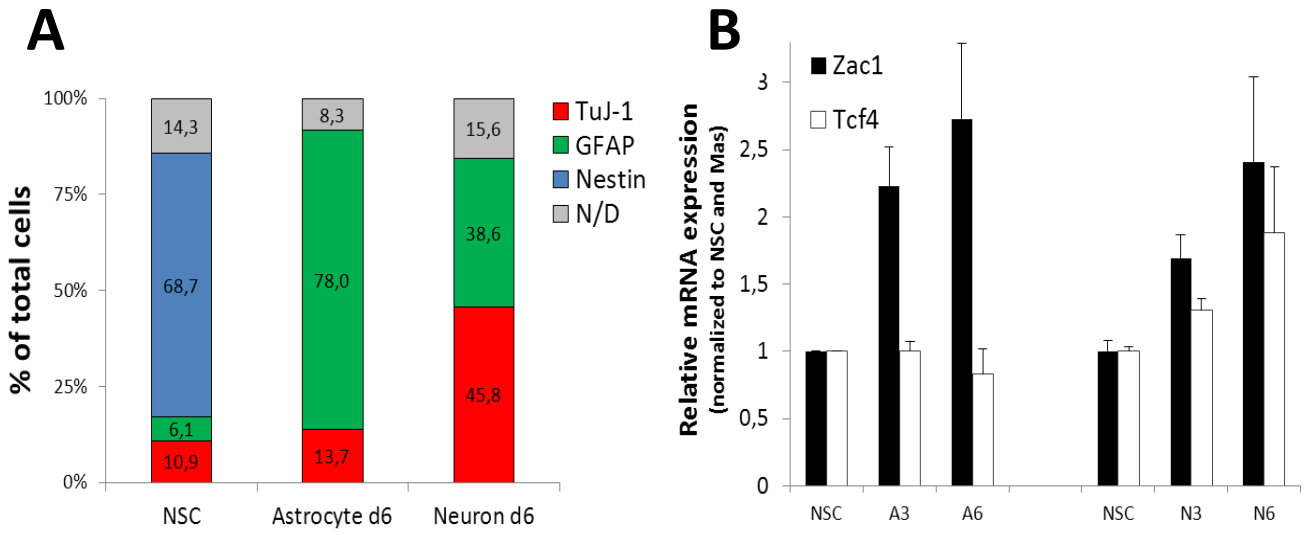


Figure 18: ***Zac1* and *Tcf4* are co-induced during neuronal differentiation of NSC** A) Distribution of NSC (Nestin), glial (GFAP) and neuronal cells (TuJ-1) among NS derived cells, grown in NSC conditions or in differentiation medium for 6 days. TuJ-1, GFAP and Nestin negative cells were counted as N/D . Bars represent the percentage of cells expressing the fate specific marker, normalized to the total cell number (nuclei stained with DAPI). Cultures were performed twice, and cells numbers were measured in 4 separate fields. B) qRT-PCR analysis showing *Zac1* expression is induced during neuronal or astroglial differentiation for 3 or 6 days (N3/A3 and N6/A6) , whereas *Tcf4* is only induced during neuronal differentiation. Bars represent the average of 3 independent experiments in duplicates; data were normalized to NSC conditions and to Mas. SD is shown.

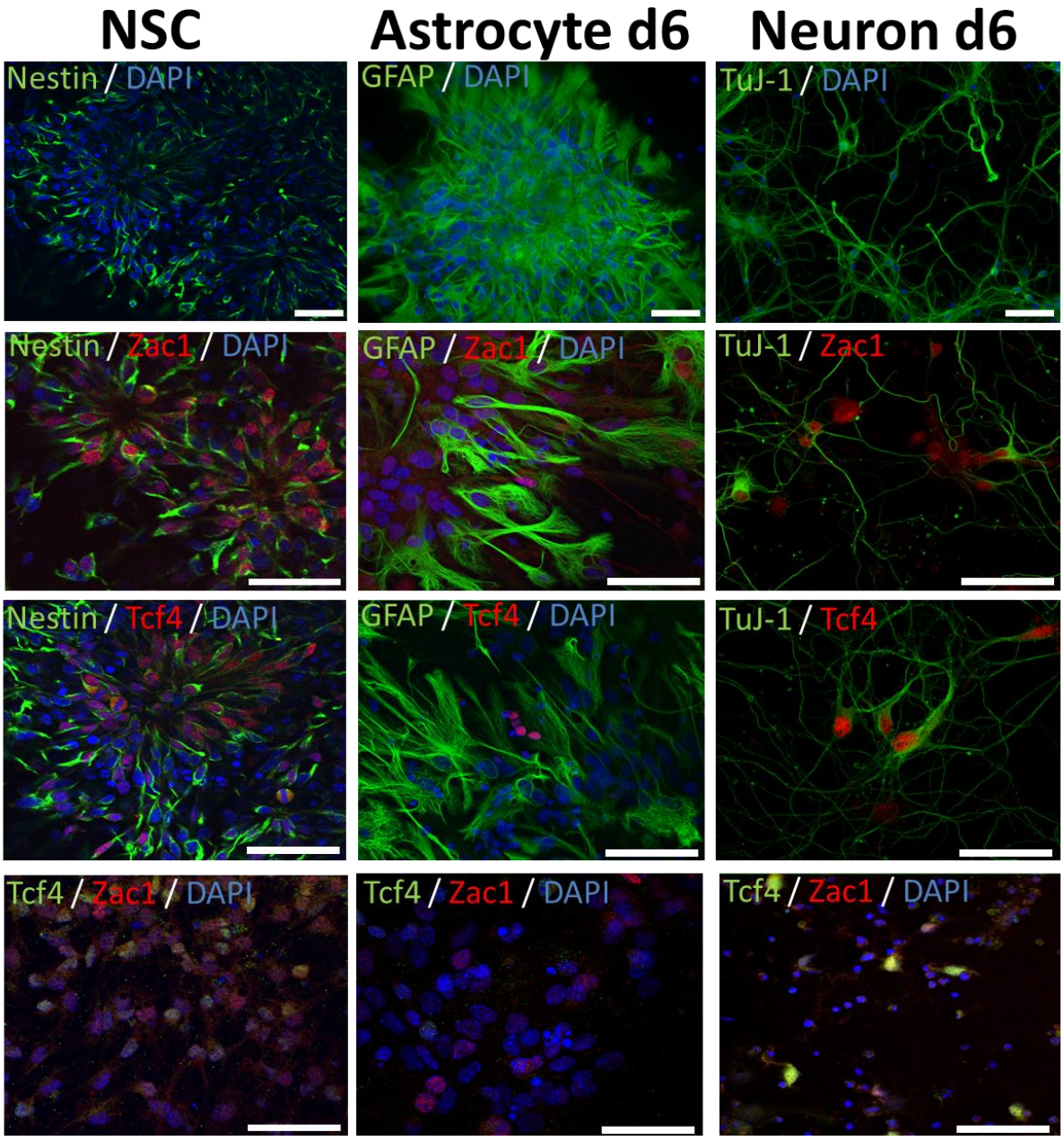


Figure 19: *Zac1* and *Tcf4* are co-expressed during neuronal differentiation of NSC. Immunocytochemistry of NSC in proliferating conditions (NSC) and after astroglial or neuronal differentiation for 6 days (Astrocyte d6 / Neuron d6). *Zac1* and *Tcf4* are co-expressed in mature neurons (TuJ-1 positive cells), and in NSC (Nestin positive cells). *Zac1* is also expressed in mature astrocytes (GFAP positive cells) in contrast to the proneural factor *Tcf4*. Scale bar: 100 µM

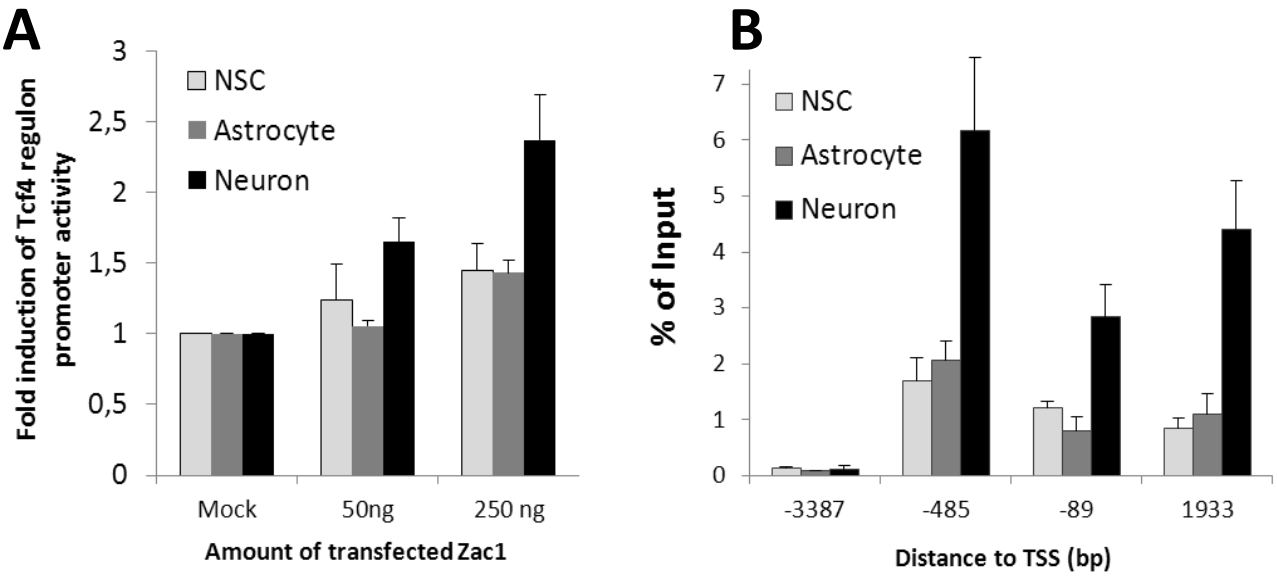


Figure 20: *Zac1* regulates *Tcf4* expression in neural progenitors and differentiating neurons. A) NSC, Astrocytes or Neurons were transfected with 250 ng of the ß-gal reporter gene, along with Mock (M) or increasing amount of pRK7-*Zac1*WT and with 500 ng of PGL-3-*Tcf4*reg. *Zac1* transfection increased promoter activity of *Tcf4* construct in all cell types, with the highest induction observed in neurons. Luciferase activity of the transfected cell extracts was determined 24 hrs after transfection and normalized to ß-gal activity values and to the mock transfected activity. Bars represent the average of 3 independent experiments with duplicates; SD is shown. B) ChIP assays were conducted in NSC, in astrocytes or in neurons (after 6-day culture in differentiation medium). *Zac1* occupies *Tcf4* proximal promoter (-485 and -89 bp) and first intron (+1933) in the 3 lineages. The strongest binding was detected in neuronal cells, suggesting that *Tcf4* locus is more accessible to *Zac1* in neuronal cells compared to NSC and Astrocytes. Values were first calculated as % of input, to which negative control (IgG) values were subtracted. Bars represent the average of 3 independent experiments with duplicates; SD is shown.

To confirm our findings *in vivo*, we co-stained coronal sections of E15 mice brains using Zac1 and Tcf4 antibody and observed the co-localization of the two proteins in distinct cells of the VZ and SVZ of the lateral ventricles, hinting to a possible co-regulation between the 2 transcription factors (*Fig. 21.A*). However, the characterization of the cells co-expressing Zac1 and Tcf4 needs to be performed. ChIP analysis of brain punches of this region confirmed that Zac1 binds strongly to *Tcf4* locus *in vivo* (*Fig. 21.B*) and validate *Tcf4* as *Zac1* target gene during neurogenesis. A second brain region where Zac1 and Tcf4 protein levels could not be detected (data not shown) surrounding the third ventricle was used as negative control for ChIP experiment. Finally, after dissection of brain regions of E15 mice containing roughly the VZ and SVZ of the lateral ventricles, as well as the developing cortex, dissociated cells were nucleofected with increasing amount of *Zac1*, which induced *Tcf4* gene expression *(Fig. 21.C)*.

Altogether, we show that *Zac1* regulates *Tcf4* expression in neural precursors and/or mature neurons during neurogenesis.

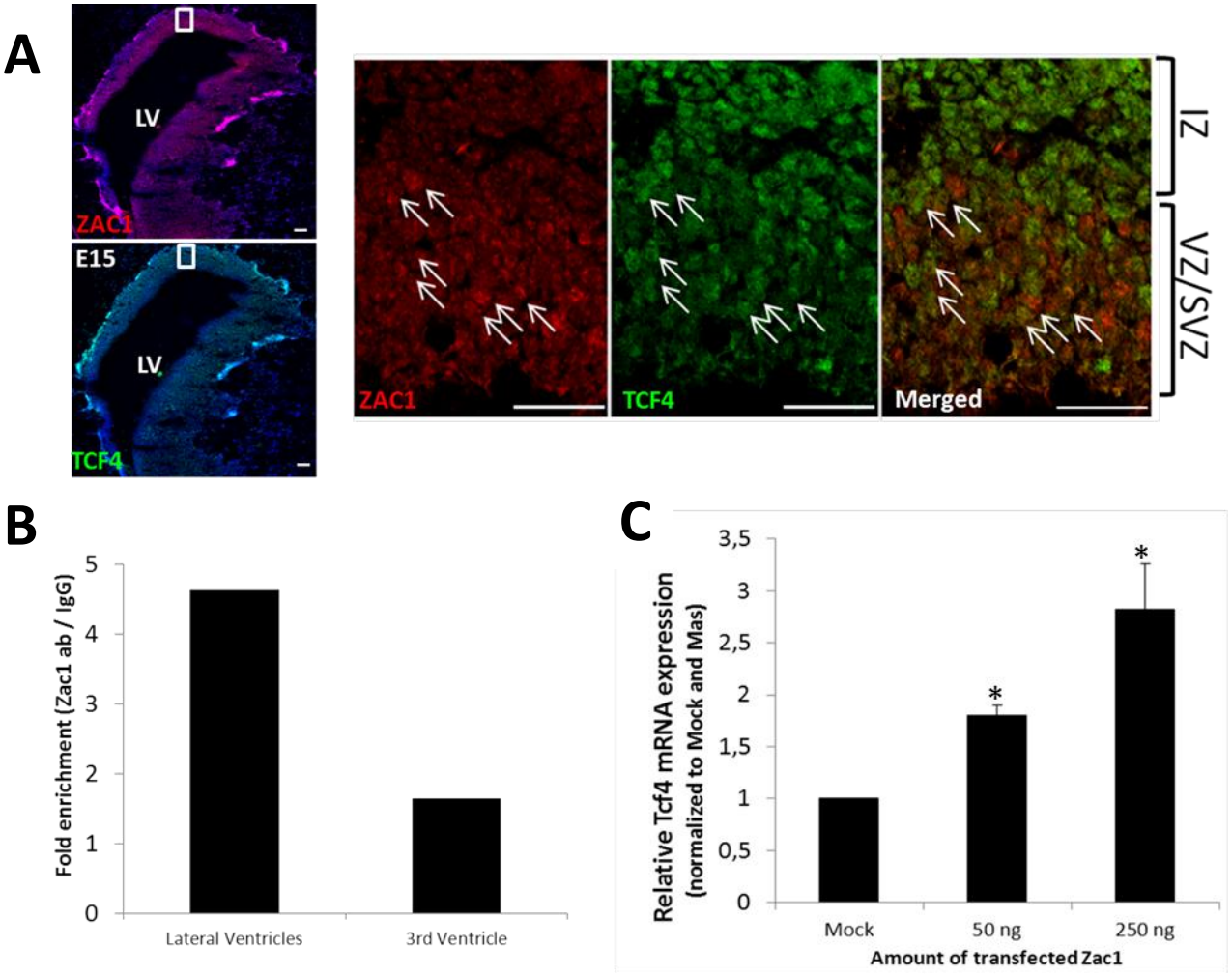


Figure 21: ***Zac1* regulates *Tcf4* in progenitors of the VZ and SVZ of the lateral ventricles of E15 mice.** A) *Zac1* and *Tcf4* immunoreactivity patterns in the region surrounding the lateral ventricles in the embryonic mouse (E15) brain. 10 µM coronal sections were obtained and incubated with guinea pig anti mouse Zac1 antibody and rabbit anti mouse Tcf4 antibody. *Zac1* and *Tcf4* are co-expressed in the neurogenic area surrounding the lateral ventricle (VZ and SVZ, but not in the Intermediate zone (IZ)). Scale bar: 100 µm. B) ChIP analysis of Zac1 occupancy at *Tcf4* promoter in the regions surrounding the lateral ventricles or the 3rd ventricle. Brain punches from 5 animals were pooled and used for 2 independent immunoprecipitations. Values were first normalized to the Input, and then calculated as fold enrichment (*Zac1* antibody value divided by the IgG value). % of Input could not be used here as the value of the IgG differed between the 2 brain regions. C) qRT-PCR analysis revealing that *Tcf4* gene expression increases in a *Zac1*-dependent manner in neural progenitor cells and/or neurons, dissected from lateral ventricule area in E15 embryos, and nucleofected with increasing amount of pRK-Zac1-GFP. Cells were harvested 48 hrs after nucleofection. Bars represent the average of 3 independent experiments performed in duplicates; SD is shown. (*) $p<0.05$ (t-test).

IV. *Zac1*, *Tcf4* and *p57^{kip2}* are co-regulated during brain development

Finally, we investigated the biological significance of the regulation of *Tcf4* gene by *Zac1*. As *Tcf4* can act as a transcription factor, we tested whether the control of the expression of *Tcf4* by *Zac1* can affect the transcription of *Tcf4* target gene. We decided to focus on the paternally imprinted gene *p57^{kip2}* for 3 reasons.

1) It is co-regulated with *Zac1* in the context of the Imprinted gene Network (Varrault et al., 2006).

2) *p57^{kip2}* plays an important role in differentiation and migration of RGC and IPC during neurogenesis (Tury et al., 2011a).

3) *Zac1*, *Tcf4* and *p57^{kip2}* are known inducers of cell cycle arrest (Matsuoka et al., 2006; Herbst et al., 2009a).

p57^{kip2} is one of the three members of the the Cip/Kip family of cell cycle inhibitors. It is expressed in proliferating progenitors in the VZ and SVZ of the telencephalon, but also in cortical precursors and mature neurons. *p57^{kip2}* expression is strongly reduced in the adult brain, except in the developing cerebellum where it is transiently expressed during the postnatal cerebellar neurogenesis. *p57^{kip2}* has been identified as a functionally relevant target recruited by the E-proteins to induce cell cycle arrest in the human neuroblastoma cell line SK-N-SH (Rothschild et al., 2006). Hence, we first determined whether *Tcf4* can regulate the expression of *p57^{kip2}* in the murine C17.2 cell line. To address this, cells were transfected with *Tcf4* expression vector and endogenous *p57^{kip2}* gene expression was measured by qRT-PCR. *Tcf4* overexpresssion induced *p57^{kip2}* by 1.5 fold confirming the role of *Tcf4* as regulator of *p57^{kip2}* gene expression in the mouse brain (*Fig. 22.C*). Previous studies showed that *p57^{kip2}* might be directly regulated by *Zac1*; hence, it is difficult to determine the relative contribution of both transcription factors to *p57^{kip2}* gene regulation. However, in contrast to *p57^{kip2}*, *Zac1* gene expression was not affected by *Tcf4* overexpression *(Fig. 22.C)*, we could therefore conclude that *Tcf4* could regulate *p57^{kip2}* expression in a *Zac1* independent manner. It is thus likely that both factors control the expression of the cell cycle regulator *p57^{kip2}*. Additional *Zac1* overexpression and knocking down approaches confirmed that the regulation of *Zac1* expression mediates the expression of *Tcf4* and of its target gene *p57^{kip2}* (*Fig. 22.A-B-D*).

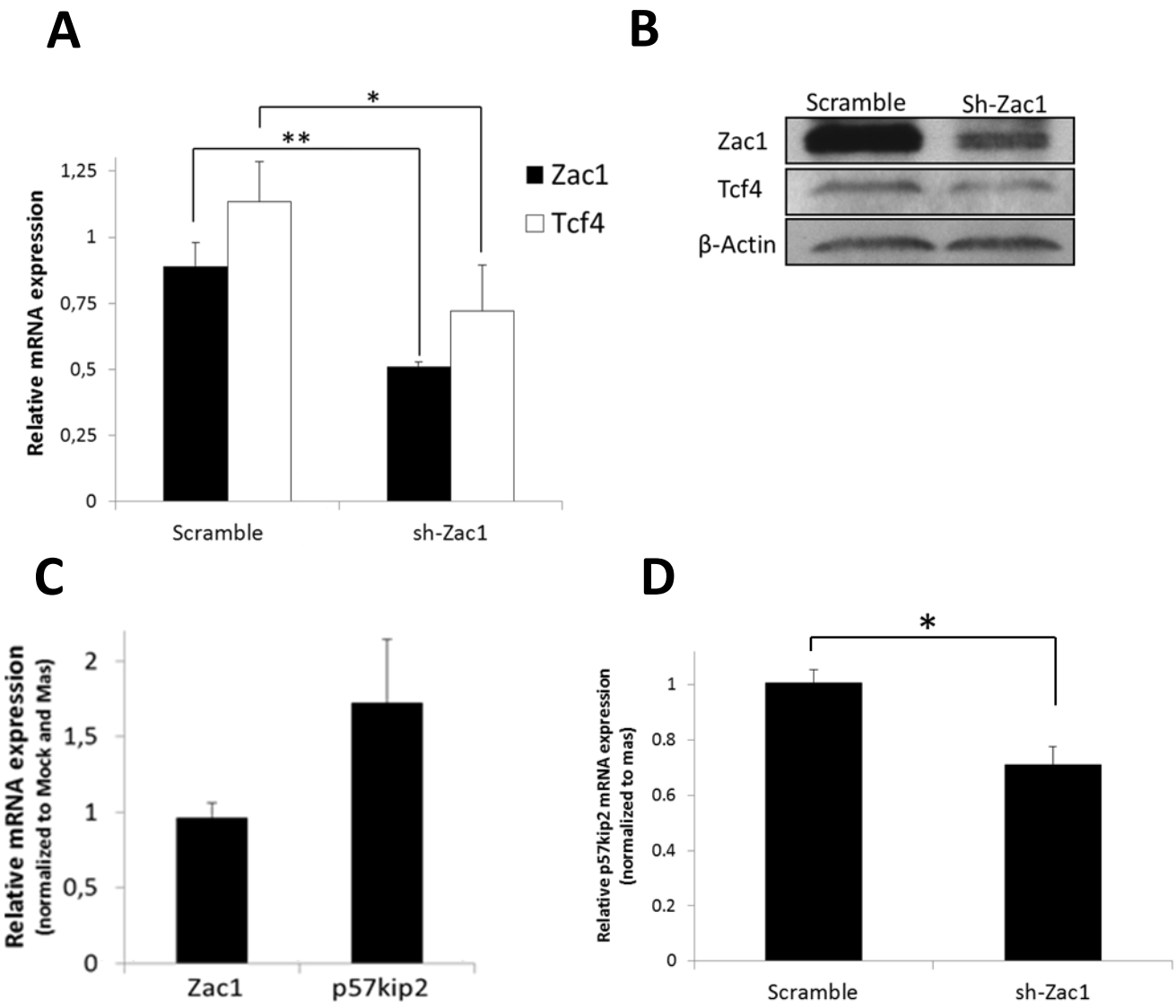


Figure 22: *Zac1* and *Tcf4* regulate *p57^{kip2}* expression in C17.2 cells. A). Knock down of *Zac1* by transfection of 500 ng of a pool of shRNA targeted against *Zac1* (shRNA-*Zac1*) for 24 hrs, resulted into a significant decrease of *Zac1* and *Tcf4-B* mRNA levels as measured by qRT-PCR. B) Immunoblot analysis of 50 µg of WCE from C17.2 cells transfected with 500 ng of scramble shRNA or shRNA-*Zac1*. The reduction of *Tcf4* and *Zac1* mRNAs after *Zac1*-knockdown translates into their increased protein expression. C) C17.2 cells were transfected for 24 hrs with 100 ng of pCDEF3-Flag-Tcf4, and expression of *Zac1* and *p57^{kip2}* was assessed by qRT-PCR. Overexpression of *Tcf4* increased *p57^{kip2}* gene expression independently of *Zac1*. D) Knock down of *Zac1* by transfection of 500 ng of a pool of shRNA targeted against *Zac1* (shRNA-*Zac1*) for 24 hrs, resulted into a significant decrease of *p57^{kip2}* mRNA levels as measured by qRT-PCR. This indicates that regulation of *Zac1* expression control *Tcf4* and its target gene *p57^{kip2}* expression. Bars represent the average of 3 independent experiments ran in duplicates; SD is shown. *p<0.05; **p<0.01 (t-test).

Finally, to verify these findings *in vivo*, we measured Tcf4 occupancy at *p57^{kip2}* promoter in two brain regions where they are both expressed (Alam et al., 2005; Ye et al., 2009): in the VZ and

SVZ of the lateral ventricles and in the developing cerebellum (*Fig. 23*) by ChIP. The two analyzed genomic regions on *p57^{kip2}* promoter contain an E-box (E-protein binding motif) and *Tcf4* proximal promoter was used as negative control of binding as no E-Box motif could be identified in this region. We observed a strong enrichment of Tcf4 at *p57^{kip2}* promoter in vivo, supporting a role for the proneural gene in the regulation of *p57^{kip2}* gene transcription.

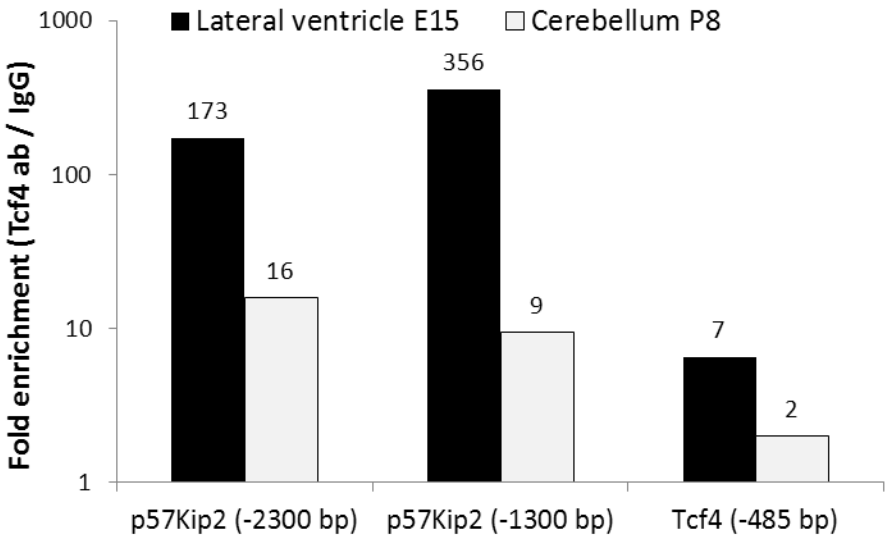


Figure 23: *Tcf4* occupies *p57^{kip2}* promoter in neural progenitors. ChIP analysis of *Tcf4* occupancy at *p57^{kip2}* promoter and *Tcf4* proximal promoter in the regions surrounding the lateral ventricles of E15 mouse brain or in the developing murine cerebellum of P8. Brain punches from 6 animals for lateral ventricle were pooled and used for each immunoprecipitation (n=2) whereas 1 whole cerebellum was used for each immunoprecipitation (n=3). Values were first normalized to the Input, and then calculated as fold enrichment (*Tcf4* antibody value divided by the IgG value) and plotted on a logarithmic scale. % of Input could not be used here as the value of the IgG differed grandly between the 3 primers.

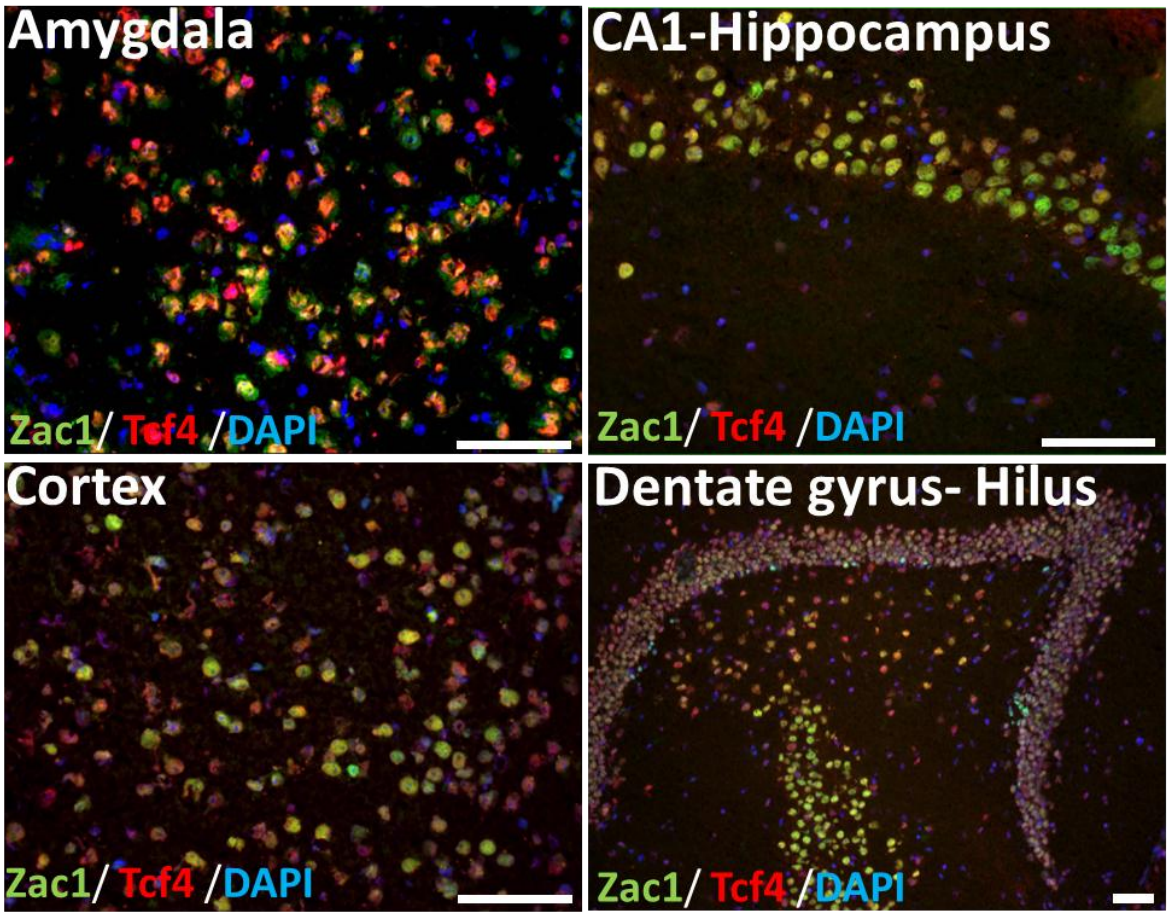
Altogether, we could show that *Zac1* is an important transcriptional activator during neurogenesis. Via the control of *Tcf4*, it might promote neuronal differentiation. By controlling *p57^{kip2}* gene expression, *Zac1* might also control the cell cycle exit of progenitor cells.

V. *Zac1* regulates *Tcf4* expression during adulthood.

In contrast to the other E-proteins (E2A and HEB) that are transiently expressed in the embryonic and early post-natal brain, *Tcf4* expression increases during neurogenesis and remains strong in adult brain, especially in the cortex, in the hippocampus, the amygdala and the cerebellum (Brzózka et al., 2010). We asked whether *Zac1* could regulate the expression of *Tcf4* during

adulthood. We found that Tcf4 and Zac1 are co-expressed in the neurons of all regions of the hippocampus, in the cortex, in the basolateral nucleus of the amygdala *(Fig. 24.A)* and in the EGL and IGL neurons of the cerebellum (data not shown). We performed ChIP analysis and confirmed that Zac1 occupies the *Tcf4* gene locus in the adult hippocampus and cerebellum, which suggests that *Zac1* might regulate *Tcf4* expression in these adult brain regions *(Fig. 24.B)*.

A



B

Brain region	Zac1/ Tcf4 expression	Zac1/ Tcf4 Colocalization	Zac1 occupancy at the Tcf4 locus
Amygdala	+ / ++	+	+
Cerebellum	++ / +++	++	+++
Cortex	+ / +++	+	++
Hippocampus	+++ / +++	+++	+++

Figure 24: *Zac1* and *Tcf4* are co-expressed in specific adult brain regions. A) *Zac1* and *Tcf4* immunoreactivity patterns in the basolateral nucleus of the amygdala, in the hippocampus CA1, Dentate gyrus and Hilus, and in the cortex. 10 µM coronal sections were obtained from 3-month old cd1 mice and incubated with guinea pig anti mouse

Zac1 antibody (1/1000) and rabbit anti mouse *Tcf4* antibody (1/1000). Scale bar: 100 µm B) Table to summarize *Zac1*, *Tcf4* expression and co-localization in 4 brain regions. In the *Zac1*/*Tcf4* expression column, is shown whether cells expressed low (+), moderate (++) and strong (+++) levels of *Zac1* and *Tcf4*, as estimated by immunohistochemistry. In the *Zac1*/*Tcf4* co-localization column is indicated whether a small subset (+), a majority (++) or all the cells (+++) co-expressed *Zac1*and *Tcf4*. ChIP analysis of *Zac1* occupancy at *Tcf4* proximal promoter in the adult mouse brain. Low (+), mild (++) and strong enrichment of *Zac1* at *Tcf4* locus was observed in all analyzed brain regions.

Additionally ectopic expression of *Zac1* in primary hippocampal (*Fig. 25*) and cerebellar granule cells of the EGL (data not shown) led to increased endogenous *Tcf4* gene expression (2 fold) and increased *Tcf4* protein levels. Altogether we suggest that *Zac1* is a transcriptional regulator of *Tcf4* in adult neurons.

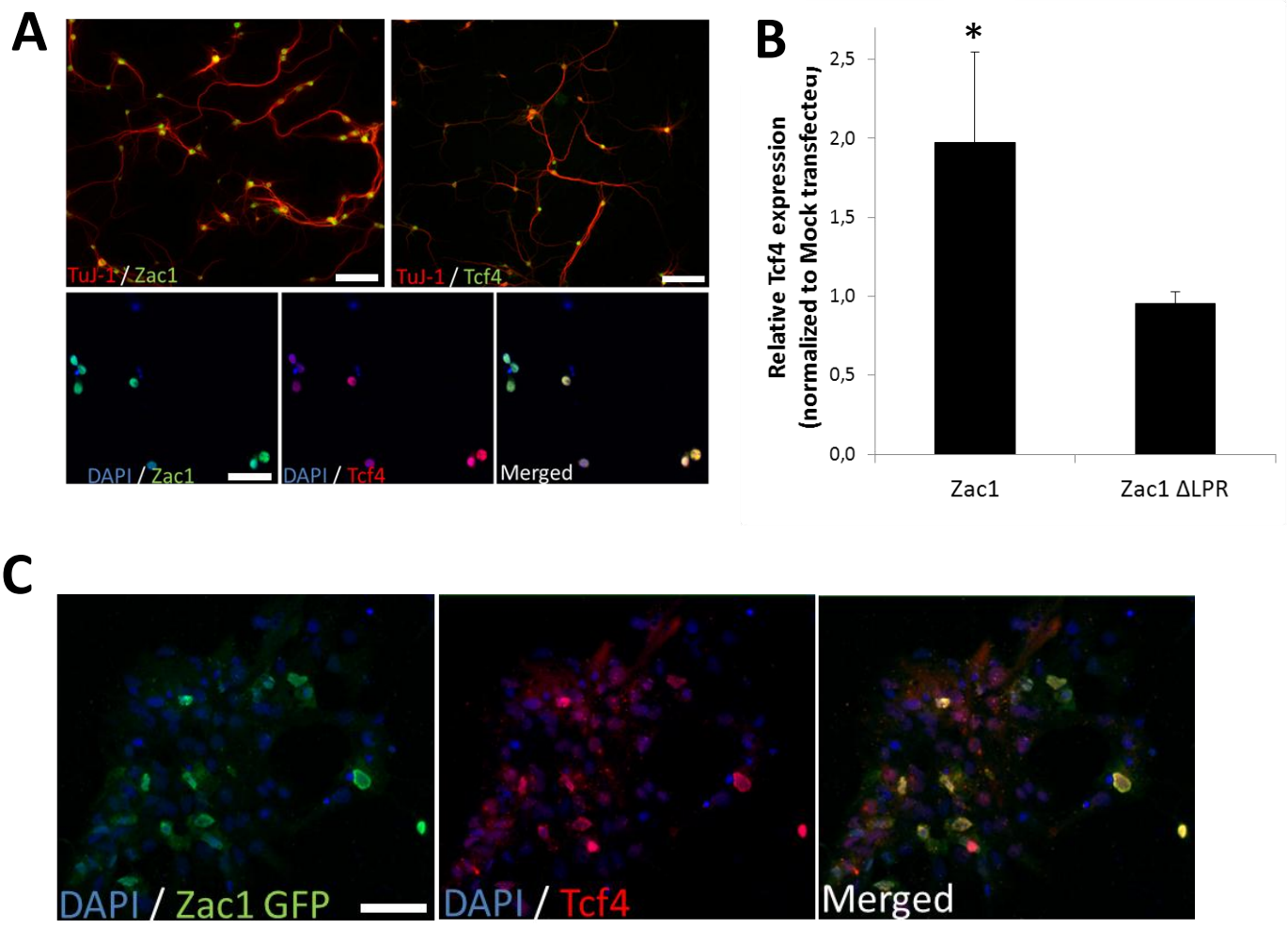


Figure 25: ***Zac1* transactivates *Tcf4* in hippocampal neurons**. A) Co-staining of Zac1 and Tcf4 of mature hippocampal neurons with anti-Zac1 and anti-Tcf4 antibodies showed that both factors are co-expressed in the nuclei of the neuronal cells (TuJ-1 positive cells). B) Hippocampal neurons were transfected with 100 ng of either *Zac1* WT (Zac1) or the transactivation defective mutant construct (Zac1 ΔLPR); *Tcf4* gene expression was measured by qRT-

PCR 48hrs after transfection and normalized first to expression of the house keeping gene Mas, then to Mock transfected conditions. *Zac1*WT overexpression induces *Tcf4* expression in hippocampal neurons, whereas deletion of the transactivator domain abolished completely *Tcf4* induction. Bars represent the average of 3 independent experiments with duplicates; SD is shown. (*) p<0,05. C) Cells were transfected with 100 ng pRK-Zac1 GFP for 48 hrs and co-stained with anti-GFP and anti-Tcf4 antibodies. *Zac1* overexpression increases Tcf4 protein levels in primary hippocampal neurons. Scale bar: 100 µm

VI. *ZAC1* might regulate *TCF4* expression in Human.

We analyzed the conservation between the mouse and human *Tcf4* promoter and first intron DNA sequences for potential *Zac1* binding sites (*Fig. 26*). Computational analysis showed that both promoter and intron sequences are highly conserved (78% identical) between the two species supporting a role for these genomic elements in the control of the gene transcription. Putative *Zac1*/*ZAC1* binding sites on *TCF4* promoter and on the first intron were conserved across species, suggesting that *ZAC1* could bind to *TCF4* promoter and first intron to regulate *TCF4* gene expression.

To examine if *Zac1*-mediated *Tcf4* induction is conserved in human, we next transfected *ZAC1* into the human neuroblastoma cell line SK-N-MC. ZAC1 increased *TCF4* mRNA in a dose dependent manner suggesting cross-species conservation *(Fig. 27).*

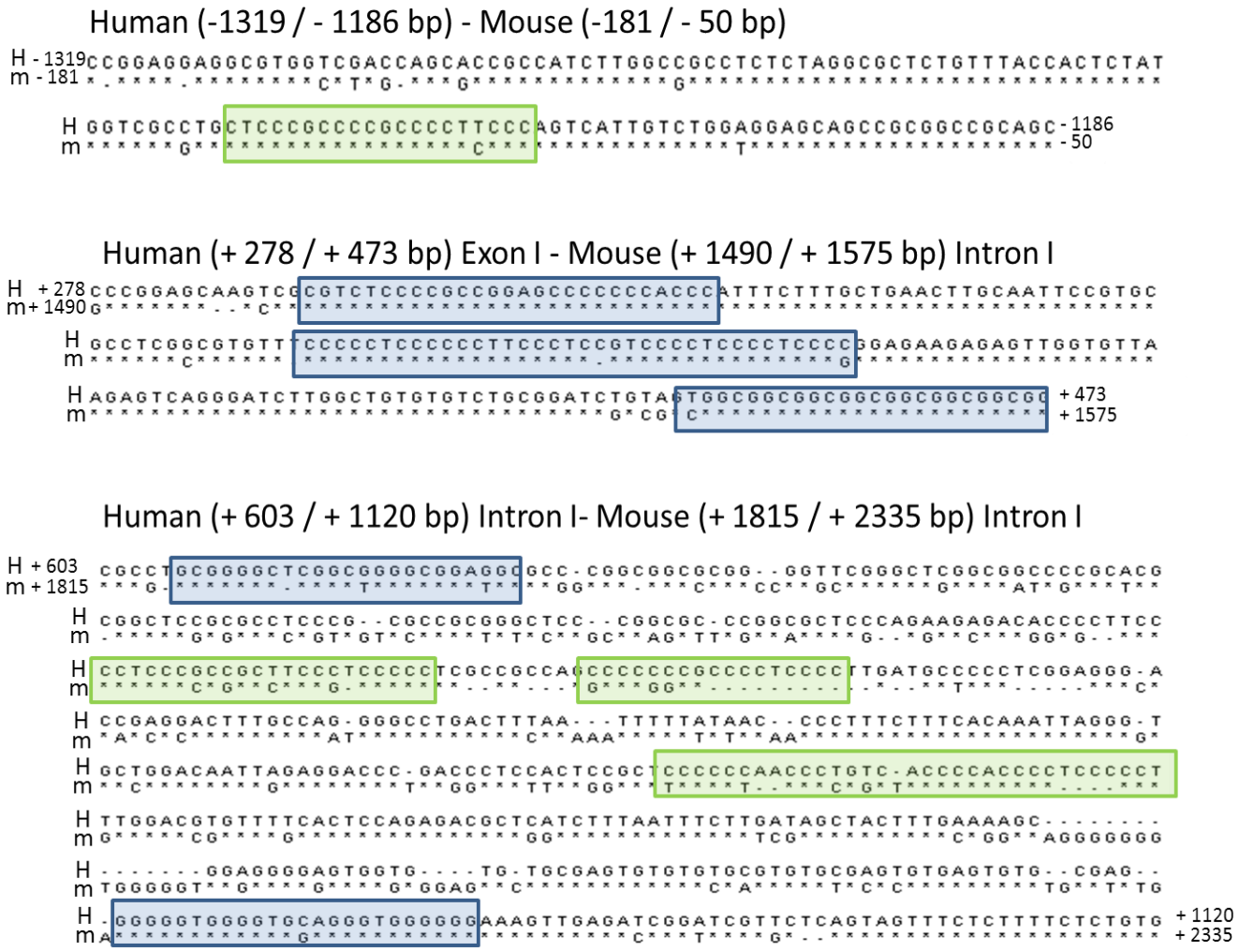


Figure 26: **Conservation of the multiple *Zac1*/*ZAC1* binding sites on *Tcf4*/*TCF4* locus.** Alignment reveals conservation of *Tcf4* promoter sequences between mouse (m) and Human (H). Identity, gaps were symbolized with (*) and (-) respectively. Relative bp positions to the transcriptional start sites are indicated. For both species, the multiple potential *Zac1*/*ZAC1* binding sites that resemble the idealized direct repeat type $(G4_{N1–12}G4)_2$ were framed with blue rectangles and binding sites previously identified in mouse were framed with green rectangles.

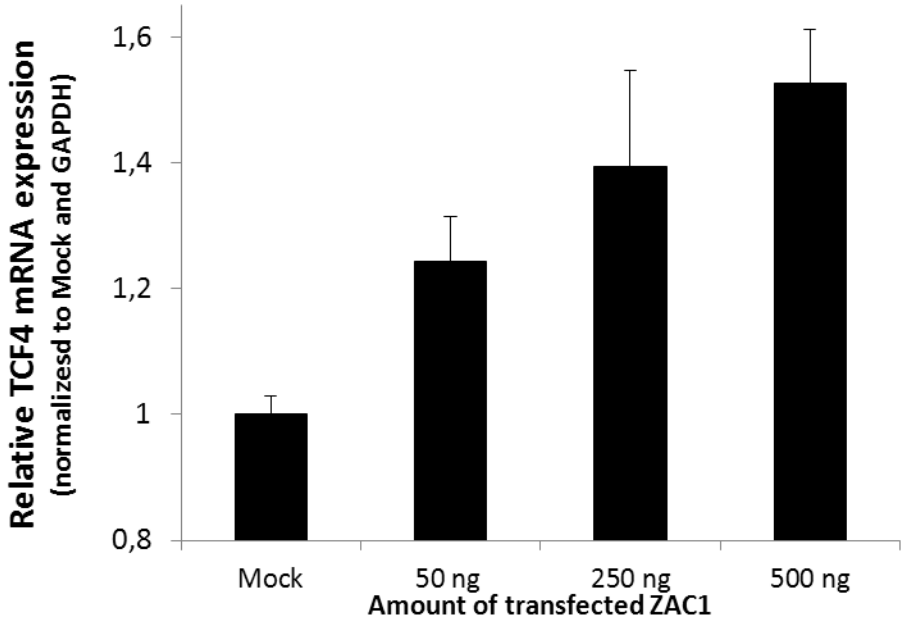


Figure 27: ***ZAC1* regulates *TCF4* expression in SK-N-MC cells.** Cells were transfected with increasing amount of PRK-Flag *ZAC1* for 24 hrs, and expression of *TCF4* was measured by qRT-PCR and normalized first to the house keeping gene GAPDH gene expression, then to Mock transfected cells. Bars represent the mean of 2 independent experiments performed in duplicates; SD is shown.

VII. Antibody validation

We have generated two rabbit polyclonal antibodies that recognize different epitopes on the Tcf4 protein. We created an antibody that could recognize total *Tcf4* proteins and one that recognizes specifically the isoform that is induced by *Zac1*, namely the isoform B *(Fig. 28).*

To validate sera immunoreactivity, we performed immunoblot and immunocytochemistry analysis. We used 2 different cell lines: the LLC-PK1 cells which can be electroporated with high efficiency and the DLD1-ITF2 cell line that stably overexpresses Flag-tagged *TCF4*-B when cultured in presence of doxycycline (Herbst et al., 2009a). Whole cell extract (WCE) of LLC-PK1, transfected with Flag-tagged *TCF4*-A or Flag tagged *TCF4*-B, were blotted with a mouse anti-Flag and the 2 rabbit anti-*Tcf4* antibodies. Immunoblot analysis confirmed that *Tcf4* and *Tcf4*-B antibodies could be used to detect *TCF4* protein level. The *Tcf4*-B antibody could detect specifically the isoform B, whereas *Tcf4* antibody could detect both isoforms. No signal was detected in the mock transfected conditions *(Fig. 29.A).* We further confirmed the specificity of the signal by blotting WCE of DLD1-ITF2 cells with the mouse monoclonal antibody Ri-3b9 that

recognizes the 2 human isoforms of *TCF4*. Strong signals were detected at the same molecular weight in the WCE of cells cultured in presence of doxycycline, whereas no signal was observed with WCE of non-induced cells *(Fig. 29.B)*. *Tcf4* protein is highly conserved between Human and mouse, and we confirmed that both sera could also be used to detect mouse *Tcf4* proteins *(Fig. 29.C)*. Finally, we validated the antibodies for immunocytochemistry experiments by co-staining LLC-PK1 cells transfected with Flag-tagged TCF4-B, with *Tcf4* and Ri-3b9 antibodies *(Fig. 29.D)*.

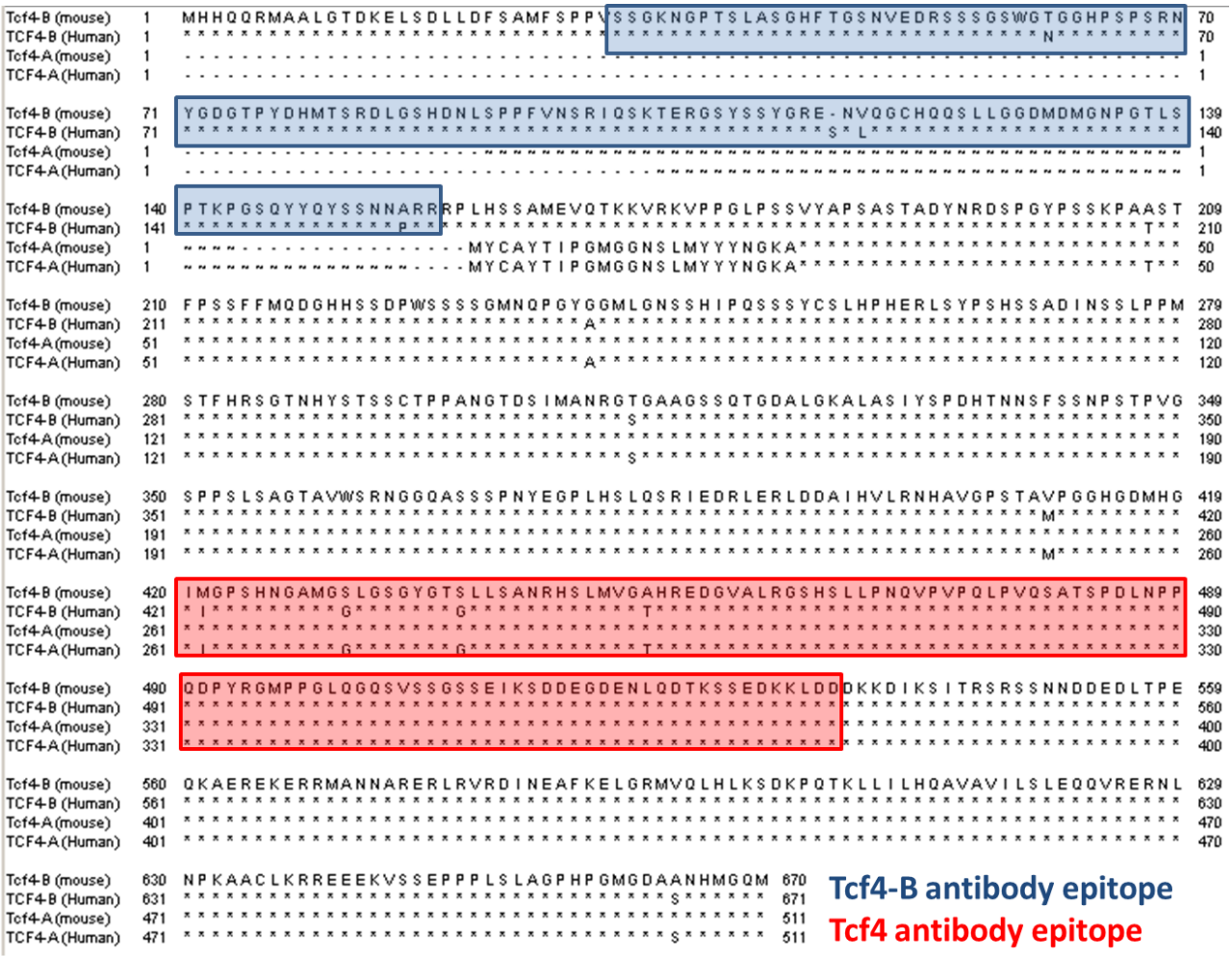


Figure 28: **Protein sequence alignment of the 2 isoforms of mouse and human Tcf4.** Epitopes of Tcf4-B and Tcf4 antibodies are framed in the blue and red box respectively. Identity and gaps were respectively symbolized with (*) and (-). Alignment showed extremely high conservation of protein sequences between the two species.

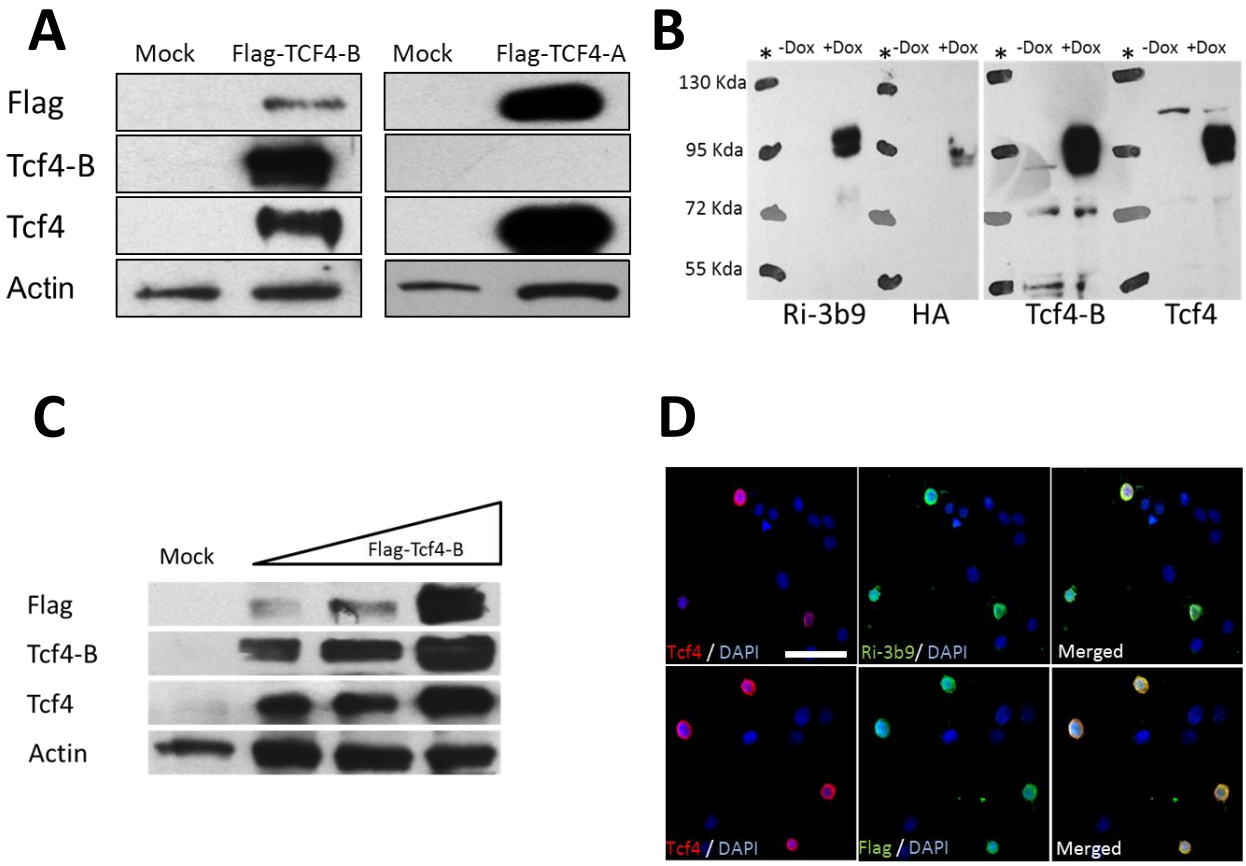


Figure 29: Validation of *Tcf4* antibodies for immunoblot and immunocytochemistry. A) Immunoblot analysis of LLCPK-1 WCE. Cells were transfected with 4 µg of PRK-Flag *TCF4*-A, PRK-Flag *TCF4*-B or 4 µg parent vector as mock. WCE were prepared 24 hrs after transfection, and blotted with mouse anti Flag (1/1000), rabbit anti Tcf4-B (1/500) and rabbit anti Tcf4 (1/1000) antibodies. B) Immunoblot analysis of DLD-1 WCE. TCF4-B expression was induced for 48 hrs by adding doxycycline (+Dox) in the growing medium. WCE of non induced cells (-Dox) and induced cells (+Dox) were blotted with mouse anti hemagglutinin HA (1/1000), mouse monoclonal anti TCF4 (Ri-3b9), rabbit anti Tcf4-B (1/500) and rabbit anti Tcf4 (1/1000) antibodies. Size marker (*) and corresponding molecular weights are shown. C) Immunoblot analysis of LLCPK-1 WCE. Cells were transfected with increasing amount of mouse Tcf4 expression vector or with PAM as mock. WCE were prepared 24 hrs after transfection, and blotted with mouse anti Flag (1/1000), rabbit anti Tcf4-B (1/500) and rabbit anti Tcf4 (1/1000) antibodies. D) Immunocytochemistry analysis of LLC-PK1 cells transfected with 1 µu PRK-Flag-TCF4-B. Cells were co-stained with a mouse anti Flag (1/1000) and with rabbit antibody anti Tcf4 (1/1000) or with the mouse monoclonal anti *TCF4* (Ri-3b9 - 1/200) and with the rabbit anti Tcf4 (1/1000). Scale bar: 100 µm.

Discussion

I. The imprinted gene *Zac1* regulates the expression of the proneural factor *Tcf4*.

The seven-zinc finger protein Zac1 is a transcriptional regulator controlling apoptosis and cell cycle arrest. It is strongly expressed in proliferative compartment of the developing brain and in the lower layers of the cortex where it might mediate control of proliferation and differentiation of neural progenitors (Alam et al., 2005; Valente et al., 2005). However, until now, no *Zac1*-regulated genes have been identified providing insight into *Zac1*-dependent pathways during brain development. Using genome-wide expression analysis, we identified the proneural gene *Tcf4* as *bona fide Zac1* target gene. *Tcf4* has been shown to mediate neuronal differentiation of specific populations in the hindbrain but also to control cellular proliferation and cell migration (Flora et al., 2007; Sobrado et al., 2009). *TCF4* plays a critical role during human brain development which was unraveled by genetic studies that identified haploinsufficiency of *TCF4* as cause of the Pitt Hopkins syndrome. We could show that *Zac1* coordinately binds to the proximal promoter and first intron of *Tcf4 in vitro* and *in vivo* and induces specifically its expression in several mouse neural cell types (embryonic stem cells, neural stem cells and specific mature neuronal populations). We could also show that *ZAC1*, the human *Zac1* orthologue could regulate *TCF4* in vitro, suggesting cross species conservation. However reporter assays and ChIP experiments in human cell line and tissue are still required to validate *TCF4* as *ZAC1* direct target gene.

Our work shows that *Tcf4* expression is regulated by *Zac1* during brain development and in specific regions of the adult brain. However its relative importance in the control of *Tcf4* gene expression remains unclear. A targeted *Zac1* knock-down approach as well as a comparison between WT and *Zac1* KO mice during development could further strengthen the hypothesis of an important contribution of *Zac1* to *Tcf4* expression.

Although *Zac1* is expressed in both glia and neurons, it seems to mediate *Tcf4* expression mainly in neuronal progenitor cells or mature neurons hinting to a cell specific control of *Tcf4* gene transcription, as suggested by chromatin immunoprecipitation and reporter assay experiments. Hence, the regulation of *Zac1* binding to *Tcf4* locus might be one of the molecular events contributing to *Tcf4* cell specific gene expression. However, the precise molecular mechanism as well as the individual roles of *Tcf4* promoter and first intron need to be addressed.

One hypothesis could be that epigenetic mechanisms regulate the *Tcf4* promoter and the first intron activity. *Tcf4* expression is regulated in human cancer by histone acetylation (Herbst et al.,

2009a) but also by CpG methylation. Indeed, methylation levels are higher in intestinal-type gastric cancer and correlate with decreased *TCF4* expression (Kim et al., 2008, Joo et al., 2010). Interestingly, the proximal promoter and the first intron of *Tcf4* harbor a CpG island, and previous studies indicated that *Zac1* cannot bind to palindromic DNA elements when their cytosines are methylated (Arima et al., 2005). It is therefore tempting to hypothesize that DNA methylation of the *Tcf4* promoter and first intron might differ between neuronal and glial cell types, and hence impair or prevent *Zac1* DNA binding.

Our computational analysis also revealed that the first intron of *Tcf4* which is highly conserved between the human and mouse genome (73% identity), contains many putative binding sites for transcription factors involved in brain development and histone modifiers (e.g Hes1, Hes5, E47, Sox2, Sox5, Stat3, Zic2, CBP, p300, Kdm5b) (Nagai et al., 2000; Fukuda and Taga, 2005; Kiefer, 2007; Xie et al., 2011). Additionally, this genomic element is a DNAseI sensitive site in mouse embryonic stem cells, in the embryonic cerebrum and cerebellum (E14.5) and adult brain (UCSC genome browser - chr18:69,503,893-69,507,335 - [http://genome.ucsc.edu/cgi-bin/hgTracks]). All these features depict enhancer elements; however the *Tcf4* first intron does not function as an enhancer *sensu stricto*. Indeed our reporter assays indicated that it does not increase promoter activity and its activation depends on its orientation relative to the naïve promoter. As its association with the naive *Tcf4* proximal promoter is required to observe *Zac1*-induced upregulation of *Tcf4* promoter activity in a cell specific manner (Fig. 16.B), we hypothesize that the intronic element functions as a regulatory element. We suggest that cell-context dependent signaling associated with specific chromatin signatures might mediate the specific activation of *Tcf4* by *Zac1*.

II. *Zac1* as regulator of cell differentiation

During brain development, the cross talk between proneural and maintenance factors governs cell fate decision and the timing of differentiation. The balance between these factors regulates the transcriptional programs that determine whether cells will pass through proliferative or neurogenic division. *Zac1* might be one of the factors influencing this equilibrium by promoting neuronal differentiation via different mechanisms.

Zac1 might counter-act the effect of the Id proteins by increasing the protein levels of the E-protein *Tcf4*. During neurogenesis, RGC proliferation is under the control of Id proteins which prevent binding of the bHLH factors to the promoter of genes involved in differentiation (Lyden et al., 1999; Jung et al., 2010). Id proteins were shown to sequester E-proteins and prevent their

association with proneural factors. Hence, *Zac1* could promote neuronal differentiation by increasing *Tcf4* levels, and thus increasing the binding of transcriptional activator complexes to neurogenic gene promoter. In this case, *Zac1* is possibly involved in the transcriptional programs controlling neuronal differentiation.

Additionally *Zac1* could counter-act the effect of Hes/Id genes by promoting cell cycle arrest of the neural progenitors. Rotschild et al (Rothschild et al., 2006), showed that Id and E-proteins compete to regulate *p57^{kip2}* expression in order to induce cell cycle arrest in developing neuroblasts. Here we suggest a novel degree of fine tuning for this mechanism by showing that *Zac1* regulates the expression of the cell cycle regulator *p57^{kip2}*. We could confirm that *Zac1* regulates *p57^{kip2}* indirectly via up-regulation of *Tcf4*. However our data also indicate that *Zac1* binds to *p57^{kip2}* promoter, suggesting a direct transactivation mechanism as indicated by previous studies (data not shown). Here, we show that both factors, Zac1 and Tcf4, might induce *p57^{kip2}* gene expression; however we could not assess the relative contribution of *Zac1* and *Tcf4*. Further experiment using *Tcf4* knock-down approaches could provide information about this regulatory mechanism. Firstly, the effects of transient silencing of *Tcf4*, by RNA interference, on p57^{Kip2} gene expression, could be determined *in vitro*. In addition, comparison of *p57^{kip2}* mRNA and protein levels in *Tcf4* WT and KO mice could indicate whether *Tcf4* is an important regulator of the cell cycle regulator *p57^{kip2}*. Finally, primary cerebellar neurons, or neural progenitors of the VZ obtained from *Tcf4* knockout mice could be transfected with Zac1 expressing plasmid. In these cells, *p57^{kip2}* expression should then be determined. Increased expression of *p57^{kip2}* upon Zac1 overexpression would indicate that *Zac1* can activate *p57^{Kip2}* via *Tcf4*-independent mechanisms.

p57^{kip2} regulates cell cycle dynamics of RGC and IPC, controls precursor pool size, neuronal differentiation, cortical size and laminar patterning (Tury et al., 2011a, 2011b; Mairet-Coello et al., 2012). *Zac1* and *p57^{kip2}* have been shown to interact in the context of an imprinting gene network that regulates early mouse development (Varrault et al., 2006) and they show a strikingly similar expression pattern during development (Alam et al., 2005; Ye et al., 2009). Interestingly, during corticogenesis, cells leaving the cell cycle early (E11- E14) generate layers 4-6 of the cortex whereas those born later (E15-E17) populate layers 2-3 (Polleux et al., 1997). As the timepoint that precursors exit the cell cycle correlates with their laminar destination, mechanisms that regulate the cell cycle machinery also impact neuronal fate. *Zac1* could thus contribute to the timing of neuronal differentiation and to their fate by activating *Tcf4* and *p57^{kip2}* expression. *p57^{kip2}* and *Tcf4* have also been shown to regulate cell migration. *Zac1* could therefore contribute

to establishing brain patterning by regulating migration of neuronal precursors. We hypothesize that *Zac1*, like proneural factors, acts via different mechanisms during neurogenesis. We suggest that it might regulate the transcriptional programs and the correct timing of differentiation, hence, controlling the fate of neural progenitors.

Functional approaches such as *in vivo Zac1* overexpression or knock down in RGC or IPC should be used to determine whether *Zac1* expression can initiate differentiation and override the effect of Id or Hes genes in the control of proliferation. Furthermore, the migration pattern of *Zac1*-overexpressing cells could be determined to confirm whether *Zac1* controls the fate of neural precursors.

III. Future directions: *Zac1*, *Tcf4* and schizophrenia

Schizophrenia is a spectrum disorder with ~1% lifetime prevalence and is a strongly inherited disease with a heritability of 80% or more. Disease onset occurs usually during adolescence, and suicide is the leading cause of premature death. Although the genetic basis for the disease has been clearly established, the underlying genetics of schizophrenia is still partially answered. It is now believed that accumulation of single gene mutations, lead to the onset of the disorder, as each single mutation identified until now, has only modest phenotypic effects. A recent meta-analysis across SNP data from several genome-wide scans revealed that the risk of schizophrenia was associated with a marker in the intron 4 of *TCF4* (Stefansson et al., 2009). Another study in a large Han Chinese cohort identified another SNP in the close neighboring of the previous study, further confirming the association between genetic variation in *TCF4* intragenic regions and schizophrenia (Li et al., 2010). Interestingly, a recent study identified the miR-137 (Ripke et al., 2011) and four of its putative targets (CSMD1, C10orf26, CACNA1C and *TCF4*) (Kwon et al., 2011) as reaching genome-wide significant association with schizophrenia. miR-137 plays a role in the control of adult neurogenesis and neuronal maturation, mechanisms through which variation at this locus contribute to brain development abnormalities in schizophrenia (Szulwach et al., 2010). These findings indicate that dysregulation of miR-137 may be a new pathway that contributes to schizophrenia, by leading to misregulation of its target genes, including *TCF4*.

The involvement of *TCF4* in schizophrenia has been recently identified and therefore, only few studies attempted to address its role in the disease progression (Brzózka et al., 2010; Lennertz et al., 2011; Quednow et al., 2011). It was shown that both transgenic mice moderately

overexpressing *TCF4* postnatally in the brain as well as individuals carrying the risk allele display reduction in sensorimotor gating (Brzózka et al., 2010), which is an endophenotype of schizophrenia. In addition, human induced pluripotent stem cells-derived neurons from schizophrenic patients express higher levels of *TCF4*, which could be reduced to control level after treatment with the antipsychotic medication, loxapine (Brennand et al., 2011). Altogether, this suggests that the fine tuning of *TCF4* gene expression is important for the development of brain circuitries involved in certain aspects of schizophrenia, which could be established during early brain development, or in the adult brain. Herein we showed that *Zac1* can regulate *Tcf4* expression pre and postnatally, and that their expression colocalize in distinct brain regions implied in the etiology of schizophrenia and involved in sensorimotor gating (hippocampus, basolateral amygdala, cortex). Hence, it would be of interest, to investigate whether modulation of Zac1 expression *in vivo* by drug treatment, could regulate the *Tcf4* gene expression and impair cognitive performance and affective behavior of animal models.

To our knowledge, there is no report of correlation between the Zac1 gene expression and antipsychotic treatments (typical and atypical). It is therefore unlikely that these drugs directly impair Zac1 expression. Interestingly, PACAP signaling has been shown to be regulated by Zac1, and impaired PACAP signaling was recently associated with the pathophysiology of schizophrenia (Hashimoto et al., 2007). PACAP Knockout mice display schizophrenia-related behavior, which could be reverted by antipsychotic treatment (Hashimoto et al., 2007). Although the direct relation between the PACAP signaling and Zac1 has been demonstrated in the cerebellum (Fila et al., 2009), this interaction has to be proven in other brain regions involved in the etiology of schizophrenia (e.g prefrontal cortex, hippocampus, amygdala and striatum). To address this, direct infusion of PACAP, or stimulation of PACAP signaling *in vivo* with lithium (Brandish et al., 2005) could be performed. Expression levels of Zac1 and its target genes could be then assessed. Ultimately the importance of Zac1 in the positive effects of PACAP signaling could be determined by increasing Zac1 expression in PACAP null mice. Further investigation on the interaction between the PACAPergic system, *Zac1*,*Tcf4* and *p57kip2* during neural development are required to determine whether *Zac1* plays a role in cognitive functions.

Besides a strong genetic origin of the disease, schizophrenia onset has been also linked to environmental factors (Sullivan et al., 2003). Indeed, the association between influenza exposure during pregnancy and schizophrenia symptoms has been confirmed by several studies and

extended to many other pathogens including other viruses (HSV-2, rubella), bacteria, and protozoa (Brown and Derkits, 2010). In animal models, prenatal immune challenge was found to disrupt brain maturation and to induce behavioral alterations related to schizophrenia (deficits in pre-pulse inhibition, latent inhibition, and amphetamine hypersensitivity (Boksa, 2010)) and display developmental and pharmacological features concordant with the disease (Shi et al., 2003; Zuckerman et al., 2003). Interestingly, *Zac1* expression is regulated through TLR3, IRF3 dependent pathways and is induced by viral infection in murine embryonic fibroblasts (MEFs), however the physiological function of this induction remains unknown (Warzée et al.,2010; Andersen et al., 2008). We could reproduce these findings in the neural stem cell line C17.2 and in hippocampal neurons, where treatment with the viral mimic, polyinosinic:polycytidylic acid (poly I:C) induced both *Zac1* and *Tcf4* expression, associated with an increased binding of *Zac1* to *TCF4* locus (data not shown). Hence, upon infection during pregnancy, *Zac1* expression might be induced in neural progenitors and neurons, and in turn up-regulate *Tcf4* gene expression, which could contribute later to the development of the behavioral alterations related to schizophrenia. To address this, *Zac1* and *Tcf4* expression should be determined throughout early embryonic development and postnatally in a mouse model of prenatal infection with the viral mimic, Polyinosinic:polycytidylic acid (poly I:C) (Meyer et al., 2010).

Conclusion

The imprinted gene *Zac1* is a regulator of progenitor cell fate during development. Zac1 is a transcription factor that is temporarily expressed in the neuroepithelia and in differentiating neuronal precursors during neurogenesis. We have shown here, that *Zac1* can regulate the expression of the E-protein *Tcf4* and represents a factor promoting neuronal differentiation. As *Zac1* activates the expression of the obligatory partner of proneural genes, it might compete with the *Id* and/or *Hes* genes to initiate transcriptional cascades required for neuronal differentiation. Besides, by controlling *Tcf4* and its target *p57^{Kip2}* gene expression, *Zac1* could also regulate the cell cycle and the fate of the neural precursors. Finally in the adult brain, *Zac1* contributes to the fine tuning of *Tcf4* expression which is important for cognitive performance and affective behavior (Fig. 30). Altogether we could show that Zac1 mediates neural progenitor cell differentiation, and could play a role in the establishment of the neuronal networks that control particular cognitive functions later in life.

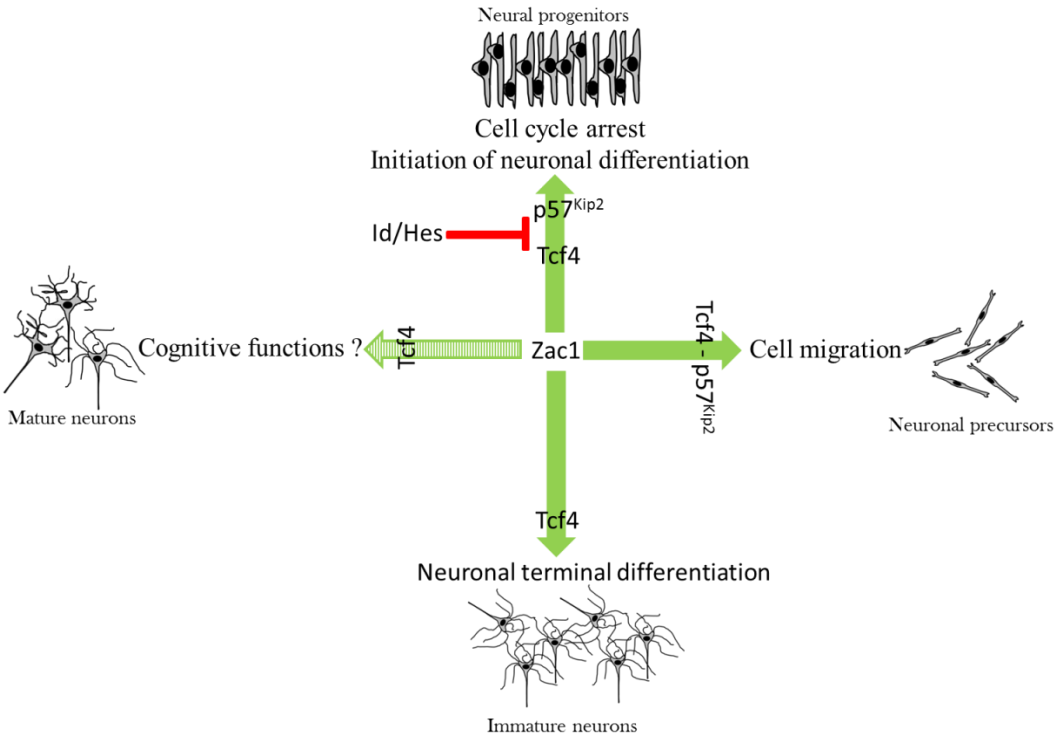


Figure 30: **Model: Zac1 regulates the Tcf4 gene and its target p57^{kip2} gene in the embryonic and adult brain.** Zac1, Tcf4 and p57^{Kip2} might compete with the *Id* / *Hes* genes to regulate neuronal precursor cell differentiation and migration. The interaction between Zac1 and Tcf4 in adult might mediate cognitive functions, like sensorimotor gating, however this question still needs to be addressed.

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Education

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Sep 2004-Jun 2007	Ecole superieure d´Ingenieurs de Luminy (ESIL), Marseilles, France. Master in biotechnology engineering, specialization in biotechnologies for Health industry, with honours (Mention Bien). Diploma student at the Psychiatry Discovery Technology Group, GlaxoSmithKline, Harlow, UK. Thesis: development of cell-based assays able to detect modulators of neurogenesis.
Sep 2002 - Jun 2004	2-year university degree (DEUG) in Biology (science de la vie et de la Terre) with Honours (mention Tres bien) at the University of Avignon, France.
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Erklärung

Hiermit erkläre ich, dass die vorliegende Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist.
Ich erkläre weiterhin, dass ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt worden ist.

Guillaume Daniel, April 2012