

Role of telomere binding protein TRF2 in neural differentiation of human embryonic stem cells

by

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Abstract

Telomere repeat binding factor 2 (TRF2) is reported to be a key component of shelterin, a multi-protein complex that binds telomeric deoxyribonucleic acid (DNA) to protect chromosome ends and maintain genome stability. However, in recent years, TRF2 has been found to also bind non-telomeric regions and to act as a protein hub, interacting with a wide range of non-telomeric proteins and thus raising the possibility that it may serve functions independent of telomere maintenance. Despite the importance of TRF2, there is little information about how TRF2 is expressed during development and whether it could have an extratelomeric role in this process. Human embryonic stem cells (hESCs) derived from pre-implantation embryos are able to differentiate into most, if not all, tissues of the adult body, thereby provide a good cell model to tackle the problem. Given the abundance of TRF2 in the human brain and its potential for extratelomeric roles, this study focused on neural differentiation. TRF2 protein levels were found dramatically increased upon differentiation of hESCs to neural progenitor cells (NPCs) and these high levels, similarly to what is observed *in vivo*, were specific to the neural lineage. Gain and loss of function approaches revealed that exogenous expression of TRF2 in hESCs induced neural differentiation while, in contrast, TRF2 knockdown in NPCs drastically hindered their ability to terminally differentiate into neurons and glia. This enhancing neural function of TRF2 is achieved through the ability of TRF2 to inhibit the proteasomal degradation of REST4, an alternative splice variant of RE1-Silencing Transcription factor (REST), which alleviates REST repression over neural genes, hence consolidating neural progenitor identity and potency. This study identifies TRF2 as a novel component of neural differentiation, suggesting its

importance in central nervous system development as well as in neurological disorders.

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Dedicated to

my mother

For her continuous love, support and assurance

Statement of originality

All experiments in this thesis were performed by me Patrick Ovando Roche unless otherwise stated in the text.

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Abbreviations

APS	ammonium persulphate
ATM	ataxia telangiectasia mutated
ATR	ataxia-telangiectasia-mutated and Rad 3-related
BCA	bicinchoninic acid
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CHX	cycloheximide
CM	conditioned medium
CNS	central nervous system
Co-IP	Co-immunoprecipitation
C-strand	cytosine rich strand
DAPI	4',6-diamidino-2-phenylindole
DDR	dna damage response
DIG	digoxigenin
D-loop	displacement loop
DMEM	dulbeccos modified eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DSB	double strand break
EB	embryoid body
EDTA	ethylene-dinitrilo tetraacetic acid
EF1 α	elongation factor-1 alpha
EGF	epidermal growth factor
ESCs	embryonic stem cells
FACS	fluorescence-activated cell sorter
FBS	foetal bovine serum
FGF	fibroblast growth factor

FGFR	fibroblast growth factor receptor
FISH	fluorescent <i>in situ</i> hybridization
FITC	fluorescein isothiocyanate
g	g force
GFP	green fluorescent protein
G-strand	guanidine rich strand
Gy	gray
HEF	human embryonic fibroblast
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hESCs	human embryonic stem cells
HR	homologous recombination
hTERC	human telomerase RNA component
hTERT	human telomerase reverse transcriptase
ICM	inner cell mass
IF	immunofluorescence
iPSC	induced pluripotent stem cell
ITS	interstitial telomeric sequences
kb	kilo base
kDa	kiloDalton
KO-DMEM	knockout Dulbecco's modified Eagle's medium
KSR	knockout serum replacement
LIF	leukaemia inhibitory factor
m	milli
M	molar
mESCs	mouse embryonic stem cells
MEFs	mouse embryonic fibroblasts
mRNA	messenger RNA
NEAA	non-essential amino acids
NHEJ	non-homologous end joining
NPCs	neural progenitor cells
NRSF	neuron restrictive silencer factor
NSCs	neural stem cells

Oligo(dT)	oligonucleotide (deoxy-thymine)
PAGE	polyacrylamide gel electrophoresis
Pax6	paired box gene 6
PBS	phosphate buffered saline
PD	population doubling
PFA	paraformaldehyde
PNA	peptide nucleic acid
Polybrene	hexadimethrine bromide
POT1	protection of telomeres 1
PVDF	polyvinylidene fluoride
P/S	penicillin/streptomycin
PLL	poly-L-lysine
PMSF	phenylmethanesulphonylfluoride
qRT-PCR	quantitative real time-polymerase chain reaction
Rap1	repressor/activator protein 1
RE-1	repressor element 1
RIPA	radio immuno precipitation assay buffer
RNA	ribonucleic acid
RPE	retinal pigment epithelium
SDS	sodium dodecyl sulphate
SGZ	subgranular zone
shRNA	short hairpin ribonucleic acid
Sox	SRY-box containing gene
SSC	saline sodium citrate
SVZ	subventricular zone
TEMED	tetramethylethylenediamine
TERC	telomerase RNA component
TERT	telomerase reverse transcriptase
TGF	transforming growth factor
TIN2	TRF1-interacting nuclear protein 2
TRF	telomere restriction fragment
TRFH	TRF homology
TRF1	telomeric repeat binding factor 1

TRF2	telomeric repeat binding factor 2
UV	ultraviolet

Chapter 1 General introduction

1.1 Telomeres and telomerase

1.1.1 Telomere structure and function

Eukaryotic telomeres are regions located at the ends of chromosomes composed of non-coding repetitive DNA and its associated protein complex, shelterin (Blackburn, 1991). In mammals, telomeric DNA is composed of conserved double stranded TTAGGG repeats which end as a single-strand 3'-overhang, which can vary greatly in length between species (Figure 1.1 A). For instance, telomeres in humans may only be a few kilobases (kb) long whereas in mice and rats may be as long as 100 kb (Meyne *et al.*, 1989; de Lange *et al.*, 1990; Kipling and Cooke, 1990). These DNA repeats are packaged with histones to form constitutive heterochromatin and have been shown to have transcriptional activity (Azzalin *et al.*, 2007; Blasco, 2007; Schoeftner and Blasco, 2008).

In 1999 Griffith *et al* used electron microscopy to show that human and mouse telomeres end in a large organised duplex loop, known as the t-loop (Figure 1.1 B), rather than in a double stranded linear form as previously thought (Figure 1.1 A). The mechanism by which these t-loops are formed is still not fully understood, but the most accepted hypothesis is that these are formed through invasion of the single-strand guanine-rich (G-strand) 3'-overhang into the double stranded DNA. The guanine-rich overhang then complementary base-pairs with the 5'-end of cytosine-rich strand (C-strand), displacing the G-strand into a displacement loop (d-loop) (Figure 1.1 B). The formation of this t-loop structure is regarded as a capping mechanism that prevents telomeric DNA ends from being detected as double strand breaks (DSB) by the DNA damage response (DDR) mechanism, protecting chromosomes against end-to-end fusions and ultimately cell senescence and genomic instability (Smogorzewska and de Lange, 2002; Takai *et al.*, 2003; Karlseder *et al.*, 2004; Bae and Baumann, 2007; Denchi and de Lange, 2007).

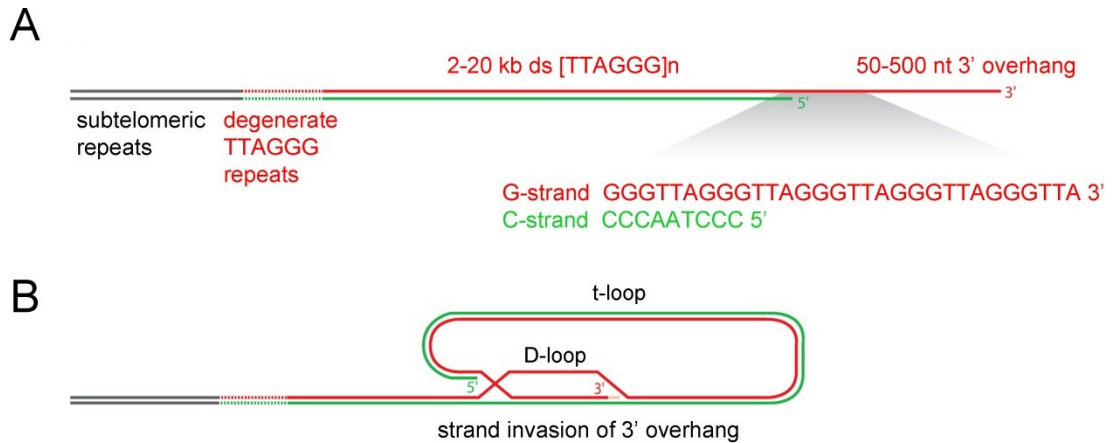


Figure 1.1 Schematic representation of telomeres and t-loops structure.

(A) Human telomeres are composed of double stranded TTAGGG DNA repeats of up to 15 Kb in length with a 3'-end G-rich overhang of approximately 50-500 nucleotides. The 5' C-rich strand features the sequence ATC. (B) Schematic of the t-loop formation. 3'-end overhang invades the double stranded DNA, forming the d-loop. Image adapted from Palm and de Lange, 2008.

Telomere length also plays an important role in genome maintenance. Given that the DNA replication mechanism is unable to replicate DNA fully to the end of the chromosomes (Olovnikov, 1971; Ohki *et al.*, 2001), each cell division results in a progressive shortening of the genomic DNA, a phenomenon known as the end replication problem. Thus to prevent the erosion of our coding DNA, telomeres act as a buffer for the gradual loss of chromosome ends. Shortening of one or more telomeres to a critical limit activates p53 and p16 DNA-damage signalling pathways resulting in induction of senescence (Kipling *et al.*, 1999; Espejel and Blasco, 2002), a permanent state of cell cycle arrest but continued viability. This process is referred to as replicative senescence and explains why primary cells can only divide in culture a certain number of times, referred to as the Hayflick limit (Hayflick and Moorhead, 1961). Therefore telomeres are thought to act as a biological clock, where replicative senescence is a mechanism of ageing and tumour-suppression (Hiyama *et al.*, 1995). This view is partly supported by some studies that have reported a direct correlation between an organism age and telomere length (Hastie *et al.*, 1990; Beneto *et al.*, 2001) and by recent suggestions that an active lifestyle may actually increase telomere length by increasing telomerase activity (Ornish *et al.*, 2013) and therefore may result in an increased lifespan. However, other studies have found no evidence of a relationship between telomere length and normal ageing (Harris *et al.*, 2012).

The precise mechanism by which replicative senescence is triggered is still unclear. Critically short telomeres, known as dysfunctional telomeres, accumulate DDR factors commonly found in DSB such as phosphorylated histone 2A.X (γ H2AX), p53-binding protein (53BP1) and ataxia telangiectasia mutated (ATM). Short telomeres that have accumulated DDR factors are termed telomere-induced foci. These are thought to occur because telomeres are too short to form the t-loop structure (Griffith *et al.*, 1999), resulting in exposure of the telomere ends and activation of ATM, which leads to activation of p53 via Chk2 (Gire *et al.*, 2004; Guo *et al.*, 2007). Activation of p53 leads to transcription activation of Cip/WAF CDK inhibitor p21 which prevents entry into S-phase and induces senescence. Interestingly it has been shown that these events can be recapitulated by inducing overexpression of dominant negative mutant TRF2, which strips endogenous TRF2 from telomeres (Smogorzewska and de Lange, 2002).

While most cell types are subjected to telomere shortening and replicative senescence, cells in the early embryo, germ cells, certain stem cell populations and cancer cells are not (Hiyama *et al.*, 1995; Wright *et al.*, 1996; Soder *et al.*, 1997; Betts and Kind, 1999). This is because telomeric shortening in these cells is counteracted by the action of telomerase, an enzyme that replenishes the TTAGGG DNA repeats at the end of the telomere, allowing self-renewal (Greider and Blackburn, 1985; Lingner *et al.*, 1997).

1.1.2 Telomerase components and function

Telomerase was first discovered and characterised in the ciliate *Tetrahymena* by Nobel Prize winners Elizabeth Blackburn, Carol Greider and Jack Szostak in 1985 (Greider and Blackburn, 1985). Human telomerase is a ribonucleoprotein complex consisting of three main components: a reverse transcriptase catalytic subunit (hTERT) (Lingner *et al.*, 1997; Nakamura *et al.*, 1997), a telomerase RNA component (hTERC also known as hTR) (Feng *et al.*, 1995) and accessory factors Dyskerin and Est1A/B. Dyskerin, a small nucleolar ribonucleoprotein, stabilises the telomerase complex (Mitchell *et al.*, 1999; Cohen *et al.*, 2007) while Est1A/B are known to bind hTERT but their function remains unknown (Reichenbach *et al.*,

2003; Snow *et al.*, 2003; Sealey *et al.*, 2011). hTERC contains the sequence CUAACCCUAAC, which is complementary to the telomere repeat unit TTAGGG (Figure 1.2) and acts as a template for the addition of TTAGGG repeats to the 3'-overhang by hTERT. The extended 3'-overhang allows DNA polymerase to function in conventional DNA replication, preventing telomere shortening and replicative senescence. Both hTERT and hTERC elements are required and sufficient for maintenance and elongation of telomeres (Linger *et al.*, 1997).

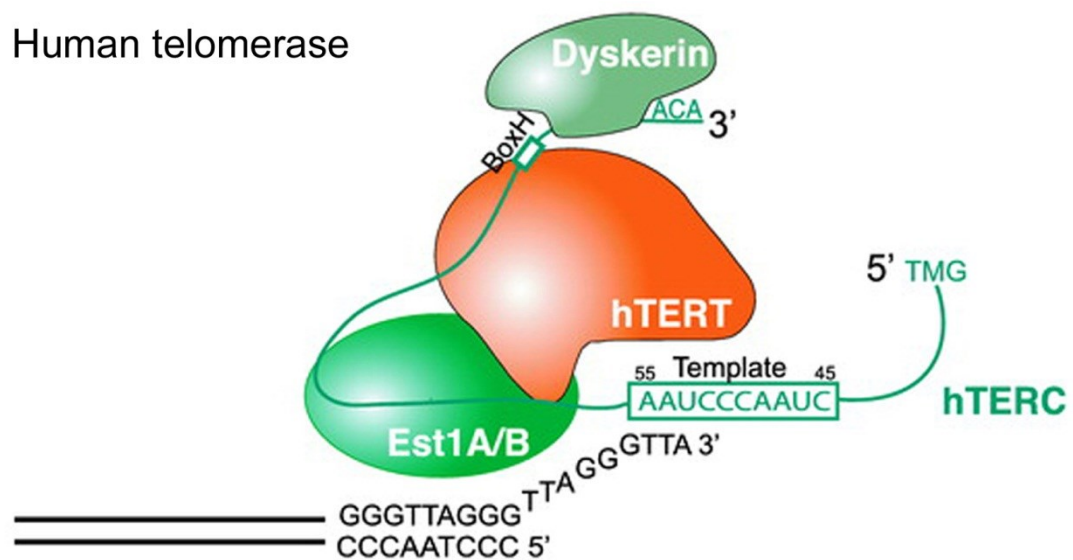


Figure 1.2 Telomerase binding to telomere 3' end overhang

Schematic representation of human telomerase, composed of hTERT, hTERC, Dyskerin and EST1A/B, binding to the 3' end of a telomere. Image adapted from Smogorzewska and de Lange, 2004.

While telomerase activity and hTERT expression in the adult organism is restricted to germ cells, T-lymphocytes, and stem cell population, almost 90% of cancers express hTERT and have telomerase activity (Blasco and Hahn, 2003). hTERC is found to be ubiquitously expressed in all cells but is also upregulated in cancer (Soder *et al.*, 1997). Mouse TERT or TERC knockout models are viable but begin to display defects such as infertility, reduced lymphocyte proliferation, reduced wound healing and premature ageing after 3-7 generations (Blasco, 2005; Sahin and

DePinho, 2010). Some of these phenotypes are associated with the loss of stem cell function and mimic human premature ageing conditions; supporting the notion that telomere function is related to ageing. Furthermore, several of human premature ageing diseases are also found to be caused by mutations in telomerase components. For example in Dyskeratosis congenita, a disease that causes bone marrow failure and skin abnormalities, involves a mutation in dyskerin or hTERC (Vulliamy *et al.*, 2001).

Overexpression of hTERT in somatic cells counteracts telomere shortening and prevents replicative senescence without necessarily inducing cell transformation (Bodnar *et al.*, 1998). Thus, hTERT overexpression has become a useful tool to “immortalise” primary cell lines so they can be studied over long periods of time. The immortalisation of primary cell lines by hTERT automatically hinted to whether this could be used to artificially increase the lifespan of whole organisms. This was attempted in different labs with very interesting results. Initially, overexpression of TERT in mice resulted in increased incidences of cancer (Gonzalez-Suarez *et al.*, 2001; Blasco, 2003). However, when these mice were crossed with mice overexpressing tumour suppressor genes, the resulting progeny revealed an increased lifespan, providing evidence that expression of TERT is important in mammalian ageing (Tomas-Loba *et al.*, 2008). Furthermore, reactivation of TERT expression in telomerase knockout mice has been shown to result in increased telomere length, attenuation of DDR activation and restoration of stem cell functionality, ultimately reversing the ageing phenotype and increasing lifespan (Jaskelioff *et al.*, 2011).

1.2 The shelterin complex

1.2.1 Components and functions of the shelterin complex

Telomere TTAGGG repeats provide a platform for specific binding of a complex of proteins called shelterin. Shelterin is a 6-protein complex, consisting of TRF1 (telomere repeat binding factor 1) (Chang *et al.*, 1995), TRF2 (Broccoli *et al.*, 1997), TIN2 (TRF1-interacting nuclear protein 2) (Kim *et al.*, 1999), Rap1 (repressor/activator protein 1) (Li *et al.*, 2000), POT1 (protection of telomere 1) (Baumann and Cech, 2001) and TPP1 (formerly known as TINT1, PTOP or PIP1) (Houghtaling *et al.*, 2004; Liu *et al.*, 2004; Ye *et al.*, 2004c).

Each of the 6 proteins interacts with telomere directly or indirectly. Telomere binding proteins TRF1 and TRF2 bind to double stranded TTAGGG repeats while POT1 only binds to single stranded 3'-overhang (Figure 1.3). TRF1, TRF2 and POT1 do not interact with each other directly, rather they are held in place by TIN2, which occupies a central position in the shelterin complex, binding to TRF1 and TRF2 directly, and to POT1 via TPP1, thus providing a link between double-stranded DNA and single-stranded DNA binding proteins (Figure 1.3) (Kim *et al.*, 1999; Kim *et al.*, 2004; Ye *et al.*, 2004a; Ye *et al.*, 2004b). Rap1 binds to TRF2 and is dependent on TRF2 for its telomeric localisation and stability while TPP1 binds POT1 and is dependent on its interaction with TIN2 and POT1 for its localisation at the 3'-overhang (Figure 1.3) (Li *et al.*, 2000; Celli and de Lange, 2005; Denchi and de Lange, 2007; Hockemeyer *et al.*, 2007). The shelterin complex has been shown to play an important role in maintaining and protecting telomere structure and functionality (de Lange, 2005). It controls the accessibility of telomerase to telomere ends and regulates telomere elongation. It is also heavily involved in inhibiting DDR activation at telomeres (Denchi and de Lange, 2007).

Diagram illustrating the structure of the telomeric DNA sequence and the associated proteins. The DNA sequence is shown as a double-stranded structure with the 3' strand (top) and the 5' strand (bottom). The 3' strand sequence is GATTGGGATTGGGATTGGGATTGGGATTGGGATTGGGATTGGG, and the 5' strand sequence is CTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCT. The proteins shown are TIN2 (orange oval), TPP1 (red oval), POT1 (red oval), TRF1 (green Y-shaped protein), TRF2 (green Y-shaped protein), and Rap1 (small green oval). The diagram shows the interaction between these proteins and the DNA sequence, with TIN2 binding to the 3' strand and TRF1/2 binding to the 5' strand. TPP1 and POT1 are also shown interacting with the 3' strand.

A detailed diagram of a cell membrane cross-section. The membrane is represented by a phospholipid bilayer with red heads and green tails. Various proteins are embedded in the membrane, including transmembrane proteins with multiple orange and red domains, and peripheral proteins. A blue and white striped structure is visible on the left side of the membrane.

(A) Telomere binding proteins TRF1 and TRF2 bind to double stranded TTAGGG repeats while POT1 binds single stranded only. Rap1 is loaded onto the double stranded DNA by TRF2 and TPP1 is loaded onto the single stranded DNA by POT1. TIN2 occupies a central position and binds to TRF1, TRF2 and TPP1, stabilising the complex. **(B)** Representation of what the t-loop structure might look like with the shelterin complex bound to it. Images adapted from Palm *et al.*, 2008.

1.2.2 Regulation of telomeres by TRF1 and TRF2

TRF1 and TRF2, the first two telomere binding proteins identified, have similar protein structures and both bind to double stranded telomeric DNA. They share a TRF homology (TRFH) domain and a C-terminus SANT/Myb DNA-binding domain connected through a hinge domain (Figure 1.4) (Hanoaka *et al.*, 2005). However, they differ in their N-terminus regions, where TRF1 has an acidic N-terminus while TRF2 bears a basic Gly/Arg rich domain (Figure 1.4). Both proteins bind to the DNA as homodimers, formed through interactions between their TRFH domain (Broccoli *et al.*, 1997).

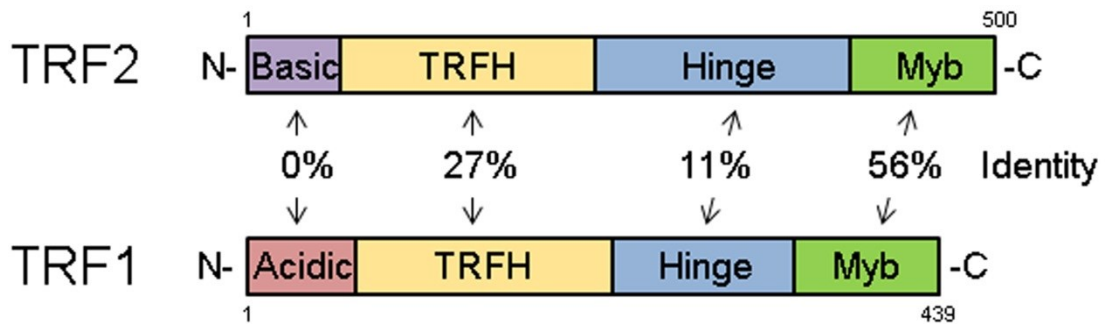


Figure 1.4 Schematic representations of the domains of TRF1 and TRF2.

TRF1 and TRF2 share a TRFH, Hinge and Myb DNA binding domain but differ in their N-terminus regions, where TRF1 has an acidic domain (red) and TRF2 bears a basic domain (purple). The sequence identity between the TRFH (yellow), Hinge (blue) and Myb domains (green) of TRF1 and TRF2 appears indicated as percentages.

TRF1 has been shown to negatively regulate telomerase-dependent telomere elongation (van Steensel and de Lange, 1997; Smogorzewska *et al.*, 2000). Overexpression of TRF1 in telomerase-positive tumour cell lines results in telomere shortening, whereas ectopic expression of a dominant negative TRF1 in these cells leads to telomere elongation. In addition, TRF1 also regulates the binding stability of TIN2 and TRF2 to telomeres, which subsequently affects functional telomere structure and chromosomal stability (Iwano *et al.*, 2004). Depletion of TRF1 has also been shown to lead to destabilisation of POT1, leading to activation of ATM and Rad3 related (ATR) DDR (Okamoto *et al.*, 2008).

Similarly to TRF1, TRF2 was also found to be a negative regulator of telomerase-dependent telomere length (Smogorzewska *et al.*, 2000). However, when TRF2 was overexpressed in TRF1-null mouse ES cells, instead of shortening, as previously reported in TRF1-wild type cells (van Steensel *et al.*, 1997; Smogorzewska *et al.*, 2000), the telomeres elongated (Okamoto *et al.*, 2008). Thus, suggesting that TRF2 telomere length regulation may be also dependent on TRF1 expression. In addition to telomere length regulation, TRF2 has been shown to have the property to aid telomere repeats to form and maintain t-loop structures *in vitro* (Griffith *et al.*, 1999; Stansel *et al.*, 2001). These structures have been proposed to be the means by which telomere ends inhibit DDR activation and non-homologous end joining (NHEJ) of chromosome ends (Smogorzewska and de Lange, 2002; Takai *et al.*, 2003; Karlseder *et al.*, 2004; Bae and Baumann, 2007; Denchi and de Lange, 2007). However, little is known about the molecular mechanisms behind these properties of TRF2. It has been proposed that TRF2 may carry these functions through its ability to inhibit Holliday junction resolution (Poulet *et al.*, 2009), which are thought to occur during d-loop formation (Stansel *et al.*, 2001), and stimulating d-loop formation (Amiard *et al.*, 2007). These functions have been proposed to be a consequence of change in topology generated by the N-terminal domain of TRF2 (Poulet *et al.*, 2012).

1.2.3 TRF1 and TRF2 protect telomeres from DNA damage response

TRF1 and TRF2 protect telomeres from DNA damage response not only through binding to telomeres themselves but also via recruitment of other proteins. Recruitment of these non-telomeric proteins is carried out predominantly by the TRFH domains of TRF1 and TRF2 (Chen *et al.*, 2008) and the hinge region of TRF2 (Okamoto *et al.*, 2013) (Figure 1.4). Crystal structure studies of TRF1 and TRF2 identified the conserved peptide docking sites F141 and F120 located in the TRFH domains of TRF1 and TRF2, respectively, which recognise the binding motifs FxLxP and YxLxP. Although both proteins have similar docking sites, their significant sequence differences (Hanoaka *et al.*, 2005) result in different protein structures surrounding the docking sites, making TRF1 bind more efficiently to FxLxP and TRF2 bind more efficiently to YxLxP (Chen *et al.*, 2008). Proteomic studies further confirmed that the core motif to achieve TRF2 binding is [Y/F]xL (Kim *et al.*, 2009).

Most of the proteins that bind to TRF1 and TRF2, particularly TRF2, are involved in DNA damage signalling and repair, especially DSB signalling and repair, such as ATM, Ku70/80, XPF/ERCC1, Apollo, MRN complex, RAD51D, PARP1, PARP2, RecQ helicases (Hsu *et al.*, 2000; Zhu *et al.*, 2000; Tarsounas *et al.*, 2004; Francia *et al.*, 2006; Hsiao *et al.*, 2006; Lenain *et al.*, 2006; Deng *et al.*, 2007). The primary mechanisms for DNA DSB repair in human cells are homologous recombination (HR) and NHEJ. Detection of DSB leads to activation of ATM (Bakkenist and Kastan, 2003). ATM activation results in phosphorylation of downstream effectors of the DDR, such as γ H2AX, MDC1, 53BP1 and p53 (Dimitrova and de Lange, 2006), consequently leading to HR or NHEJ DNA DSB repair. NHEJ is an error-prone DNA damage repair mechanism and can often lead to the introduction of mutations (van Gent and van der Burg, 2007), which in the case of the telomere ends can lead to incorrect telomere structure, telomere bridging, cell senescence and apoptosis (Karlseder *et al.*, 1999; Denchi and de Lange, 2007).

TRF2 interaction with ATM on telomeres can prevent ATM-dependent DDR activation and inhibit NHEJ of chromosome ends; however the precise molecular mechanism has yet to be elucidated. TRF2 binds to a region of ATM containing residue serine 1981 (S1981), which is activated by autophosphorylation in response to DNA damage. Therefore, TRF2 could act as a specific inhibitor of ATM at the telomeres (Karlseder *et al.*, 2004; Bakkenist and Kastan, 2003). It has been shown that TRF2 overexpression in cells with very short telomeres inhibited S1981 phosphorylation which otherwise would have been activated (Karlseder *et al.*, 2004). However, it is still unknown if this effect is due to the formation of t-loops upon overexpression of TRF2 or as a direct repression of ATM autophosphorylation. Deletion of TRF2 usually results in loss of the t-loop structure, resulting in the recognition of the telomere ends as DSB, therefore leading to ATM activation and resulting in NHEJ initiation. Recent reports have revealed that TRF2 inhibits DDR in a two step-mechanism where TRF2 blocks DDR by, first, dimerization of its TRFH domains which inhibits ATM activation, and secondly, by inhibiting 53BP1 localization to the telomeres through suppression of chromatin ubiquitination by RNF168, an E3 ubiquitin ligase (Okamoto *et al.*, 2013). Furthermore, it has been suggested that very small amounts of TRF2 at the telomeres are sufficient to inhibit

end joining of chromosome ends (Cesare *et al.*, 2013). In addition, in 2005 Tanaka *et al* showed that TRF2 was rapidly phosphorylated upon X-ray irradiation, most likely through binding partner ATM, and that this phosphorylated form did not bind to the telomeres but to the sites of DNA damage. This indicates a possible extra-telomeric role of TRF2 in DNA damage response. It is possible that TRF2 is not only required for telomere capping, as suggested by deletion and overexpression experiments, but also for non-telomeric functions related to DNA damage response which may be masked upon de-capping of the telomere by TRF2 deletion.

1.2.4 TRF2 potential for extra-telomeric roles

Despite the importance of TRF2 in genomic stability, little is known about the expression and involvement during embryonic development and adult tissue homeostasis. This is because the majority of studies were performed in either tumour cell lines or primary/immortalized fibroblasts which may not be adequate representatives of normal tissue development. Furthermore TRF2 knockout mice exhibited embryonic lethality, restricting further interrogation of TRF2 functions during development (Celli and de Lange, 2005). However, TRF2 has recently been found to bind to other non-telomeric regions in chromosomes (Bradshaw *et al.*, 2005; Simonet *et al.*, 2011; Yang *et al.*, 2011) and to interact with many non-telomeric proteins (Chen *et al.*, 2008; Zhang *et al.*, 2008; Kim *et al.*, 2009; Giannone *et al.*, 2010; Okamoto *et al.*, 2013), raising the possibility of TRF2 having alternative functions independent of telomere maintenance.

1.2.5 TRF2 role in neurogenesis

Two research groups have proposed that TRF2 has a role in neurogenesis. Zhang and colleagues in their first study showed that in rat non-dividing cells TRF2 inhibition enhanced the morphological, molecular and biophysical differentiation of already differentiated neurons while in dividing human and rat cells, SH-SY5Y and astrocytes respectively, it induced cell senescence (Zhang *et al.*, 2006). According to their study this indicated that TRF2 in addition to protecting cells from DNA damage responses it also had a role in neural differentiation. In their second study they proposed a mechanism whereby TRF2 regulates neurogenesis. They showed that TRF2 inhibition in SH-SY5Y and Ntera2 dividing cells induced neural differentiation and proposed that this was due TRF2 stabilising the neural master repressor REST. Inhibition of TRF2 resulted in degradation of REST protein and derepression of its neural lineage target genes, ultimately leading to acquisition of a neuronal phenotype (Zhang *et al.*, 2008). In their latest study, Zhang *et al* propose that in nondividing neural cells TRF2 switches its full length expression to a unique short nontelomeric isoform (TRF2-S) that sequesters REST from the nuclei to the cytoplasm so neural gene expression can be maintained in mature neurons (Zhang *et al.*, 2011). Their studies propose that TRF2 and TRF2-S have opposing effects on REST. Their model proposes that in neural progenitors expression of full length TRF2 maintains REST protein levels and therefore limits neural differentiation while upon differentiation into mature neurons TRF2-S expression occurs and results in REST sequestration to the cytoplasm allowing neural gene expression in the mature neurons to continue (Figure 1.5).

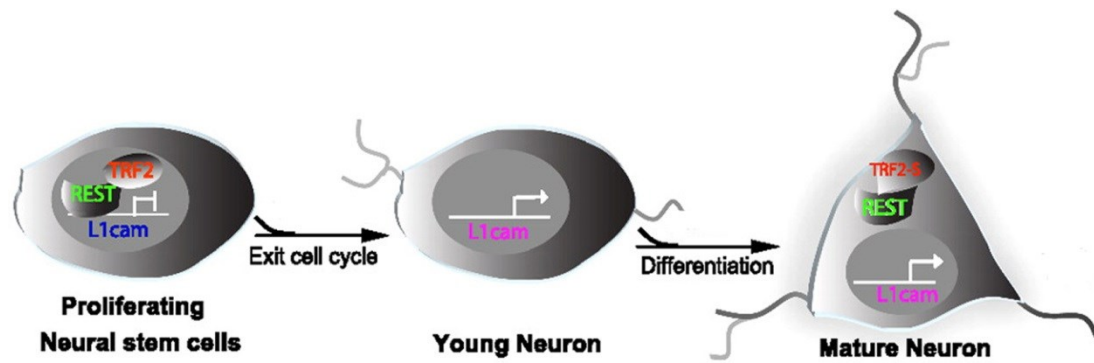


Figure 1.5 Zhang and colleagues TRF2 model of neural differentiation regulation.

Full length TRF2 and TRF2-s can both bind to REST but have opposing effects. TRF2 stabilizes REST in proliferating neural stem cells to inhibit neural gene expression (e.g. L1cam) and maintain the pluripotent state, while TRF2-s sequesters REST from the nuclei of mature neurons to allow neural gene expression in mature neurons.

While Zhang and colleagues suggested that TRF2 regulates neurogenesis through protein-protein interactions, a second research group suggested that TRF2 could regulate neurogenesis through protein-DNA interactions. Through ChIP-Seq experiments Simonet and colleagues found that in the tumor cell line BJ-HELTRa, TRF2 binds near the genic regions of neural lineage genes that are regulated by REST. In fact TRF2 was found bound near the REST binding sites of these genes. Therefore this second group proposed that perhaps TRF2 could be regulating neural gene expression through a TRF2-mediated synergistic interaction between the TRF2 and the REST binding sites (Simonet *et al.*, 2011).

While these studies were carried out in cancer cell lines which may not reflect the normal situation *in vivo*, they revealed a potential extra-telomeric role of TRF2 in neurogenesis. Since TRF2 expression has been found to be very abundant in human brain tissue (Jung *et al.*, 2004), it is plausible to think that TRF2 may have a role in human neurogenesis.

1.3 Neural induction and adult neurogenesis of the central nervous system

1.3.1 Neural induction

Development of the vertebrate central nervous system (CNS) is one of the earliest events in embryonic germ layer induction and a multi-step process that has long been thought to follow formation of the embryonic ectoderm, one of the three primary germ cell layers (i.e. endoderm, mesoderm and ectoderm) (Hamburger, 1988). The CNS is a complex tissue both in terms of cell type and number of cells. Furthermore, billions of neurons have to interact in a very precise manner in order to form functional neuronal networks. Development and maintenance of such a complex dynamic structure involves highly controlled gene expression and signalling pathways. CNS development in vertebrate embryos starts with the formation of the neuroepithelium from the neuronal ectoderm and finishes with the formation of different brain regions responsible for different cognitive functions (Waddington, 1939; Morange, 2001; Stiles, 2008).

The first insights into the mechanism of neural induction were obtained from studies carried out in amphibian embryos. Two major findings were obtained: the discovery of the organizer and its role in neural induction (Spemann and Mangold, 1924), and the discovery of the mechanisms that lead to neural induction (Sasai and De Robertis, 1997). Spemann and Mangold, discovered that when they transplanted a dorsal blastopore lip from one donor embryo to the ventral side of a host embryo this caused the initiation of a second neural induction site. Therefore, the dorsal blastopore lip was proposed as the region that induces and organises the CNS, and was named “Spemann’s organizer” (Spemann and Mangold, 1924). They proposed that the organization of the vertebrate embryo results from a succession of cell-cell inductions rising from the organizer, which organizes neighbouring cells and instructs their positional fate (anteroposterior and dorsoventral) and differentiation (neural tissue, notochord, somites). Following this study an “organizer” was found in

other embryos such as fish, birds and mammals (Waddington, 1934; Oppenheimer, 1936; Beddington, 1994).

The discovery of the organizer in the various species prompted investigation in the signalling mechanisms that give rise to this neural-inducing region. Experiments in *Xenopus laevis* provided insights into the mechanisms that underlie the phenomenon of neural induction in vertebrates. Noggin, found to be excreted by the organizer, was the first isolated factor shown to induce neural tissue formation (Smith and Harland, 1992; Lamb *et al.*, 1993). Similarly to noggin, follistatin (Hemmati-Brivanlou and Melton, 1994) and chordin (Sasai *et al.*, 1994) were also identified to induce neural tissue formation. Throughout studies of chordin, it was found that its sequence showed considerably homology to the *Drosophila melanogaster* gene *short gastrulation*, which had been previously reported to act as an antagonist to the gene *decapentaplegic*, which is a homologous of vertebrate genes bone morphogenetic protein (BMP) 2 and 4 (Holley *et al.*, 1995). BMP2 and 4, expressed in the ventral part of the embryo (Dale *et al.*, 1992; Jones *et al.*, 1992), are members of the transforming growth factor (TGF) superfamily and have been shown to inhibit neural induction and promote epithelial growth. When BMP4 was added to dissociated animal cap (roof region of the embryo that gives rise to ectodermal derivatives) cells, neural induction was inhibited (Wilson and Hemmati-Brivanlou, 1995). Furthermore, during *Xenopus laevis* embryo development BMPs messenger RNA (mRNA) expression, which is ubiquitously expressed at first, is cleared from the neural plate as the organizer begins to form (Fainsod *et al.*, 1994). Therefore, since BMPs are found to normally prevent embryonic ectoderm development, these data indicated that chordin may induce neural development by inhibiting BMP signalling pathway. In fact, in addition to chordin, follistatin and noggin were also found to bind and inhibit the activity of BMP-4 and BMP-2 (Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996; Iemura *et al.*, 1998; Sasai *et al.*, 1995). Noggin and chordin have high affinity to bind to BMP ligands and to prevent their binding to BMP receptors, whereas follistatin mainly binds to activin and its antagonizing effects on BMPs may be through a different mechanism (Zimmerman *et al.* 1996; Sasai *et al.* 1995). These BMP inhibitors are expressed in the organizer region of *Xenopus* embryos and can induce neural markers in blastula stage animal cap cells (Hemmati-Brivanlou and Melton, 1994). Furthermore, dominant negative forms of the BMPs introduced into

early *Xenopus* embryos also promote neurogenesis (Hawley *et al.*, 1995). Taken together, it is implied that the suppression of BMP signalling by BMP antagonists secreted from the organizer is sufficient to induce neural differentiation and that this double gradient that emanates from opposite poles of the embryo aids dorsal-ventral formation.

While neural induction in amphibians is largely considered to be dependent on BMP signalling and secretion of BMP antagonists, further experiments in chick and *Xenopus* embryos have questioned this hypothesis and have led to the proposal that BMP inhibition is not sufficient for neural induction, suggesting that fibroblast growth factors (FGFs) may be involved in embryo neurogenesis (Lamb and Harland, 1995; Launay *et al.*, 1996; McGrew *et al.*, 1997).

FGFs are a large class of secreted diffusible glycoproteins that bind to four classes of extracellular receptors (called FGFR1-4) to mediate their effects (Launay *et al.*, 1996). In *Xenopus* it has been shown that overexpression of dominant negative forms of FGF receptors in animal cap cells blocks the ability of noggin or chordin to induce neuralization (Lamb and Harland, 1995; Launay *et al.*, 1996), indicating the requirement of FGF for neural induction. Furthermore, it has been shown that blocking BMP signals at the gastrula stage does not result in neural induction; neural induction was only observed when BMP was inhibited prior gastrula stage (Wawersik, 2006). However, when FGF activation was accompanied by BMP inhibition at the gastrula stage neural induction occurred (Wawersik, 2006). This suggested that FGF signalling occurred prior to BMP signalling and therefore is also important for neural induction. In fact, FGF has been shown to be required for neural induction independently of BMP expression (Linker and Stern, 2004; Delaune *et al.*, 2005). Studies in chick have shown that FGF signalling can repress expression of BMP mRNA (Wilson *et al.*, 2000; Streit *et al.*, 2000) and downregulate BMP signalling (Pera *et al.*, 2003). In addition, in zebrafish, it has been proposed that both BMP inhibition and FGF signalling can act as neural inducers, with BMP antagonism inducing anterior and FGFs inducing posterior neural fates (Kudoh *et al.*, 2004). In contrast with these studies, how is neural induction regulated in the mouse embryo is less clear. Double mutants for the BMP antagonists chordin and noggin are still able to form a neural plate but fail to maintain forebrain markers at later stages (Bachiller

et al., 2000), raising the question of what is relieving BMP inhibition in the mouse embryo. Given that inhibition of FGF signalling has been shown by several reports to result in neural tissue formation in mice and that presence of FGF leads to inhibition of neural fate (Sun *et al.*, 1999; Ciruna *et al.*, 1997; Burdsal *et al.*, 1998; Di-Gregorio *et al.*, 2007), it is possible that FGF does not act as a neural inducer in mice. While in *Xenopus*, chick, and zebrafish embryos, FGFs and BMP antagonists may play a role in neural induction, in mice the mechanism of neural induction appears to be different.

Overall, the current evidence indicates that FGFs can play a role in promoting acquisition of neural fate. Although the mechanisms might vary between species, the abrogation of BMP signalling remains the central event that precedes this acquisition.

1.3.2 Establishment of the anterior-posterior axis

Primary Neurulation

Formation of the CNS in vertebrate embryos starts with the allocation of a group of ectodermal precursor cells that will give rise to the entire CNS (Hemmati-Brivanlou and Melton, 1997). The discovery of the organizer in amphibian by Spemann and Mangold in the 1920's initiated the search for a homologous structure in vertebrates. Therefore, soon after the equivalent region was discovered in most vertebrate species. In birds and mammals the organizer was named "Hanns node" and "the node" respectively (Waddington, 1933; Waddington, 1936). Recent studies in the chick embryo have shown that neural induction starts before formation of the organizer, likely by the FGF signalling pathway. The neural plate area is established by FGF8 activity coming from the primary endoderm and the suppression of BMP signalling stabilises neural differentiation rather than initiates (Linker *et al.* 2009). During gastrulation, these molecular interactions together with the activity of Hox genes leads to the formation of the anterior-posterior and dorso-ventral axes of the embryo including the generation of the three germ layers: ectoderm, mesoderm and endoderm. During this process, the neural plate forms along the anterior-posterior axis of the embryo in the medial region of the embryo as a result of planar and

vertical signalling (Figure 1.6 A). The neural plate which starts as a single layer of neuroepithelial cells goes through a process of thickening known as apicobasal thickening and pseudostratification, where cells become thicker while maintaining a single layer of cells (Smith and Schoenwolf, 1989; Keller *et al.*, 1992). This thickening of the ectoderm plate has been shown to be an intrinsic property that occurs when neuroepithelial cells have been induced to become neural and is a result of the “interkinetic movement” of the nuclei of these cells which migrate up and down the anterior-posterior axis during cell cycles (Shoenwolf, 1988; McConnell, 1995). Following formation of the neural plate, the neuroepithelial cells change in shape and rearrange to form neural folds which establish a through like space called the neural groove (Figure 1.6 B). The neural plate becomes narrower along the anterior-posterior axis so the neural folds can bend enough to be brought together at the dorsal midline (Figure 1.6 C) to finally form the neural tube (Figure 1.6 D) (Voiculescu *et al.*, 2007).

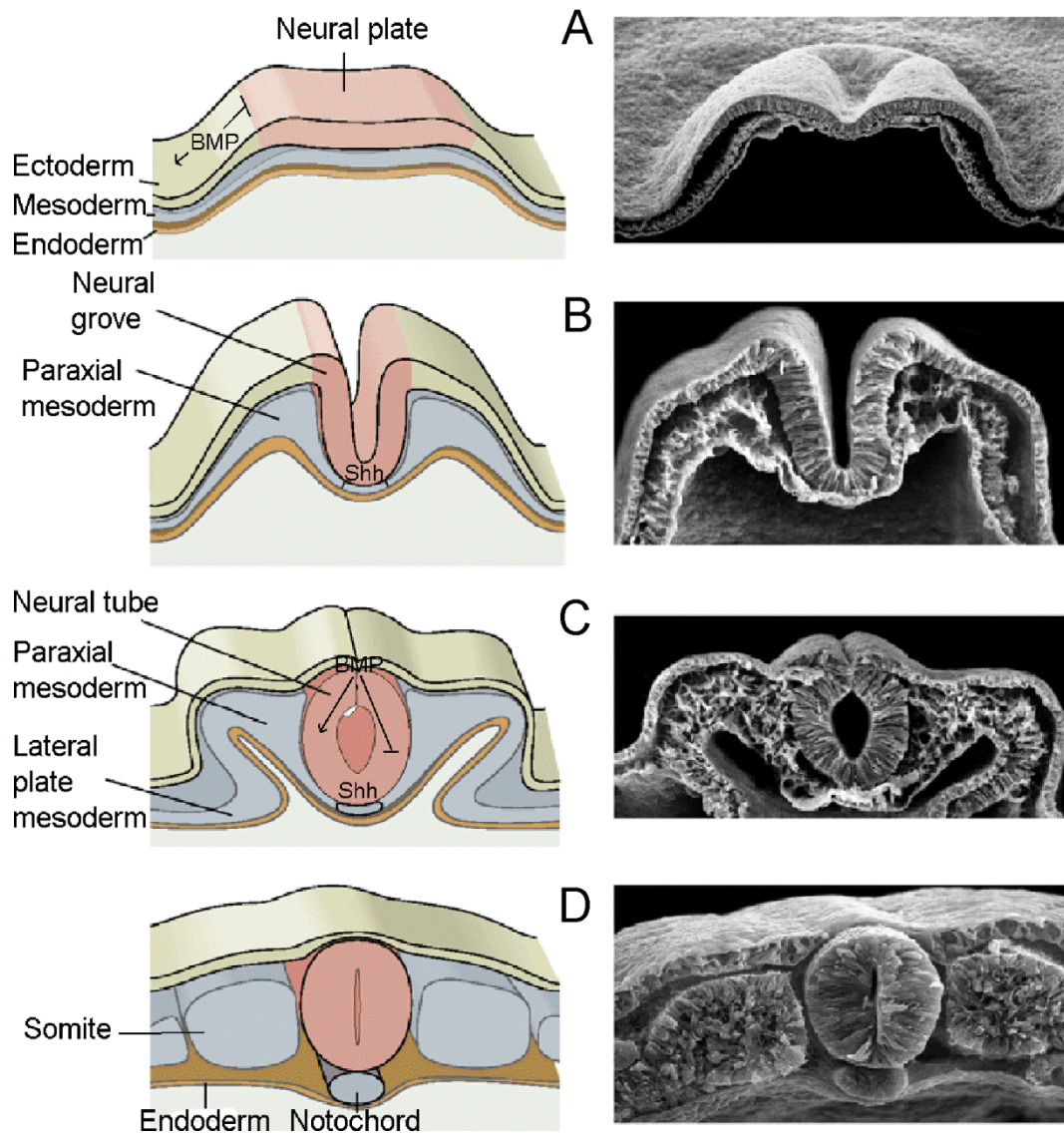


Figure 1.6 The process of neural induction.

Left side of the figure is a schematic representation of chick neural tube development shown on the right hand side by electron micrographs. **(A)** Early in embryogenesis the three germ cell layers (ectoderm, mesoderm and endoderm) lie close to each other. The ectoderm gives rise to the neural plate (pink), the precursor of the central nervous system. Process starts with the inhibition of BMP signalling in the neural plate region. **(B)** The neural plate then buckles at its middle to produce the neural groove. **(C)** Extrinsic signals from BMP and Shh together with the expression of intrinsic factors finally gives rise to the neural tube by closure of the dorsal neural folds. **(D)** The neural tube finally lies over the notochord and is flanked by somites, a group of mesodermal cells that give rise to muscle and cartilage. Figure adapted from E. R. Kandel, 2013, *Principles of Neuroscience*, 5th ed., McGraw-Hill.

Secondary Neurulation

The posterior region of the neural tube develops after the anterior region and in a different manner. In the posterior neural tube, cells condense to form an epithelial cord of cells known as the medullary cord (Shoenwolf and Delongo 1980; Shoenwolf 1984). The outer cells of the medullary cord then undergo a process of pseudostratification similar to that of the neural plate during primary neurulation. The pseudostratified cells then become polarized and form and fuse to form small lumina around a central core of mesenchymal cells. These inner cells are removed during cavitation. Finally cavitation results in the formation of a single secondary lumen which will join the primary lumen of the rostral neural tube.

Once the neural tube is completely formed and closes, the future CNS consists of a single layer of neuroepithelial cells that line the lumen of the neural tube. The neuroepithelial cells then undergo symmetric proliferation to produce daughter stem cells which will then divide asymmetrically and give rise to more specialised postmitotic neuron/glia type of cells that migrate out of the neural tube and will contribute to the formation of the CNS (Chenn and McConnell, 1995). These cells build up 6 new distinct cell layers: the ventricular (VZ), subventricular (SVZ), intermediate, subplate, cortical plate and marginal zone (Molnar *et al.*, 2006).

The lumen of the neural tube is the developing VZ of the brain. Neural development of the VZ proliferating cells proceeds following an “inside out” principle where neurons are born in the VZ, migrate to the cortical plate and then localize further out.

Young neurons migrate from the VZ to other regions of the developing brain to build up the mature brain and spinal cord. In order for the correct type of neuron to arrive to the correct brain region radial glial cells, which differentiate from neuroepithelial cells at the beginning of neurogenesis, guide and support the newly generated neurons out of the VZ (Rakic, 1972). Neurons migrating along radial glial cells form columns, which could produce a highly ordered cortex based on the pattern of underlying neural tube. In addition to acting as a guidance of newly generated neurons, radial glial cells, are a source of neural progenitors that have a more restricted fate than neuroepithelial cells and will replace these later in differentiation.

(Campbell and Gotz, 2002). As a consequence, most of the neurons in the brain are derived, either directly or indirectly, from radial glial cells. In addition, radial glial cells mainly generate neurons when isolated during early neurogenesis (Anthony *et al.*, 2004) while producing both neurons and astrocytes when isolated in late neurogenesis (Malatesta *et al.*, 2003).

The AP patterned neural tube further develops into different domains of the developing and adult brains; forebrain, midbrain, hindbrain and spinal cord, successively. The boundaries of each domain defined by morphology and marker genes are being identified and further subdivide the CNS into distinct regions.

1.3.3 Adult neurogenesis

The early studies of Altman and Das in the 1960's provided the first evidence of neurogenesis in the adult brain (Altman and Das, 1965). Almost 30 years after their study, multipotent neural stem cells (NSCs) were successfully derived from mammalian brain (Reynolds and Weiss, 1992; Richards *et al.*, 1992). Later with the introduction of bromodeoxyuridine, a nucleotide analog that can be used to trace proliferating cells, the neuroscience field demonstrated that neurogenesis was likely in humans (del Rio and Soriano, 1989; Kuhn *et al.*, 1996; Eriksson *et al.*, 1998). It is now well established that adult neurogenesis occur in most mammals throughout adulthood and in two neurogenic areas of the adult mammalian CNS: the subventricular zone (SVZ) of the lateral ventricles (Lois and Alvarez-Buylla, 1994) and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Kuhn *et al.*, 1996; Taupin and Gage, 2002), however, neurogenesis declines with ageing in both areas (Molofsky *et al.*, 2006).

During development, NSCs give rise to all the neurons of the mammalian CNS. They are the source of neurons, astrocytes and oligodendrocytes (Doetsch *et al.*, 1999; Alvarez-Buylla *et al.*, 2001). Two different types of NSCs are present in the SVZ and SGZ. These have been identified according to marker expression, morphology, proliferation and differentiation potential (Zhao *et al.*, 2008). These are type B and type C cells in the SVZ and type 1 and 2 in the SGZ. Type 1 and type B cells have

radial processes and ramifications at their end, these express markers such as GFAP, Nestin, Blbp and Sox2. Type 2 and type C cells are non-radial cells that have very short processes. Type 2 cells maintain the expression of Sox2 and Nestin and some express doublecortin, a neuroblast marker, while type C cells are negative for GFAP but express Dlx2, Mash1 and epidermal growth factor (EGF) receptor. Cell dividing ablation studies have revealed that radial type NSCs (type 1 and type B) are slow growing infrequent dividing cells or quiescent cells that can generate more actively dividing NPCs (type 2 and type C) (Doetsch et al, 1999; Ahn and Joyner, 2005). However, studies at the single cell level instead of population level, suggest that GFAP positive radial cells are NSCs that can differentiate into neurons and astrocytes (Seri *et al.*, 2004), while another study proposed that non-radial NPCs that are Sox2 positive have the potential to self-renew and give rise to neurons and astrocytes (Suh *et al.*, 2007).

SVZ adult neurogenesis

In the adult SVZ type B dormant NSCs line the lateral ventricle and give rise to actively proliferating type C NPCs (Figure 1.7) (Doetsch *et al.*, 1999). These type C cells are able to self-renew and differentiate into neuroblasts (type A cells) which then migrate through a dense glial tube along the rostral migratory stream (RMS) for several days until they reach the olfactory bulb (Lois and Alvarez-Buylla, 1994, Lois *et al.*, 1996). Once there the neuroblasts differentiate into olfactory GABAergic, dopaminergic or glutamatergic interneurons and integrate into the neuronal circuit (Carleton *et al.*, 2003; Brill *et al.*, 2009). However, it has recently been proposed that type B NSCs, similarly to type C NPCs, also have the ability to asymmetrically divide into more specialised glial type of cells (Morrens *et al.*, 2012). Finally, it is important to note that recent findings have suggested that SVZ adult neurogenesis is largely absent in humans and only present in early stages of infancy (Sanai *et al.*, 2011).

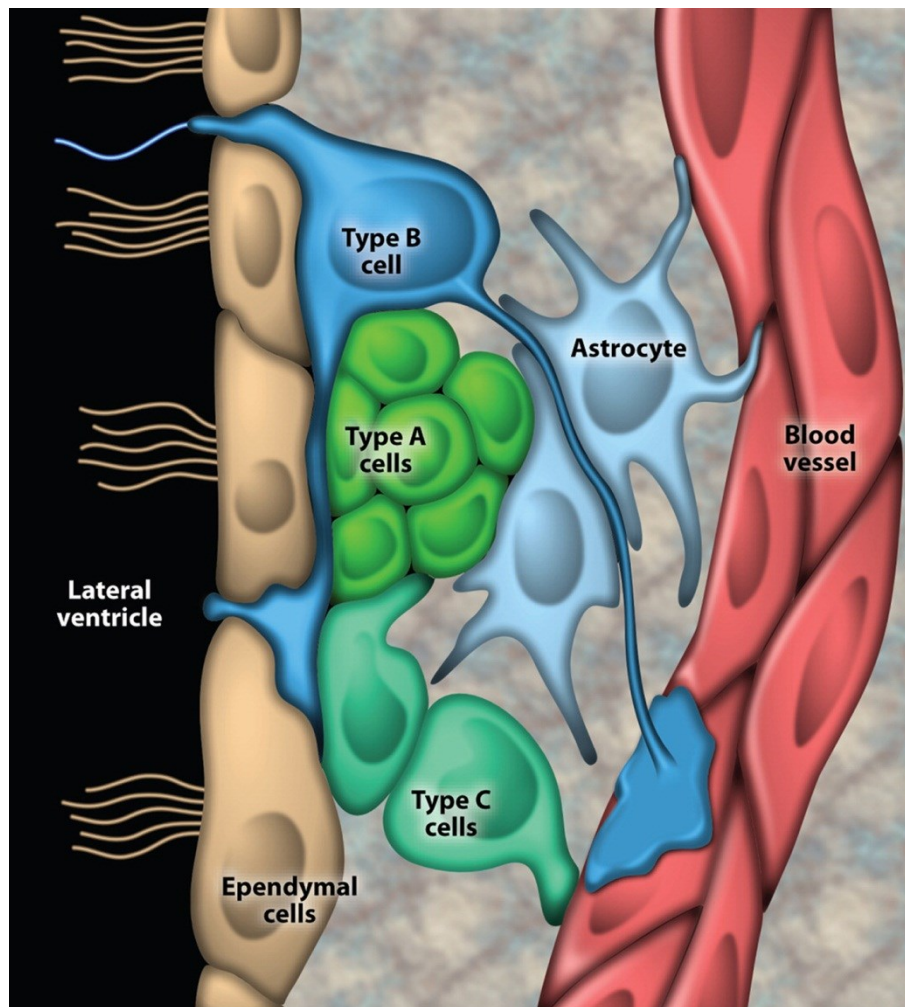


Figure 1.7 Adult neurogenesis in the subventricular zone (SVZ).

Different types of NSCs can be observed in the SVZ, these can be classified depending self-renewal and differentiation capabilities. Type B NSCs are GFAP positive NSCs located at the SVZ of the lateral ventricles that can asymmetrically divide to give rise to another type B cell and Type C cell. Type B cells have long processes that allow them to contact the lateral ventricles and blood vessels. The actively dividing type C cells give rise to neuroblast type A cells that migrate to the olfactory bulb. Figure adapted from Suh *et al.*, 2009.

SGZ adult neurogenesis

The hippocampus is a cortical structure found in both hemispheres of the mammalian brain where hippocampal neurons from the granular zone of the dentate gyrus, and cornu ammonis (CA) form a trisynaptic circuitry that plays a central role in cognitive functions such as learning and memory (Milner *et al.*, 1998) (Figure 1.8). The process by which type 1 NSCs give rise to specialised granular zone neurons is a

multi-step process that starts with either their symmetrical or asymmetrical division. If cells divide symmetrically they will give rise to two radial type 1 NSCs whereas if they divide asymmetrically, they can either self-renew and give rise to a NSC and a non-radial NPC or divide into two non-radial NPCs (Bonaguidi *et al.*, 2011; Encinas *et al.*, 2011). Following this step the newly generated NPCs or type 2 cells, can give rise to actively proliferating neuroblasts also called type 3 cells. The neutrally committed neuroblast then will start to mature and extend its dendrites and axon to reach the molecular layer and hilus, respectively. Once fully mature the new neuron will be able to excite CA3 pyramidal cells and join the trisynaptic circuitry (Zhao *et al.*, 2006; Toni *et al.*, 2008).

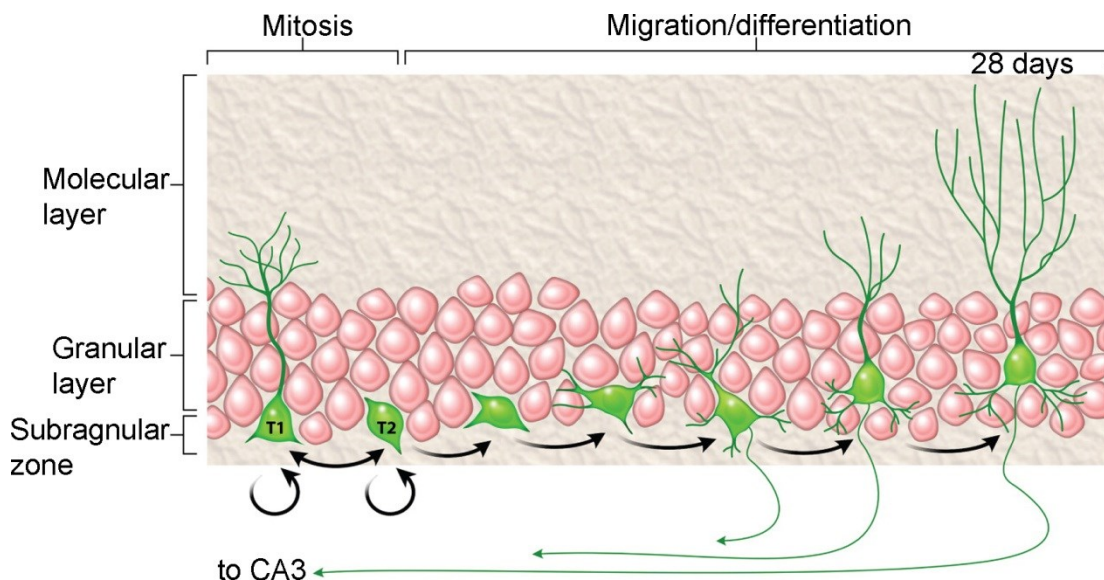


Figure 1.8 Stages of adult neurogenesis in the subgranular zone (SGZ).

Adult NSCs are located at the SGZ of the dentate gyrus of the hippocampus. Two different types of NSCs (green cells) can be distinguished: relative quiescent radial type 1 NSCs and actively dividing non-radial type 2 NSCs. These NSCs proliferate and give rise to granular neurons (pink cells). Granular neurons can be found in the granular layer and have been shown to connect the dentate gyrus, cornu ammonis 3 (CA3), and interneurons in the hilus of the hippocampus. Figure adapted from Suh *et al.*, 2009.

1.4 Neurodegenerative disorders

Recent studies have suggested that certain symptoms associated with neurodegenerative diseases may arise as a result of impaired adult neurogenesis (Lazic *et al.*, 2004; Winner *et al.*, 2004; Rodriguez *et al.*, 2008). While various neurodegenerative diseases are characterised by the death of neurons as a consequence of accumulation of toxic proteins, numerous studies in mouse models of neurodegenerative disease have shown abnormal levels of adult neurogenesis which could have contributed to the development of the condition. In Parkinson's disease mouse models levels of adult neurogenesis are heavily downregulated at both the SVZ and SGZ (Winner *et al.*, 2004). Similarly, Huntington's disease models display impaired olfactory function as a consequence of reduced adult neurogenesis at the SVZ (Lazic *et al.*, 2004). Interestingly in certain Alzheimer's disease models, the number of NSCs present was significantly reduced and linked to the presence of amyloid- β plaques in hippocampal neurons indicating that there may be an association between the accumulation of toxic proteins and disruption of adult neurogenesis (Rodriguez *et al.*, 2008). Therefore studying the mechanisms that regulate NSC and NPCs *in vivo* and *in vitro* could potentially provide novel therapeutics for treating neurodegenerative diseases.

1.5 Neural stem cells

Neural stem cells are defined by their ability to self-renew and differentiate into the three main CNS cell lineages: neurons, astrocytes and oligodendrocytes (Gage, 2000). Neural stem cells in early stages of development have the capacity of producing early-born neurons while neural stem cells in later stages of development are more restricted to a gliogenic potential (i.e. producing non-neuronal astrocyte or oligodendrocyte populations) (Temple, 2001). A similar neurogenesis to gliogenesis potential transition is preserved when neuroectodermal cells are cultured or embryonic stem cells (ESCs) are differentiated along the neural lineage (Li *et al.*, 2004). Therefore it has been suggested that the mechanisms regulating neuronal and glial lineage development is retrained *in vitro*, and highlights the feasibility of using ESCs as a tool to study neural differentiation processes.

The balance between stem cell maintenance and differentiation is regulated by complex regulating network which acts at different cellular levels and where intrinsic and extrinsic regulators play a key role. Fully elucidating the roles of each regulator will aid in understanding the biology of NSCs and create hope in using these cells for regenerative medicine.

1.5.1 Extrinsic regulators

NSCs ability to self-renew, proliferate and differentiate is known to be regulated by their niche, a local microenvironment that provides nurturing factors and stem cell to stem cell interactions that allows the maintenance of their characteristics (Fuchs *et al.*, 2004; Morrison and Spradling, 2008). Some of these nurturing factors present in the niche include several signalling molecules such as BMP, wnt, Notch, Sonic hedgehog and fibroblast and epidermal growth factors.

Fibroblast and epidermal growth factors

In order to generating a sufficient number of neural stem cells, it is assumed that cell proliferation may be predominant in the early phases, and that more cells differentiate during later stages. This implies that there is higher probability for generating two undifferentiated daughter cells at early stages (symmetric division), and later division favour the production of neurons and later glial cells (asymmetric division). Neural stem cells in the developing neocortex undergo both symmetrical and asymmetrical divisions (Cai *et al.*, 1997). Several pathways that interact to regulate cell proliferation have been identified. Perhaps the best understood are those triggered by growth factors. All neural stem cells respond to multiple growth factors, but the exact subset of growth factors acting at a specific stage may be unique for a particular stage of neural stem cell differentiation. Early neural stem cells respond solely to FGF2 [also known as basic fibroblast growth factor (bFGF)], and loss of FGFs or FGF receptors leads to a significant reduction in neural stem cell proliferation (Raballo *et al.*, 2000). Later appearing neural stem cells require either FGF2 or EGF for proliferation (Tropepe *et al.*, 1999). *In vivo* and *in vitro* experiments have shown that presence of EGF increases NSCs proliferation

(Reynolds and Weiss, 1992; Bain *et al.*, 1995). It is well known that cell self-renewal is tightly linked to this potential. Self-renewal is essential for neural stem cells because it is required for the cells to perpetuate themselves, so that at least one of the daughter cells retains the molecular characteristics of the original cells. It is important to note that while a process of self-renewal occurs, neural stem cells may undergo changes in their abilities to produce different progeny during development (Shen *et al.*, 1998).

Bone morphogenetic proteins

BMPs of the TGF- β protein family of extracellular ligands are important for many steps of neural induction and differentiation, including the initial specification of the neural plate region as well as neural crest and tube formation (Streit and Stern, 1999). In mouse ESCs (mESCs) BMPs synergizes with leukaemia inhibitory factor (LIF) to maintain self renewal (Nakashima *et al.*, 1999; Ying *et al.*, 2003). Furthermore, in mice, BMP2/4 inhibits neurogenesis of mouse neuroepithelial cells (Di-Gregorio *et al.*, 2007). In contrast, addition of BMPs to hESCs has been shown to induce differentiation (Xu *et al.*, 2002), while addition of BMP antagonists such as noggin induces neural differentiation (Pera *et al.*, 2004; Gerrard *et al.*, 2005).

It is important to note that extrinsic factors may collaborate with intrinsic factors to maintain and determine the functional characteristics of neural stem cells (Faux *et al.*, 2001; Nagao *et al.*, 2007). Extrinsic factors may regulate intrinsic factors by various cascades with stimulatory or inhibitory effects that will lead to the maintenance of the NSCs characteristics or to their differentiation. These may be through transcription factors, signalling pathways or epigenetic modifications.

1.5.2 Intrinsic factors

Extensive studies have revealed that intrinsic regulators as well as extrinsic regulators are required for the regulation of NSC multipotency. They are important for the development of the embryonic CNS as well as for the maintenance of NSCs. Some of these include but are not limited to: members of the SRY-box containing

genes (Sox) and basic helix-loop-helix (bHLH) genes, paired box protein 6 (Pax6) and neuron restrictive silencer factor (NRSF) also known as REST.

Sox2

Sox genes are a group of transcription factors belonging to the family of high mobility group of DNA binding protein that play a role in maintaining the undifferentiated state of NSCs both *in vivo* and *in vitro*. Sox2 is the most extensively examined as it has been shown to be essential for the maintenance of NSCs. In vertebrate, Sox2 is expressed in NSCs from early development to adulthood (Pevny and Placzek, 2005). Sox2 is highly expressed in quiescent NSCs of the SGZ and is downregulated upon post-mitotic differentiation (Suh *et al.*, 2007), consistent with the essential role of Sox2 in maintenance of NPCs identity (Graham *et al.*, 2003). Ectopic expression of Sox2 in chick neural tube inhibits post-mitotic neural differentiation and allows the maintenance of neural progenitor characteristics (Bylund *et al.*, 2003). Conditional deletion of Sox2 in Nestin expressing NSCs leads to a marked reduction of type 1 and type 2 cells of the adult hippocampus (Favaro *et al.*, 2009). In ESCs it has been shown that ectopic expression of Sox2 promotes neuroectoderm fate (Kopp *et al.*, 2008) and that low Oct4 and high Sox2 expression is required for ESCs to differentiate into the neuroectoderm lineage (Thomson *et al.*, 2011). In fact, ectopic expression of Sox2 has been shown to be sufficient to reprogram mouse and human fibroblasts into induced NSCs (Ring *et al.*, 2012), further supporting Sox2 importance in NSC identity. Taken together, these results suggest that Sox2 may play a critical role in the maintenance and priming of neuronal differentiation of cultured NSCs. While the mechanism behind this dual role is still largely unknown a recent report has proposed that Sox2 carries these functions by maintaining optimal levels of Lin28 transcription (Cimadamore *et al.*, 2013) required for NSCs proliferation and differentiation potential (Zhao *et al.*, 2010).

Pax6

Pax6 is a member of the paired box family of transcription factors which are crucial for the development of specific tissues. Pax6 is a highly conserved transcription factor involved in the development of the CNS (Hanson and Van Heyningen, 1995). Mutation in human Pax6 leads to aniridia, a disease characterized by mild mental retardation and loss of the iris in the eye, indicating the importance of this gene in neural development (Jordan *et al.*, 1992). Pax6 mutant mice display impaired cerebral cortex development, thinner ventricular zone, slower cell proliferation and a reduction in new-born neurons (Fukuda *et al.*, 2000). Given that Pax6 is highly expressed in neural NSCs and NPCs of the CNS (Simpson and Price, 2002), it is possible that an impairment of its expression results in the neurological disorders observed both in human and mice. In addition, Pax6 controls the expression of bHLH transcription factors such as Mash1 (Marquardt *et al.*, 2001), which has been shown to be involved in neuronal differentiation of retinal stem cells (Philips *et al.*, 2005; Xu *et al.*, 2007). Taking all this into account, it has been proposed that Pax6 functions in cortical development to prevent precocious neuronal differentiation and depletion of the progenitor pool, as well as in the maintenance of retinal stem cells indirectly by the regulation of Mash1.

BHLH genes

Basic helix-loop-helix genes (named for a shared basic helix-loop-helix tertiary DNA structure that defines their DNA binding domain) are a family of transcription factor encoding genes that are important to define the neural or glial fate. Two sets of bHLH genes are involved in neural differentiation and have opposite functions. One set function as repressors while the other set act as activators (Bertrand *et al.*, 2002). A good example of repressors and activators of bHLH would be HES genes and Mash1, respectively. HES genes are expressed in NSCs to suppress neuronal differentiation. Overexpression of the HES gene HES1 inhibits neuronal differentiation in the CNS (Ishibashi *et al.*, 1994) while, mutations in HES1 or HES5 induce neuronal differentiation (Ohtsuka *et al.*, 1999). In contrast, Mash1 and Neurogenin have been shown to be highly expressed in differentiating neurons

(Kageyama *et al.*, 2005). Ectopic expression of either these two genes in NSCs results in neuronal fate restriction rather than glial induction while cells from Mash1:Neurogenin double knockout mice become glial cells (Nieto *et al.*, 2001). All together, these data indicate that there is a fate switch regulated by bHLH activator/repressors that controls the fate of NSCs as well as their maintenance (Hatakeyama *et al.*, 2004).

1.6 The repressor element 1-silencing transcription factor

In addition to signalling pathways and transcription factors regulating neurogenesis, research in the past decade has shown that regulation of tissue-specific gene expression is also regulated by epigenetic modulators. Hence it is not surprising that epigenetic regulators are one of the key molecular mechanisms involved in controlling the various steps of neurogenesis.

REST, also known as NRSF, is a 116-kiloDalton (kDa) zinc finger containing protein that is composed of a DNA-binding domain and two repressor domains: one at the N-terminal and the other at the C-terminal of the protein (Figure 1.9) (Ballas *et al.*, 2005). REST was first identified as a protein that binds to a 21-base pairs (bp) DNA sequence consensus known as repressor element 1 (RE-1), to repress expression of neural specific genes *Nav1.2* and *SCG10* (Chong *et al.*, 1995; Schoenherr and Anderson, 1995). Later genome-wide analyses by four independent groups have found that the RE1 target site of REST is present in a wide variety of genes (Bruce *et al.*, 2004; Jones and Pevzner, 2006; Mortazavi *et al.*, 2006; Wu and Xie, 2006), which generate a complete set of genes that contain RE-1 binding motif and may be regulated by REST, including many genes that are involved in neurogenesis. Several of them have been validated to be REST target genes or have been shown to be indirectly regulated by REST, these include BDNF (Timmusk *et al.*, 1999), Synaptosomal associated protein, Snap25 (Bruce *et al.*, 2004), L1 cell adhesion molecule, L1CAM (Kallunki *et al.*, 1997), Mash1 (Ballas *et al.*, 2005), GFAP (Kohyama *et al.*, 2010) neurogenin 2, Ng2 and Sox2 (Singh *et al.*, 2008).

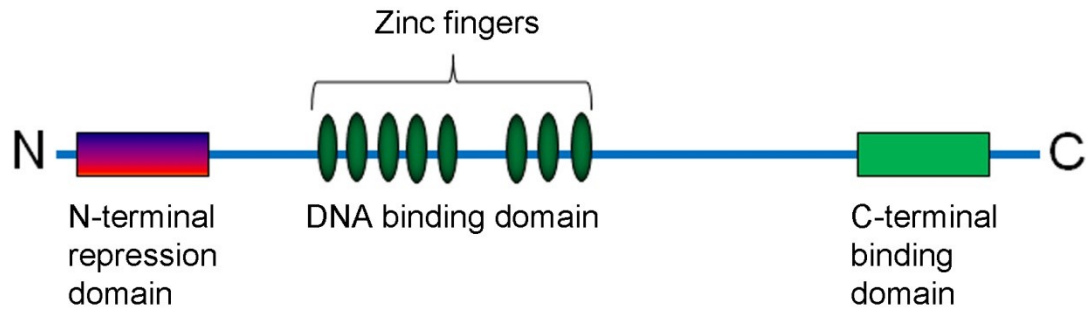


Figure 1.9 Schematic representation of REST domains.

Full length REST contains a middle DNA binding domain composed of eight zinc finger proteins (green ovals) and an N-terminal (red-blue rectangle) and C-terminal (green rectangle) repression domains.

Although genome-wide analysis has indicated that REST may have wider functions than originally thought, REST first well known main function is to suppress neuronal gene expression in non-neuronal cells of the brain as an aid to inhibit neuronal differentiation. This was based on the observations that REST expression was rarely observed in neurons and was more limited to non-neuronal cells, and that a large proportion of its target genes are involved in neural differentiation (Ballas *et al.*, 2005). However, REST was also found to be expressed in certain mature neurons in adults (Griffith *et al.*, 2001; Shimojo and Hersh, 2004), suggesting that its repression is regulated by an unknown mechanism or that its expression is maintained in a specific type of neurons. Regarding whether REST is required to maintain pluripotency of ESCs or multipotency of NPCs there is much controversy in the literature. While some groups indicate that REST is not required for pluripotency others indicate that it is required (Singh *et al.*, 2008; Buckley *et al.*, 2009; Jorgensen *et al.*, 2009; Covey *et al.*, 2012;). Furthermore, since most of these studies were carried out in mouse ESCs and NPCs; this makes it even more difficult to ascertain whether REST is required to maintain self-renewal in hESCs and human NPCs.

Truncated isoforms of REST have been observed in both rat (Palm *et al.*, 1998) and human tissue (Palm *et al.*, 1999; Coulson *et al.*, 2000). In humans, two variants of REST termed hREST-N4 and hREST-N62 were only detected in neuronal tissues. These transcripts are generated by alternative splicing of a neuron-specific exon (exon N) located between exons V and VI (Figure 1.10). Exon N is 62 bp in length

and contains a premature stop codon. Alternative splicing of REST into hREST-N62 leads to the insertion of exon N, which under the presence of a premature stop codon, results in an insertion of 13 amino acids (VGYGYHLVIFTRV) upon translation (Figure 1.10), while splicing of REST into hREST-N4 results in no amino acid insertion only a stop codon. hREST-N4 and hREST-N62 retain the N-terminal repression domain and five out of the eight zing fingers of full length REST. This neuron specific splicing of REST has been observed in human, mouse and rat (Palm *et al.*, 1998, 1999).

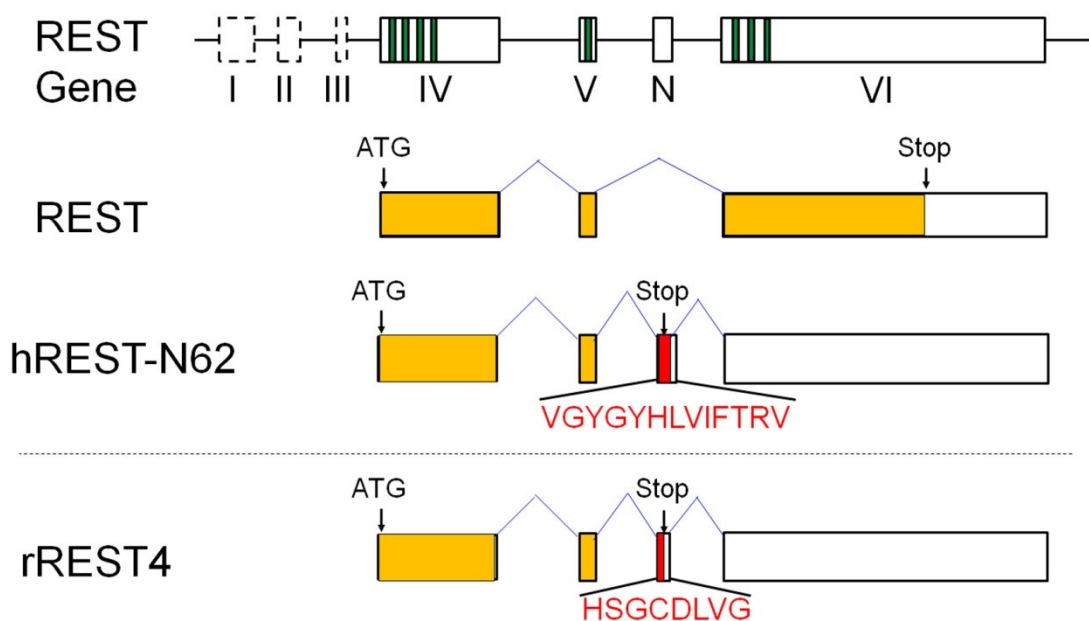


Figure 1.10 Representation of human REST gene transcripts and rodent rREST4 transcript.

Introns are shown as dotted boxes and exons as continuous line boxes. Exon numbers appear in roman numbers I to VI under the respective exon boxes. The neuron specific exon is located between exons V and VI and is indicated with a N. Vertical green bars indicate zinc finger motifs. The open reading frames of each transcript are shown in filled yellow boxes while empty boxes denote no transcription. The long dotted line across the figure separates human from rodent REST transcripts. Note that, similarly, human hREST-N62 and rodent rREST4 transcripts encode five out of the 9 zinc finger motifs encoded by full length REST but lack its C-terminal repression domain.

Although no function has been ascribed to hREST-N62, the rodent protein orthologous to this REST variant known as rodent REST4 (rREST4) has been shown to counteract the repressive role of REST in the regulation of its target genes, particularly during neurogenesis (Palm *et al.*, 1999; Shimojo *et al.*, 1999; Tabuchi *et al.*, 2002; Uchida *et al.*, 2010; Raj *et al.*, 2011). Whilst the protein sequence encoded by exon N differs between hREST-N62 and rodent rREST4, both retain the REST N-terminal domain and 5 of the 9 zinc-finger DNA binding domains, but lack the C-terminal repression domain (Figure 1.10).

1.6.1 Post-translational regulation of REST expression in NPSCs

Expression of REST is high in ES cells, decreases during neural induction, and decreases further upon formation of neurons (Ballas and Mendel, 2005). Therefore it is assumed that in order to achieve neural differentiation of ES cells, REST repression needs to be alleviated. In fact it has been shown that activation of REST target genes in NSCs is sufficient to cause neuronal differentiation (Su *et al.*, 2006), further supporting this hypothesis. However, how REST is regulated to allow neural differentiation is still unclear. Two studies in mouse NSCs have shown that REST is regulated post-translationally by the E3 ubiquitin ligase β -TrCP (Guardavaccaro *et al.*, 2008; Westbrook *et al.*, 2008). β -TrCP binds and polyubiquitinates REST so that ubiquitinated REST can be recognised by the proteasome for degradation. Therefore, the presence of β -TrCP in NSCs reduces REST protein levels and allows neuronal differentiation. Furthermore, another study has found that the neural specific Ser/Arg repeat-related protein of 100 KDa nSR100 promotes the alternative splicing of REST into rREST4, thus contributing to REST inactivation during mouse NPC differentiation (Raj *et al.*, 2011). However, in this study they showed that as a consequence of the induced splicing of REST into rREST4, REST protein levels were greatly downregulated therefore it remains unclear if the promotion of neuronal differentiation is due to the reduction of REST protein or the previously reported antagonistic effect of rREST4 over REST repression activity. Interestingly, this study claims that the nSR100 dependent switch from REST to rREST4 is an independently acting mechanism to that of β -TrCP and suggests that the downregulation of REST is a consequence of the alternative splicing which acts as an additional mechanism.

Recently, in human NPCs it has been found that deubiquitylase HAUSP can antagonise the ubiquitination of β -TrCP on REST and hence stabilises the expression of REST to maintain NPC identity (Huang *et al.*, 2011). These results suggest that regulation of REST may depend on the species or type of cell, which is line with the findings observed in *in vivo* neural development.

1.6.2 Mechanism of REST repression on its target genes

REST mediated repression is coordinated by the recruitments of two separate co-repressor complexes, mSin3 and CoREST, to their N-terminal and C-terminal repressor domains, respectively (Figure 1.11) (Andres *et al.*, 1999; Roopra *et al.*, 2000). At the N-terminal domain, mSin3a binds to REST (Grimes *et al.*, 2000) and recruits chromatin remodelling enzymes, histone deacetylases (HDACs) 1, 2, 4 and 5 (Huang *et al.*, 1999; Nakagawa *et al.*, 2006). At the C-terminal domain, REST recruits Co-REST which in turn recruits HDACs 1 and 2 (You *et al.*, 2001), H3K4 demethylase LSD1 (Shi *et al.*, 2004) and BRG1, a member of the SWI/SNF chromatin remodelling family (Battaglioli *et al.*, 2002; Ooi *et al.*, 2006). BRG1 has been shown to stabilise REST binding to the RE1 sites *in vivo* (Ooi *et al.*, 2006), which is thought to occur by BRG1 changing the chromatin environment around the RE1 site to allow REST gain better access (Watanabe *et al.*, 2006). In addition to chromatin remodelling, REST has been shown to regulate gene expression more directly by affecting the basal transcription machinery. REST has been shown to inhibit transcription initiation by interacting with TATA binding protein and RNA polymerase II-bound small C-terminal domain phosphatases (Murai *et al.*, 2004; Yeo *et al.*, 2005).

These findings indicate that in order for REST to repress its target genes, other repressors and chromatin remodelling factors are required to be recruited co-ordinately to its N-terminal and C-terminal domains. Interestingly it has been reported that overexpression of REST mutants lacking either of its repression domains results in partial repression of REST (Andres *et al.*, 1999; Huang *et al.*, 1999; Grimes *et al.*, 2000), which suggests that at least some of the REST-regulated genes require both REST repressor domains to be repressed. This may indicate that repression by REST can occur at different levels of intensity. It is also worth

indicating that overexpression of REST mutants lacking either of the repressor domains of full length REST has a similar effect on REST than the presence of REST4 in cells, which lacks the C-terminal repression domain (Tabuchi *et al.*, 2002; Shimojo and Hersh, 2004).

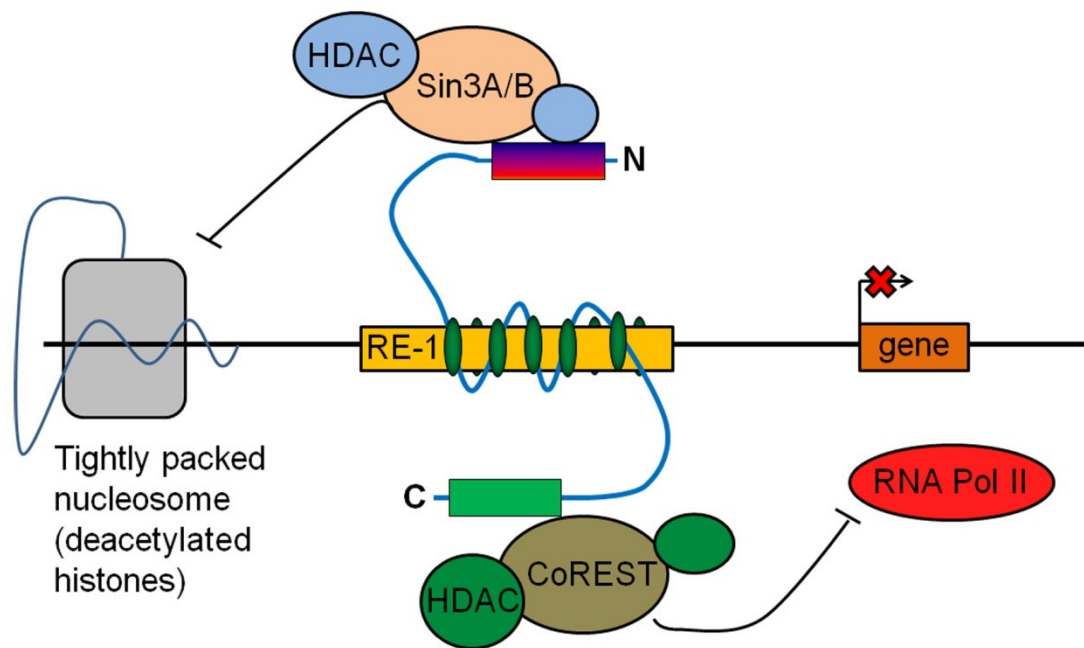


Figure 1.11 REST mechanism of repression.

REST binds the RE1 element of target genes using its DNA binding domain. REST interacts with Sin3A/B at its N-terminal and CoREST at its C-terminal region to recruit histone deacetylase (HDAC) and associated proteins to the promoter region. While REST interaction with Sin3A/B results in epigenetic changes that limit accessibility of DNA transcription factors, REST interaction with CoREST inhibits transcription start by blocking RNA pol II binding (RNA Pol II).

1.7 Embryonic stem cells

ESCs were first isolated from the inner cell mass (ICM) of pre-implantation mouse embryo in 1981 (Evans and Kaufman, 1981; Martin 1981). Building on this research and in the later derivation of non-human primate ESCs, the first hESCs line was derived also from the ICM of pre-implantation embryos (Thomson *et al.*, 1998). Following the isolation of hESCs, several lines have been derived in multiple laboratories throughout the world (Thomson *et al.*, 1998; Reubinoff et al 2000; Amit *et al.*, 2002; Richards *et al.*, 2002). HESCs, similarly to mouse ESCs (mESCs), grow

in colonies and have a high nucleus to cytoplasmic ratio. They are able to differentiate into the three embryonic germ layers by embryoid body formation (Itskovitz-Eldor *et al.*, 2000) and can form teratomas when injected into nude mice (Tzukerman *et al.*, 2003; Lensch *et al.*, 2007), reflecting their potential to differentiate *in vivo*.

1.7.1 Regulation of self-renewal and pluripotency in undifferentiated embryonic stem cells

ESCs self-renewal and pluripotency is governed by key intrinsic and extrinsic factors. The best studied intrinsic factors are Oct4, Nanog and Sox2, which play essential roles in both mESCs and hESCs. Oct4, Nanog and Sox2 are highly expressed in ESCs and downregulated upon differentiation (Rogers *et al.*, 1991; Reubinoff *et al.*, 2000; Chambers *et al.*, 2003; Fong *et al.*, 2008). They form a regulatory circuit to maintain expression of pluripotent genes and to repress expression of lineage-specific genes (Boyer *et al.*, 2005; Chew *et al.*, 2005; Rodda *et al.*, 2005). The significant roles of these factors have been further confirmed by their ability to reprogram somatic cells into induced pluripotent stem cells (iPSCs) (Takahashi *et al.*, 2007; Yu *et al.*, 2007; Nakagawa *et al.*, 2008; Yamanaka *et al.*, 2008).

Extrinsic factors, such as growth factor signaling pathways, are also very important for regulating self-renewal of ESCs. However, unlike the intrinsic factors, which seem to be regulated by very similar means in both mESCs and hESCs, the signalling pathways required to maintain the ESCs characteristics in mESCs and hESCs differ considerably. Although, initially mESCs and hESCs were maintained throughout their co-culture with MEFs, the extrinsic factors that are contributing to retain their undifferentiated state may be different. While maintenance and propagation of mESCs properties requires leukaemia inhibitory factor (LIF) (Smith *et al.*, 1988; Williams *et al.*, 1988), hESCs self-renewal has been shown to require the presence of FGF2/bFGF (Reubinoff *et al.*, 2000). This is likely to be because of the lack of LIF receptors (LIFR)/gp130 complexes in hESCs (Brandenberger *et al.*, 2004), which in mESCs bind LIF and mediate pluripotency through the activation of signal transducer and activator of transcription 3 (STAT3) (Yoshida *et al.*, 1994). In fact,

BMP and LIF has been shown to be sufficient to maintain self-renewal and pluripotency in mESCs without the need of serum or feeder layers (Ying *et al.*, 2003), while hESCs are currently thought to be mainly maintained by FGF signalling. Fibroblast growth factor receptor (FGFR) 1, the receptor of FGF2, is abundantly expressed in hESCs in contrast to differentiated cells (Brandenberger *et al.*, 2004; Carpenter *et al.*, 2004). Other FGFRs, including FGFR2, FGFR3 and FGFR4, also appear to be enriched in the undifferentiated hESCs (Dvorak and Hampl, 2005).

1.7.2 Potential applications of hESCs

While the generation of hESC lines has raised major ethical issues, hESCs provide a new useful cell source for basic science and to some extent, translational medicine. In the context of basic science research, the property of hESCs to differentiate into most if not all cell types of the human body makes them very useful to study human embryonic development. In fact, many studies have demonstrated that differentiation of hESCs into specific cell types recapitulates, to a large extent, the normal development of those cells *in vivo* (Gerrard *et al.*, 2005; Hay *et al.*, 2008; Zhang *et al.*, 2010; Lancaster *et al.*, 2013). hESCs have particularly been an important tool in illuminating key mechanisms underlying neuronal development (Chamberlain *et al.*, 2008; Crook and Kobayashi, 2008). These cells allow investigators to assess aspects of human neurogenesis that otherwise would not be possible due to technical and ethical obstacles in obtaining human embryonic and foetal tissues. Furthermore, it has been shown that the mechanisms that regulate embryonic and adult neurogenesis in vertebrates can be very different between species; therefore hESCs provide a unique tool to advance our understanding in human development. A full understanding of the mechanisms that regulate the development of the different cells types in the human body is required prior to using hESCs in translational medicine.

In the translational medicine context, hESCs have been reported to partially restore impaired cardiac functions in several animal models of myocardial infarction (Caspi *et al.*, 2007; Laflamme *et al.*, 2007). Furthermore, two recent food and drug administration (FDA)-approved hESC therapy trials for spinal cord injury and

macular degeneration used hESC-derived oligodendrocyte cells and retinal pigment epithelium (RPE) cells, respectively, for treating the conditions (Lu *et al.*, 2009; Sharp *et al.*, 2010). However, while hESCs have provided some success in translational medicine, a range of ethical and technical issues such as embryo destruction upon hESCs derivation or immune rejection of the transplanted cells/tissue, has massively halted their application to patients. The direct reprogramming of somatic cells to a hESC-like cell type known as iPSCs by the groups of Yamanaka and Thomson in 2007 (Takahashi *et al.*, 2007; Yu *et al.*, 2007) raised the possibility of circumventing the immune rejection and ethical problems of hESCs. Ectopic expression of four pluripotency intrinsic factors (Oct3/4, Sox2, c-Myc, and Klf4 or Oct3/4, Sox2, Nanog, and Lin28) was found to be enough to reprogram human fibroblasts into iPSCs. iPSCs largely resemble hESCs in terms of pluripotency, surface markers, morphology, proliferation, feeder dependence, global transcriptomic profile and epigenetic status, promoter activities, telomerase activities, and *in vivo* teratoma formation (Takahashi *et al.*, 2007; Yu *et al.*, 2007). These findings indicate that patient specific iPSCs could be generated with translational medicine intentions; however, high incidence of cancer, low reprogramming efficiency and the use of retroviruses, have slow down the use of these cells. Recently, several studies have managed to use less factors and non-viral means to produce iPSCs albeit with a relatively low reprogramming efficiency still (Yusa *et al.*, 2009; Meng *et al.*, 2012; Churko *et al.*, 2013), which provides hope to use these cells for translational medicine purposes in the near future.

1.8 Neural differentiation of human embryonic stem cells

Owing to their unique properties, ESCs provide a good *in vitro* model to study early development and lineage fate regulation. Several protocols have been developed to drive hESCs differentiation toward neural lineages, either through cell aggregation or via monolayer differentiation (Shulz *et al.*, 2003, Gerrard *et al.*, 2005, Chambers *et al.*, 2009). These studies have shown that inhibition of the SMAD signalling pathway, a family of transcription factors, is critical for neural differentiation initiation. Our laboratory has previously demonstrated that it is necessary to block the BMP signalling pathway during initial differentiation of hESCs to block SMADs

1, 5 and 8 signalling and induce neural differentiation, from which we have developed a monolayer culture system to efficiently generate NPCs from hESCs (Figure 1.12) (Gerrard *et al.*, 2005). While recent investigations have proposed that additional inhibition of SMADs 2 and 3 with the inhibitor SB431542 induces a more rapid conversion of hESCs to NPCs (Chambers *et al.*, 2009), our laboratory did not find major differences between the dual or single inhibition protocols and therefore uses the single inhibition as the method of choice for neural differentiation. Treatment of hESCs with the BMP antagonist noggin progressively and efficiently generates a morphologically distinct neural-like population of cells that express neuroectodermal markers, such as Pax6, Musashi1 and Sox2 but not mesoderm and endoderm lineage markers. Following neural initiation (N1) and neural progenitor formation, noggin can be replaced by bFGF/EGF and NPCs can be maintained in these conditions for an extended time (N2 and N3). However, continuous culture of NPCs results in the progressive shift from a bipolar morphology to triangular shape morphology. By passage 15, all cells exhibited triangular morphology and remained constant, without significant change (N3). It is also noticeable that early NPCs (N2) are more like neuronal progenitors and mainly generate neurons upon further differentiation, while late NPCs (N3) are able to differentiate to both neurons and glia, thus NSC-like, though their differentiation preference is gradually shifted toward glial differentiation as other researchers have found in NSC cultures (Tropepe *et al.*, 1999; Raballo *et al.*, 2000; Gerrard *et al.*, 2005).

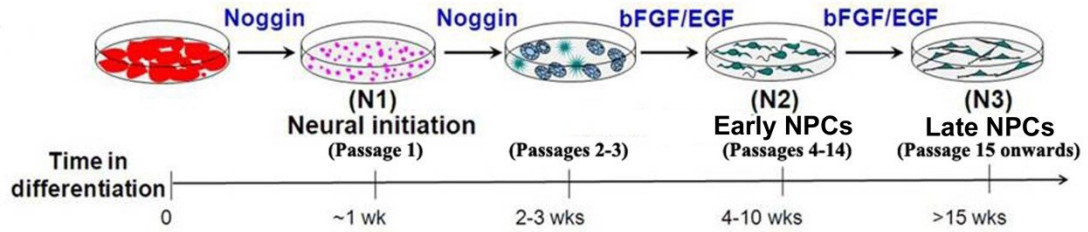


Figure 1.12 Schematic representation of the different stages of neural differentiation.

From left to right, small clusters of hESCs were cultured with N2B27 media supplemented with the BMP antagonist noggin for approximately one week, until the cells formed neural tube-like structures (N1 neural initiation, passage 1-2). Following N1 stage, noggin is replaced by bFGF (FGF2) and cultured in these conditions until bipolar early NPC morphology was observed (N2 Early NPCs, passages 4-14). Continuous culture of NPCs in bFGF progressively shifts cells from a bipolar morphology to a triangular morphology typical of late NPCs (N3 Late NPCs, passage 15 onwards). Once this morphology was observed cells were cultured with bFGF and EGF. Following N3 stage cell morphology did not vary.

1.9 Aims of the project

Knockout studies in mice have indicated that TRF1 and TRF2 may play an important role during embryonic development (see sections 1.2.4 and 1.2.5). However, due to embryonic lethality, it remains unclear what roles they play in development. Furthermore, TRF2 has been suggested to be involved in neurogenesis (Jung *et al.*, 2004; Zhang *et al.*, 2008) and preliminary data in our laboratory showed that TRF2 protein may be dramatically upregulated during neural differentiation. Thus, elucidating its role in normal neural development, particularly in brain function may aid in the improved procurement of human neural cells for potential use in regenerative therapies. Therefore, in this project, I explored TRF2 function during neural differentiation using hESCs as an *in vitro* system.

1.9.1 Hypothesis

TRF2 may play an important role in the neural differentiation of hESCs.

1.9.2 Aim and Objectives

The overall aim of my project is to investigate whether TRF2 plays a role in neural differentiation of hESCs and, if so, to explore the possible molecular mechanisms. The specific objectives are to:

- 1. Characterise TRF2 and TRF1 expression patterns in hESCs, their neural derivatives and other cell lineages.**

This will help to determine if TRF2 is differentially expressed during neural differentiation of hESCs.

- 2. Investigate the role of TRF2 in hESC neural differentiation.**

Carry out gain or loss of TRF2 function experiments in hESCs and NPCs to determine the effect TRF2 in telomere regulation and neural differentiation.

- 3. Explore the molecular interactions of TRF2 during hESC neural differentiation.**

Immunoprecipitation of TRF2 in hESCs and their neural progenitor derivatives may shed some light on the binding partners of TRF2 during neural differentiation and aid in the determination of a molecular mechanism.

- 4. Investigate the molecular pathway where TRF2 may play a role in neural differentiation.**

Carry out appropriate experiments to determine how TRF2 is affecting neural differentiation.

Chapter 2 Materials and methods

2.1 Cell biology materials and methods

2.1.1 Cell culture reagents

<u>Item</u>	<u>Supplier</u>	<u>Catalogue Number</u>
0.01% Poly-L-lysine (PLL)	Sigma-Aldrich	P4707
0.02% ethylene-dinitrilo tetraacetic acid (EDTA)	Sigma-Aldrich	E8008
100X L-Glutamine	Life Technologies	25030-024
100X Non-Essential Amino Acids (NEAA)	Life Technologies	11140-035
100X Penicillin/Streptomycin (P/S)	Life Technologies	15140-122
2% Gelatin	Sigma-Aldrich	G1393
20% Bovine serum albumin (BSA)	Sigma-Aldrich	A9418
2-mercaptoethanol	Life Technologies	31350-010
Accutase	Sigma-Aldrich	A6964
B27 Supplement	Life Technologies	17504-044
bFGF	Peprtech	100-18C
Collagenase	Life Technologies	17104-019
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D2650
DMEM:F12 (1:1)	Life Technologies	21331-020
Dulbeccos modified eagles medium (DMEM)	Life Technologies	11960-044
EGF	Peprtech	100-15

<u>Item</u>	<u>Supplier</u>	<u>Catalogue Number</u>
Foetal bovine serum (FBS)	Sigma-Aldrich	F7524
Hexadimethrine bromide (Polybrene)	Sigma-Aldrich	H9268-5G
Knockout Dulbecco's modified eagles medium (KO-DMEM)	Life Technologies	10829-018
Knockout serum replacement (KSR)	Life Technologies	10828-028
Laminin	Sigma-Aldrich	L2020
Matrigel	Life Technologies	12760021
Neurobasal medium	Life Technologies	21103-049
Noggin	R&D systems	719-NG-050
PBS without Ca ²⁺ & Mg ²⁺	Life Technologies	14190-169
phosphate buffer saline (PBS)	Life Technologies	14190-094
Puromycin	Sigma-Aldrich	P8833
TryLE Express	Life Technologies	12604-013
Trypsin	Sigma-Aldrich	T3924
Y-27632 ROCK inhibitor	Reagents direct	53-B85

2.1.2 Cell culture media and stock solutions

KSR medium

Component	Amount (ml)	Final Concentration
KO-DMEM	400	--
KO-SR	100	20%
10ng/ml bFGF	0.2	4ng/ml
100X NEAA	5	1X
2-mercaptoethanol	1	0.1mM
100X L-Glutamine	5	1X

N2B27 medium

Component	Amount (ml)	Final Concentration
DMEM/F12	250	50%
Neurobasal medium	250	50%
100X N2	2.5	0.5X
50X B27	5	0.5X
100X L-Glutamine	1	1X

Mouse embryonic fibroblast (MEF) medium

Component	Amount (ml)	Final Concentration
DMEM	440	--
FBS	50	10%
100X L-Glutamine	5	1X

Embryoid body (EB) differentiating medium

Component	Amount (ml)	Final Concecntration
KO-DMEM	400	--
FBS	100	20%
100X NEAA	5	1X
2-mercaptoethanol (50 mM)	1	0.1mM
100X L-Glutamine	1	1X

bFGF stock solution

Component	Amount	Final Concentration
FGF2	250 µg	10 µg/ml
PBS	25 ml	--
20% BSA	250 µl	0.20%

Collagenase stock solution

Component	Amount	Final Concentration
Collagenase IV	20,000 units	200 units/ml
KO-DMEM	100 ml	--

2.1.3 Primary and secondary antibodies

<u>Primary antibodies</u>	<u>Type</u>	<u>Company</u>	<u>Catalogue Number</u>	<u>IF Dilution</u>	<u>WB Dilution</u>
β-Actin	mouse monoclonal	Sigma-Aldrich	A5441	1:5000	1:5000
β-tubulin III (Tuj1)	mouse monoclonal	Sigma-Aldrich	T8660	1:1000	-
Caspase 3	rabbit polyclonal	Cell Signalling	9662	1:1000	-
GFAP	rabbit antiserum	DAKO	Z0334	1:500	-
HA	mouse monoclonal	Sigma-Aldrich	H3663	-	1:1000
HA	rabbit polyclonal	Sigma-Aldrich	H6908	-	1:1000
HNF4α (H-171)	rabbit polyclonal	Santa Cruz	sc-8987	1:50	1:200
Muscle actin (1E12-s)	mouse monoclonal	Hybridoma Bank	1E12	1:50	-
Myc	mouse monoclonal	Santa Cruz	SC-40	-	1:1000
Nestin	mouse monoclonal	Chemicon	MAB 5326	1:200	1:500
Oct4	rabbit polyclonal	Abcam	ab19857	1:200	1:500
Oct4	mouse monoclonal	Santa Cruz	sc-5279	1:250	1:1000

<u>Primary Antibody</u>	<u>Type</u>	<u>Company</u>	<u>Catalogue Number</u>	<u>IF Dilution</u>	<u>WB Dilution</u>
p21 (Cip1/WAF1)	mouse monoclonal	DAKO	M7202	-	1:250
Pax-6	mouse monoclonal	Hybridoma Bank	-	1:50	-
Pax-6	rabbit polyclonal	Millipore	AB2237	1:500	1:1000
Phospho Histone H3 (Ser10)	rabbit polyclonal	Millipore	05-806	1:200	-
RAP1	Rabbit polyclonal	Bethyl labs	A300-306A	-	1:1000
REST	rabbit polyclonal	Millipore	07-579	-	1:1000
REST4	Rabbit monoclonal	Epitomics	2417-1	-	1:2000
Sox1	rabbit polyclonal	Abcam	ab87775	1:500	1:1000
Sox2	rabbit polyclonal	Abcam	ab97959	1:500	1:1000
Sox2	goat polyclonal	R&D System	AF2018	1:400	-
SSEA4	mouse monoclonal	Hybridoma Bank	MC-813-70	1:5	-
Tra-1-81	mouse monoclonal	Santa Cruz	sc-21706	1:50	-
TRF1	mouse monoclonal	Millipore	04-638	-	1:1000
TRF2	mouse monoclonal	Millipore	05-521	1:500	1:500
TRF2 (H-300)	rabbit polyclonal	Santa Cruz	sc-9143	1:30	1:200
γ -H2A.X (S139)	rabbit polyclonal	Upstate	07-164	-	1:500

<u>Secondary antibodies</u>	<u>Company</u>	<u>Catalogue Number</u>	<u>IF Dilution</u>	<u>WB Dilution</u>
Goat Anti-Mouse IgG Alexa Fluor 488	Life Technologies	A11001	1:400	-
Goat Anti-Rabbit IgG Alexa Fluor 488	Life Technologies	A11008	1:400	-
Goat Anti-Rabbit IgG Alexa Fluor 568	Life Technologies	A11011	1:400	-
Goat Anti-Mouse IgG Alexa Fluor 568	Life Technologies	A11004	1:400	-
Goat anti-mouse Light chain specific	Jackson Labs	115-035-174	-	1:25000
Mouse anti-rabbit Light chain specific	Jackson Labs	211-032-171	-	1:25000

2.1.4 Preparation of mouse embryonic fibroblast conditioned medium

MEFs isolated from embryonic day 13.5 (E13.5) CD-1 mouse were grown and expanded in MEF culture medium for up to 3 passages. Cells were trypsinised, collected in 50 ml falcon tubes (Corning) by centrifugation (Hettich Universal 320 centrifuge), irradiated with 40 grays (Gy) (IBL 637 Cell irradiator), and seeded with MEF medium at $\sim 80,000$ cells/cm² in 0.5% gelatin coated T225 cell culture flasks (Corning). Cells were allowed to attach and recover overnight. The following day MEF media was replaced with 150 ml KSR medium supplemented with 4 ng/ml human bFGF, and incubated for 24 hours. The next day the 150 ml KSR MEF conditioned media (CM) was collected into sterile 150 ml collection bottles (Corning) and stored at -80C until required. MEFs were fed again with KSR media as the day before for another collection next day. The procedure can be repeated for 6 days maximum. On the day of use in hESCs cell culture, CM was defrosted, mixed with 1X L-glutamine and filtered to remove any MEF cells. Fresh bFGF was added into the medium (8 ng/ml) prior use for hESCs cell culture.

2.1.5 Preparation of matrigel coated plates

Growth factor-reduced matrigel was slowly thawed on ice at 4°C overnight and diluted 1:2 with ice cold KO-DMEM. This 1:2 diluted matrigel-KO-DMEM solution was aliquoted in 1 ml volumes and stored at -20°C until required. To prepare matrigel coated plates, frozen stock matrigel solution was slowly defrosted at 4°C overnight and then diluted 1:15 in ice cold KO-DMEM (final working concentration 1:30), which was then plated into cell culture plates and incubated overnight at 4°C. In emergency instances, the coating could be shortened to 3 hour at room temperature. Matrigel coated plates can be maintained at 4°C for up to a week before use.

2.1.6 Human embryonic stem cell culture

The hESCs used in this project were the H1 and H7 lines, two of the first hESC lines derived in 1998 (Thomson *et al.*, 1998). These were obtained from WiCell Research Institute (Madison, VA). hESCs were grown and maintained in an undifferentiated state on matrigel-coated plates with MEF-CM supplemented with 8 ng/ml bFGF (Xu *et al.*, 2001). They were routinely passaged in 6-well plates (Corning) at 1:3 ratio by incubating for 5-10 minutes with 200 U/ml collagenase IV at 37°C. The collagenase was then washed off from the cells and the cells mechanically scrapped with a 5 ml strippette (Corning) to generate small clumps of cells that were subsequently plated into new matrigel-coated wells.

2.1.7 Neural differentiation of human embryonic stem cells

Human embryonic stem cells were differentiated into neural NPCs using the laboratory published protocol (Gerrard *et al.*, 2005). Confluent hESCs were treated with 0.02% ethylene-dinitrilo tetraacetic acid (EDTA) to reduce the adhesion, flushed with culture medium, and broken into small clumps which were then split at a 1:5 ratio and plated onto PLL/laminin coated 6-well plates (passage 1). The differentiating cells were cultured in N2B27 medium supplemented with 100 ng/ml noggin. Upon confluence, cells were split 1:3 using collagenase IV and scraping (as

with hESC culture). Following the formation of neural rosettes approximately at passages 2-3, noggin was replaced with bFGF and EGF (20 ng/ml each). NPCs were propagated with TrypLE and maintained in the same culture conditions (See Figure 1.12 for a schematic representation). For further differentiation into neurons and glia, growth factors were withdrawn from the N2B27 medium for 1-2 weeks.

2.1.8 Fibroblast differentiation of human embryonic stem cells

Fibroblast differentiation of hESCs was induced by replacing hESC culture medium with DMEM supplemented with 10% FBS and 1% L-glutamine. The hESCs colonies were allowed to differentiate over three weeks into human embryonic fibroblasts (HEFs), passaging them with trypsin when required.

2.1.9 Differentiation of hESCs by Embryoid Body formation

Differentiation of hESCs via EB formation was induced by culturing hESCs in suspension and in EB differentiation medium. A confluent well of a 6-well plate (Corning) of hESCs was treated with 1 ml collagenase IV and treated as in hESCs propagation, except small clumps of cells were generated in 2 ml of EB differentiation medium. Cell clumps were transferred to one well of a low attachment plate (1:1 split). 2 ml of differentiation medium were added to make up a total volume of 4 ml per well. After overnight culture in suspension, ES cells formed floating aggregates known as EBs. To change the medium, EBs were transferred into a 15-ml tube and allowed to settle for 5 min. Supernatant was then replaced with fresh differentiation medium (4 ml/well), and the EBs transferred again into a low attachment 6 well plate for further culture. Medium was changed every 2 to 3 days. EBs were kept in culture for 7 days and then dissociated in a 24-well plate containing 0.5% gelatine coated glass coverslips and 1 ml differentiation medium. The dissociated EBs were allowed to further differentiate in these conditions for 1-2 more weeks, then fixed for immunofluorescence (IF) or harvested for ribonucleic acid (RNA) or protein extraction.

2.1.10 mESCs and human hepatoblasts

Protein lysates of mESCs, mouse epiblast stem cells (mEpiSCs) and mouse NPCs (mNPCs) were a kind gift of Professor Meng Li's laboratory (Cardiff University, Stem Cell Neurgensis group). The H1 hESCs-derived hepatoblast lysates used in this thesis were a kind gift of Jason Yu (Imperial College London, Stem Cell Differentiation group) who differentiated the cells and provided me with the protein lysate.

2.1.11 hESCs and NPCs transduction

Human embryonic stem cells were split 24 hours prior to infection with accutase and plated into matrigel-coated plate under hESCs culture conditions and supplemented with 10 μ M Rock inhibitor Y-27632 (Watanabe *et al.*, 2007). Medium was replaced with fresh hESC media with no Rock inhibitor and 20 μ l of concentrated virus in a total volume of 1 ml medium containing 8 μ g/ml Hexadimethrine bromide (Polybrene). For packaging and production of lentiviruses see section 2.2.3. Cells were selected for their respective selection marker resistance 48-72 hours after infection. Surviving cells were then cultured and propagated as in routine hESC culture. NPCs were infected similarly to hESCs with the following modifications. NPCs were split with TripLE and no Rock inhibitor was added. Same dilution of viruses was tested and 4 μ g/ml Polybrene used for the infection. Infection was carried out under NPC culture conditions. The lentivector pLVTHM containing green fluorescent protein (GFP) complementary DNA (cDNA) was used as a control for infection (Appendix, Figure A2), GFP expression was visualised by fluorescent microscopy (Nikon Eclipse TE 2000-U).

2.2 Molecular biology materials and methods

2.2.1 Molecular biology reagents

<u>Item</u>	<u>Supplier</u>	<u>Catalogue Number</u>
100 bp DNA ladder	NEB	N0468S
1 kb DNA ladder	NEB	N0467S
2-Mercaptoethanol	Sigma-Aldrich	M6250
30% bis/acrylamide	VWR	100639
Agarose	Sigma-Aldrich	A9539
Ammonium persulfate (APS)	VWR	443073E
BCA protein assay kit	Fisher	23227
BSA	Sigma-Aldrich	A7906
Calcium Phosphate Transfection Kit	Sigma-Aldrich	CAPHOS-1KT
Chloroform solution	Sigma-Aldrich	25666
Chondroitin sulphate from shark cartilage	Sigma-Aldrich	C4384-1G
CL-XPosure X-ray film	Fisher	34090
Colcemid	Life Technologies	15212012
DNase I	Sigma-Aldrich	AMPD1-1KT
Dynabeads Protein G	Life Technologies	10003D
Glass coverslips	VWR	631-0149
Glass slides	VWR	631-1558
Goat serum	Sigma-Aldrich	G9023
Hexadimethrine bromide (Polybrene)	Sigma-Aldrich	H9268-5G

<u>Item</u>	<u>Supplier</u>	<u>Catalogue Number</u>
Immobilon-P membrane, PVDF	Millipore	IPVH-000-10
Lipofectamine LTX plus reagent	Life Technologies	15338030
Luminata Forte Western blot substrate	Millipore	WBLUF0500
MG132	Calbiochem	474791
Normal rabbit IgG & mouse IgG	Santa Cruz	sc-2027/2025
Oligo dT 12 18 Primer	Life Technologies	18418012
One Taq DNA polymerase	NEB	M0480L
Page Ruler Plus protein ladder	Fermentas	SM1811
PCR-graded water	Life Technologies	10977-023
Phase lock gel tubes	5 Prime	2302820
Phenol solution	Sigma-Aldrich	P4557
Phenylmethanesulfonyl fluoride solution (PMSF)	Sigma-Aldrich	93482
Positively charged nylon membrane	Roche	
Protease Inhibitor	Sigma-Aldrich	P8340
Protein A/G plus agarose beads	Santa Cruz	sc-2003
QIAfilter Plasmid Maxi Kit	Qiagen	12263
QIAquick gel extraction kit	Qiagen	28704
Resolving gel buffer	Bio-Rad	161-0798
Restriction enzymes	NEB	Various
RNase OUT	Life Technologies	10777019
Site-directed Quickchange mutagenesis kit	Agilent	200519
Stacking gel buffer	Bio-Rad	1610799

<u>Item</u>	<u>Supplier</u>	<u>Catalogue Number</u>
SuperScript II RT	Life Technologies	18064014
SYBR® Green JumpStart™ Taq ReadyMix™	Sigma-Aldrich	S4438
T4 DNA ligase	NEB	M0202L
TAM1 Competent Cells	Active Motif	11096
Telomere PNA FISH Kit/FITC	DAKO	K5325
TeloTAGGG Telomere Length Assay kit	Roche	12209136001
Tetramethylethylenediamine (TEMED)	VWR	443083G
Thermanox coverslips	Thermo Scientific	174950
Tri reagent	Sigma-Aldrich	T9424
Triton X-100	Sigma-Aldrich	T8787
Ubiquitin Aldehyde	R&D Systems	U-201
Vectashield	DAKO	H-1200

2.2.2 Construction of expression vectors

All original plasmids were obtained from Addgene unless indicated and all restriction enzymes as well as modification enzymes were purchased from New England Biolabs. Lentiviral vectors for overexpressing and knocking down TRF2 were derived from pLVTHM (Addgene 12247, Wiznerowicz and Trono, 2003). TRF2 cDNA was isolated from pLPC-TRF2 (Addgene 18002, Wang *et al.*, 2004). Briefly, site-mutagenesis was performed to mutate stop codon of PURO cDNA in pPUR (Clontech) which enable the insertion of a 2A foot and mouth disease virus sequence PCR fragment downstream of PURO. An Agilent site directed mutagenesis kit was used following the manufacturer instructions. The PURO-2A fragment was then assembled with TRF2 cDNA in pBluescript KS II (-) (Stratagene) to generate

PURO-2A-TRF2 fusion protein fragment, which subsequently substituted GFP in pLVTHM to generate a TRF2-expressing lentiviral vector. The knockdown plasmid was generated by replacing scramble short hairpin ribonucleic acid (shRNA) in pLVTHM with a duplex oligonucleotide targeting TRF2 mRNA 5'-CGC GCA GGA GCA TGG TTC CTA ATA ATA CTG CAG TAT TAT TAG GAA CCA TGC TCC TGT TTT T -3' and 5'-GCA AAA ACA GGA GCA TGG TTC CTA ATA ATA CTG CAG TAT TAT TAG GAA CCA TGC TCC TG -3'. The lentiviral vector pLCMV-Neo-Myc-REST was a kind gift from Dr Shideng Bao (Huang *et al.*, 2011) and was used to generate both REST4 and REST4 mutant expression vectors. REST4 expression vector was generated by subcloning REST *XbaI/BamHI* fragment from pLCMV-Neo-Myc-REST into pBluescript KS II (-), where the *NdeI/BamHI* fragment containing REST C-terminal domain was then replaced with an oligonucleotide duplex containing REST4 exon N coding sequence (5'-TAT GCG TAC TCA TTC AGT GGG GTA TGG ATA CCA TTT GGT AAT ATT TAC TAG AGT GTG AG-3' and 5'-GAT CCT CAC ACT CTA GTA AAT ATT ACC AAA TGG TAT CCA TAC CCC ACT GAA TGA GTA CGCA-3'). The resulting REST4 cDNA was then inserted back into pLCMV-Neo-Myc-REST with *XbaI* and *BamHI* replacing full length REST. The REST4 mutant vector was generated by replacing exon N sequence with a stop codon using the oligonucleotide duplex 5'-TAT GCG TAC TCA TTC AGGT-3' and 5'-GAT CCT CAA CCT GAA TGA GTA CGC ATG AG-3'. pCS2-HA-TRF2 was generated by cloning TRF2 cDNA into pCS2-HA. All constructs were verified by sequencing (Beckman Coulter Genomics, UK).

2.2.3 Packaging and production of lentivirus

Lentiviral packaging of pLVTHM or its modified versions (see section 2.2.2) was carried out using the helper construct pCMVΔ8.91 (Addgene 12263) and the envelope construct pVSV-G (Addgene 8454). HEK (Human embryonic kidney) 293T cells were used for lentivirus packaging, these were cultured and maintained in DMEM with 1% glutamine and containing 10% FBS. Briefly, HEK 293T cells were transfected in a 3:2:1 DNA ratio of lentivector (15 µg), helper construct (10 µg) and envelope constructs (5 µg) using CAPHOS kit (Sigma-Aldrich) following the manufacturer's instruction. Lentiviral particles were harvested from the cell culture

medium 48 and 72 hours post transfection, centrifuged at 290 g (Hettich Zentrifugen Universal 320) and filtered using a 0.45 µm sterile filter. 4 µl (20 mg/ml stock) Polybrene and 4µl (20 mg/ml stock) chondroitin sulphate were added to the virus supernatant and vortexed briefly. Samples were then incubated at 37°C in a humidified CO₂ incubator for 20 minutes. Viruses were then spun at 10000 g (Beckman Coulter Avanti J-20 XPI) at room temperature for 30 min. This produced a pellet containing the vector polybrene-chondroitin sulphate complex. Supernatant was discarded and the complex resuspended in 400 µl fresh DMEM by gently pipeting up and down until pellet was dissolved. Lentiviruses were immediately stored at -80°C until use.

2.2.4 Telomere fluorescent in situ hybridization and immuno-fluorescent in situ hybridisation

To prepare metaphase spreads, cells were treated with 0.5 µg/ml colcemid at 37°C for 4 hours, harvested, resuspended in 0.8% sodium citrate and incubated at 37°C for 15 minutes. Cells were then fixed in methanol-acetic acid (3:1 ratio) and dropped onto a clean glass slide. Fluorescent in situ hybridisation (FISH) was carried out using a telomere peptide nucleic acid (PNA) FISH kit from DAKO following the manufacturer's instructions. For immuno-FISH experiments, cells grown on coverslips were first subjected to the IF protocol as indicated in section 2.2.6 of materials and methods. Cells were then incubated in 3.7% formaldehyde, dehydrated in cold ethanol series and incubated with denatured PNA probe at 85°C for 5 minutes. Hybridisation were performed by incubating the cells overnight at 37°C, followed by washing in 70% formamide and 2X saline sodium citrate (SSC) for 5 minutes each. Finally, the cells were dried and mounted with VECTASHIELD solution containing 4,5-diamidino-2-phelylindole (DAPI).

2.2.5 Telomere length assay

Telomere lengths were measured by telomere restriction fragment (TRF) southern blotting analysis using Roche's TeloTAGGG telomere length assay kit, according to the manufacturer protocol. Genomic DNA was isolated from cell pellets using phenol-chloroform extraction and phase lock gel tubes. 2 µg of genomic DNA were digested with 0.75 µl of *RsaI* and *HinfI* each, in a total volume of 25 µl at 37°C overnight. Fragmented DNA was separated on a 0.8% agarose gel at 100V for 20 minutes and then 30V for ~16 hours. Following separation the gel was depurinated in 0.25 M HCl, denatured in 1.5 M NaCl/0.5 M NaOH, neutralised in 0.5 M Tris/1.5 M NaCl and blot onto a positively charged nylon membrane overnight by capillary transfer. The membrane was cross-linked to the DNA by ultraviolet (UV) irradiation, pre-hybridized at 42°C for 1 hour with digoxigenin (DIG) easy hybridisation solution and hybridised to a DIG-labelled (AATCCC)_n telomere-specific probe overnight. After stringent washes, non-specific antibody binding was blocked by incubation with blocking reagent for 30 minutes and then the membrane was incubated with a sheep anti-digoxigenin antibody (diluted 1:10,000) conjugated to alkaline phosphatase. Finally the membrane was washed, treated with a chemiluminescent alkaline phosphatase substrate and exposed to X-ray film for different times.

2.2.6 Immunoblotting and IF

For immunoblotting, cells were lysed in cold radio immuno precipitation assay (RIPA) buffer [150 mM NaCl, 50 mM Tris HCl pH 8.0, 12 mM sodium deoxycholate, 0.1 % sodium dodecyl sulphate (SDS), 1 % NP-40] supplemented with protease inhibitor cocktail and 0.2 mM PMSF. Protein lysates were assayed for their protein concentration with a bicinchoninic acid (BCA) protein assay kit (Pierce). 20 µg of total protein from each extract were separated in 7% SDS-polyacrylamide gel and electroblotted onto polycylnidene fluoride (PVDF) membranes. Quantification of the immunoblots was carried out using Quantity One software (Bio-Rad). For IF, hESCs were grown on matrigel coated Thermanox coverslips while NPCs and their derivatives were grown on PLL/laminin coated glass coverslips. They were fixed in 4% paraformaldehyde (PFA) for 10 min and blocked and permeabilised with PBS

containing 0.3% triton X-100, 10% goat serum and 2.5% bovine serum albumin 30 min. They were then incubated with primary antibodies for 1 h, followed by fluorescent conjugated secondary antibodies for 30 min with washing in between. After mounting the coverslips with VECTASHIELD solution containing DAPI, cells were visualized and captured with a Leica SP5 confocal microscope. Primary and secondary antibodies are listed in section 2.1.3.

2.2.7 Fluorescence activated cell sorting

The analysis of the cell surface antigen Tra-1-81 was performed as follows: cells were harvested by trypsinisation and 1.5×10^6 cells were washed twice in fluorescence-activated cell sorting (FACS) buffer (PBS, 2% FBS), resuspended in primary antibody (normal mouse IgG or mouse anti-Tra-1-81 antibody) diluted in FACS buffer and incubated 30 min at 4°C. Cells were then washed twice in FACS buffer and incubated with specific anti-mouse IgG Alexa Fluor 568. Finally, cells were washed twice and then resuspended in 100 µl of FACS buffer for analysis. For Pax6 intracellular staining, cells were harvested by trypsinisation, washed in FACS buffer and 1.5×10^6 cells fixed (10 min, 37°C) with 0.1% PFA in PBS. Cells were then washed and permeabilised (30 min, 4°C) with cold 90% methanol. After washing once, cells were incubated for 30 min at room temperature with primary antibody diluted in FACS buffer with 0.1% Triton X-100. Cells were again washed twice before incubating for 30 min with secondary antibody diluted in FACS buffer with 0.5% Triton X-100. The cells were finally washed twice and resuspended in 600 µl of FACS buffer before analysis on a FACScalibur flow cytometer (BD Biosciences) with CellQuest software. The profile of stained cells was compared to unstained cells and cells stained with the secondary antibody only. A list of antibodies and reagents used can be found in sections 2.1.3 and 2.2.1.

2.2.8 Immunoprecipitation

Cell extracts were prepared in ice cold lysis buffer [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) Ph 7.4, 10 mM KCl, 100 mM NaCl₂, 1mM EDTA, 0.1mM ethylene glycol tetraacetic acid (EGTA), 0.1 % triton x100, 0.5 % NP-40] supplemented with protease inhibitor cocktail and 0.2 mM PMSF and pre-cleared by centrifugation at 15,000 g for 30 minutes at 4°C. 1 mg extracts from NPCs or 1 mg extracts from HEK293T transfectants were incubated with 2-3 µg appropriate antibodies at 4°C for 1-3 h with rotation. Immunocomplexes were captured at 4°C with either 20 µl Protein A/G Plus agarose beads for 1 h or 50 µl protein G Dynabeads for 3 hours. Immunoprecipitates were eluted with 2 X Laemmli buffer (150 mM Tris-Cl (pH 6.8), 4% SDS, 20% glycerol, 0.02% bromophenol blue) for 10 min, then analysed by immunoblotting.

2.2.9 REST4 ubiquitination assay

HEK293T cells stably carrying either pLVTHM-PURO (Control) or pLVTHM-PURO-2A-TRF2 (TRF2-Ov) constructs were co-transfected with pLCMV-Neo-Myc-REST4 and HA-Ubiquitin. Following treatment with 20 µM MG132 for 5 h, cells were lysed in cold lysis buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP40) supplemented with protease inhibitor cocktail, 0.2 mM PMSF and 1 µM ubiquitin aldehyde and centrifuged at 15,000 g for 15 min at 4°C. 1 mg lysate was subjected to immunoprecipitation followed by immunoblotting as described in sections 2.2.8 and 2.2.6 respectively.

2.2.10 Quantitative real time-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from one confluent well of a 6-well plate using Tri Reagent following the manufacturer's instructions. Briefly, cells grown on a 6-well plate (Corning) monolayer were washed twice with PBS and disrupted by adding 1 ml TRI reagent. Samples were allowed to stand for 5 minutes at room temperature prior to the addition of 0.2 ml chloroform. Samples were mixed by shaking and centrifuged at 15110 g (Hettich Mikro 200K) for 15 minutes at 4°C. The aqueous phase was

transferred to a new tube and 0.5 ml isopropanol added for 10 minutes to precipitate RNA. Samples were centrifuged at 12000 g for 10 minutes, the supernatant discarded and the pellet washed with 75% ethanol. After air-drying, the pellet was resuspended in PCR graded water. Concentration of RNA was measured by a Nanodrop spectrophotometer. 2 µg of RNA were treated with deoxyribonuclease I (DNase I) to remove any traces of genomic DNA in a final volume of 10 µl. First-strand cDNA was synthesised from total RNA using oligo(dT) (oligonucleotide deoxy-thymine) primers and Superscript II reverse transcriptase following the manufacturer's indications. Final cDNA was obtained in a volume of 20 µl, diluted 1/10 and stored at -20°C until use. PCR was carried out in an Opticon 2 thermal cycler (Bio-Rad) with the following thermal cycling conditions: initial denaturing at 94°C for 2 minutes followed of 39 cycles of 95°C for 15 seconds, annealing at 60°C for 30 seconds, extending at 72°C for 30 seconds and melting curve of 60°C to 95°C with readings every 0.5°C. Gene expression was normalized using the housekeeping genes GAPDH and HPRT as endogenous controls. Fold differences were calculated using the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001; Bustin and Mueller 2005). For a list of primers used see appendix Table A1.

Chapter 3 TRF2 expression during neural differentiation of hESCs

3.1 Introduction

TRF2 is a well-known key component of the shelterin complex which protects chromosome ends, regulates telomere length and, ultimately, maintains genome stability (Smogorzewska *et al.*, 2000; Bradshaw *et al.*, 2005; Palm and de Lange, 2008). However, in addition to these functions TRF2 has been shown to interact with a wide range of non-telomeric proteins, acting as a protein hub and thus raising the possibility that it may serve functions independent of telomere maintenance. Despite its importance in many physiological and pathological functions, there is little information about how TRF2 is expressed during development and whether it plays an extratelomeric role in this process. One of the reasons for this lack of information is the unavailability of early developmental materials. Moreover, TRF2 knockout in mice causes early embryonic death which restricts further interrogation of TRF2 functions during development (Celli and de Lange, 2005). hESCs provide a good solution to overcome this problem since they are capable of prolonged growth in culture whilst retaining their capacity to differentiate into most, if not all, tissues in the adult body. Additionally, many studies have demonstrated that differentiation of hESCs into specific cell types recapitulates, to a large extent, the normal development of those cells *in vivo* (Gerrard *et al.*, 2005; Hay *et al.*, 2008; Zhang *et al.*, 2010; Lancaster *et al.*, 2013). Given that hESCs have the ability to mimic development *in vivo* and that TRF2 protein is very abundant in the human brain (Jung *et al.*, 2004), this study was particularly focused on the differentiation of hESCs to neural progenitor cells, neurons and glia.

The aim of this chapter is to characterise the expression of TRF2 during the neural differentiation of hESCs to ascertain its abundance and specificity to this lineage. TRF1 expression was also characterised given its close structural and functional resemblance to TRF2.

3.2 Results

3.2.1 *TRF2 protein levels are upregulated upon differentiation of hESCs to NPCs.*

In order to explore the role of TRF1 and TRF2 during hESC neural differentiation, hESCs from both H1 and H7 lines were differentiated into their neural derivatives using our laboratory established protocol (Gerrard *et al.*, 2005). Neural differentiation was prompted by culturing the hESCs with N2B27 medium supplemented with BMP antagonist noggin. Following formation of NPCs (~passage 4) noggin was replaced with bFGF and NPCs were able to grow in this condition for over several months (Wu *et al.*, 2010). RNA and protein were extracted at various passages and analysed for the expression of TRF2.

First, the mRNA expression of *TRF1* and *TRF2* was analysed in hESCs and their NPC derivatives. *TRF1* mRNA was found being highly expressed in hESCs but was radically reduced following neural differentiation and maintained at low levels throughout the NPC culture, whereas the expression of *TRF2* transcripts did not exhibit an obvious pattern of change during neural differentiation (Figure 3.1 A). These results were consistent with our lab previous genome-wide RNA sequencing results (Wu *et al.*, 2010) and published data (Miura *et al.*, 2004; Skottman *et al.*, 2005), indicating that *TRF1* and *TRF2* transcription is controlled by different mechanisms.

In contrast to the mRNA levels, TRF1 protein did not show a considerable reduction upon neural differentiation. More surprisingly, in both H1 and H7 hESC lines, TRF2 protein levels were low in hESCs, but dramatically increased following neural differentiation and were maintained at these elevated levels through the culture of NPCs (Figure 3.1 B). TRF2, upon prolonged SDS-polyacrylamide gel electrophoresis (PAGE) separation, was detected as two bands of 65 and 69 kDa as previously reported (Bilaud *et al.*, 1997; Broccoli *et al.*, 1997). The discrepancy between mRNA and protein levels of TRF1 and TRF2 indicates that the expression of both proteins may be regulated at post-transcriptional levels, which is in line with previous findings (Chang *et al.*, 2003; Fujita *et al.*, 2010).

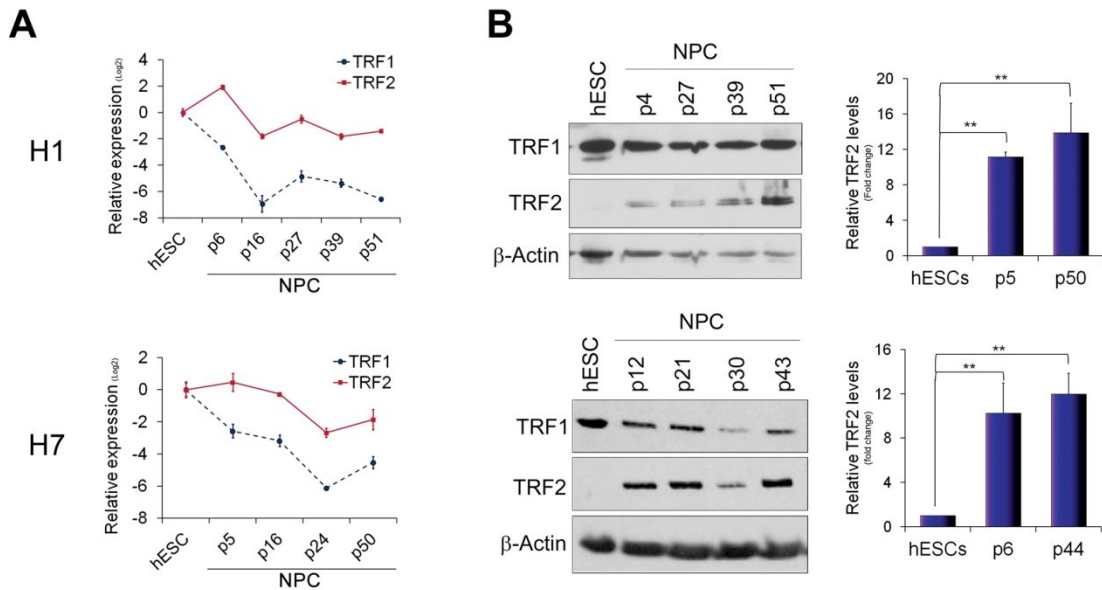


Figure 3.1 Expression of TRF1 and TRF2 in hESCs and their neural derivatives at both mRNA and protein levels.

(A) Expression of *TRF1* and *TRF2* mRNAs by qRT-PCR during the neural differentiation of H1 (top panel) and H7 hESCs (bottom panel) and subsequent culture of their progenitors. Passage numbers post-differentiation are indicated. Data are presented as mean $\pm$ SD of 6-9 PCRs from three independent experiments. (B) Immunoblotting showing TRF1 and TRF2 protein levels during the neural differentiation of H1 hESCs (top panel) and H7 hESCs (bottom panel). Left panels are representative images; right panels are from the densitometry analysis of the protein levels as mean $\pm$ SD of three experiments. Representative bottom panel immunoblot image was generated and provided by Miss Sarah Testori (Imperial College London). Statistical significance was tested by paired Student's t-test, ** indicates $P < 0.005$.

Given the importance of TRF2 in genomic stability, its abundance was further analysed using immunocytochemistry with specific antibodies in H1/H7 hESCs and their neural derivatives. TRF2 protein levels in undifferentiated Oct4 positive hESCs was much lower than that observed in Sox1 positive NPCs (Figure 3.2). These results further supported the previous finding (Figure 3.1 B). In addition, they indicated that the hESCs and NPCs analysed by immunoblotting were a relatively homogenous population of undifferentiated cells as shown by the abundance of Oct4 and Sox1 positive cells, respectively (Figure 3.2). Quantification of Sox1 or Oct4 positive cells was not carried out in this occasion as our laboratory routinely maintains and differentiates hESCs to NPCs with an efficiency greater than 95% as previously

reported (Gerrard *et al.*, 2005). Following the analysis of H1 and H7 hESCs and their NPC derivatives via western blotting and quantitative real-time PCR (Figure 3.1) all subsequent experiments were carried out in H1 hESCs and their derivatives unless otherwise stated in the text.

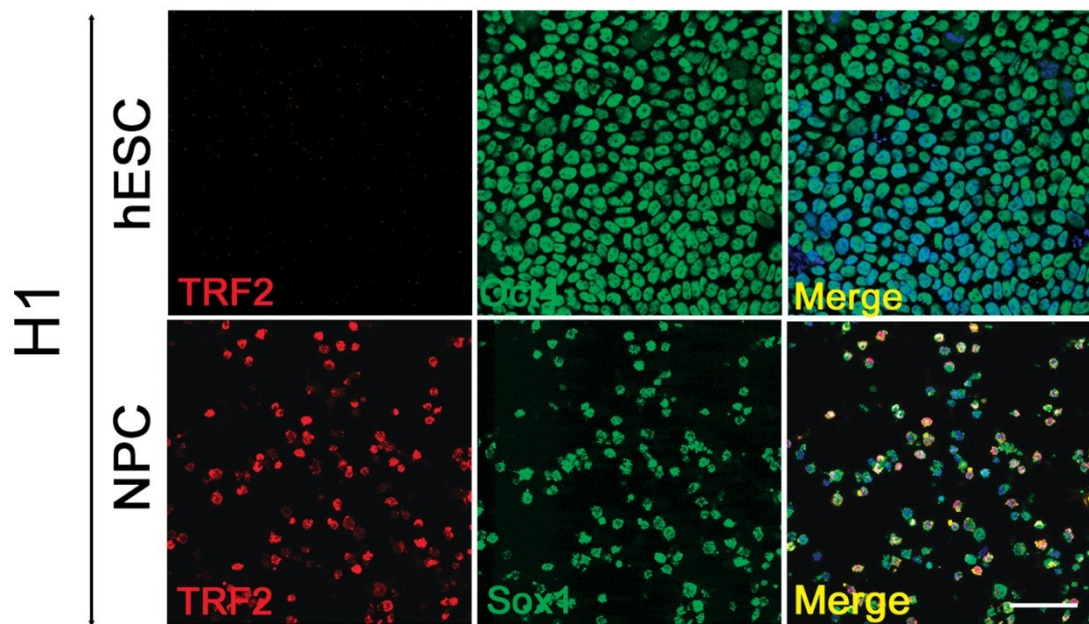


Figure 3.2 TRF2 is differentially expressed in NPCs and hESCs.

Representative IF images of H1 hESCs and H1 hESCs-derived NPCs using antibodies against TRF2, Oct4 and Sox1. Data shows considerably higher TRF2 expression in Sox1 positive NPCs (lower panels) compared to Oct4 positive hESCs (upper panels). Nuclei are shown by DAPI staining (blue). Scale bar = 50 μ m. Data shown is representative of two experiments.

Although TRF2 proteins were predominantly located in cell nuclei of the NPCs as they are in hESCs (Figure 3.3 A), they appeared not to be restricted solely to the telomeres (Figure 3.3 B). Since TRF2 is well known to bind to telomeres, its expected immunocytochemistry pattern of localization is punctuated (Takai *et al.*, 2010). While hESCs showed the expected pattern of TRF2 localization, NPCs exhibited localization throughout the nuclei. This suggested that in NPCs TRF2 proteins were potentially binding to non-telomeric regions of DNA or interacting with non-telomeric proteins, raising the possibility of extra-telomeric roles.

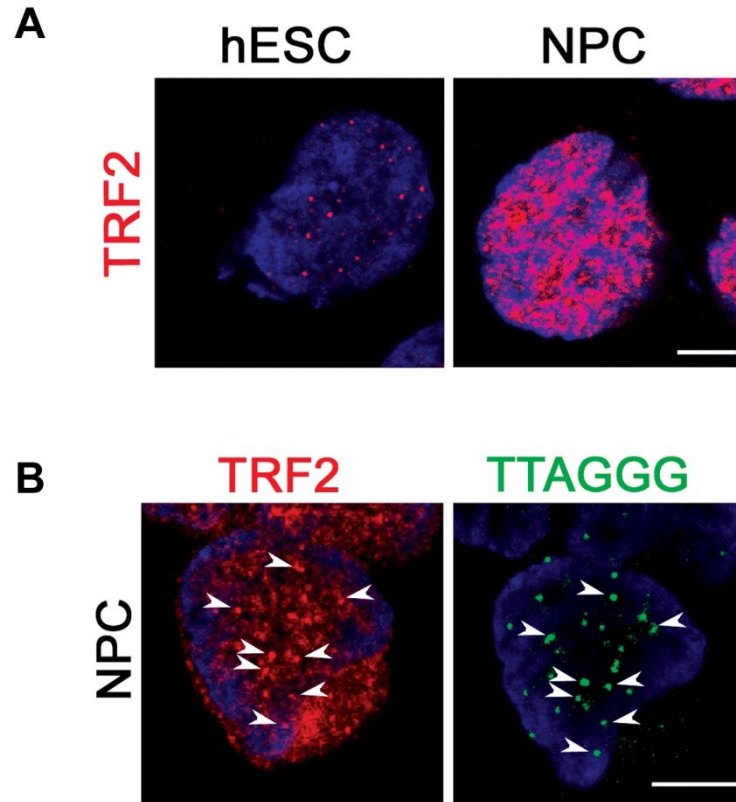


Figure 3.3 Localization of the telomere binding protein TRF2 in hESCs and NPCs.

(A) TRF2 IF of H1 hESCs and H1 hESC-derived NPCs. Scale bar = 5 μ m. Data shown is representative of the independent experiments. **(B)** Immuno-FISH double staining of TRF2 and telomeres in H1 NPCs. TRF2 protein was stained by TRF2 antibody (left) and telomeres by telo-FISH in NPCs (right). Only a proportion of TRF2 signals show co-localization with telomeres (arrowheads). Scale bar = 5 μ m. Data is representative of two independent experiments. Nuclei are shown by DAPI staining (blue).

Overall these results indicate that whilst TRF1 protein levels are relatively similar between hESC and NPCs at various stages of the neural differentiation process, there is in contrast, a considerable upregulation of TRF2 protein. Additionally TRF2 appears to be abundantly distributed throughout the nuclei of the NPCs rather than being confined to the telomeres only, which suggests a potential extra-telomeric role.

3.2.2 Differential TRF2 protein levels are specific to the neural lineage

Since TRF2 exhibited a more distinctive change during the neural differentiation of hESCs, subsequent studies were focused on TRF2. We asked whether TRF2 upregulation is a general phenomenon in hESC differentiation or is more restricted to neural lineages. To address this question, hESCs were differentiated via EB formation to the three germ layers. Differentiation of hESCs by EB formation generates cells of all three germ layers (Itskovitz-Eldor *et al.*, 2000) while losing the hESC characteristics, such as downregulation of Oct4 expression, a pluripotent marker.

Following 14 days in EB differentiation medium, no Oct4 positive cells were detected, indicating that these cells had been differentiated (Figure 3.4, top panel). High levels of TRF2 protein were mainly detected in Sox2 positive cells (Figure 3.4, middle panels) but not in HNF4 α (endoderm marker) positive cells (Figure 3.4, lower panel), showing its specificity in neural progenitors.

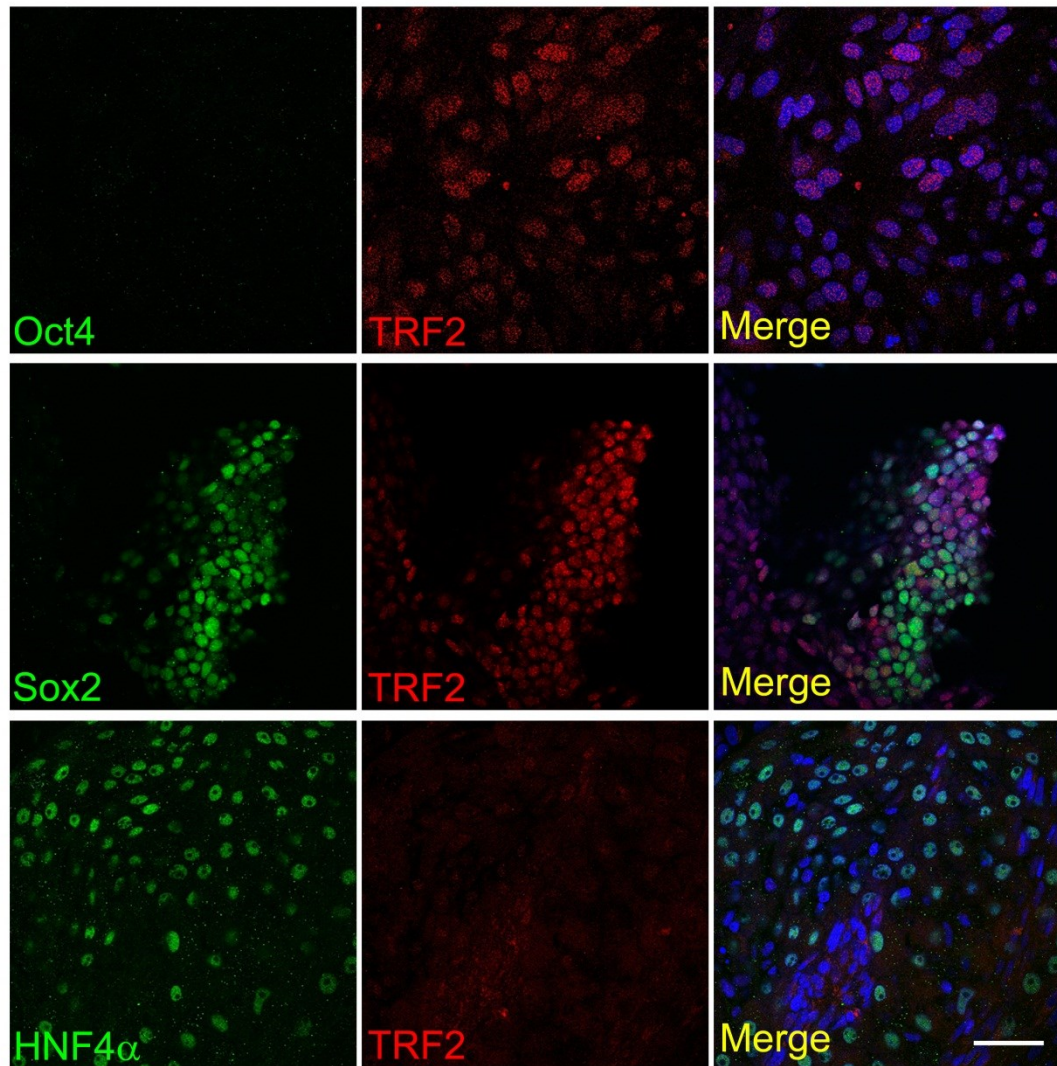


Figure 3.4 TRF2 differential expression is restricted to the neural lineage.

H1 hESCs were differentiated through embryoid body (EB) formation for 14 days and immunostained with TRF2 and indicated antibodies. Nuclei are shown by DAPI staining (blue). Scale bar represents 50 μ m. Images are representative of a single experiment carried out in duplicate.

To further corroborate these findings, hESCs were specifically differentiated to neural, hepatic or fibroblast lineages where the majority of the cells displayed their lineage-specific morphology and gene expression (Figure 3.5 A-B). The differentiated fibroblasts (FC) did not express any hESC pluripotent markers such as *Nanog*, neither markers of the ectoderm or endoderm lineage such as *Sox1* and *Sox17* respectively. On the other hand, undifferentiated H1 hESCs expressed high levels of *Nanog*, while their NPC and hepatoblast cell (HPC) derivatives expressed high levels

of *Sox1* and *Sox17* respectively. At the protein level, only NPCs expressed high levels of TRF2 protein (Figure 3.5 C).

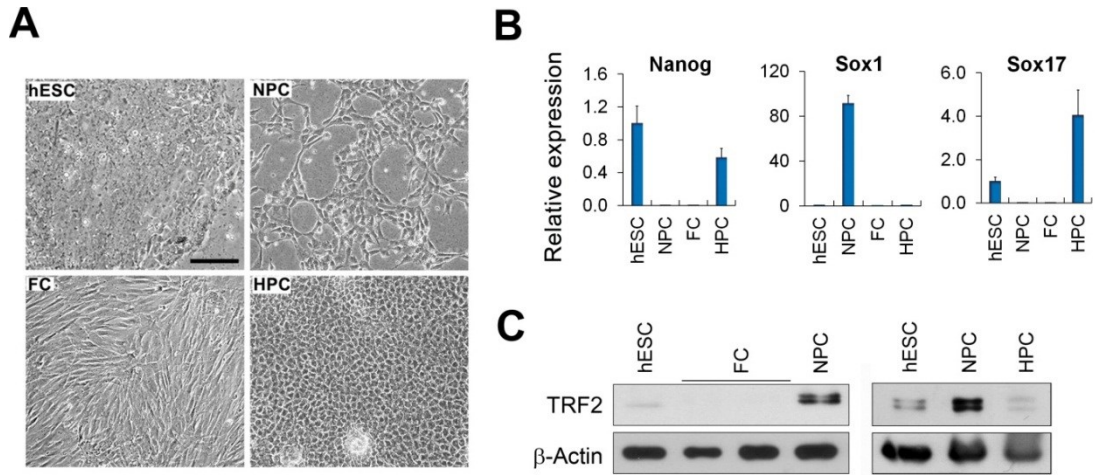


Figure 3.5 Comparison of TRF2 expression in hESCs derived NPCs, fibroblast and hepatoblasts.

(A) Phase-contrast images of H1 hESCs and their derived progenies: NPCs, FC, fibroblast cells; HPC, hepatocyte progenitor cells. Scale bars represent 50 μ m. (B) Expression of lineage-specific marker genes in the cells of (A) by qRT-PCR. Data is presented as mean $\pm$ SD of 6 RT-PCRs from three independent experiments. (C) TRF2 protein levels in the cells of (A) by immunoblotting. Images are representative of a single experiment produced in duplicate.

In order to explore whether TRF2 upregulation also happens during neural differentiation of mouse ESCs (mESCs), TRF2 protein levels in mESCs and mouse NPCs (mNPCs) were analysed. Interestingly, TRF2 protein was found at lower levels in mESCs than in hESCs and did not exhibit any upregulation upon neural differentiation (Figure 3.6 A). Therefore, TRF2 levels are much lower in mouse NPCs than in human NPCs, highlighting yet another difference between human and mouse biology.

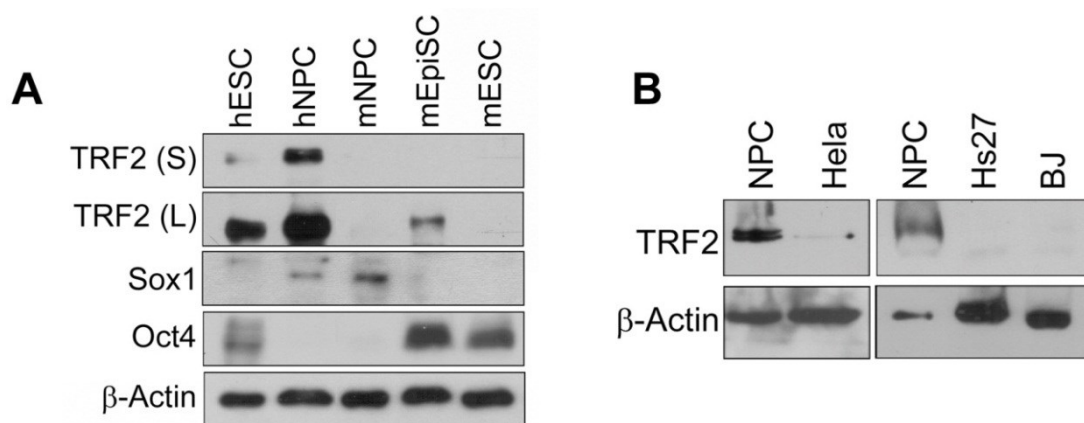


Figure 3.6 TRF2 protein levels in mouse stem cells and other cell types.

(A) Comparison of TRF2 protein abundance between mESCs, mouse epiblast stem cells (mEpiSC), H1 hESCs and their respective NPC derivatives as indicated by immunoblotting, showing that TRF2 protein levels are not upregulated upon neural differentiation of mESCs. TRF2 (S) and TRF2 (L) indicate short and long exposure times to the x-ray film. Images are representative of two independent experiments. (B) High levels of TRF2 protein as observed in human NPCs, were not detected in other cell lines. Western blotting of HeLa cancer cells, Hs27 foreskin fibroblasts and BJ5ta immortalised fibroblasts revealed no dramatic increases of TRF2. Images are representative of two independent experiments.

In addition, the abundance of TRF2 protein in other cell types including a cancer cell line (HeLa), human foreskin fibroblasts (Hs27) and an immortalised human skin fibroblasts cell line (BJ5ta) was also analysed. Abundance of TRF2 protein levels was still restricted to human NPCs as the levels of TRF2 protein in HeLa, Hs27 and BJ5ta cells was almost undetectable by western blotting (Figure 3.6 B).

To address whether the abundance of TRF2 protein was specific to human NPCs or if it was also present in their derived post-mitotic neurons or glia, early and late NPCs were further differentiated into neurons and glia by withdrawal of growth factors. 14 days following differentiation, the high levels of TRF2 protein present in early and late NPCs were downregulated to levels similar to that of hESCs. Co-IF of TRF2 and the neural marker Tuj1 in NPCs, with and without growth factors, revealed that the high levels of TRF2 protein were only present in NPCs where protein levels of Tuj1 were low (Figure 3.7 A, upper panel). This was further supported by immunoblotting (Figure 3.7 A, lower panel). Similarly, co-staining of late NPCs with TRF2 and the glia marker GFAP showed a dramatic decrease of TRF2 protein

following differentiation into glia (Figure 3.7 B, upper panel). TRF2 downregulation in late NPCs was also supported by TRF2 immunoblotting (Figure 3.7 B, lower panel).

Taken together, these results demonstrate that TRF2 protein expression is dynamically regulated during neural differentiation and neuron/glia formation with higher levels restricted to human NPCs.

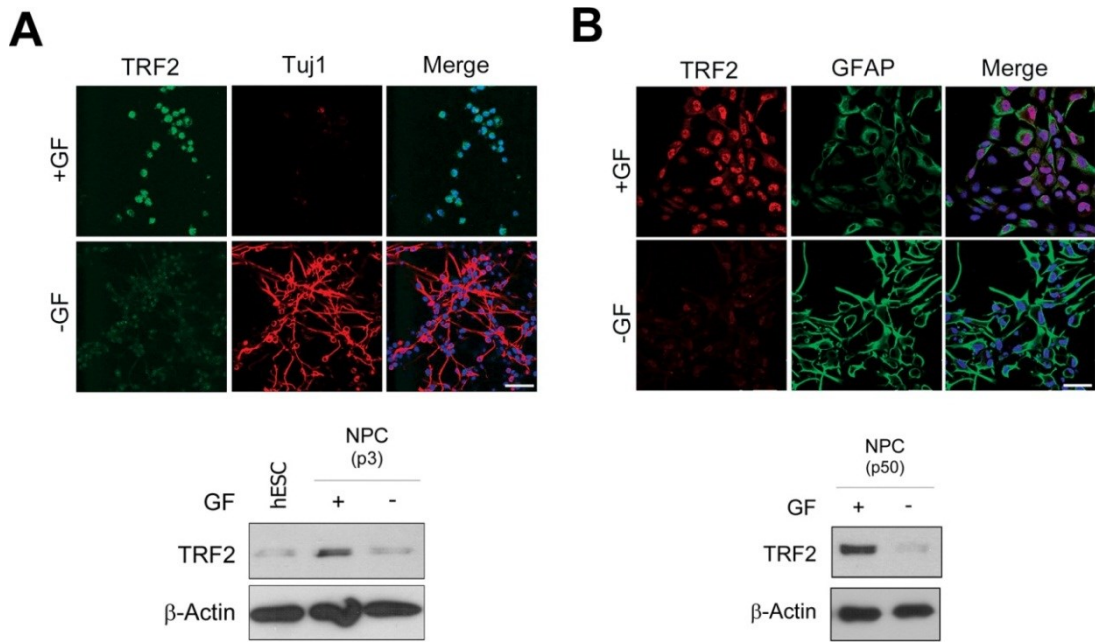


Figure 3.7 TRF2 protein levels are downregulated following differentiation of human NPCs into post-mitotic neurons and glia.

H1 hESC-derived NPCs from early (**A**) and late (**B**) neural differentiation were further differentiated for 14 days by the withdrawal of bFGF/EGF growth factors (GF). Figure shows IF of the resulting cells with the indicated antibodies (upper panel) and immunoblotting of TRF2 (lower panel). Nuclei are shown by DAPI staining (blue). Scale bar = 50 μ m in all microscopic images. IF images of (A) and B are representative of a single experiment carried out in triplicate while immunoblotting images are representative of a single experiment.

3.2.3 TRF2 protein levels are not dependent on telomere length

A previous study in our group demonstrated that hTERT was dynamically expressed during H1 and H7 hESC neural differentiation and during the culture and differentiation of their NPC derivatives (Sheldon *et al.*, 2013 under revision). hTERT was highly expressed in undifferentiated hESCs but dramatically downregulated upon neural differentiation of both H1 and H7 hESC lines. However, during extended culture of NPCs, *hTERT* expression was upregulated again, accompanied by the activation of functional telomerase. The reactivation of telomerase was neither associated with a transformed phenotype nor affected the characteristics of NPCs (Sheldon *et al.*, 2013 under revision).

Since TRF2 has been reported to regulate telomere length in tumour cell lines and was suggested to act as a measuring unit of telomere length (Smogorzewska *et al.*, 2000; Palm and de Lange, 2008), we speculated whether the differential expression of TRF2 detected in both H1 and H7 hESCs derived NPCs was related to the changes in telomere length previously observed in our lab. To answer this question, the telomere length of H1 hESCs and their NPC derivatives was analysed by southern blotting. Repeated telomere length assays indicated that hESCs have long stable telomeres of approximately 10 to 12 Kb in length (Figure 3.8 B). Upon neural differentiation, the high expression of telomerase observed in hESCs is dramatically downregulated and consequently telomeres shortened with cell proliferation (Figure 3.8 A-B). Quantitative RT-PCR analyses showed that *hTERT* was indeed expressed at high levels in undifferentiated hESCs and was downregulated after neural differentiation (Figure 3.8 A). However, with extended culture, *hTERT* expression became upregulated again to levels similar to that of hESCs (Figure 3.8 A) as previously observed in our group (Sheldon *et al.*, 2013 under revision). Concurrently, *hTERT* expression reactivation was also followed by gradual telomere elongation (Figure 3.8 B) to a relatively stabilised length which was in line with the expression of *hTERT*. These dynamic changes of telomere length were notably different from the levels of TRF2 protein (Figure 3.1 B vs Figure 3.8 B).

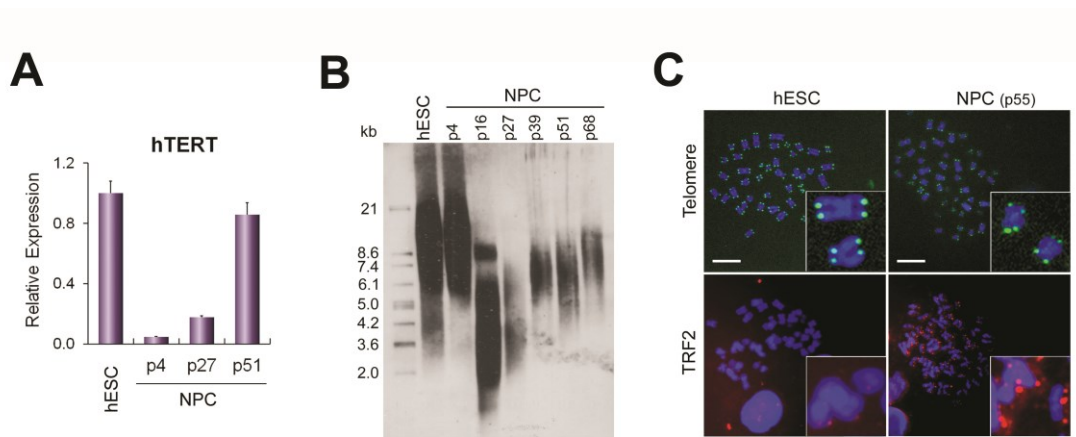


Figure 3.8 No correlation between TRF2 protein levels and telomere length or telomerase expression in NPCs.

(A) Expression of telomerase catalytic subunit *hTERT* mRNA in H1 hESCs and their neural progenitors by qRT-PCR. Data is presented as mean $\pm$ SD of three experiments. (B) Dynamic changes of telomere length in H1 hESCs and their NPC derivatives by southern blotting. Image is representative of two independent experiments. (C) Comparison of telomere length (upper panel, by telomere FITC-FISH) and TRF2 protein accumulation at the telomeres (lower panel, by IF) in hESCs and their neural progenitor derivatives (p55). Inserts are the same images but with a higher magnification. Images are representative from the analysis of 15 chromosomal spreads of two independent experiments. Scale bars= 10 μ M.

Comparative analysis of telomere length and protein levels of TRF2 in hESCs and their NPCs derivatives (Figure 3.1 B vs Figure 3.8 B) revealed no correlation. TRF2 protein expression was low in hESCs, which have long telomeres. However, in early differentiated NPCs at passage 4 (p4), which have similar telomere length as hESCs, TRF2 was dramatically upregulated. Furthermore, while telomeres were significantly shorter in p27 NPCs, and re-lengthened to longer length at p50, TRF2 protein levels remained at relative stable higher levels. Similarly, TRF1 protein expression did not seem to correlate with telomere length either as TRF1 protein levels did not change dramatically upon shortening or re-lengthening of the telomeres (Figure 3.1 B vs Figure 3.8 B). Taken together, these results suggest that TRF2 protein levels may not be dependent on telomere length as previously suggested (Smogorzewska *et al.*, 2000).

Although the telomere length assays and western blotting data suggested that TRF2 protein expression did not correlate with telomeres length in these cells, it was not clear whether the TRF2 protein accumulated in NPCs with shorter telomeres was telomere bound or not (Figure 3.3 B). In order to address this TRF2 IF and telomere FISH were carried out, in parallel, in hESCs and p55 NPCs chromosomal spreads (Figure 3.8 C). Telomere length was assessed by examining the intensity of the fluorescein isothiocyanate (FITC) telomere probe signal, where longer telomeres bind more probe and therefore emit a higher intensity signal. While hESCs and late NPCs (p55) revealed similar telomere lengths (Figure 3.8 C top panels), it appeared that NPCs had more TRF2 bound to their telomeres than hESCs (Figure 3.8C lower panels). These results reflect those obtained by TRF assay, where longer telomeres do not necessarily accumulate more TRF2 protein. Over 15 chromosomal spreads from different preparations were examined and similar staining patterns were observed.

These results have demonstrated that TRF1 and TRF2 protein levels may be independent of telomere length and telomerase levels. In addition, they also indicate that the sustained growth and proliferation of hESC-derived NPCs may be attributed to the reactivation of telomerase.

3.3 Discussion

In this chapter, the expression of TRF2 was characterised in H1/H7 hESCs, their neural derivatives and other cell types. In the course of this characterisation, it was found that TRF2 is differentially expressed during neural differentiation and that this differential expression may be specific to the neural lineage. Furthermore, we found that the mRNA expression of TRF1 and TRF2 did not correlate with their protein levels, suggesting that they may be regulated post-transcriptionally.

The expression of TRF1 and TRF2 protein observed during neural differentiation did not correspond with that of mRNA levels, suggesting that TRF1 and TRF2 may be regulated at the post-transcriptional level. Indeed, TRF1 stability and localisation to telomeres is regulated by several post-translational modifications. In human cells, TRF1 is poly ADP-ribosylated by tankirase-1, a poly(ADP-ribose) polymerase, resulting in TRF1 depletion from telomeres. Following depletion, unbound TRF1 is ubiquitinated for proteosomal degradation by Fbx4, an F-box protein that functions as substrate for ubiquitin E3 ligases (Lee *et al.*, 2006). However, when bound to the telomere, TIN2 stabilises TRF1 by protecting it from poly (ADP-ribosyl)ation by tankirase 1 and by sequestering its degradation motif from Fbx4 (Zeng *et al.*, 2006). In addition, Pin1 has been identified as another factor being able to deplete TRF1 from telomeres and to promote its degradation (Lee *et al.*, 2009). As for TRF2, a recent study in fibroblasts (Fujita *et al.*, 2010), has reported that TRF2 can be ubiquitinated for degradation by Siah1 (a p53-inducible E3 ubiquitin ligase) which is induced by activated p53 upon critical shortening of the telomeres. Upregulation of Siah1 results in a degradation of TRF2 protein while *TRF2* mRNA expression remains unchanged, explaining the differences in TRF2 mRNA and protein expression. However, these reported post-translational regulations of TRF1 and TRF2 are telomere length dependent and in our experiments the protein levels of TRF1 and TRF2 did not seem to correlate with telomere length. H1 hESCs which have long and stable telomeres had low levels of TRF2 protein while their derivative NPCs had high levels of TRF2 protein regardless whether their telomeres were short or long. Furthermore, TRF1 protein levels did not seem to change dramatically upon telomere shortening or re-lengthening. These results prompt two questions: are

the TRF2 protein levels observed in hESCs enough to ensure t-loop formation? And why shorter telomeres accumulate equal or more protein levels of TRF1 or TRF2? Regarding the low levels of TRF2 protein in hESCs, a recent publication has indicated that you only require few molecules of TRF2 at the telomere to protect it from DDR activation (Cesare *et al.*, 2013), explaining why our hESCs show such low levels of TRF2 protein. As for the high levels of TRF1 or TRF2 protein in NPCs with short telomeres, we propose that since TRF2 is mainly responsible for the formation of telomere t-loop structure and TRF1 aids in this function, it is possible that some cell types have high levels of TRF1 and TRF2 protein to ensure proper t-loop formation upon telomere shortening. In fact, a study by (Karlseder *et al.*, 2004) reported that upon overexpression of TRF2 in cells with short telomeres, increased protection of the telomeres was observed. Since TRF1 and, TRF2, specially, are crucial for the formation of the t-loop, it is possible that during hESC differentiation to NPCs maintenance of their protein levels upon telomere shortening occurs as a as an effort to maintain t-loop structure, avoiding DNA damage response and, ultimately, cell apoptosis. This could suggest that TRF1 and TRF2 are regulated posttranscriptionally by a telomere length independent pathway.

It is important to note that while western blotting and southern blotting may be good assays to measure protein and telomere length, they may not be specific and sensitive enough to detect changes at the telomere level and therefore to address if TRF1 and TRF2 proteins are telomere length dependent in our cell types we would need to use more sensitive techniques such as quantitative FISH or telomere qRT-PCR.

Differentiation of H1 and H7 hESCs to their NPC derivatives resulted in a dramatic increase of TRF2 protein expression while TRF1 expression remained to similar levels. Remarkably, the IF of TRF2 in NPCs showed large quantities of TRF2 protein that were not restricted to the telomeres (Figure 3.3 B). This was surprising as immunostaining of shelterin members is well known for revealing a punctuate pattern of localisation that corresponds to the telomeres (Li and de Lange, 2003; Ye *et al.*, 2004b; Hockemeyer *et al.*, 2007). The pattern of protein localization shown in NPCs indicated that TRF2 could potentially be located elsewhere in the nuclei of these cells, perhaps carrying out functions independent of telomere protection and regulation as it has been suggested in several reports (Bradshaw *et al.*, 2005; Yang *et*

al., 2011; Chen *et al.*, 2008; Zhang *et al.*, 2008; Kim *et al.*, 2009; Giannone *et al.*, 2010; Simonet *et al.*, 2011).

Differentiation of hESCs via embryo body formation and direct differentiation revealed no increase of TRF2 protein levels in other lineages. Furthermore, differentiation of NPCs to neurons and glia resulted in a downregulation of TRF2 protein.. These results suggest that the differential expression of TRF2 may be specific to the neural lineage and moreover to NPCs. However, a previous report has shown that TRF2 was also expressed in the cytoplasm of terminally differentiated neural cells (Jung *et al.*, 2004). While this data may seem contradictory to ours, a later report has shown that some mature neurons can express a truncated ~35 KDa version of TRF2, termed TRF2 short (TRF2-s), in their cytoplasm (Zhang *et al.*, 2011). Since we only detect a single 66 KDa TRF2 band by immunoblot (see appendix Figure A1) and we have shown by immunofluorescence that TRF2 is present only in the nuclei of hESCs and NPCs (Figure 3.3), it is very likely that the TRF2 cytoplasm staining detected in neurons by Jung and colleagues is in fact TRF2-s and not full length TRF2. While these findings could benefit from further characterisation, specially the embryoid IF data which requires more lineage specific markers, these findings are consistent with previous reports in the sense that they suggest that TRF2 may have an extratelomeric role in neural differentiation (Jung *et al.*, 2004; Zhang *et al.*, 2008; Simonet *et al.*, 2011). Thus, the observed nontelomeric localization and specific differential expression of TRF2 in NPCs raised the possibility that TRF2 could have a role in neural differentiation.

Given the specific and differential TRF2 protein expression observed upon the differentiation of H1 and H7 hESCs to NPCs, this protein was investigated with more attention. Interestingly, a recent study has found that TRF2 transcription is activated by the Wnt/ β -catenin signalling pathway (Diala *et al.*, 2013). This study revealed that TRF2 contains six putative binding sites of the TCF-LEF family of transcription factors. Furthermore they demonstrated that activation of the canonical Wnt signalling pathway triggers the translocation of β -catenin to the nucleus where it subsequently binds to members of the TCF-LEF family of transcription factors which ultimately activate gene expression of TRF2. Given that it has been shown that Wnt signalling is sufficient to extinguish BMP4 mRNA expression (Baker *et al.*, 1999), it

is possible that during neural differentiation of hESCs the Wnt signalling pathway is activated to help inhibit BMP signalling and induce neural differentiation, resulting in increased expression of TRF2 which could then be post-translationally regulated by the telomere dependent Siah1-p53 pathway or an alternative unknown telomere independent pathway.

Chapter 4 TRF2 induces differentiation of hESCs to the neural lineage and regulates their ability to produce functional NPCs

4.1 Introduction

In chapter 3, the expression of telomere binding proteins TRF1 and TRF2 during the differentiation of hESCs to the neural lineage was analysed. It was found that in contrast to TRF1, TRF2 protein expression was dramatically upregulated upon differentiation to NPCs. Surprisingly this differential expression was specific to NPCs, not restricted to telomeres and independent of telomere length. Since only TRF2 showed significantly dynamic changes in this process, the subsequent investigations were focused on TRF2. Given its abundance in the human brain (*Jung et al., 2004*) and its dramatic protein increase upon differentiation of hESCs to NPCs, TRF2 may indeed play a role in the neural differentiation process. In this context, it has been previously reported that overexpression of TRF2 in COS cells induces neurite-like processes (*Jung et al., 2004*). However, another study has shown that inhibition of TRF2 promotes neural differentiation in NTERA2 human embryonic carcinoma cells (*Zhang et al., 2008*). It is unclear whether the discrepancies are attributable to the differences in the two cell types used in these studies. COS cells are a fibroblast-like immortalised cell line derived from monkey kidney tissue and as such they are not derived from human tissues nor have the same potential to differentiate to the neural lineage as a human pluripotent stem cell line would. NTERA2 cells are a human carcinoma stem cell line derived from a malignant testicular cancer and as such may not reflect the normal neural development situation *in vivo*. Nonetheless, these two cell types are unlikely to reflect normal human embryonic development. Given the contradictory results of the two studies, it is necessary to investigate this issue in a cell model system that is closer to normal embryonic development; hESCs provide a good cell source for such studies.

The aim of this chapter is to use gain or loss of TRF2 function approaches to investigate whether modification of TRF2 expression affects the self-renewal of hESCs and their ability to differentiate to the neural lineage. I hypothesised that overexpression of TRF2 in the low TRF2 expressing hESCs would induce their differentiation towards NPCs and that inhibition of TRF2 in hESCs may hinder their neural differentiation to NPCs.

4.2 Results

4.2.1 TRF2 overexpression in hESCs compromises their self-renewal and induces expression of neural markers

Since TRF2 is significantly upregulated during neural differentiation of hESCs, we speculated that TRF2 may play a role in this process. Gain- and loss- of function approaches were applied to address this question, whereby TRF2 was ectopically expressed in H1 hESCs by lentiviral transduction in the first instance (Figure 4.1 A). The lentivector that gave rise to the lentiviral particles was generated from pLVTHM backbone, in which *PuroR* (puromycin resistant gene) and *TRF2* cDNAs were linked with a 2A foot and mouth disease virus sequence. This allows the expression of *PuroR* and *TRF2* to be controlled by the same promoter and translated as one fusion protein first, then cleaved at the 2A peptide to produce two functional proteins. This approach ensures that cells resistant to puromycin treatment definitely express TRF2. In addition, the elongation factor-1 alpha (EF1 α) promoter was replaced in the original vector with a cytomegalovirus (CAG) enhanced promoter to maximise the transgenic expression levels. Same lentivector containing *PuroR* cDNA only was used as the control. Efficiency of transduction of hESCs was assessed using original pLVTHM containing GFP cDNA (appendix, Figure A2). After optimisation of the transduction efficiency, hESCs transduced with control or TRF2 overexpressing (TRF2-Ov) lentivirus were selected with puromycin in hESC CM culture conditions. Following 2 weeks in selection, several colonies in the TRF2-Ov plates revealed neural rosette-like structures (Figure 4.1 B, top right panel), which became more evident upon continuous culture with the appearance of bipolar neural progenitors (Figure 4.1 B, bottom right panel). By contrast, no such colony was found in the controls (Figure 4.1 B, top and bottom left panels). These phenotypic changes were reproducible in repeated experiments.

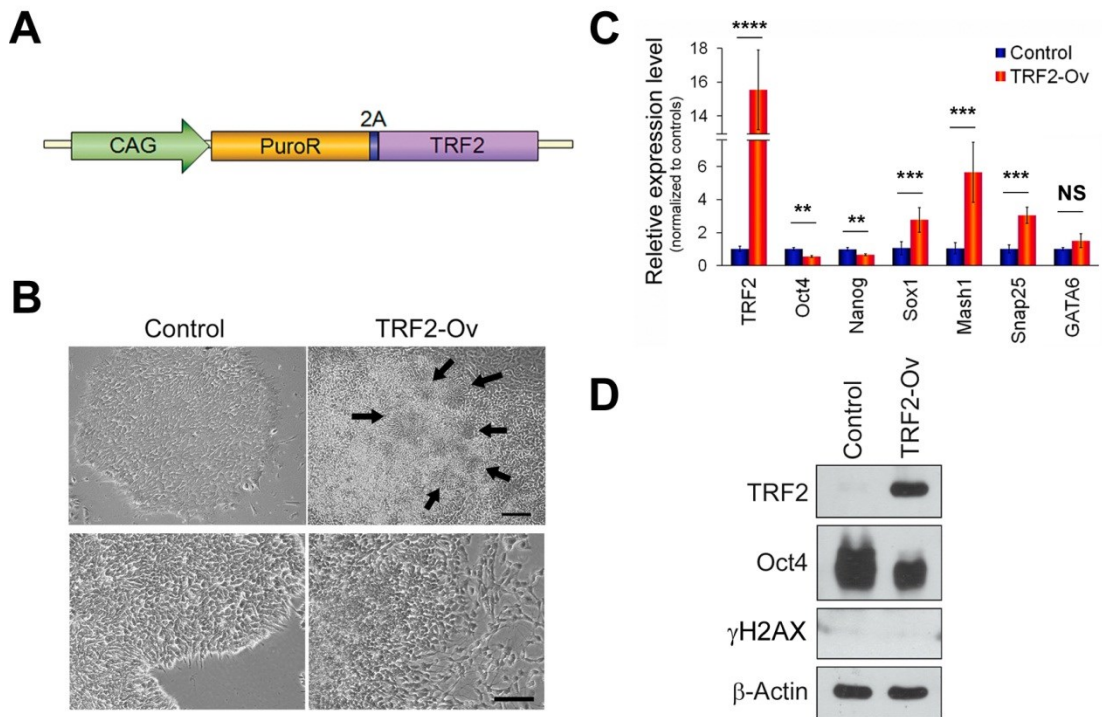


Figure 4.1 TRF2-overexpression in hESCs induces neural differentiation.

(A) Schematic map of TRF2-expressing lentiviral vector. CAG, CAG promoter; PuroR, puromycin resistant gene; 2A, 2A foot and mouth disease virus sequence; TRF2, TRF2 cDNA. (B) Representative phase-contrast images of control and TRF2-overexpressing hESCs 2 passages post-infection. Arrows point to neural rosette-like structures. Scale bar = 100 μ m. Images are representative of two independent experiments. (C) TRF2 overexpression in hESCs mainly increased the expression of neural marker genes but reduced the expression of pluripotent genes as detected by qRT-PCR in cells 5 passages after infection. Data are presented as mean $\pm$ SD of 6 repeats of 2 independent experiments. Statistical significance was tested by paired Student's t-test, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, NS $p > 0.05$ (non-significant). (D) Immunoblot showing indicated protein expression in control and TRF2-overexpressing hESCs 5 passages after infection. Images are representative of two independent experiments.

Successful TRF2 overexpression via lentiviral infection resulted in a dramatic increase of TRF2 expression compared to control, as determined by qRT-PCR and western blotting (Figure 4.1 C and D). Analysis of gene expression in TRF2-Ov hESCs revealed a downregulation of pluripotent markers, *Oct4* and *Nanog*, and an upregulation of neural markers *Sox1*, *Mash1* and *Snap25* (Figure 4.1 C). No significant change of expression was observed for the endoderm marker *Gata6* (Figure 4.1C). Expression of the neural transcription factors *Sox1* and *Mash1* increased three and five fold, respectively while expression of the neural membrane

protein *Snap25* increased three fold (Figure 4.1 C). At the protein level, TRF2 overexpression was readily detected in TRF2-Ov hESCs (Figure 4.1 D). Overexpression of TRF2 had a dramatic effect in the expression of Oct4 protein which was heavily downregulated (Figure 4.1 D). These results indicate that these cells were differentiating toward neural lineages as a consequence of TRF2 overexpression. In addition, increase of γ H2AX protein levels was not detected in these cells (Figure 4.1 D and Figure A3).

4.2.2 TRF2-Ov hESCs differentiate predominantly to the neural lineage

Since TRF2-Ov hESCs exhibited a concession of self-renewal and a tendency to differentiate to neural lineage, we questioned whether this overexpression was inducing neural lineage differentiation specifically or all three lineages. To answer this question these transgenic cell lines were characterised further. Flow cytometry of pluripotent surface marker Tra-1-81 staining clearly revealed that control hESCs displayed one predominant peak of 80% of cells positive for Tra-1-81 while TRF2-Ov cells produced two clear populations with only 57% of them expressing Tra-1-81 and the rest being negative (Figure 4.2 A, top panels). As such, over 40% TRF2-Ov cells compared to only 20% of control did not express the hESC cell surface marker and therefore were considered as differentiated cells. When analysing with anti-Pax6 antibody, I found that 17.5% of TRF2-Ov hESCs expressed the NPC marker compared to only 4.5% of control (Figure 4.2 A, lower panels). This indicated that the overexpression of TRF2 in hESCs increased the proportion of NPCs by almost four fold. Only 2.5% of TRF2-Ov hESCs expressed the endoderm lineage marker Gata6 compared to 1.4% of control indicating less than a fold increase (Figure 4.2 A, bottom panels).

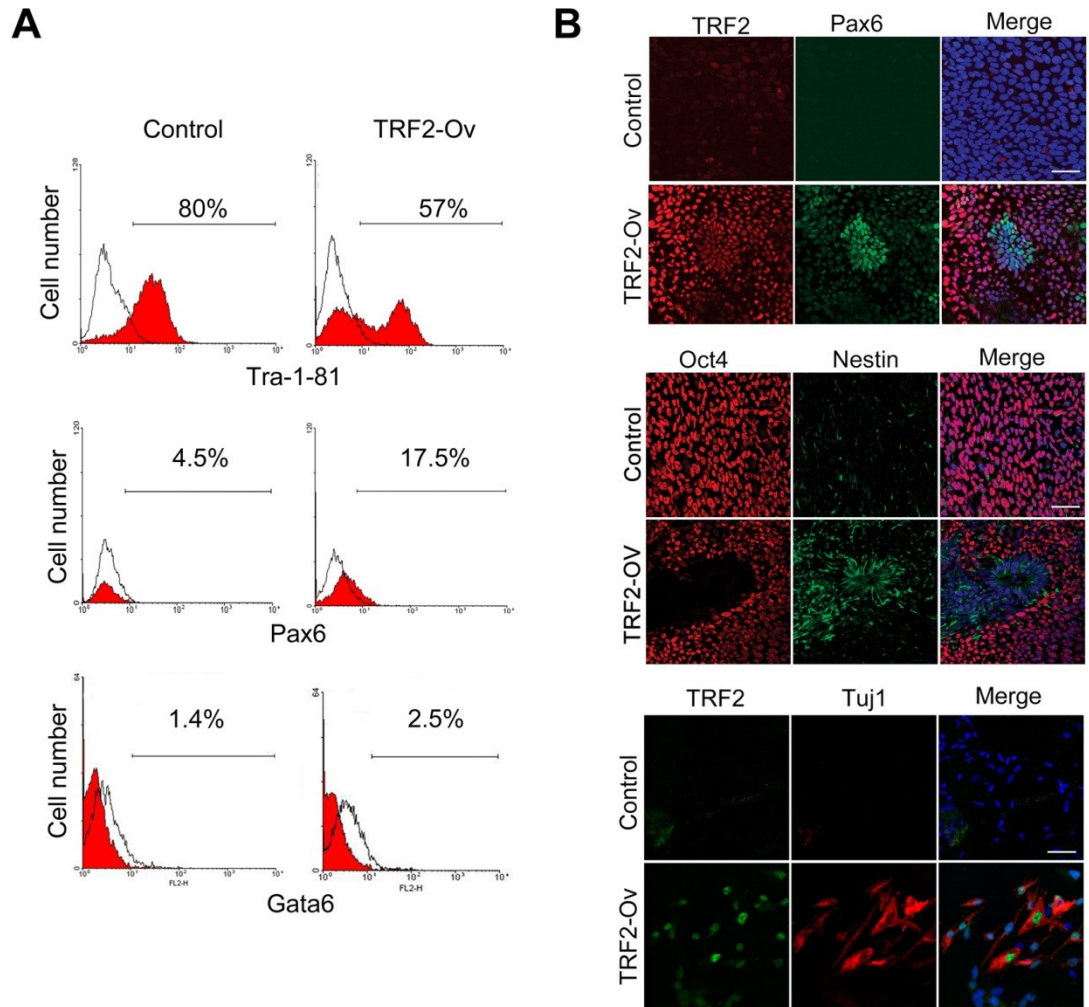


Figure 4.2 TRF2 overexpressing hESCs differentiate into neurons in hESC culture conditions.

(A) Flow cytometry analysis of Tra-1-81, Pax6 and Gata6 protein expression in control and TRF2-overexpressing hESCs (passage 11) shows decreased Tra-1-81 and greatly increased Pax6 positive population in TRF2 overexpressing cells. Gata6 positive cell number increased slightly in TRF2-Ov hESCs. (B) IF with indicated antibodies depicting areas of neural differentiation in TRF2-overexpressing hESCs (passage 5). This is shown by positive staining of Pax6, Nestin and Tuj1. Nuclei are shown by DAPI staining (blue). Scale bar = 50 μ m. Images are representative of two independent experiments.

To support the flow cytometry data IF of NPCs with markers Pax6 and Nestin was carried out (Figure 4.2 B). Similarly to the immunoblot and flow cytometry results, TRF2 overexpression affected the self-renewal of hESCs and induced neural differentiation, as shown by the decreased proportion of Oct4 positive hESCs and the increase of Nestin and Pax6 positive cells (Figure 4.2 B, top and middle panel sets). In fact, under hESCs self-renewing culture conditions, the TRF2-Ov hESCs were able to differentiate into Tuj1 positive neurons (Figure 4.2 B, bottom panel sets). It is also worth mentioning that the protein expression of Pax6 and Nestin found in the TRF2-Ov cells was detected in cells which exhibited a neural rosette like structure similar to that previously observed under phase contrast conditions (Figure 4.1 B, top right panel vs Figure 4.2 B, middle panel set).

When subjecting hESCs into neural differentiation conditions shortly after transduction (Passage 1), and prior observing a neural differentiation phenotype, the TRF2-Ov hESCs differentiated faster than controls. Protein expression of Oct4 in the TRF2-Ov hESCs was much further downregulated than control 12 days following neural induction (Figure 4.3 A). This was further supported by IF of the same cells using Oct4 and Pax6 antibodies. IF revealed that after 12 days in neural differentiation conditions, control cells still remained majorly pluripotent, as shown by the Oct4 positive staining, while most cells in the TRF2-Ov cells became NPCs as indicated by positive Pax6 and negative Oct4 protein expression (Figure 4.3 B).

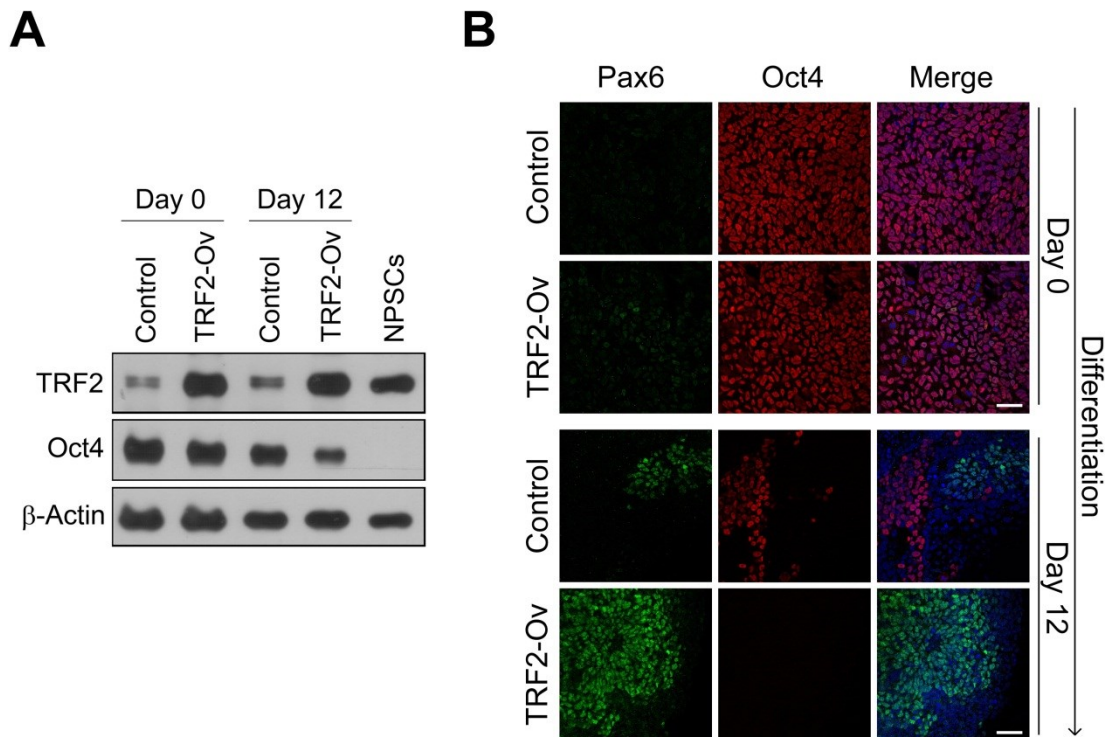


Figure 4.3 Ectopic expression of TRF2 in hESCs accelerates their differentiation into NPCs under neural differentiation conditions.

Control and TRF2-ov hESCs were subjected to neural differentiation for 12 days, one passage after their transduction and selection. Cell extracts were analysed by immunoblotting **(A)** and showed accelerated downregulation of Oct4 protein in TRF2-Ov hESCs only. Image is representative of a single experiment. **(B)** IF with Pax6 and Oct4 antibodies in cells of **(A)** showing clear downregulation of Oct4 and upregulation of Pax6 in TRF2Ov hESCs following 12 days in neural differentiation conditions. Nuclei are shown by DAPI staining (blue). Scale bars = 50 μ m. Images are representative of a single experiment done in triplicate.

4.2.3 Differentiation to the neural lineage is unlikely to be a consequence of telomere shortening

A recent publication has reported that ES cells with short telomeres have unstable differentiation (Pucci *et al.*, 2013). Given that TRF2 regulates telomere length and that when overexpressed in cancer cell lines it shortens telomeres, we questioned whether the differentiation observed was a consequence of telomere shortening. To answer this question genomic DNA was extracted from control and TRF2-Ov hESCs and telomere length assays carried out on both DNAs. It was found that TRF2-Ov did not result in telomere shortening during the first 5 passages (Figure 4.4 left panel).

However, over continuous culture in hESCs self-renewing conditions, telomeres showed a slight shortening of the telomeres at passage 14 (Figure 4.4, right panel).

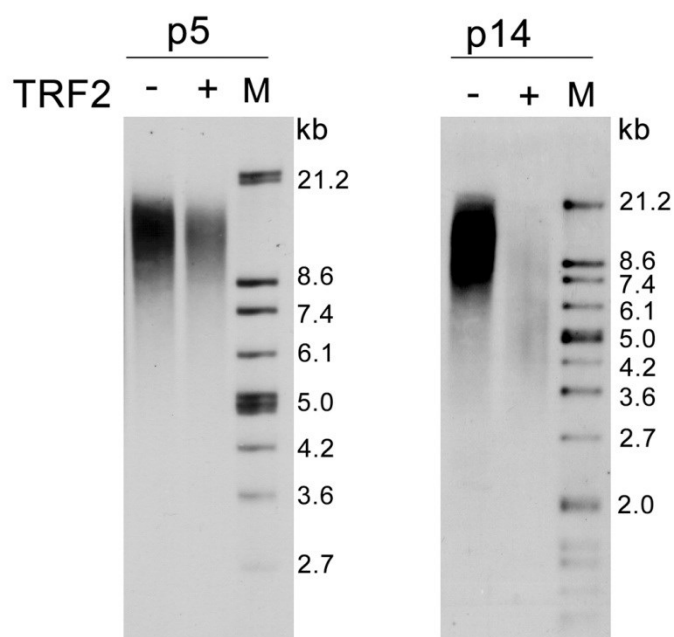


Figure 4.4 Neural differentiation of hESC is not a result of telomere shortening.

Comparison of telomere length in control and TRF2-overexpressing hESCs at two indicated passages by southern blotting shows that telomere length shortening occurs in TRF2-overexpressing hESCs only after 5 passages. Images are representative of three independent experiments.

Since the neural differentiation appeared as early as passages 2-5 while telomere attrition was not detected in TRF2-Ov hESCs before passage 5, it is unlikely that the differentiation is a consequence of telomere shortening. However, it is important to note that alternative highly sensitive techniques to measure telomere length may show shortening of the telomeres over TRF2-Ov during the first 2-5 passages. These results suggest that TRF2 plays a positive role in promoting neural differentiation of hESCs, which is likely to be independent from its roles in regulating telomere length and telomere protection.

4.2.4 TRF2 knockdown in hESCs does not affect their self-renewal

To further address the role of TRF2 in hESCs, TRF2-deficient hESCs were generated by shRNA-knockdown. TRF2 knockdown lentivector (TRF2-Sh) was generated by inserting a shRNA sequence specifically targeting TRF2 into pLVTHM-CAG-PURO and the lentivector and the lentivector without the shRNA was used as the control. hESCs were infected with both control and TRF2-Sh viruses and selected with puromycin 48 hours post infection. No striking differences were observed between control and TRF2-Sh cells 2 weeks after selection (Figure 4.5 A). Gene expression revealed a successful ~50% knockdown of *TRF2* transcripts, which had no significant effect in the expression of pluripotent markers *Oct4*, *Nanog* and *Sox2* (Figure 4.5 B). These gene expression results were further supported by immunoblotting with Oct4 and TRF2 antibodies (Figure 4.5 C). These results demonstrated that under self-renewing CM culture conditions, TRF2 knockdown, unlike TRF2 overexpression, did not seem to have an effect on hESC self-renewal and pluripotency.

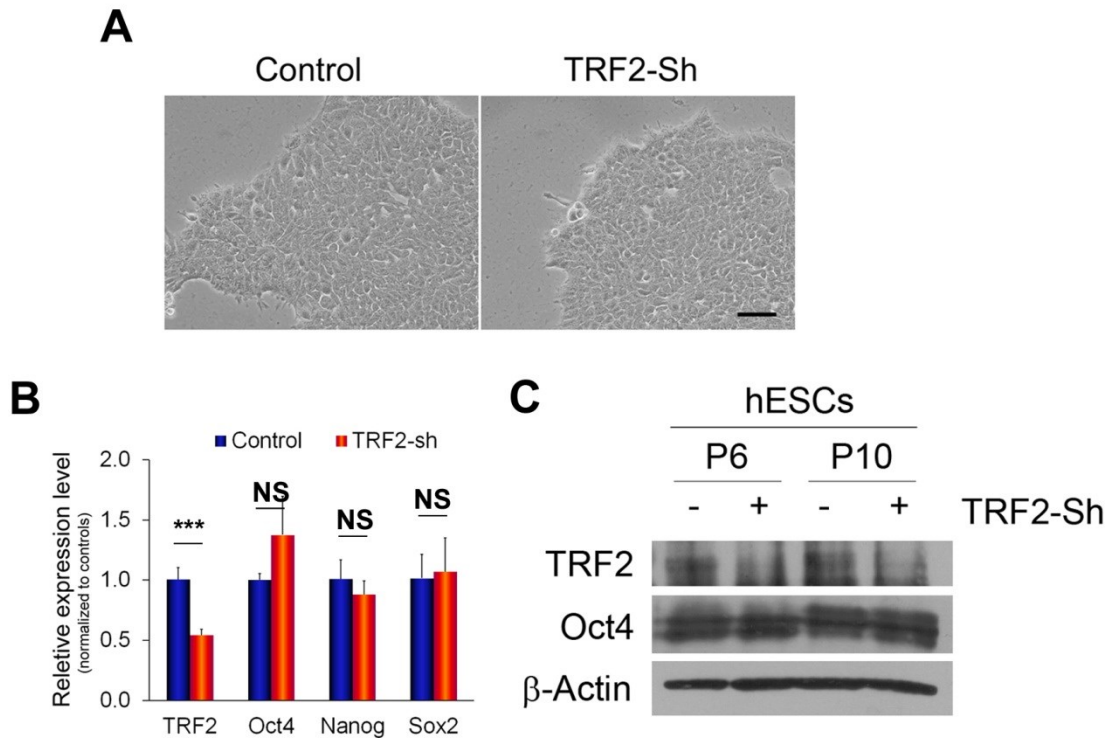


Figure 4.5 TRF2 knockdown in hESCs does not affect their self-renewal.

(A) Phase-contrast images of H1 hESCs overexpressing puromycin only (Control) and puromycin with TRF2 shRNA (TRF2-Sh). Scale bar = 100 μ m. (B) Quantitative real-time PCR expression analysis of the indicated genes, showing no-significant differences in gene expression. Data are presented as mean $\pm$ SD of 6 repeats of 2 independent experiments. Statistical significance was tested by paired Student's t-test, *** $p \leq 0.001$, NS $p > 0.05$ (non-significant). (C) Immunoblot analysis with the indicated antibodies of NPCs 6 and 10 passages following selection. Oct4 protein levels were not significantly affected by TRF2 knockdown. Images are representative of a single experiment carried out in duplicate.

4.2.5 TRF2 knockdown in hESCs affect their ability to differentiate to the neural lineage

To support the above data further, control and TRF2-Sh hESCs were differentiated by embryoid body formation to the three germ layers. Cells were grown in suspension for 7 days, plated on coverslips, cultured a further 7 days, and stained with the appropriate antibodies. Interestingly despite both cells exhibited similar staining for HNF4 α (endoderm) and muscle actin (mesoderm), they revealed striking differences in Sox2 and Tuj1 (ectoderm) staining, a NPCs and neural marker respectively (Figure 4.6 A). Expression of Sox2 and Tuj1 was hardly detectable in

the EB differentiated TRF2-Sh cells. In order to verify that the Sox2 positive cells in the control samples were not undifferentiated hESCs, control and TRF2-Sh cells were co-stained with Sox2 and TRF2. Since we previously found that only hESC-derived NPCs express high levels of TRF2 (Figure 3.4), it was expected that if these cells were hESCs they would be TRF2 negative and if they were NPCs they would be TRF2 positive. The control cells co-expressed high levels of TRF2 and Sox2 while TRF2-Sh cells were hardly positive for TRF2 or Sox2 staining. These results reveal that the differentiation process does not affect TRF2 knockdown levels and that upon TRF2 knockdown, no Sox2 positive NPCs are formed (Figure 4.6 B).

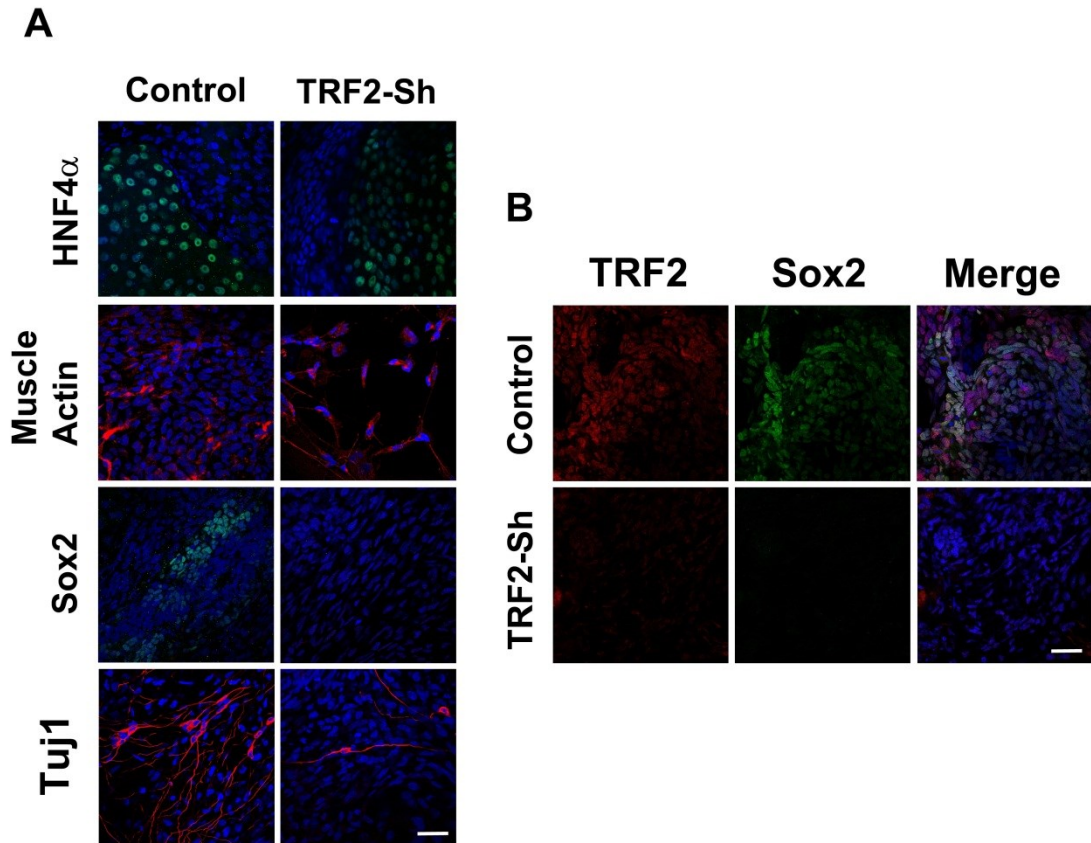


Figure 4.6 TRF2 knockdown in hESCs affected their ability to differentiate to the neural lineage.

IF of control vs TRF2-Sh EBs with endoderm (HNF4 α), mesoderm (Muscle Actin) and ectoderm (Sox2 and Tuj1) lineage markers (**A**) and TRF2/Sox2 co-staining (**B**). Nuclei are shown by DAPI staining (blue). Scale bar = 40 μ m in all microscopic images. Images are representative of a single experiment carried out in duplicate.

To further test if TRF2 knockdown affects the ability of hESCs to specifically differentiate into the neural lineage, control and TRF2-Sh hESCs were differentiated to NPCs using the same protocol used to differentiate wild type hESCs. A clear difference was observed in their morphology within 5 passages of differentiation (Figure 4.7 A). The control cells displayed a homogenous population of NPC morphology, whereas TRF2-Sh cells were heterogeneous in cell morphology with a proportion of them revealing a flatter and rounder phenotype that differed from the typical triangular or bipolar NPC morphology (Figure 4.7 top and bottom right panels). Gene expression analysis showed that the expression of NPC markers, *Sox1*, *Sox2* and *Mash1*, was heavily downregulated in the TRF2-Sh cells after neural differentiation while expression of the neural markers *Tuj1* and *Snap25* did not significantly change (Figure 4.7 B). This differentiation did not affect the levels of TRF2 knockdown (Figure 4.7 B).

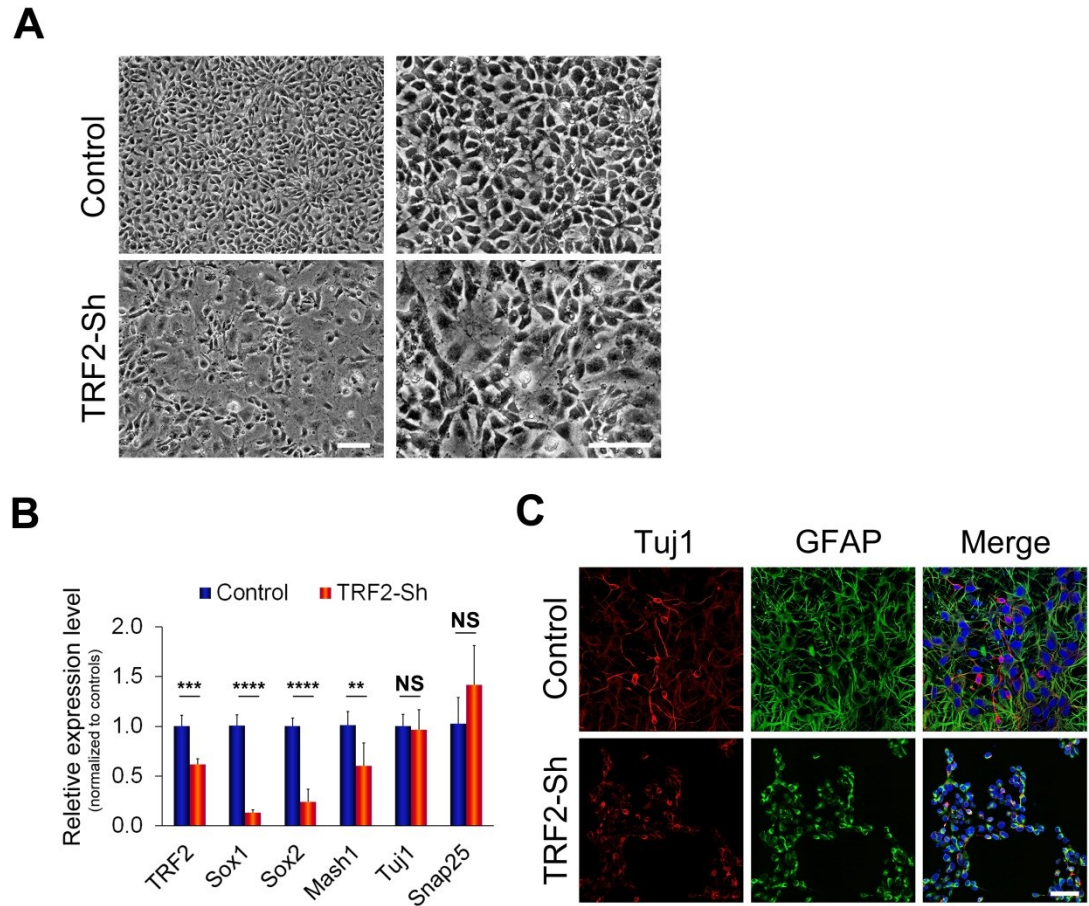


Figure 4.7 TRF2 knockdown in hESCs affects the ability of their derived NPCs to terminally differentiate into neurons or glia.

(A) Phase contrast images of control and TRF2-Sh hESC-derived NPCs. Left panel images scale bar is 100 μ M right panel images 50 μ M. **(B)** qRT-PCR gene expression analysis of *TRF2*, NPC marker genes (*Sox1*, *Sox2* and *Mash1*) and neural marker genes (*Tuj1* and *Snap25*). Data are presented as mean $\pm$ SD of 6 repeats of 3 independent experiments. Statistical significance was tested by paired Student's t-test, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, NS $p > 0.05$ (non-significant). **(C)** IF of terminally differentiated control and TRF2-Sh hESC-derived NPCs with Tuj1 and GFAP makers. Nuclei are shown by DAPI staining (blue). Scale bar= 40 μ M in all images. Images are representative of two independent experiments.

When these NPCs were allowed to further differentiate into post-mitotic neurons and glia by the withdrawal of growth factors, only few of the TRF2-Sh NPCs managed to terminally differentiate as indicated by little protein expression of Tuj1 and GFAP markers, as well as little neuron or glia typical cell processes (Figure 4.7 C). In contrast, control NPCs showed clear expression of these markers and cell processes.

4.2.6 TRF2 knockdown effect on neural differentiation is unlikely to be due telomere attrition

Given that TRF2 is well known to be important for telomere length regulation and DNA damage protection, we questioned whether the knockdown had affected these cells in either of these ways. Telomere length assay showed no clear differences on telomere length. While TRF2-sh hESCs seem to show a higher molecular weight than control hESCs, control hESCs do show faint bands at approximately the same molecular weight. Therefore the difference in genomic DNA loading could be giving the impression TRF2-sh hESCs have longer telomeres. In order to be able to address this question further telomere length assays need to be performed (Figure 4.8 A). Furthermore, chromosomal spreads of at least 10 spreads from two independent experiments revealed no differences between control and TRF2-Sh hESCs. Chromosomes were of the correct number with no apparent NHEJ of the telomere ends (Figure 4.8 B). However, these results would benefit greatly by carrying out FISH on the chromosomal spreads of both cell lines, as in this way a more accurate distinction of NHEJ of telomere ends could have been achieved and quantified. However, due to time constraints this was not possible.

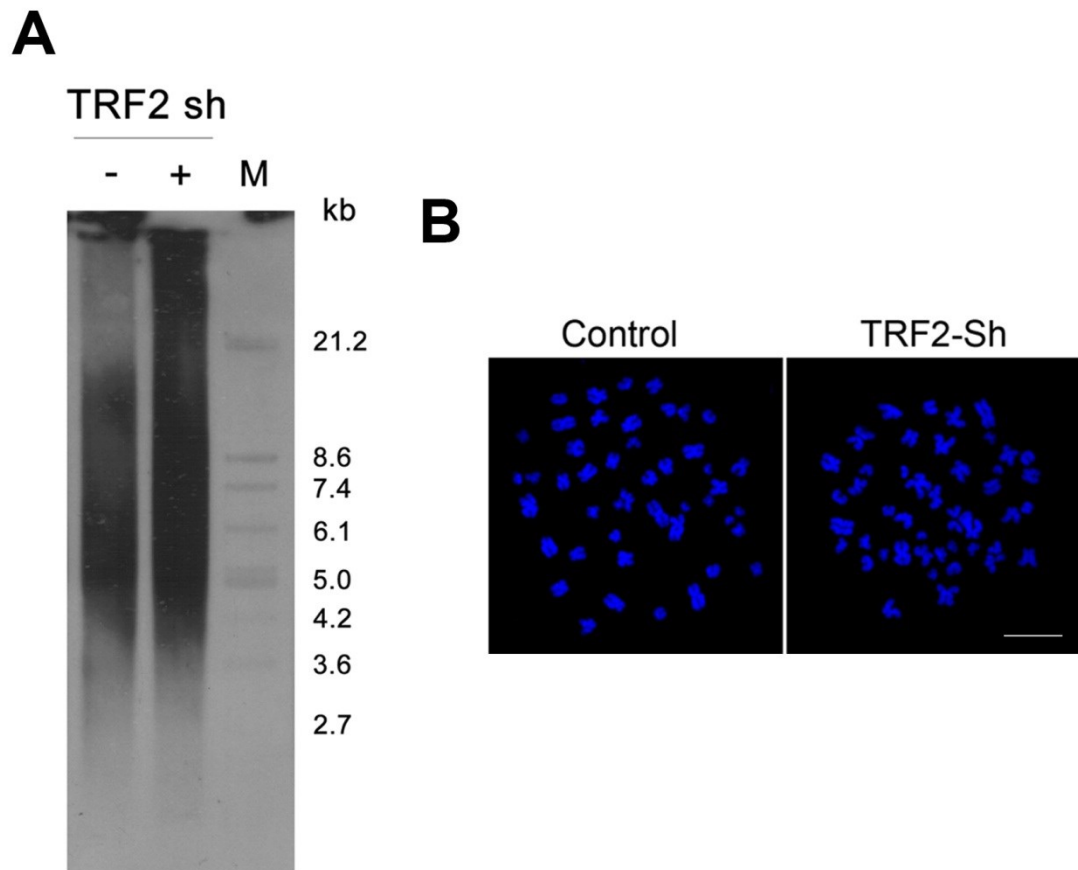


Figure 4.8 TRF2 knockdown in hESCs does not affect telomere length or induce non-homologous telomere end joining.

(A) Southern blotting assessing telomere length of control vs TRF2-Sh cells. Both control and TRF2-Sh cells showed similar telomere lengths despite differences in genomic DNA loading. Image is representative of a single experiment. (B) Chromosomal spreads of control and TRF2-Sh hESCs counterstained with DAPI which show no obvious non-homologous end joining of telomere ends. Images are representative of two independent experiments. Scale bar= 10 μ M.

Altogether, knockdown of TRF2 in hESCs does not affect their self-renewal or ability to differentiate into cells of the mesoderm and endoderm germ layers. However, it appears to hinder hESCs to differentiate into functional NPCs. Furthermore, NPCs derived from these cells exhibit a dramatic reduction in their ability to generate post-mitotic neurons and glia.

4.3 Discussion

The previous chapter described how TRF2 protein is specifically abundant in NPCs and that its localization is both telomeric and extratelomeric, suggesting an extratelomeric function within the neural lineage. These results were consistent with previous findings showing that TRF2 levels were specifically abundant in human brain tissue (Jung *et al.*, 2004), and with previous suggestions that TRF2 may play a role in neurogenesis (Zhang *et al.*, 2008; Simonet *et al.*, 2011). To explore this possibility further, gain and loss of function approaches were used to determine the importance of TRF2 in hESCs. Ectopic expression of TRF2 in hESCs promoted neural differentiation. TRF2 overexpression in hESCs resulted in the increase of neuronal marker expression at both the mRNA and protein level without clear changes in Gata6 expression (Figure 4.1 C and Figure 4.2). Since TRF2 overexpression has been shown to diminish the repair of single strand breaks in human fibroblasts (Richter *et al.*, 2007), which can result in DNA damage accumulation (Ward and Chen, 2001), and since DNA damage has been recently reported to induce differentiation of neural progenitors (Schneider *et al.*, 2013), the protein levels of γ H2AX were analysed via western blotting and IF to ensure the neural differentiation phenotype observed following TRF2 overexpression was not a consequence of DNA damage accumulation. No dramatic changes of γ H2AX were observed upon TRF2-Ov in hESCs (Figure 4.1 D and Figure A3). Thus, while these results could benefit of additional analysis, our available data suggests that the induction of neuronal differentiation was likely to not be a result of DNA damage. Furthermore, it is also worth mentioning that in order to terminally differentiate NPCs, bFGF and EGF, which promote NPC growth *in vitro*, need to be removed from the medium to allow terminal differentiation. However, our TRF2-Ov hESCs managed to differentiate into neurons under these self-renewing conditions (Figure 4.2 C). This suggested that the increase of TRF2 protein levels has a potent effect in inducing neural differentiation. In fact when control and TRF2-Ov hESCs were introduced in neural differentiation conditions, one passage following transduction and prior TRF2 overexpression inducing neural differentiation, TRF2-Ov hESCs differentiated to NPCs at a much faster rate.

Given that TRF2 is important in the maintenance of telomere length and recent reports have indicated that ESCs with short telomeres have unstable differentiation (Pucci *et al.*, 2013), telomere length in control and TRF2-Ov hESCs was analysed at different passages. The results indicated that the neural differentiation observed in the TRF2-Ov hESCs was unlikely to be a consequence of telomere shortening, as neural differentiation was observed as early as passages 2-5 and at this stage no shortening of the telomeres was observed. Shortening of telomeres was only observed after passage 5 following neural differentiation, indicating that telomere shortening was much more likely to be the consequence of neuronal differentiation and not the other way around. However, it is important to note that the uneven loading of the sample's genomic DNA makes the data difficult to interpret. The experiment would need to be repeated or an alternative more sensitive telomere length analysis technique such as q-FISH or telomere qRT-PCR should be used to fully address this question.

Since TRF2 overexpression induces neural differentiation, we questioned whether TRF2 knockdown would conversely inhibit neural differentiation. TRF2 knockdown in hESCs did not affect their self-renewal neither had an obvious detrimental effect (Figure 4.5). It is possible that this may be due to the fact that ESCs are derived from pre-implantation embryos and TRF2 knockout mice only resulted in embryonic lethality at a later embryonic stage (Celli and de Lange, 2005). TRF2 knockdown was confirmed by qRT-PCR and western blotting of two different passages. TRF2 knockdown did not affect the expression of pluripotent markers *Oct4*, *Nanog* and *Sox2* significantly and cells appeared no different from control under the microscope. *Oct4* was not affected at the protein level either. However, when these cells were differentiated to the three germ cell lineages by EB formation, significant events unfolded. While TRF2-Sh hESCs were able to differentiate to the endoderm and mesoderm similarly to control hESCs, differentiation to the ectoderm lineage was impaired. Expression of *Sox2*, a NPCs marker, was greatly reduced and in addition so was the expression of the neuronal marker *Tuj1*. To confirm these *Sox2* positive cells were NPCs and not undifferentiated hESCs, which do not express high levels of TRF2, co-staining with *Sox2* and TRF2 antibodies was carried out, resulting in a strong positive staining of both markers in control cells only. It is likely that the reduction on the neural marker *Tuj1*, observed in TRF2-Sh EB-derived cells, is a

consequence of the reduced number of Sox2 positive NPCs as Sox2 has been extensively reported to be critical in maintaining NPCs self-renewal and multipotency (Graham *et al.*, 2003; Suh *et al.*, 2007). It is possible that the knockdown had an effect on the differentiation of viable NPCs and consequently resulted in a reduction of postmitotic neurons. To see if this was the case, control and TRF2-Sh hESCs were differentiated to NPCs by the same protocol used to differentiate wild type hESCs to NPCs. Differentiation of TRF2-Sh hESCs to NPCs appeared hindered as a large proportion of the cells did not display the triangular/bipolar morphology typical of NPCs, instead cells were flatter and of a rounder shape. Gene expression analysis confirmed that the expression of NPCs markers *Sox1*, *Sox2* and *Mash1* was heavily downregulated but neural markers *Tuj1* and *Snap25* were not significantly affected. The non-significant change in neuronal marker expression observed could be due to the presence of growth factors during the hESCs to NPCs differentiation, which inhibit neuronal differentiation and therefore the expression of these markers. To investigate if TRF2 knockdown could also affect the ability of the derived NPCs to differentiate to neurons or glia these cells were allowed to further differentiate into neurons or glia by withdrawal of growth factors. Interestingly, only control cells displayed strong Tuj1 and GFAP staining with typical neuron or glia processes. This indicated that TRF2 knockdown in hESCs affects their ability to differentiate to healthy NPCs that have the capability to further differentiate into neurons and glia.

TRF2 knockdown has been well reported in cancer cells to induce NHEJ of the chromosome ends leading to cell senescence and apoptosis (Karlseder *et al.*, 1999; Denchi and de Lange, 2007). It could be that the detrimental effects observed on differentiation upon TRF2 knockdown are a consequence of DNA damage and NHEJ; however this would not explain why the detrimental effects occur specifically in the neural lineage. Nevertheless, the telomere lengths and chromosomal spreads of both control and TRF2-Sh hESCs were analysed. It was unclear whether telomere length was affected upon TRF2 knockdown, at an initial glance there appears to be no dramatic changes on telomere length even 10 passages after TRF2 knockdown. However, to confirm this we would need to use more sensitive techniques to measure telomere length which could not be achieved due time constraints. TRF2 knockdown did not appear to result in NHEJ of telomere ends; chromosomes were of the correct

number and appeared without NHEJ. This could be due to the level of TRF2 knockdown achieved. A recent publication has shown that only high levels of TRF2 knockdown result in NHEJ of chromosome ends (Cesare *et al.*, 2013). Therefore while TRF2 knockdown may not have been sufficiently efficient to lead to NHEJ of chromosome ends, it was efficient enough to suggest that TRF2 has a role in neural differentiation.

Chapter 5 TRF2 is required to maintain NPCs differentiation potential

5.1 Introduction

The previous results chapters demonstrated that TRF2 is specifically upregulated in NPCs. Ectopic expression of TRF2 in hESCs induced their differentiation to the neural lineage under hESC self-renewing conditions, while TRF2 knockdown affected their ability to differentiate into NPCs that give rise to neurons and glia. Given that high TRF2 protein expression is abundant in NPCs and that modulation of its expressions in hESCs affects neural differentiation, we attempted to further elucidate its role in NPCs. In the developing CNS, NPCs exist at different developmental stages. The developmental stage of a NPC alters its differentiation potential. NPCs in their early stages of CNS development have more potential to differentiate into neurons while NPCs in late stages are more prone to give rise to glia (Temple, 2001). In addition to their more limited differentiation potential, NPCs have shorter telomeres than hESCs and express lower levels of telomerase. Interestingly, it has recently been reported that mouse ES cells with short telomeres display unstable differentiation (Pucci *et al.*, 2013). This phenotype was shown to be only rescued upon re-lengthening of the telomeres by introduction of telomerase catalytic subunit TERT. The aim of this chapter is to carry out a similar approach of gain or loss of TRF2 function in late stage NPCs, taking particular interest in telomere length and differentiation potential.

5.2 Results

5.2.1 TRF2 knockdown in NPCs hinders their ability to terminally differentiate into neurons and glia

In the previous chapter, TRF2 knockdown in hESCs affected their ability to differentiate into proper NPCs, rendering the resulting TRF2-Sh hESC-derived NPCs unable to generate neurons and glia (see section 4.2.5 for details). To further validate the function of TRF2 in NPCs, TRF2 knockdown was carried out directly in hESC derived NPCs using the same TRF2 shRNA-expressing lentivirus particles. Following infection and selection in puromycin, the surviving TRF2-Sh NPCs exhibited evident TRF2 deficiency as measured by qRT-PCR, WB and IF (Figure 5.1 B-D), displaying almost 75% reduction at the mRNA level. Although these cells did not show distinct changes in morphology (Figure 5.1 A), they revealed significant downregulation of two important NPCs markers, *Sox2* and *Mash1*, by qRT-PCR. In addition, *Snap25*, a cell membrane protein often related to neural synaptic vesicle, was also heavily downregulated upon TRF2 knockdown. In agreement with these mRNA data, western blotting and IF also showed obvious decrease in Sox2 and Nestin protein levels (Figure 5.1 C and E). TRF2 knockdown also triggered activation of DNA damage response as shown by the increase of γ H2AX (Figure 5.1 C). These results indicate that TRF2 deficiency indeed modified the gene expression profile of NPCs, particularly those that are important in neurogenesis, which could consequently affect the differentiation potential of these cells.

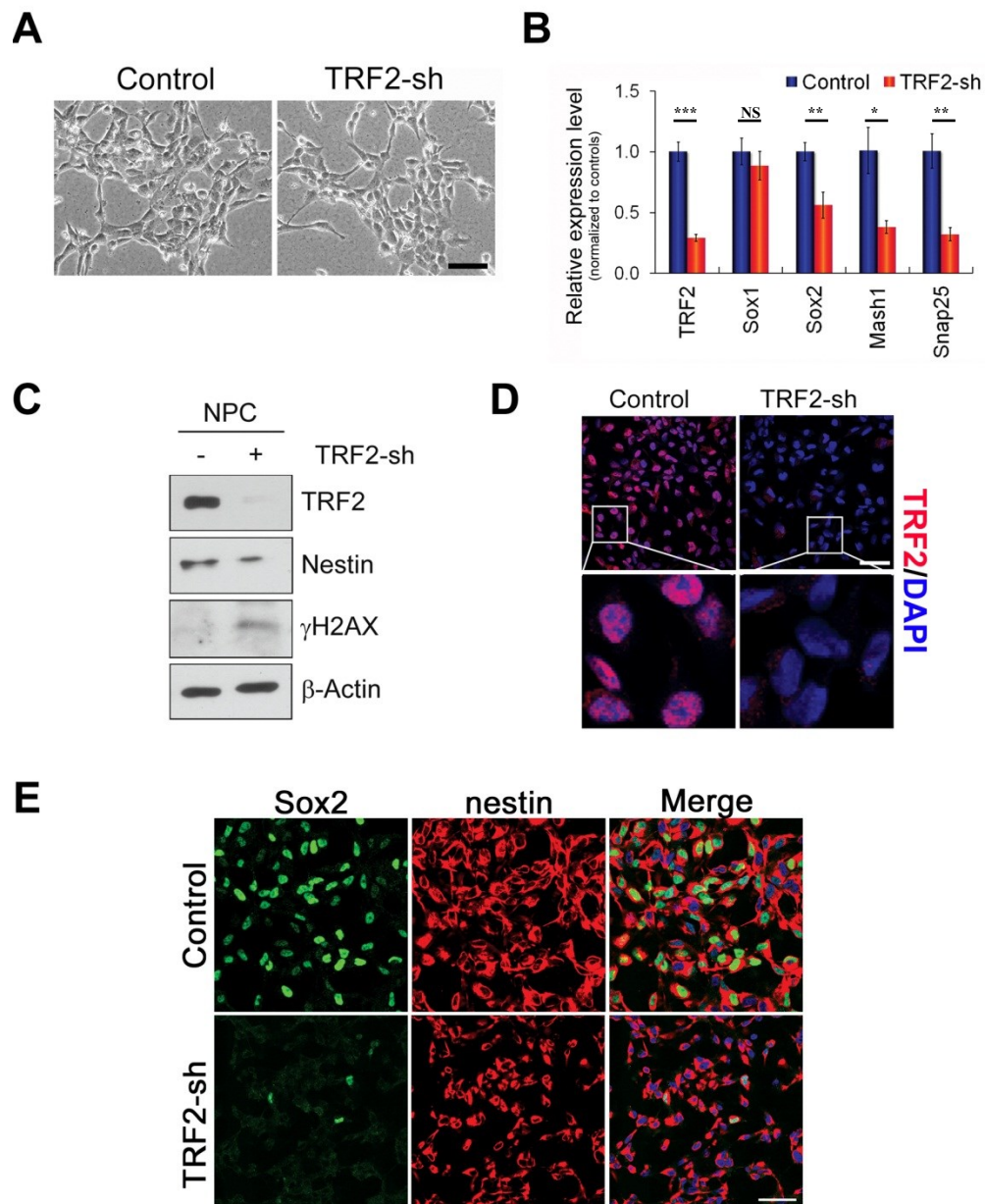


Figure 5.1 TRF2 knockdown in NPCs affects their marker expression.

(A) Phase contrast images of control and TRF2-Sh NPCs following selection. Scale bar= 100 μ m. (B) Gene expression analysis of NPCs markers (*Sox1*, *Sox2*, *Mash1*) neural marker *Snap25* and *TRF2* by qRT-PCR. Error bars represent standard derivation from 6 repeats of two independent experiments. Statistical significance was tested by paired Student's t-test, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, NS $p > 0.05$ (non-significant). (C) Immunoblotting of control and TRF-Sh NPCs with the selected antibodies. Image is representative of two independent experiments. (D) IF with TRF2 antibody showing the evident reduction of TRF2 in cell nuclei after TRF2-shRNA knockdown. Nuclei are shown by DAPI staining (blue). White squares magnify the area they surround as shown in the bottom panels. Scale bar = 50 μ m. Images are representative of two independent experiments. (E) Sox2/Nestin co-IF in control and TRF2-Sh NPCs showing a significant downregulation of both markers upon TRF2 knockdown. Nuclei are shown by DAPI staining (blue). Scale bar= 40 μ m. Images are representative of three independent experiments.

To validate the functional effect of TRF2 knockdown, these cells were further differentiated into neurons and glia by withdrawal of growth factors, subsequently revealing different differentiation potentials. Control cells developed long and multiple processes of interconnecting outgrowths, whereas the TRF2-sh cells appeared flatter, with fewer outgrowths (Figure 5.2 A). These phenotypic differences were reflected by the lack of Tuj1 and GFAP staining in the TRF2-sh cells (Figure 5.2 B, lower panels). These results indicate that TRF2 deficiency significantly diminishes the capability of NPCs to further differentiate into neurons and glia, and as such, TRF2 knockdown may therefore compromise the neuropotency of neural progenitors, leaving them unable to further differentiate. These results are in line with the previously generated gene expression data where several neural progenitor and neuronal markers, particularly the neural transcription factors, Sox2 and Mash1, were expressed at considerably lower levels (Figure 5.1 B). As these transcription factors are essential for the maintenance of neural progenitors, this deficiency in expression may therefore account for their hindered ability to differentiate into neurons and glia.

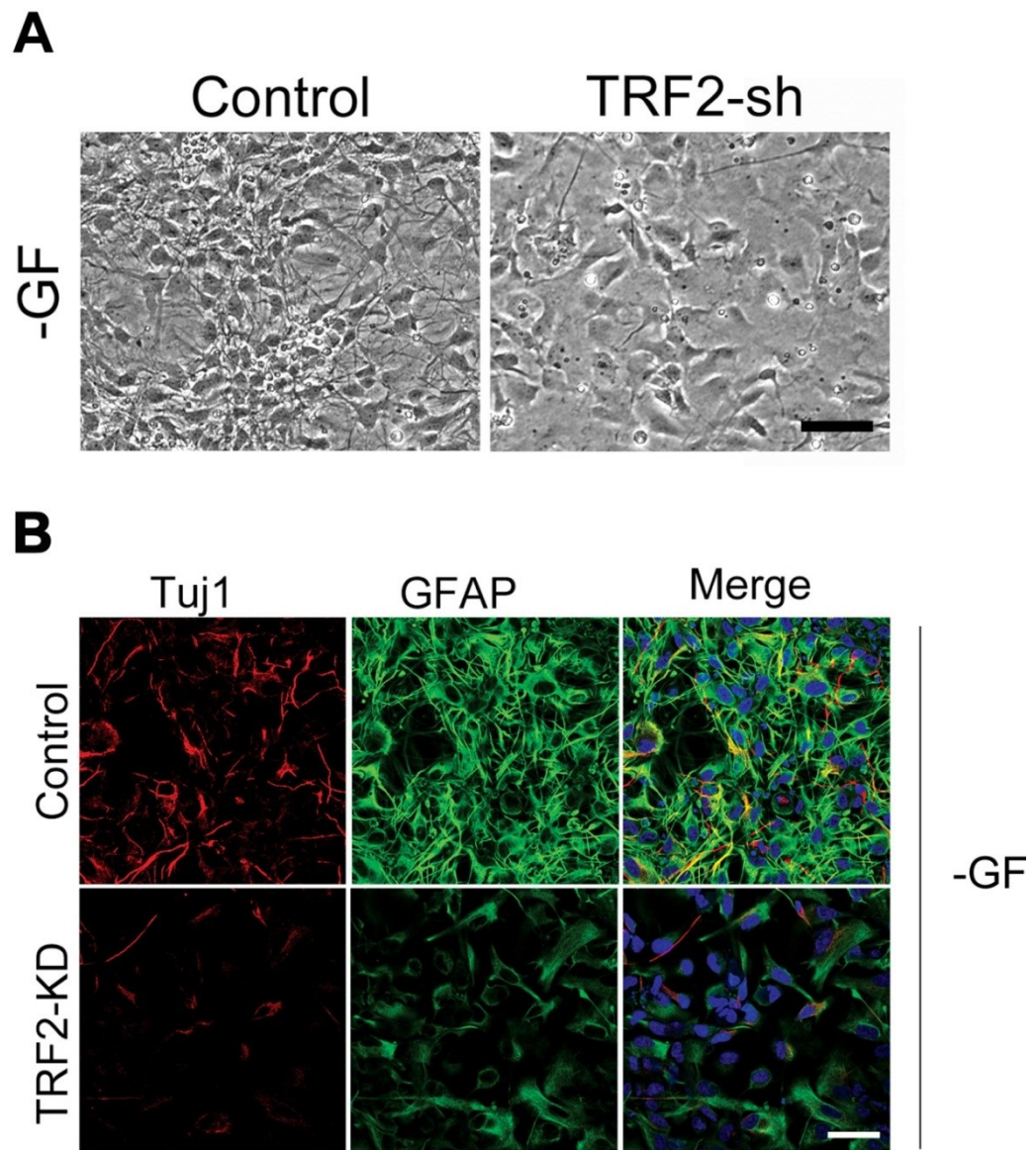


Figure 5.2 TRF2 knockdown in NPCs affects their ability to terminally differentiate into neurons and glia.

(A) Phase-contrast images of control and TRF2-Sh NPCs terminally differentiated for 10 days in NPC culture medium without growth factors (-GF). Scale bar= 100 μ m. (B) IF of terminally differentiated control and TRF2-Sh NPCs with neuronal marker Tuj1 and glial marker GFAP. Nuclei are shown by DAPI staining (blue). Scale bar= 50 μ m. Images are representative of three independent experiments.

5.2.2 TRF2 knockdown in NPCs results in slight telomere lengthening but no obvious telomere NHEJ

Telomere length in these TRF2-sh NPCs was found to be partially elongated (Figure 5.3 A), which is similar to that observed in telomerase positive cells (Takai *et al.*, 2010). It has been demonstrated in some cells that TRF2 plays an important role in telomere structure and protection and that lack of TRF2 leads to unprotected telomere, resulting in chromosomal end joining (Karlseder *et al.*, 2004; Denchi and de Lange, 2007; Karlseder *et al.*, 2009). However, no obvious end-to-end chromosome fusions were detected in at least 10 spreads from two independent experiments of control and TRF2 deficient NPCs (Figure 5.3 B), even though TRF2 knockdown was shown to trigger activation of DNA damage response indicated by the increase of γ H2AX (Figure 5.1 C). Similarly to the chromosomal spreads analysed in the previous chapter (Figure 4.3), these experiment would benefit greatly from FISH analysis as this technique would more clearly indicate if control or TRF2-sh NPCs contain any NHEJ of their telomere ends and help to properly address this question. However, due to time constraints this was not possible.

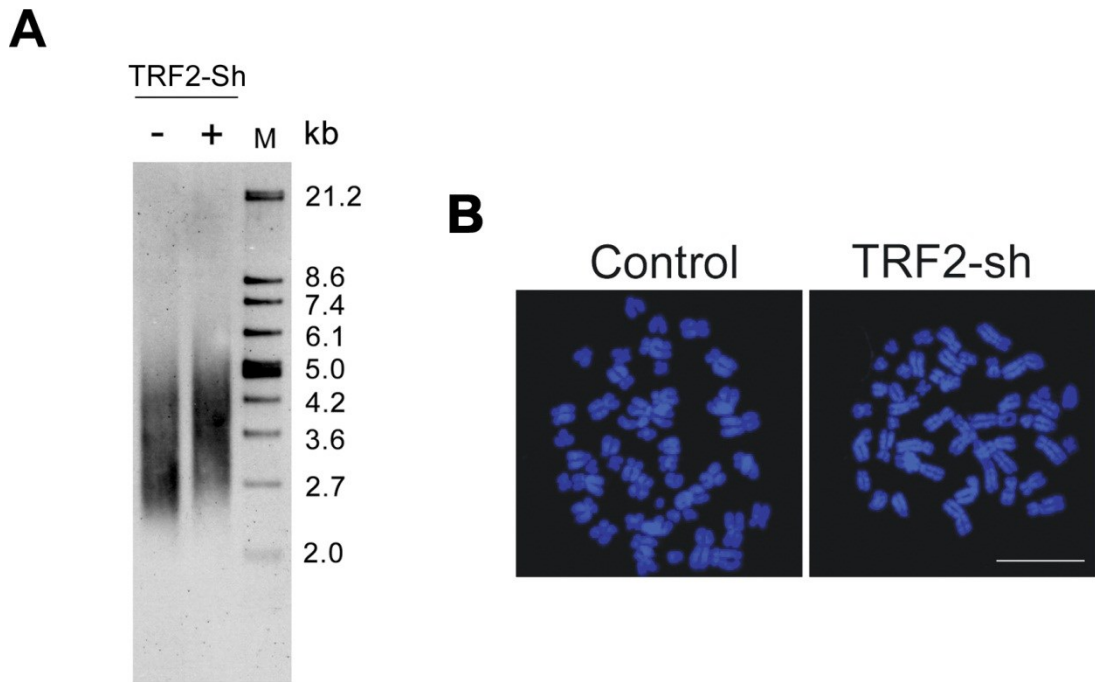


Figure 5.3 TRF2 knockdown does not induce NHEJ in NPCs but results in telomere re-lengthening.

(A) Telomere repeat fragment (TRF) southern blotting assay of control and TRF2-Sh NPCs. TRF2 knockdown in NPCs increases telomere length. Images are representative of two independent experiments. **(B)** Chromosomal spread analysis of control and TRF2-Sh NPCs revealing no obvious NHEJ of telomeres upon TRF2 knockdown. Images are representative of two independent experiments each carried out in triplicate. Scale bar= 10 μ M.

Since TRF2 is well known for its telomere protection function we questioned whether TRF2 knockdown had increased their apoptosis or affected their proliferation. Control and TRF2-Sh NPCs were seeded at a density of 5×10^5 per 6 well plates and passaged every three days. Cells were counted at each split and population doubling (PD) calculated. While control cells proliferated at a rate of 5.2 PD per 6 days of cell culture, TRF2-Sh NPCs only proliferated at a rate of 4.1 PD (Figure 5.4 A). Furthermore, IF of control and TRF2-Sh NPCs with the active mitosis marker phospho-H3, revealed 0.9% and 0.6% positive staining of the respective cells (Figure 5.4 B). Staining with the apoptosis marker Caspase 3 did not show marked differences between control and TRF2-Sh NPCs. All this together indicates that the slower proliferation observed in TRF2-Sh NPCs may be a consequence of a reduced active mitosis rather than cell death.

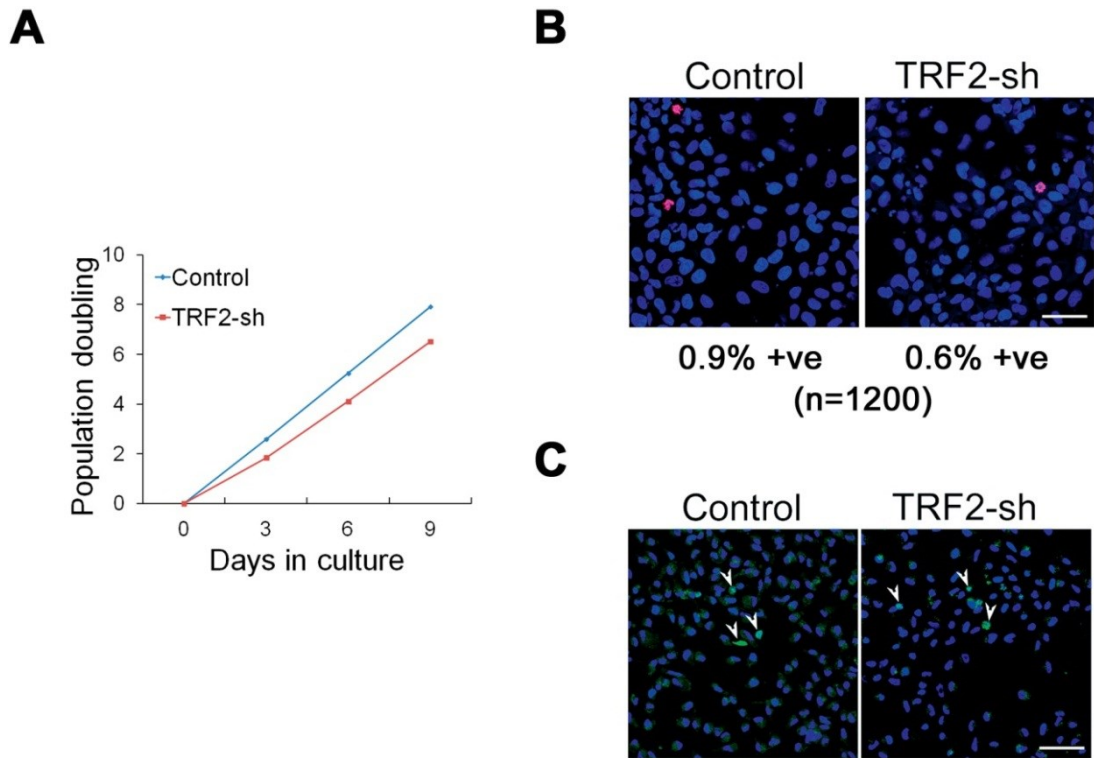


Figure 5.4 Proliferation and apoptosis analysis in TRF2-knockdown NPCs.

(A) Growth curve of control and TRF2-Sh NPCs. Image is representative of four independent experiments carried out in triplicate each. Error bars indicate standard deviation of three repeats. (B) Representative images of phospho-H3 antibody staining in both control and TRF2-Sh NPCs, quantitative data is shown below. Scale bars= 30 μ m. Experiment was carried out once in triplicate. (C) IF with Caspase 3 antibody in the two cell types. Scale bars= 50 μ m. Nuclei are shown by DAPI staining (blue) in all IF images. Experiment was carried out once in triplicate.

5.2.3 Ectopic expression of TRF2 in NPCs does not hinder their ability to terminally differentiate into neurons and glia

Chapter 3 revealed that the upregulation of non-telomere bound TRF2 was exclusively detected in NPCs and that this upregulation was dramatically downregulated to levels similar to that of hESCs upon further differentiation of these cells into neurons or glia. We hypothesised that downregulation of TRF2 protein levels may be required for NPCs to properly differentiate into neurons and glia. To address this question TRF2 was overexpressed in NPCs using lentiviral particles. Once NPCs were transduced and selected, the resulting cells were analysed for changes in their differentiation potential. TRF2 protein expression was greatly

increased in TRF2-Ov NPCs when compared to control NPCs (Figure 5.5 C). While these cells appeared morphologically normal under phase-contrast microscopy (Figure 5.5 A), in-depth gene expression analysis revealed that the expression of neuronal and glial markers was significantly affected. For example, expression of neuro-markers *NeuroD2* and *Tuj1* increased 6-fold and 2-fold respectively and expression of the glial marker *GFAP* also increased by 2-fold.

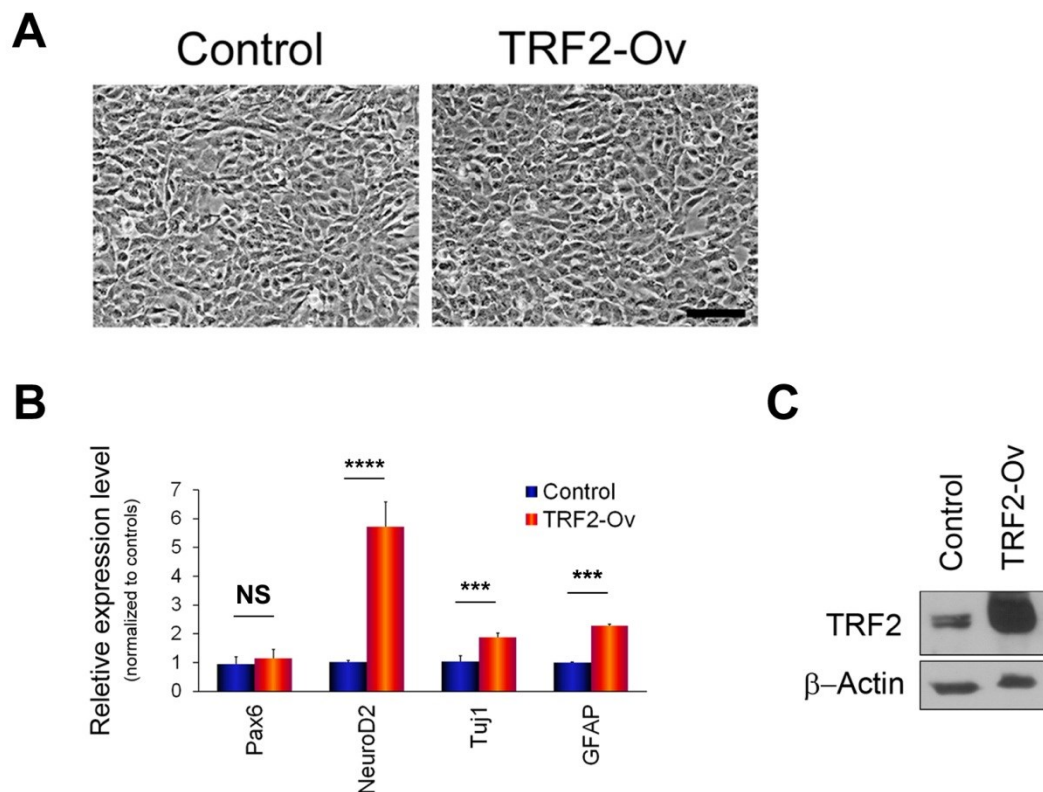


Figure 5.5 TRF2 overexpression in NPCs increases expression of neuronal and glial markers

(A) Phase-contrast images of control vs TRF2-Ov NPCs. Scale bar 100 μ m. (B) Gene expression analysis of NPCs (*Pax6*), neuronal (*NeuroD2*, *Tuj1*) and glial (*GFAP*) markers by qRT-PCR. Error bars represent standard derivation from 6 repeats of three independent experiments. Statistical significance was tested by paired Student's t-test, *** $p \leq 0.001$, **** $p \leq 0.0001$, NS $p > 0.05$ (non-significant). (C) Immunoblotting of TRF2 in control and TRF2-Ov NPC protein lysates. Image is representative of three independent experiments.

Given that NPCs were being cultured in NPC culture conditions, it is possible that the effect of TRF2-Ov may have been masked greatly by the presence of growth factors in the media. Therefore, the growth factors from the media were withdrawn and the cells allowed to terminally differentiate for 7 days prior analysis.

Interestingly, no morphological differences were observed under light microscopy. Both control and TRF2-Ov cells showed terminal differentiation into the typical morphology of neurons and glia (Figure 5.6 A). Gene expression analysis of *Tuj1* and *GFAP* did not show significant differences between control and TRF2-Ov terminally differentiated cells (Figure 5.6 B). Co-staining of TRF2 and Tuj1 or TRF2 and GFAP in NPCs under self-renewing conditions did not reveal significant differences in neuronal or glial staining (Figure 5.6 C-D, upper panels). Furthermore, both control and TRF2-Ov NPCs were able to terminally differentiate into post-mitotic neurons or glia, indicating that a reduction of TRF2 protein expression is not required for their differentiation (Figure 5.6 C-D, lower panels).

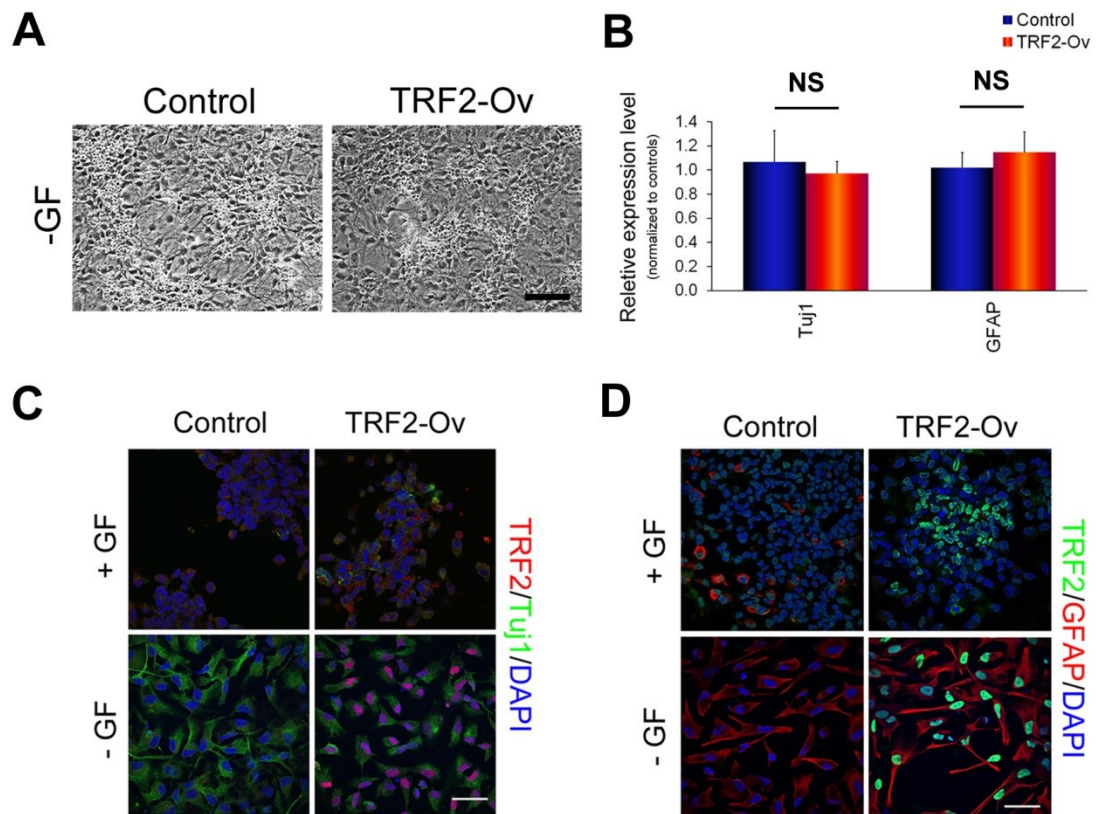


Figure 5.6 TRF2 overexpressing NPCs can terminally differentiate into neurons and glia with no significant changes in their marker expression.

(A) Light microscopy images of control and TRF2-Ov terminally differentiated NPCs. Scale bar= 100 μ m. (B) qRT-PCR of genes Pax6, Tuj1 and GFAP. Error bars represent standard derivation from 6 repeats of two independent experiments. Statistical significance was tested by paired Student's t-test, NS $p > 0.05$ (non-significant). (C) IF of control and TRF2-Ov NPCs with and without growth factors (+/-GF), with TRF2 and neuronal marker Tuj1. Images representative of a single experiment carried out in duplicate. (D) IF of genetically modified NPCs with and without growth factors with TRF2 and glial marker GFAP. Nuclei are shown by DAPI staining (blue) in all IF images. Scale bar= 30 μ m in all IF images. Images representative of a single experiment carried out in duplicate.

5.2.4 Exogenous expression of TRF2 in NPCs accelerates telomere shortening but does not results in an obvious NHEJ of telomere ends phenotype

Next, telomere length and chromosomal spreads of both control and TRF2-Ov NPCs were analysed. As previously reported and observed in TRF2-Ov hESCs, TRF2 overexpression in NPCs also resulted in increased shortening of telomeres compared to control (Figure 5.7 A). However, this did not seem to hinder their ability to

terminally differentiate into neurons or glia (Figure 5.6 C-D) and did not result in NHEJ of telomere ends (Figure 5.7 B).

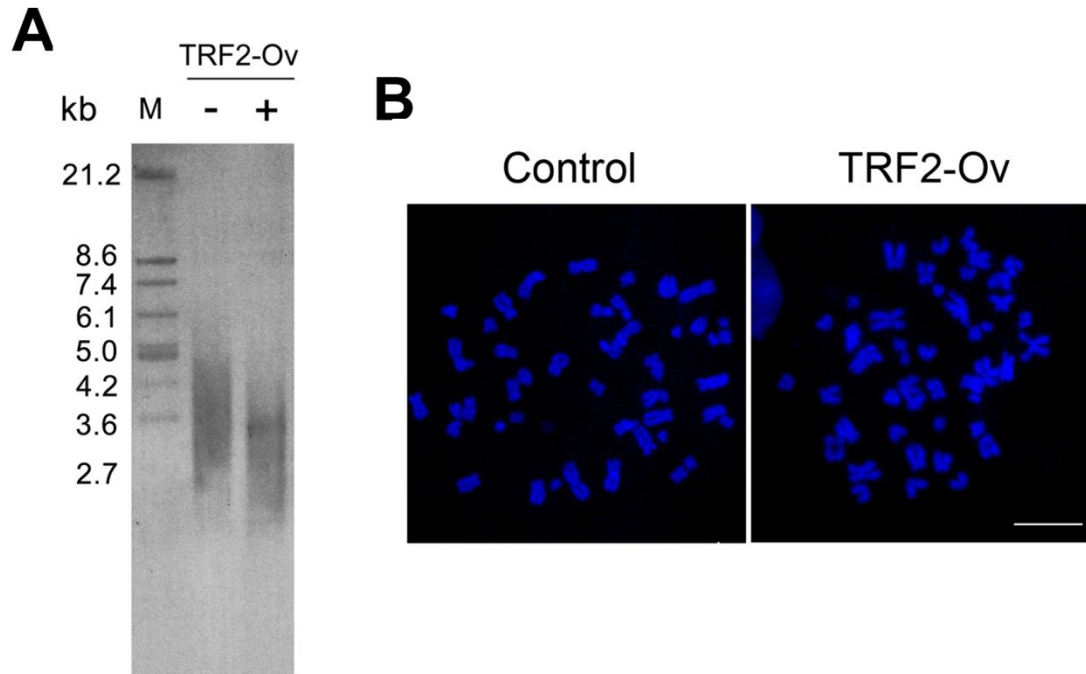


Figure 5.7 Ectopic expression of TRF2 in NPCs induces telomere shortening but no NHEJ.

(A) Telomere length analysis by southern blotting of control and TRF2-Ov NPCs genomic DNA. Image is representative of two independent experiments. **(B)** Chromosomal spread preparation of both cell types indicating no formation of NHEJ of chromosomal ends. Scale bar= 10 μ M. Images are representative of two independent experiments carried out in duplicate.

5.3 Discussion

In this chapter the effects of gain and loss of TRF2 function in NPCs were addressed to support the data obtained in hESCs and to further explore the possible role of TRF2 in neural differentiation. TRF2 knockdown in NPCs affected the expression of key neural progenitor markers at both the mRNA and protein levels and hindered their ability to terminally differentiate into neurons and glia in a similar fashion to that observed in TRF2-Sh hESC-derived NPCs. Interestingly, a recent publication using the embryonic carcinoma stem cell line NTera2 showed that knockdown of TRF2, via dominant inactive TRF2 overexpression, resulted in induction of neuron formation instead of hindering neural differentiation (Zhang *et al.*, 2008). While the discrepancy in neural induction phenotypes may be attributed to different cell types used, it is worth mentioning that accumulation of DNA damage can induce differentiation to the neural lineage in some cell types (Schneider *et al.*, 2013). Therefore, since TRF2 depletion is known to result in uncapping of the telomeres leading to accumulation of DNA damage and cell senescence (van Steensel *et al.*, 1998; Karlseder *et al.*, 1999; Karlseder *et al.*, 2004), it is possible that depletion of endogenous TRF2 in NTera2 cells induced neural differentiation as a consequence of DNA damage accumulation. While DNA damage accumulation as a neural induction mechanism could explain the phenotype observed in NTera2 cells, it could not explain the phenotype observed in our TRF2-Sh NPCs as these cells revealed hindered ability to differentiate. However, it is possible that instead of inducing neural differentiation accumulation, DNA damage accumulation in our cells triggered cell senescence, explaining why these cells could not differentiate. To address this possibility we analysed TRF2-sh NPCs with DNA damage (γ H2AX), cell proliferation (H3 phosphorylation) and cell senescence markers (Caspase 3). TRF2-sh NPCs revealed increased expression of γ H2AX, slower proliferation rate and a decrease in H3 phosphorylation. These results suggested that the increase of γ H2AX DNA damage signals could have resulted in p53 activation which in turn stalled cell cycle progression in an attempt to repair the damaged DNA, explaining the decrease in H3 phosphorylation and cell proliferation. However, while these results may explain why TRF2-sh NPCs proliferate slightly slower, they did not explain TRF2-sh NPCs inability to differentiate as these cells revealed no increases

in Caspase 3 expression or NHEJ of telomeres, indicating these cells were not genomically unstable or senescing. While the proliferation, cell senescence and chromosomal spread experiments presented here could benefit from further characterisation, such as caspase 3 quantification or FISH, a recent study has reported that accumulation of DNA damage and NHEJ at telomeres, as a consequence of TRF2 knockdown, is directly dependent on the levels of TRF2 depletion (Cesare *et al.*, 2013), indicating that perhaps the TRF2 knockdown approach used in our cells did not induce enough DNA damage to result in cell senescence. This report indicated that very small levels of TRF2 protein are sufficient to inhibit NHEJ of telomeres and DDR activation, which is in line with the levels of TRF2 protein expression observed in hESCs (Figure 3.3 A). Therefore, this could explain why TRF2-Sh NPCs do not go into NHEJ or senescence and indicate that the hindered neural differentiation observed is unlikely to be due DNA damage accumulation. Altogether, the different cell types and approaches to knockdown TRF2 used in our study and Zhang *et al* could explain the discrepancies observed. It is likely that given their testicular carcinoma origin, Ntera2 cells use a different mechanism to regulate neural differentiation to that of normal human NPCs.

TRF2 overexpression in NPCs was carried out using the same lentiviral particles used to overexpress TRF2 in hESCs. While TRF2 overexpression in NPCs resulted in an increase of expression of neuronal markers that reminisce to the effects of TRF2 overexpression in hESCs, morphological changes were not observed. Since the presence of growth factors bFGF and EGF could have greatly masked the potential neural differentiation effect of TRF2 overexpression these were withdrawn from the media. Following growth factor withdrawal, both control and TRF2-Ov NPCs differentiated into neurons and glia normally, without revealing any distinct differences in morphology, or marker expression. This indicated that TRF2 downregulation, which is observed in wild type NPCs upon differentiation to neurons or glia (Figure 3.7), is not required for the terminal differentiation of NPCs.

Telomere elongation or shortening as a result of TRF2 knockdown or TRF2 overexpression in NPCs, respectively, did not seem to be the cause of the neural differentiation phenotypes observed. It has been recently published that ES cells with short telomeres have unstable differentiation (Pucci *et al.*, 2013). The NPCs used in

this study were of short telomere length and upon TRF2 overexpression this length became even shorter. However, these cells still had the ability to terminally differentiate normally into neurons and glia unlike the TRF2-Sh NPCs which had increased telomere length upon TRF2 knockdown. This indicated that telomere length in NPCs is unlikely to be a factor contributing to the detrimental effect observed in neural differentiation upon TRF2 knockdown.

Altogether these results suggest that TRF2 is regulating neural gene expression in NPCs through an unknown mechanism and that this mechanism, according to our available data and the literature, is likely to be independent of telomere length and telomere DNA damage accumulation.

Chapter 6 TRF2-mediated stabilization of human REST4 is vital for neural differentiation and maintenance of neural progenitors

6.1 Introduction

TRF2 has been previously reported to inhibit neuronal differentiation of human embryonic carcinoma NTERA2 cells by interacting with REST and stabilising its protein levels (Zhang *et al.*, 2008). REST is a master transcriptional repressor that regulates the expression of hundreds of genes, many of which are critical for neural differentiation and the development of the central nervous system such as *Mash1*, *Snap25*, *NeuroD2*, *Tuj1*, *GFAP* and *Sox2* (Lietz *et al.*, 2003; Bruce *et al.*, 2004; Ballas and Mendel, 2005; Singh *et al.*, 2008; Kohyama *et al.*, 2010). Several splice variants of REST have been discovered in rodent and human (Palm *et al.*, 1998, 1999). In human one of these isoforms has been previously reported to be generated by the alternative splicing of a neural-specific exon (exon N) located between exon IV and V to produce a 62 nucleotide insertion in the REST mRNA, containing an in-frame stop codon that causes premature translation termination and synthesis of a truncated REST protein known as hREST-N62 (Palm *et al.*, 1999). In rodent, a similar isoform of REST termed rREST4 has very similar protein structure to hREST-N62 and has been shown to antagonize the repressive function of REST on its target genes, in neural tissues (Palm *et al.*, 1999; Shimojo *et al.*, 1999; Tabuchi *et al.*, 2002; Uchida *et al.*, 2010; Raj *et al.*, 2011). Rodent rREST4 like hREST-N62, both retain the REST N-terminal domain and 5 of the 9 zinc-finger DNA binding domains, but lack the C-terminal repression domain. As such, here hREST-N62 variant has been termed human REST4 (REST4) given the similarity in protein structure to rodent rREST4 (Figure 6.1 C), posit its involvement in regulating neural differentiation through a similar mechanism.

The previous chapters demonstrated that TRF2 plays a critical role in the neural differentiation of hESCs and maintenance of NPCs multipotency. Given the critical importance of REST and rREST4 in neural differentiation, we questioned whether TRF2 could be regulating neural differentiation of hESCs through either of these factors. To address this question the protein levels of REST and REST4 were analysed during neural differentiation of hESC. TRF2-Ov hESCs and TRF2-Sh NPCs were also analysed to address the relationship between TRF2 and REST/REST4. The aim of this chapter is to decipher the mechanism underlying the role of TRF2 in regulating neural differentiation of hESCs.

6.2 Results

6.2.1 TRF2 protein affects REST4 but not REST expression in hESCs and NPCs.

Given the critical importance of REST in neural differentiation and that TRF2 has been previously suggested to stabilise REST (Zhang *et al.*, 2008), REST and TRF2 protein expression was first analysed in hESCs and their neural derivatives to ascertain if their expression correlates. Surprisingly, even though TRF2 was clearly upregulated upon differentiation of hESCs to NPCs, no significant difference in REST protein levels was observed (Figure 6.1 A). Overexpression or knockdown of TRF2 in hESCs and NPCs, respectively, did not result in REST mRNA or protein level changes either (Figure 6.2 A-C). To further validate this lack of TRF2-REST correlation TRF2 was also overexpressed in HEK293T cells, revealing no changes in REST protein expression (Figure 6.2 D).

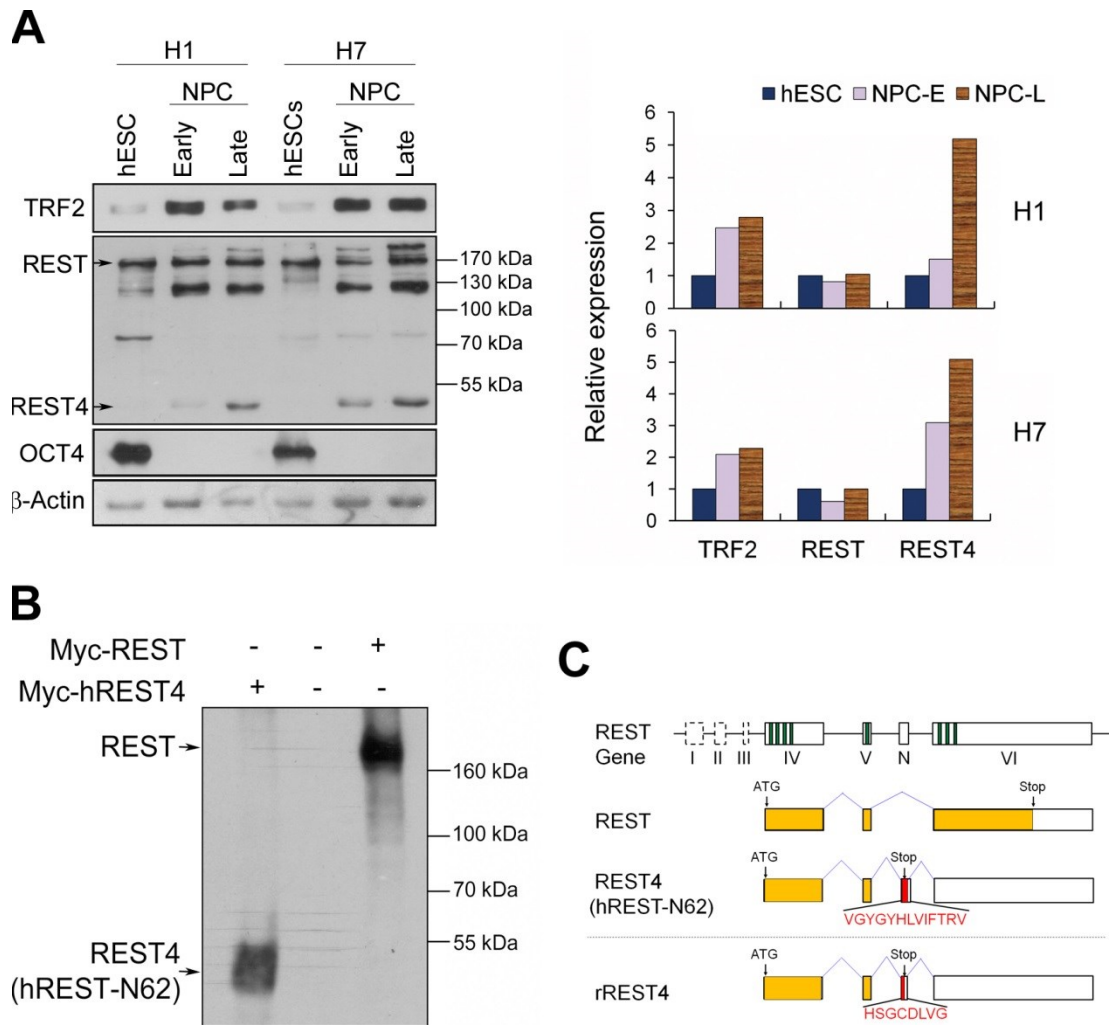


Figure 6.1 Neural differentiation of H1 and H7 hESCs does not result in a downregulation of REST but upregulation of its splice variant human REST4.

(A) Cell extracts isolated from H1 and H7 hESCs and their early and late NPCs were analysed by immunoblotting with indicated antibodies. Quantitative analysis is presented as a histogram (right panels). Arrows in immunoblot point to REST and REST4 human proteins. Immunoblot image is representative of two independent experiments. (B) Immunoblotting of HEK293T cell extracts overexpression human REST and REST4. Arrows point to REST and REST4 human proteins which are of the same molecular weight as the endogenous REST and REST4 proteins detected in (A). (C) Schematic map of human REST gene comparing the structural similarity between human REST, human REST4 (also known as hREST-N62) and rodent rREST4. Open and colored boxes represent exons and coding regions, respectively. Specific amino acid sequence derived from exon N expression is shown in red. Long dotted line across schematic map separates human from rodent REST transcripts.

It is noteworthy, that the relative stable REST protein expression observed during neural differentiation of hESCs is different from that reported during mESC neural differentiation, which shows a downregulation of REST. This reduction is thought to alleviate REST repression on neural genes, hence promoting neural differentiation (Ballas *et al.*, 2005). These results raised the question as to how REST-mediated repression on neural genes is reduced during hESC neural differentiation. It was noticed that the REST antibody, raised against the N-terminus of human REST (Epitomics antibody, see section 2.1.3 for details), consistently detected a ~40-kDa band in NPCs, which is lower in molecular weight than that of REST (Figures 6.1 A and 6.7 A). Although the antibody also detected a couple of other bands, these did not appear when using other batches of the antibody, and were therefore considered to be non-specific (Figures 6.1 A, 6.6 C, 6.7 A). Interestingly, this ~40kDa protein exhibited a pattern of upregulation similar to that of TRF2 during neural differentiation (Figure 6.1 A histograms) and is of a similar molecular weight to the predicated size of hREST-N62, a human REST isoform that is orthologous to the rodent neural-specific REST isoform rREST4 (Palm *et al.*, 1998; Palm *et al.*, 1999; Shimojo *et al.*, 1999). Correspondingly, immunoblotting with the REST antibody on HEK293T cell extracts overexpressing human REST and hREST-N62 revealed the presence of only two bands of ~170-200 KDa and ~40 kDa, respectively (Figure 6.1 B). hREST-N62 is generated by the alternative splicing of a neural-specific exon (exon N) located between exon IV and V to produce a 62 nucleotide insertion in the REST mRNA, containing an in-frame stop codon that causes premature translation termination and synthesis of truncated REST protein (Figure 6.1 C) (Palm *et al.*, 1999). Although no function has been ascribed to hREST-N62, the rodent protein orthologous rREST4 has been shown to counteract the repressive role of REST in the regulation of its target genes, particularly during neurogenesis (Shimojo *et al.*, 1999; Palm *et al.*, 1999; Tabuchi *et al.*, 2002; Uchida *et al.*, 2010). Whilst the protein sequence encoded by exon N differs between REST-N62 and rodent rREST4, both retain the REST N-terminal domain and 5 of the 9 zinc-finger DNA binding domains, but lack the C-terminal repression domain (Figure 6.1 C). Hence, we have appropriately termed this hREST-N62 variant as human REST4 (REST4) (Figure 6.1 C) and given the similarity in protein structure to the rodent rREST4, posit its involvement in regulating neural differentiation through a similar mechanism.

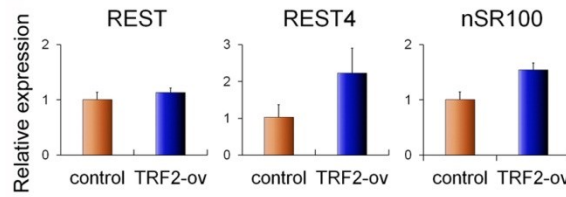
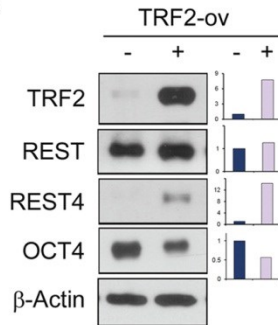
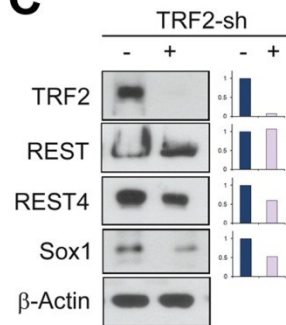
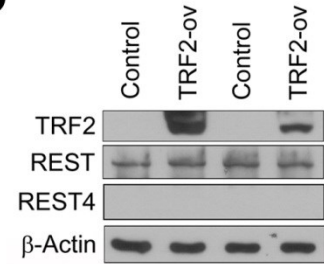
A**B****C****D**

Figure 6.2 Modulation of TRF2 protein levels affect the expression of REST4 but not REST.

(A) mRNA expression analysis of *REST*, *REST4* and *nSR100* in TRF2 overexpressing hESCs and control by qRT-PCR with sequence specific primers. Data are presented as mean $\pm$ SD of three independent experiments. Comparison of protein expression in TRF2 overexpressing H1 hESCs (B) and TRF2 knockdown H1 NPCs (C) by immunoblotting with the indicated antibodies. (D) REST and REST4 protein expression analysis in two different HEK293T TRF2 infected cell lines by immunoblotting. No difference in REST expression was detected and REST4 was not detected as it is expressed only in neural lineage tissues. Immunoblot images are representative of at least two independent experiments.

In line with the neural differentiation data, REST4 was also found to be clearly upregulated in TRF2-Ov hESCs (Figure 6.2 A-B), which was also accompanied with the upregulation of the REST splicing regulator nSR100 transcripts (Figure 6.2 A). In contrast, TRF2 knockdown in NPCs reduced REST4 protein level (Figure 6.2 C). These results demonstrate that there is a positive correlation between TRF2 and REST4 protein expression in hESCs and NPCs. However, REST4 protein expression was not detected in TRF2-Ov HEK293T cells, indicating that TRF2 probably is not directly involved in regulation of REST4 transcription and REST alternative splicing.

Taken together, these data suggests that TRF2 may act to enhance the expression of REST4 protein, subsequently affecting the neural differentiation of hESCs and the preservation of NPC neuropotency. Full immunoblots for figure 6.2 C and D can be found in the appendix (Figures A4 and A5).

6.2.2 TRF2 specifically interacts with REST4

It has been widely demonstrated that REST4 functions to counteract the repressive role of REST in the regulation of gene expression, particularly during neurogenesis (Palm *et al.*, 1999; Shimojo *et al.*, 1999; Raj *et al.*, 2011). Hence, we hypothesised that TRF2 may promote neural differentiation of hESCs by regulating the expression of REST4. Although a positive correlation between TRF2 and REST4 protein expression was observed, it remained unclear how TRF2 may affect REST4 expression. Since overexpression of TRF2 did not increase REST in hESCs as well as HEK293T cells, and did not induce REST4 expression in HEK293T cells, it seems unlikely that TRF2 enhances REST4 expression through direct regulation of REST transcription or alternative splicing. Given that TRF2 acts as a protein hub and thus interacts with many proteins (Kim *et al.*, 2009; Okamoto *et al.*, 2013), it is conceivable that TRF2 may affect REST4 through protein-protein interaction. For these reasons, the potential ability of TRF2 to interact with REST4 was first addressed endogenously in NPCs using co-immunoprecipitation (co-IP) approaches (Figure 6.3 A). The telomere binding protein Rap1, a well-known TRF2 interacting partner (Li *et al.*, 2000), was used as positive control of the co-IP. Immunoprecipitation of TRF2 in NPCs clearly showed a co-IP of RAP1 protein indicating that the reaction conditions were optimal to detect protein-protein interactions. Indeed, TRF2 immunoprecipitation in NPCs showed that endogenous REST4 but not REST could be detected in TRF2 immunoprecipitates (Figure 6.3 A).

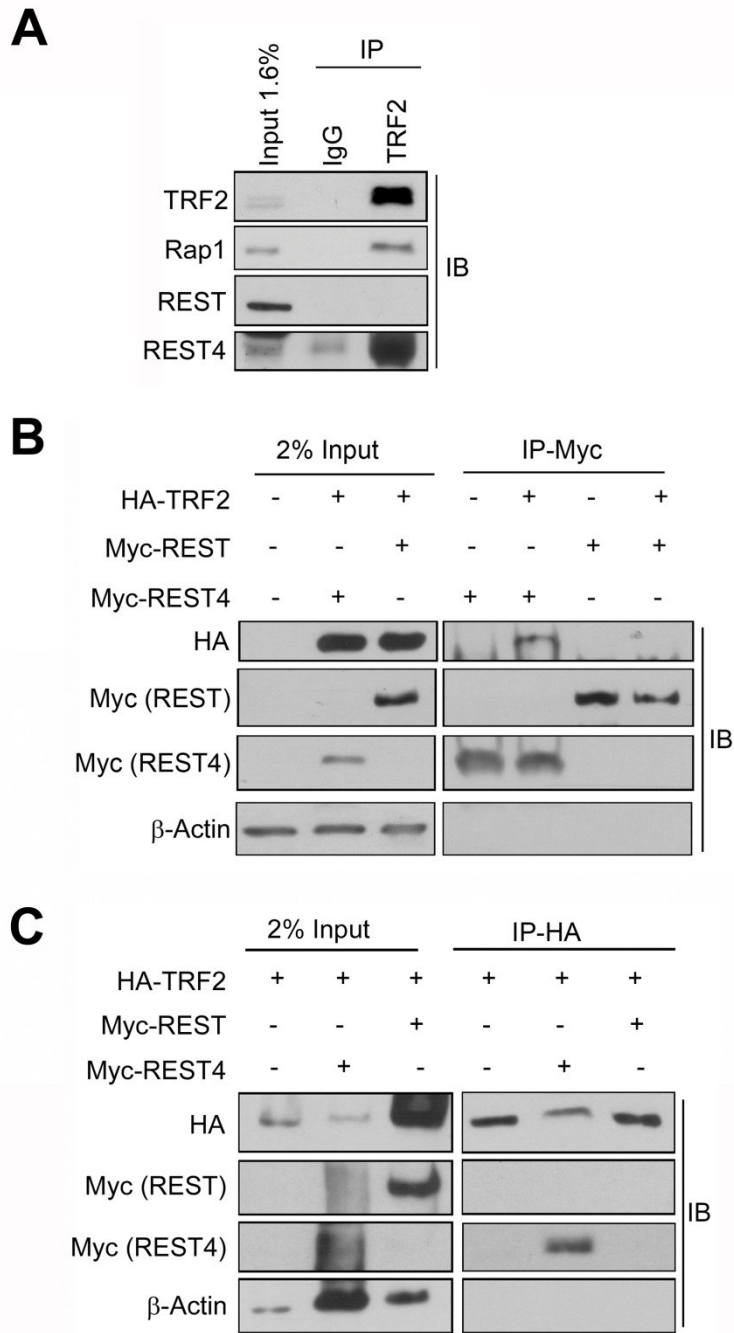


Figure 6.3 Interaction between TRF2 and REST4 by co-immunoprecipitation

(A) TRF2 immunoprecipitates or whole cell lysate from NPCs were analysed with indicated antibodies. Rap1 is used as positive control for the interaction. (B-C) TRF2 interacts with REST4 but not REST proteins. HEK293T cells were transfected with HA-tagged TRF2 (HA-TRF2), Myc-tagged REST (Myc-REST) and Myc-tagged REST4 (Myc-REST4) expression vectors individually or combined as indicated for 48 hours. HA immunoprecipitates and whole cell lysates (B) or Myc immunoprecipitates and whole cell lysates (C) were analysed by immunoblotting with HA or Myc antibodies. All three immunoblots are representative images of at least two independent experiments each.

Next, to validate this interaction I transiently overexpressed TRF2, REST and REST4 epitope-tagged proteins in HEK293T. Myc-tagged REST (Myc-REST) or REST4 (Myc-REST4) together with HA-tagged TRF2 (HA-TRF2) were transiently expressed in HEK293T cells and their cell extracts were subjected to co-IP analysis. In these experiments, I showed that HA-TRF2 could only be detected in the myc-REST4 immunoprecipitates and in the immunoprecipitates of myc-REST (Figure 6.3 B); similarly, only myc-tagged REST4 could be detected in the HA-TRF2 immunoprecipitates (Figure 6.3 C). Full immunoblots of figures 6.3 A-C can be found in the appendix (Figures A6-A8).

REST4 is largely considered as a truncated REST with almost complete homology except for the 13 amino acids (AAs) at the C-terminus of REST4, which are encoded by exon N. Hence, the 13 AAs are unique to the REST4 and interestingly, they contain the core consensus of TRF2 binding motif [Y/F]XL (Figure 6.4 A) (Chen *et al.*, 2008; Kim *et al.*, 2009). Therefore, it is plausible that the fact that TRF2 can only interact with REST4 and not REST may be attributed to the 13-AA peptide. To address whether this 13-AA peptide is critical for the interaction between REST4 and TRF2, a mutant REST4 (REST4m) expression vector was generated by introducing an early stop codon upstream of these 13 AAs (Figure 6.4 A). When HA-TRF2 with either wild-type myc-REST4 or mutant myc-REST4m were ectopically expressed in HEK293T cells, only myc-REST4 was detected in HA-TRF2 immunoprecipitates (Figure 6.4 B), indicating that the 13 AAs at the C-terminus of REST4 are essential for the interaction between TRF2 and REST4. These data demonstrate that TRF2 interacts with REST4 but not REST.

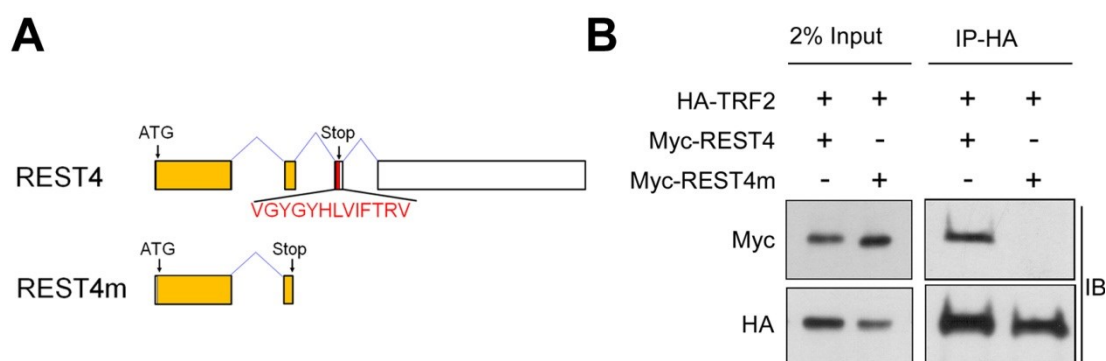


Figure 6.4 TRF2 interacts with REST4 through its exon N protein sequence.

(A) Schematic representation of REST4 and REST4 mutant (REST4m) mRNA. In red is the amino acid sequence corresponding to exon N. An early stop codon was introduced to eliminate the sequence from REST4m. (B) Co-immunoprecipitation of REST4 and REST4m proteins in HEK293T cells co-transfected with HA-TRF2 and Myc-REST4 or HA-TRF2 and Myc-REST4m expression vectors for 48 hours. HA immunoprecipitates or whole lysates were analysed by immunoblotting with Myc and HA antibodies. Immunoblot image is representative of two independent experiments.

6.2.3 TRF2 protects REST4 from ubiquitination and proteasomal degradation

Although it was ascertained that TRF2 interacts with REST4 but not REST, it remained unclear how TRF2 can enhance REST4 protein level. Given that TRF2 interacts with REST4 at the protein level and that TRF2 is unlikely to be involved in its transcription regulation, it was hypothesised that TRF2 regulates REST4 protein levels at the posttranslational level; e.g. TRF2 could protect REST4 from proteasomal degradation. It is unknown whether REST4 protein is affected by proteasome degradation system, however REST has been reported to be regulated by ubiquitin-mediated proteasomal degradation (Westbrook *et al.*, 2008; Zhang *et al.*, 2008; Huang *et al.*, 2011). Thus, tests were run to observe if REST4 was proteasome degradation dependent. NPCs were treated with either proteasome inhibitor MG132 or protein translation inhibitor cyclohexamide (CHX) for 6 hours. The cell extracts were then analysed by immunoblotting for REST4 protein levels. Blocking protein degradation with MG132 resulted in an accumulation of REST4 protein while blocking protein translation resulted in degradation of REST4 (Figure 6.5 A). These results suggested

that REST4 protein is indeed degraded by the proteasome. Since proteasome-mediated protein degradation requires ubiquitination of the protein, (Baumeister *et al.*, 1998), the possibility that TRF2 may affect ubiquitination of REST4 was investigated. HEK293T cells stably overexpressing TRF2 were generated and used to ascertain if TRF2 is able to protect REST4 from ubiquitination and consequently proteasomal degradation. Myc-REST4 and HA-tagged ubiquitin (HA-Ub) were ectopically expressed in control and TRF2-Ov HEK293T cells for 48 hours and then the whole cell lysates were subjected to IgG and Myc immunoprecipitation (Figure 6.5 B).

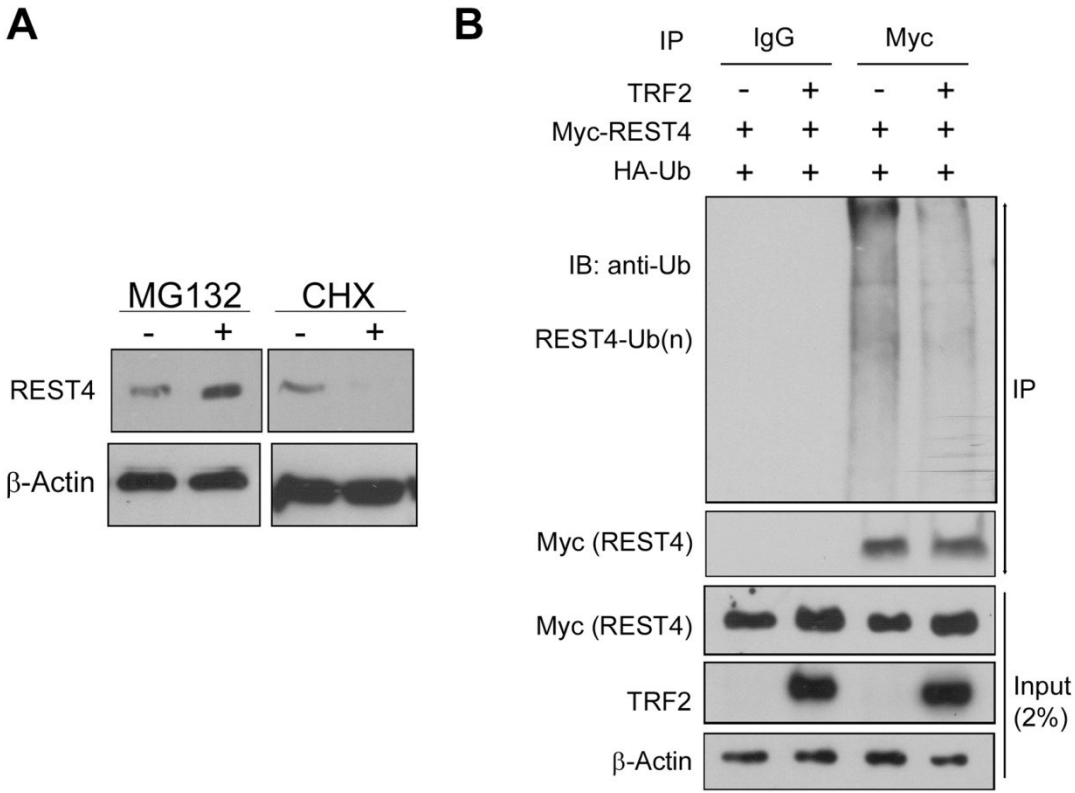


Figure 6.5 TRF2 protects REST4 from proteasome dependent degradation.

(A) REST4 protein can be degraded by a proteasome-dependent mechanism. hESC-derived NPCs were treated with either proteasome inhibitor MG132 or protein translation inhibitor CHX for 6 hours. The cell extracts were then analysed by immunoblotting for REST4 protein levels. Image representative of two independent experiments. **(B)** Control and TRF2 stably overexpressing HEK293T cells were co-transfected with Myc-REST4 and HA-tagged ubiquitin (HA-Ub) expression vectors. Myc immunoprecipitates (IP) or whole cell lysates (input) were immunoblotted with the indicated antibodies. Immunoblot is representative of two independent experiments.

Upon pull-down of Myc-REST4 protein and ubiquitin immunoblotting of the immunoprecipitates, it was found that high levels of TRF2 reduced the ubiquitination of REST4 and therefore could potentially prevent its proteasome-mediated degradation (Figure 6.5 B). Therefore, while the identity of the E3 ubiquitin ligase targeting REST4 remains to be elusive, these results demonstrate that high levels of TRF2 enhance REST4 protein expression by protecting it from ubiquitination-proteasomal degradation, which consequently functions in promoting neural differentiation of hESCs and maintenance of NPCs.

6.2.4 REST4 overexpression in hESCs induces neural differentiation

Given the structural similarity between rodent rREST4 and human REST4 as well as their specific expression in neural tissues, we proposed that REST4 could have a similar function to that of its rodent ortholog in regulating neural differentiation. Since we found that TRF2 stabilizes REST4 we hypothesised that perhaps the accumulation of REST4 protein is the causative of the observed neural differentiation. To validate this hypothesis, REST4 was ectopically expressed (REST4-ov) in hESCs. Following overexpression it was found that these cells exhibited compromised self-renewal and increased cell differentiation, particularly towards the neural lineage even under hESC culture conditions. Exogenous expression of REST4 resulted in a downregulation of ESC markers Oct4 and Sox2 while increasing the expression of NPC markers Pax6 and Nestin (Figure 6.6 A-D). In addition, when subjected to neural differentiation, REST4-ov hESCs, like TRF2-ov hESCs, showed a quicker and more efficient neural differentiation (Figure 6.6 A and E). These results indicate that the presence of REST4 in hESCs counteracts the pluripotency of hESCs, possibly through affecting the repression of neural genes by REST.

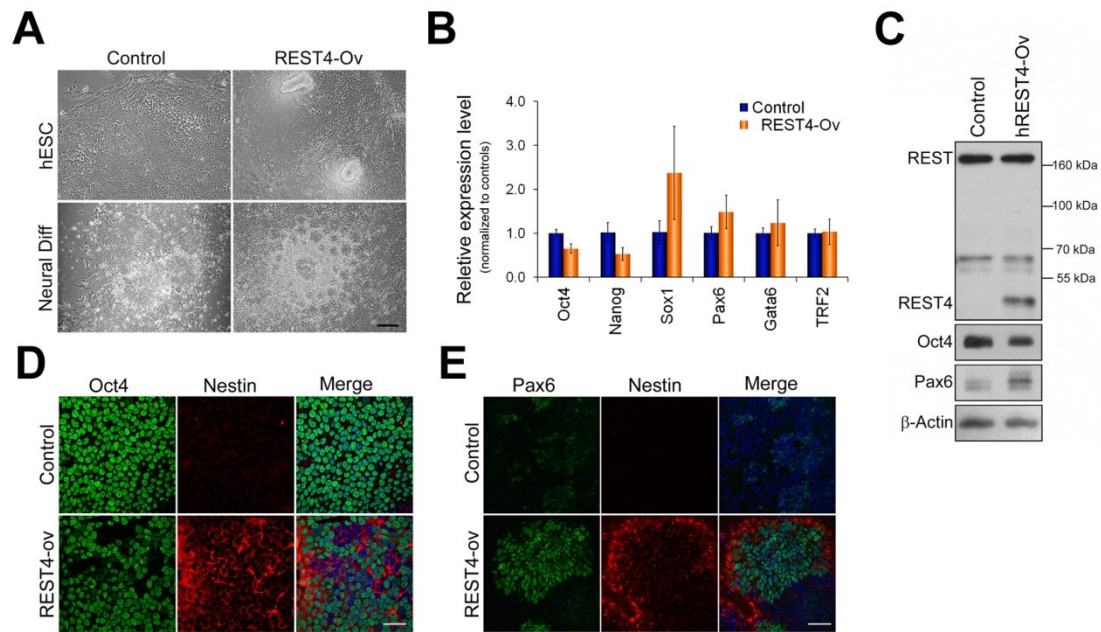


Figure 6.6 REST4 overexpression in hESCs hinders their self-renewal and promotes neural differentiation.

(A) Phase-contrast images of control and REST4-ov hESCs in hESCs culture conditions (upper panels) as well as in neural differentiation conditions (lower panels). (B) Gene expression analysis by qRT-PCR of the indicated genes. Data are presented as mean $\pm$ SD of 8 repeats of 2 independent experiments. (C) Immunoblotting showing REST4 overexpression results in a downregulation of Oct4 and an increase of Pax6 protein. (D-E) Immunofluorescence of control and REST4-ov hESCs under hESC culture conditions (D) and 13 days under neural differentiation conditions (E) with the indicated antibodies. Scale bars represent 100 μ m in A and 50 μ m in D and E.

6.2.5 REST4 overexpression in TRF2-sh NPCs rescues their neural differentiation deficiency

While the overexpression of human REST4 in hESCs revealed that this protein has a similar function in promoting neural differentiation as its rodent counterpart, the current data could not directly attribute the neural differentiation deficiency effects observed in TRF2-sh hESCs and TRF2-sh NPCs to a downregulation of REST4. To address this directly, REST4 was overexpressed in TRF2-sh NPCs to attempt to rescue the neural differentiation deficiency phenotype. Overexpression of REST4 in TRF2-sh NPCs resulted in an increase of REST4 protein similar to that of control without affecting TRF2 or REST protein levels (Figure 6.7 A). REST4

overexpression was able to rescue some of the NPC marker gene expression and neuron and astrocyte formation lost upon TRF2 knockdown (Figure 6.7 B and C).

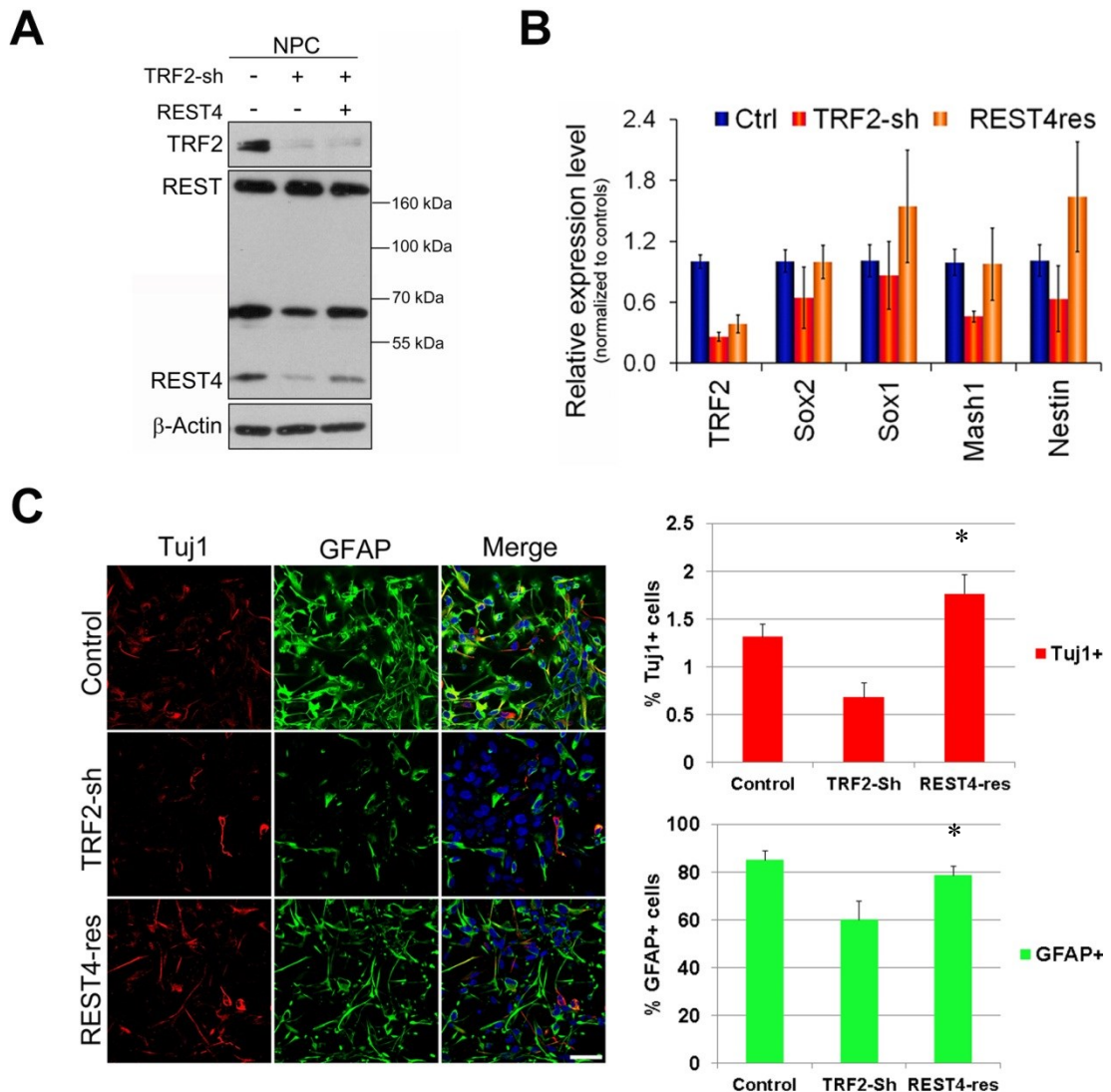


Figure 6.7 REST4 overexpression rescues neural differentiation deficiency of TRF2-sh NPCs.

(A) Immunoblotting of exogenous expression of REST4 in TRF2-sh NPCs shows an increase of REST4 protein without affecting TRF2 or REST protein levels. (B) Gene expression analysis by qRT-PCR of the indicated genes in control, TRF2-sh and REST4 rescue (REST4 res) NPCs. Data are presented as mean $\pm$ SD of 9 repeats of 3 independent experiments. (C) Immunofluorescence staining showing an increase in Tuj1 and GFAP positive cells upon REST4 overexpression in TRF2-sh NPCs (REST4-res). Quantitative analysis of the immunostaining is presented as percentage in histograms (right panels). Data is representative of 10 randomly chosen fields of three independent experiments. Statistical significance was tested by paired Student's t-test, * $p \leq 0.05$

REST4 overexpression in TRF2-sh NPCs (REST4-res) resulted in a significant increase in the numbers of Tuj1 and GFAP positive cells compared to TRF2-sh NPCs (figure 6.7 C). Cells recovered similar expression levels to control cells as well as the formation of long interconnecting processes typical neuron and glia processes.

Overall, these data suggest that TRF2 may act upstream of human REST4 to enhance its expression, subsequently affecting the neural differentiation of hESCs and the preservation of NPC neuropotency.

6.3 Discussion

The previous chapters demonstrated that TRF2 is upregulated after neural differentiation and maintained at high levels in NPCs. Furthermore, they revealed that the high levels of TRF2 promote neural differentiation of hESCs and play an important role in maintaining the neuropotency of NPCs. However, the molecular mechanisms underlying these phenotypes remained unknown. In this chapter, steps were taken to attempt to decipher the molecular mechanisms thorough which TRF2 may regulate neural differentiation of hESCs and maintenance of NPCs. Experiments were focused on the relationship between TRF2 and REST because a recent report has shown that TRF2 stabilises REST in NTera2 cells (Zhang *et al.*, 2008) and because several of the genes affected by modulation of TRF2 expression are target genes of REST (Lietz, 2003; Bruce *et al.*, 2004; Ballas *et al.*, 2005; Singh *et al.*, 2008; Kohyama *et al.*, 2010). Interestingly, when H1 and H7 hESCs were differentiated to NPCs, the levels of REST protein were not downregulated while TRF2 protein levels were dramatically increased (Figure 6.1). This was interesting because it has been proposed that mESCs require a downregulation of REST in order to become NPCs (Ballas *et al.*, 2005) and because an increase of TRF2 protein levels did not result in an increase of REST protein abundance as it would have occurred if TRF2 stabilises REST in these cells. Furthermore, REST protein levels did not change when the expression of TRF2 in hESCs, NPCs and HEK293T cells was modulated (Figure 6.2 B-D).

REST was not downregulated following differentiation of H1 and H7 hESCs to NPCs. This raised the question as to how REST-mediated repression on neural genes is mitigated during hESC neural differentiation. It was observed that the protein levels of REST splice variant hREST-N62, here termed human REST4 for its similarity to rodent rREST4, increased following differentiation of H1 and H7 hESCs to NPCs and that its expression was noticeably affected upon modulation of TRF2 expression in hESCs and NPCs. REST4 protein expression seemed to correlate with the expression of TRF2 protein in these cells. Given that rodent rREST4 has been extensively reported to antagonise the repression activity of REST (Shimojo *et al.*, 1999; Palm *et al.*, 1999; Tabuchi *et al.*, 2002; Uchida *et al.*, 2010), it was

plausible to think that TRF2 could regulate neural differentiation in hESCs and NPCs through REST4. Co-IP experiments of endogenous TRF2 in NPCs and ectopically expressed epitope-tagged TRF2 in HEK293T cells confirmed that unlike NTERA2 cells (Zhang *et al.*, 2008) TRF2 interacts with REST4 but not REST. Furthermore, TRF2 was shown to protect REST4 from proteasome dependent degradation explaining the correlation in protein expression of these two proteins in hESCs and NPCs. Since REST4 has extensively been shown to counteract REST activity and we have shown that REST4 is regulated by TRF2, it is possible that the increase of TRF2 expression results in the accumulation of REST4 protein, which in turn antagonizes the repression of REST, explaining the change in expression of REST target genes upon TRF2 protein level changes. However, to address this possibility we first needed to establish that REST4 can indeed induce neural differentiation in a similar fashion to that of rodent rREST4 and that the neural differentiation deficiency observed in TRF2-sh hESCs and NPCs is a consequence of REST4 downregulation. To answer these questions REST4 was overexpressed in hESCs and TRF2-sh NPCs. The results revealed that the exogenous presence of REST4 in hESCs induced these cells to differentiate to the neural lineage in a similar fashion to that of TRF2-ov hESCs. Furthermore, REST4 overexpression in TRF2-sh NPCs resulted in a partial rescue of the neural differentiation deficiency observed suggesting that REST4 is the downstream partner of TRF2 and that the TRF2-mediated stabilization of REST4 is critical for the neural differentiation of hESCs as well as the maintenance of NPC multipotency.

As a whole, these results suggest that repression of REST on its target genes is reduced in the neural differentiation of hESCs, mouse ES cells and NTERA2 cells, but is regulated through different mechanisms. The coordinated balance of REST4 and REST is important for human neural differentiation and NPC maintenance for which high levels of TRF2 protein are crucial.

Chapter 7 General Discussion

Increasing evidence indicates that TRF2 is not only an important factor for functional telomere maintenance but also may have extra-telomeric functions. Despite the importance of TRF2, its expression and involvement during embryonic development and adult tissue homeostasis remains largely unknown. In human, TRF2 has been reported to be highly abundant in brain tissues (Jung *et al.*, 2004), suggesting an involvement in neurogenesis. Furthermore, preliminary data in our laboratory showed that TRF2 protein levels are dramatically upregulated during neural differentiation of hESCs. Thus, elucidating its role in neural development, particularly in brain function may aid in the improved procurement of human neural cells for potential use in regenerative therapies. This study, has demonstrated a novel extra-telomeric function of TRF2 in the neural differentiation of hESCs and maintenance of NPCs which is independent of its role in regulating telomere length and protecting telomeres.

7.1 TRF2 is a vital factor for hESC neural differentiation and maintenance of NPCs properties

TRF2 protein is dramatically upregulated upon neural differentiation of hESCs and is maintained at high levels in cultured NPCs. The upregulation appears to be specific to hESC neural differentiation as neither the differentiation of hESCs to other lineages nor differentiation of mESCs to neural progenitors revealed similar increases. These results are consistent with the *in vivo* data which shows that TRF2 is much more abundant human brain than in other tissues (Jung *et al.*, 2004), indicating that TRF2 may have an important function within neural tissues. While this previous study showed accumulation of TRF2 mainly in the cytoplasm of neurons, in contrast with this study which shows clear nuclei staining, it is likely that this is the result of the in-house made anti-TRF2 polyclonal antibody used detecting an isoform of TRF2 known as TRF2 short (TRF2-s) which is known to accumulate in the cytoplasm of mature neurons (Zhang *et al.*, 2011). The anti-TRF2 monoclonal antibody used in this study only detects the 66 KDa form of TRF2 which is nuclei specific (see

appendix Figure A1). Unfortunately, Jung and colleagues did not describe whether the human brain tissue used in their immunoblotting was a NPC rich region of the brain such as the SVZ of the lateral ventricles or the SGZ of the dentate gyrus in the hippocampus. Therefore in their study it is not possible to ascertain if the abundance of TRF2 in brain tissue is due to the cumulative expression in neurons, high expression in NPCs, or both. Nevertheless, the fact that TRF2 is abundant in human brain compared to other tissues that its overexpression in COS cells induced neurite like processes and that our data suggested high specific expression in human NPCs prompted us to question whether it has a role in neural differentiation. In answering this question it was found that similarly to previous published data (Jung *et al.*, 2004), ectopic expression of TRF2 resulted in induction of neural processes. Overexpression of TRF2 in hESCs promoted neural differentiation, whilst knockdown of TRF2 impeded neural progenitor generation and maintenance, ultimately hindering the production of neurons and glia.

7.2 TRF2-mediated REST4 stability is critical for differentiation and maintenance of neural progenitors

In deciphering the molecular mechanisms underlying the role of TRF2 in neural differentiation, it was discovered that the differential expression of TRF2 during neural differentiation correlates with the expression of hREST4 protein, a truncated REST generated by the alternative splicing of exon N from the REST gene. Although two transcripts, hREST-N4 and hREST-N62, can be generated by alternative splicing of exon N at different sites and both lead to premature termination of the REST protein, only hREST-N62 produces a truncated REST protein with the additional 13 AAs at the C-terminus necessary for interaction with TRF2 (Palm *et al.*, 1999). Since hREST-N62 transcript was similar in structure, molecular weight and neural tissues/cells expression specificity as rodent rREST4 (Palm *et al.*, 1998, 1999), this protein was termed here as human REST4 (REST4). Rodent rREST4 has been widely reported to counteract the repression of REST on target genes, promoting neural gene expression and neurogenesis (Shimojo *et al.*, 1999; Tabuchi *et al.*, 2002; Uchida *et al.*, 2010; Raj *et al.*, 2011) as it lacks the C-terminal repression domain of REST and is unable to interact with CoREST (Andres *et al.*, 1999; Ooi *et al.*, 2007).

Here it was demonstrated that REST4 is also able to promote the neural differentiation of hESCs, possibly through affecting the repression on neural genes by REST. Although overexpression of 'hREST4' in murine neuroblastoma cells was found not to reduce the repression of REST on human synapsin promoter-driven luciferase expression (Magin *et al.*, 2002) , this was performed using only a single promoter in a tumour cell line, which may not be the case across all cell lines. Moreover, here it was demonstrated that TRF2 interacts with REST4 to protect it from ubiquitin-mediated degradation by the proteasome, hence positively regulating neural progenitor formation and maintenance.

In a previous study, Zhang *et al* reported that TRF2 inhibits neuronal differentiation in embryonic carcinoma NTera2 cells by stabilising REST. In their system, overexpression of a dominant negative TRF2 promoted REST ubiquitination and degradation, enhancing neuron formation (Zhang *et al.*, 2008). This project has taken this study into a more physiological context and demonstrated that TRF2 is also involved in regulating the neural differentiation process of hESCs. However, we show that TRF2 enhances REST4 expression during this process, which counteracts the repression of REST on neural genes to promote neural progenitor formation. The discrepancy of the two studies could be attributed to the two different cell types used where TRF2, REST and REST4 are differentially expressed. We have shown that TRF2 and REST are expressed at considerably higher levels in NTera2 cells than in hESCs, which may not reflect their physiological levels in normal pluripotent cells. This could affect their interaction and function due to the intricacies of protein complex formation. Nonetheless, the fact that high-TRF2 expressing NTera2 cells are more readily differentiated into neural lineages than hESCs further supports the idea of TRF2 promoting neural differentiation.

Reducing REST repression on its target genes plays an important role in controlling neural gene expression and neural differentiation. During neural differentiation of mESCs, repression of REST is alleviated by a considerable reduction of REST protein in which REST undergoes proteasomal degradation through β -TrCP-mediated ubiquitination (Ballas and Mendel, 2005). However, in both H1 and H7 hESCs, no such reduction of REST during their differentiation into NPCs was observed, probably due to the abundance of HAUSP deubiquitylase and low level of

β -TrCP in human NPCs (Huang *et al.*, 2011). Instead, hESCs exhibit a clear upregulation of REST4 following neural differentiation. This upregulation is due, at least in part, by the increase of TRF2 protein levels. Therefore, we propose a different mechanism regulating neural differentiation of hESCs: high levels of TRF2 stabilise REST4, enhancing its accumulation. This counteracts the repression of REST on neural target genes, leading to the induction of neural differentiation (Figure 7.1). The findings in this project are further supported by the notion that alternative splice variants play a major role in the regulation of gene expression, which is particularly prominent in the central nervous system (Castle *et al.*, 2008; Licatalosi *et al.*, 2006).

Overall, these results suggest that repression of REST on its target genes is reduced in the neural differentiation of both human and mouse ESCs but is regulated through different mechanisms. The balance of REST4 and REST is important for human neural differentiation and NPC maintenance for which high levels of TRF2 are crucial.

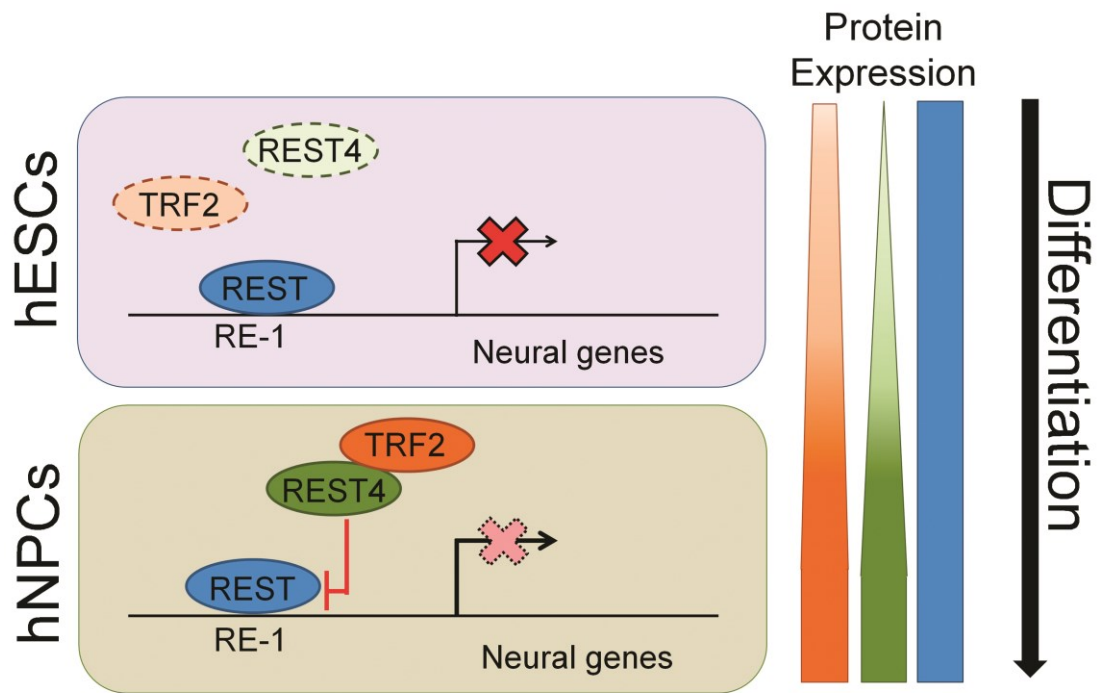


Figure 7.1 Schematic model for the role of TRF2, REST and REST4 in regulating neural differentiation of hESCs and maintenance of NPCs.

Oval cartoons indicate the abundance of the indicated proteins. Dotted ovals indicate low protein levels while continuous line ovals indicate high protein levels. Transcription is indicated with an arrow. Red cross indicates transcription is highly repressed while pink dotted cross indicates activation of some transcription. In this model, upregulation of TRF2 protein results in stabilisation of REST4, enhancing its accumulation. This subsequently counteracts the repression of REST on neural target genes, leading to the induction of neural differentiation.

7.3 TRF2-REST4 mechanism of neural induction

While TRF2 stabilisation of REST4 may indeed result in derepression of REST target genes, the mechanism behind this remains to be elucidated. Given that REST4 lacks the C-terminal repression domain of full length REST, different mechanisms can be proposed. One mechanism may result from a competition between REST and REST4 for the RE1 site of the target gene. Binding of REST4 would result in some derepression, as lacking the C-terminal domain would render REST4 unable to inhibit RNA pol II binding. An alternative mechanism is that REST4 could outcompete REST for Sin3A/B binding as REST4 retains the N-terminal repression domain. In this alternative mechanism REST4 would not bind to the RE1 site but

instead would inhibit recruitment of Sin3A/B to the RE1 site by REST, leading to a reduced repression. Both of these mechanisms are equally possible as it has been reported that overexpression of truncated forms of REST (i.e. lacking either the N-terminal or C-terminal repression domain) results in partial derepression of REST target genes (Andres *et al.*, 1999; Huang *et al.*, 1999; Grimes *et al.*, 2000).

7.4 Concluding remarks and future work

The work in this thesis has demonstrated that TRF2 plays a critical role in the differentiation and maintenance of human NPCs. It also has unravelled possible underlying mechanisms that the coordinated balance of REST4 and REST is important for human neural differentiation and NPC maintenance for which high levels of TRF2 are crucial. This work has contributed to understanding the roles of TRF2, particularly its role in neural differentiation and development, which may impact on future investigations:

1- This study has shown that TRF2 is critical for the neural differentiation of hESCs and maintenance of NPC multipotency. It has also shown that TRF2 regulates these functions by stabilizing REST4 and that REST4 has the ability to induce neural differentiation. While previous reports have indicated that the REST4 rodent ortholog, rREST4, induces neural gene expression by antagonizing the repression of REST on its target genes, this has not been fully addressed for human REST4. The TRF2-REST4 model of neural differentiation regulation presented in this study makes the assumption that REST4 induces neural differentiation by antagonizing REST in a similar manner to what has been reported for rodent rREST4. Therefore this model would benefit from additional experiments that address whether REST4 antagonizes REST. As discussed in section 7.3, REST4 could potentially antagonize REST by binding directly to the RE1 site of its target genes or by sequestering Sin3A/B from the RE1 sites. ChIP analysis of REST binding to the RE1 of its target genes in control and REST4-ov hESCs as well as in control and TRF2-sh NPCs would address if changes in REST4 protein levels affect REST binding and therefore would answer whether the first potential mechanism is correct. To address the second potential mechanism a Sin3A/B immunoblot analysis of REST4 immunoprecipitates

would answer whether REST4 could potentially be sequestering Sin3A/B from the RE1 sites.

2- Assuming the experiments to address the molecular mechanism of REST4 antagonism over REST were successful and since NPCs are essential for the proper development and function of the central nervous system, this study would attempt to address whether there is a link between neurodegenerative diseases and the expression of TRF2, REST and REST, as it has been shown that impaired neurogenesis can contribute to some types of neurodegenerative diseases.

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Appendix

Table A1. Primers for the amplification of human transcripts by qRT-PCR.

Gene name	Forward primer 5'-3'	Reverse primer 5'-3'
<i>AFP</i>	TGGGACCCGAACTTTCCA	GGCCACATCCAGGACTAGTTTC
<i>GAPDH</i>	TCTGCTCCTCCTGTTGACA	AAAAGCAGCCCTGGTGACC
<i>Oct4</i>	TCGAGAACCGAGTGAGAGGC	CACACTCGGACCACATCCTTC
<i>GAPDH</i>	TCTGCTCCTCCTGTTGACA	AAAAGCAGCCCTGGTGACC
<i>Gata6</i>	ACTTGAGCTCGCTGTTCTCG	CAGCAAAAATACTTCCCCCA
<i>GFAP</i>	CACCACGATGTTCTCTTGA	GTGCAGACCTTCTCCAACCT
<i>HPRT</i>	TCCTTGGTCAGGCAGTATAATCC	GTCAAGGGCATATCCTACAACAAA
<i>hTERT</i>	TCAACCGCGGCTTCAAG	TCCAGAAACAGGCTGTGACACT
<i>Nanog</i>	TGATTTGTGGGCCTGAAGAAAA	GAGGCATCTCAGCAGAAGACA
<i>Nestin</i>	GAGGGAAGTCTTGGAGCCAC	AAGATGTCCCTCAGCCTGG
<i>NeuroD2</i>	GGGGAACAATGAAATAAGCG	GCACGTCCGAGAGAAGG
<i>Pax6</i>	TCCGTTGGAAGTATGGAGT	GTTGGTATCCGGGGACTTC
<i>REST</i>	TGTCCTTACTCAAGTTCTCAGAAGA	GAGGCCACATAACTGCACTG
<i>Sox1</i>	AACACTGAAGCCCAGATGGA	GCAGGCTGAATTCGGTTCTC

Gene name	Forward primer 5'-3'	Reverse primer 5'-3'
<i>Sox2</i>	CACACTGCCCCTCTCACACAT	CATTTCCCTCGTTTTTCTTTGAA
<i>TRF1</i>	AATTTGTTGAACCCTGCCAC	ACAGGGTTGAGGTCAGCCTA
<i>TRF2</i>	CCGTTCTCAACCAACCCCTC	GCTGCCTGAACTTGAAACAGT
<i>Sox17</i>	GGCGCAGCAGAATCCAGA	CCACGACTTGCCCAGCAT
<i>Snap25</i>	CTGTCTTTCCTTCCCTCCCT	GGGTCAGTGACGGGTTTG
<i>Mash1</i>	GGAGCTTCTCGACTTCACCA	CTAAAGATGCAGGTTGTGCG
<i>REST4</i>	CATACACAACAGTGAGCGAG	CCAAATGGTATCCATACCCC
<i>nSR100</i>	GAACTGGGTGCCACCAGAGG	CCCCTGGAGGAAGGTGGTGG
<i>Tuj1</i>	AGTGTGAAAAGTGC GACTGC	ACGACGCTGAAGGTGTTTCAT

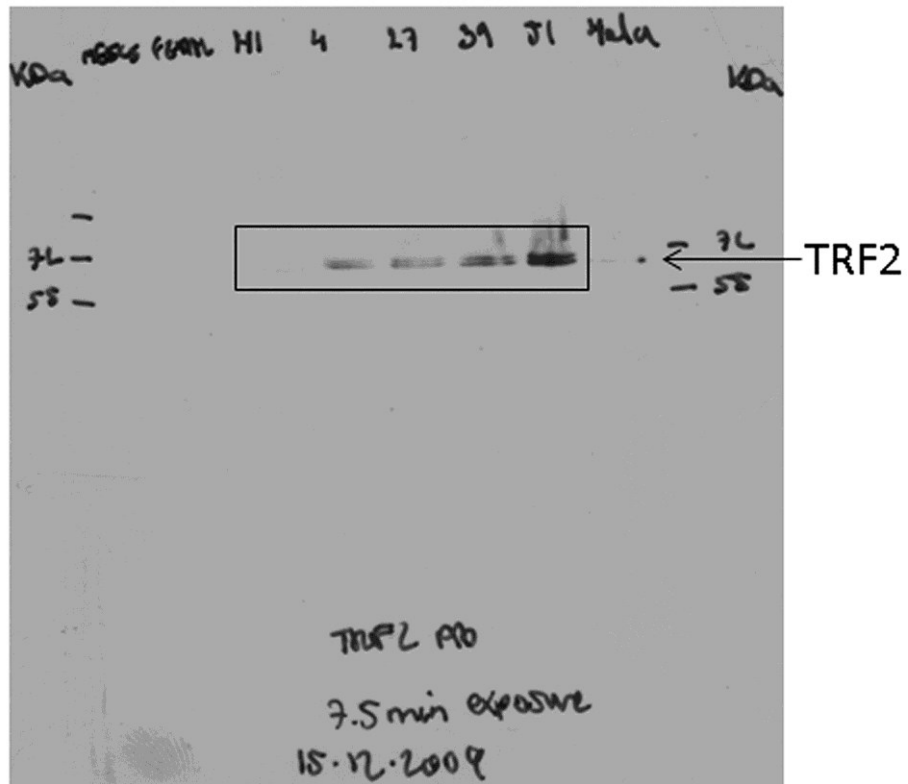
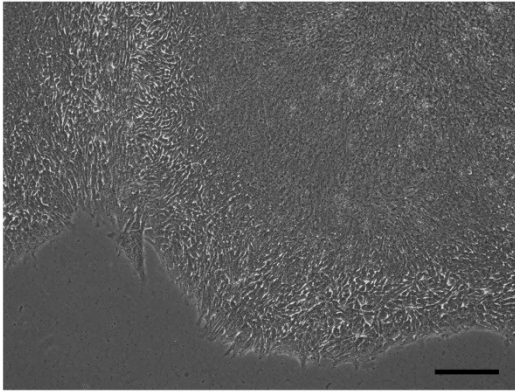


Figure A1. Full TRF2 immunoblot corresponding to Figure 3.1 B.

Uncropped TRF2 immunoblot of H1 hESCs and their neural derivatives revealing a single TRF2 band of 66 KDa.

Phase contrast



GFP

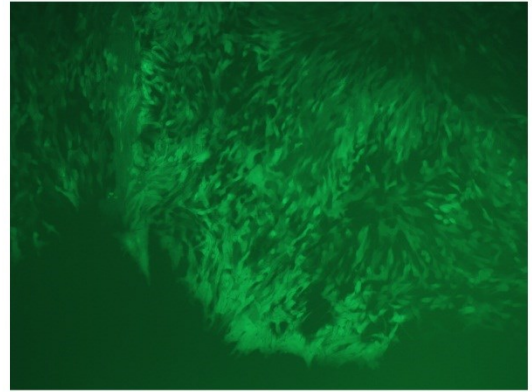


Figure A2. H1 hESCs infected with pLVTHM virus 48 hours post transduction

H1 hESCs were analysed for GFP expression 48 hours after transduction with pLVTHM-GFP virus particles. Left panels show the typical morphology of a hESCs colony and right panel reveals the expression of GFP under fluorescence of the same transduced colony. Scale bar= 100 μ m in both images.

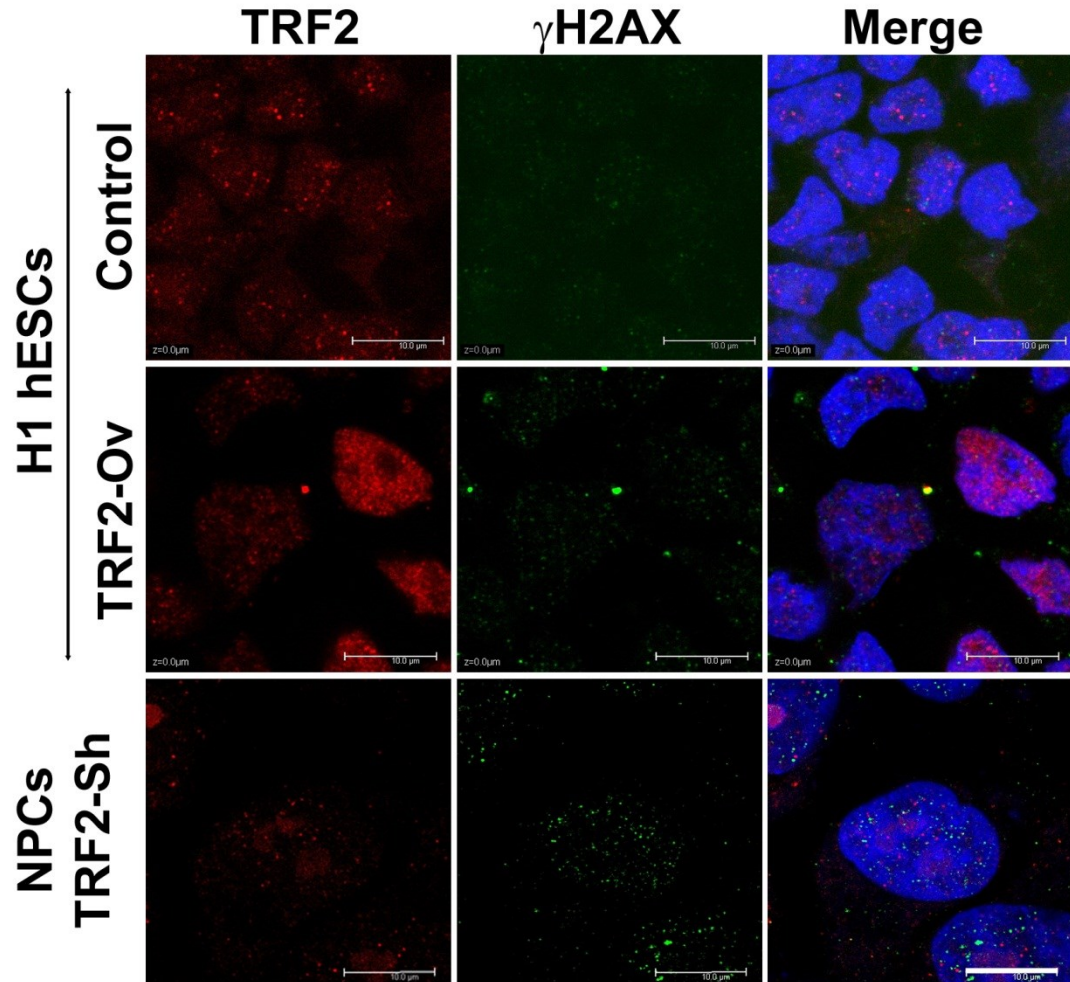


Figure A3. Overexpression in hESCs does not result in an increase of γ H2AX foci.

Immunostaining with indicated antibodies showing TRF2-ov hESCs do not have an increase of γ H2AX foci compared to control. TRF2-sh NPCs were used as a γ H2AX staining positive control. Scale bar = 10 μ M.

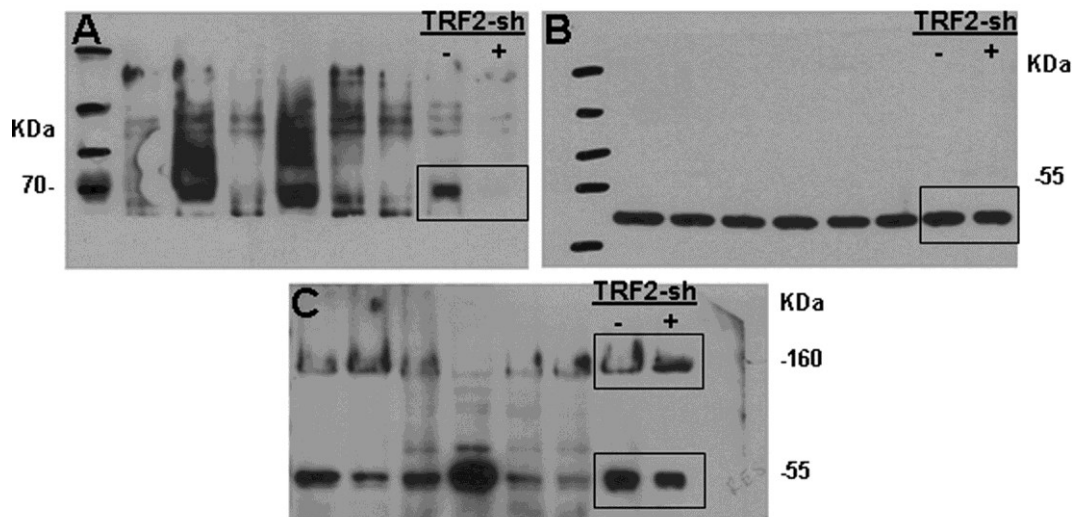


Figure A4. Full TRF2 immunoblot corresponding to Figure 6.2 C.

(A) Full TRF2 immunoblot. (B) Full β-Actin immunoblot. (C) Full REST immunoblot. Black rectangles indicate area selected for figure 6.2 C.

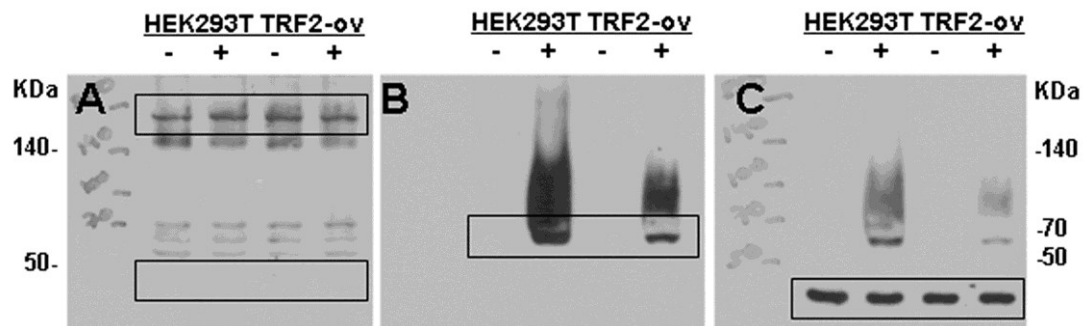


Figure A5. Full immunoblots corresponding to Figure 6.2 D.

(A) Full REST immunoblot. (B) Full TRF2 immunoblot. (C) Full β-Actin immunoblot. Black rectangles indicate area selected for figure 6.2 D.

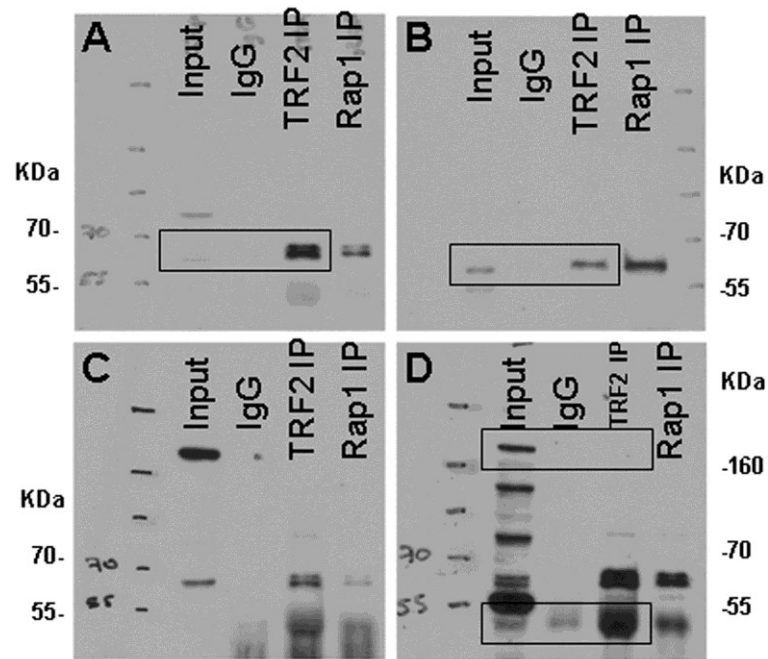


Figure A6. Full immunoblots corresponding to Figure 6.3 A.

(A) Full TRF2 immunoblot. (B) Full Rap1 immunoblot. (C) Full REST immunoblot. (D) Full REST immunoblot upon long exposure. Black rectangles indicate area selected for figure 6.3 A.

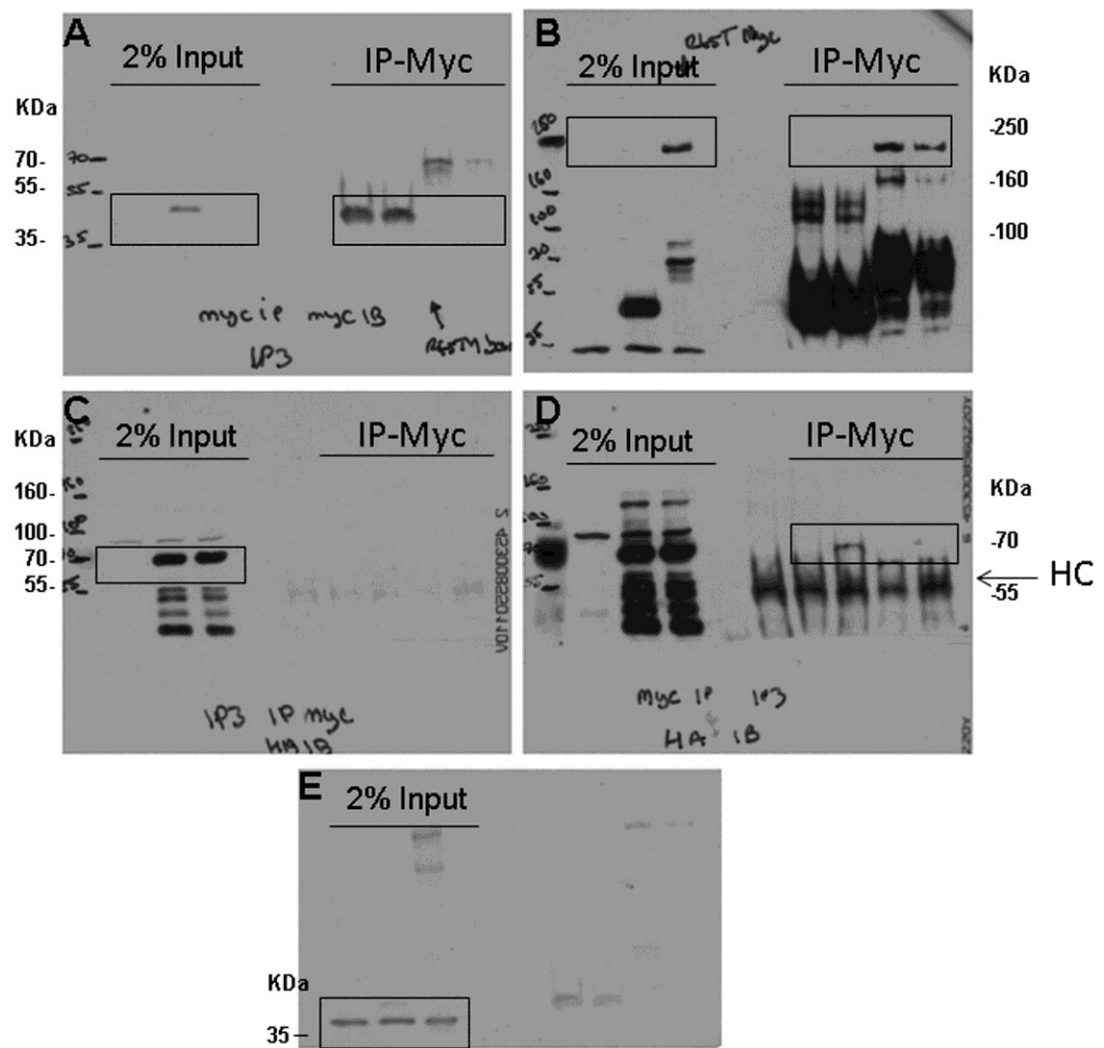


Figure A7. Full immunoblots corresponding to Figure 6.3 B.

(A) Full Myc immunoblot. (B) Full Myc immunoblot upon long exposure. (C) Full HA immunoblot. (D) Full HA immunoblot upon long exposure. (E) Full β -Actin immunoblot. Black rectangles indicate area selected for figure 6.3 B. HC = IgG heavy chain.

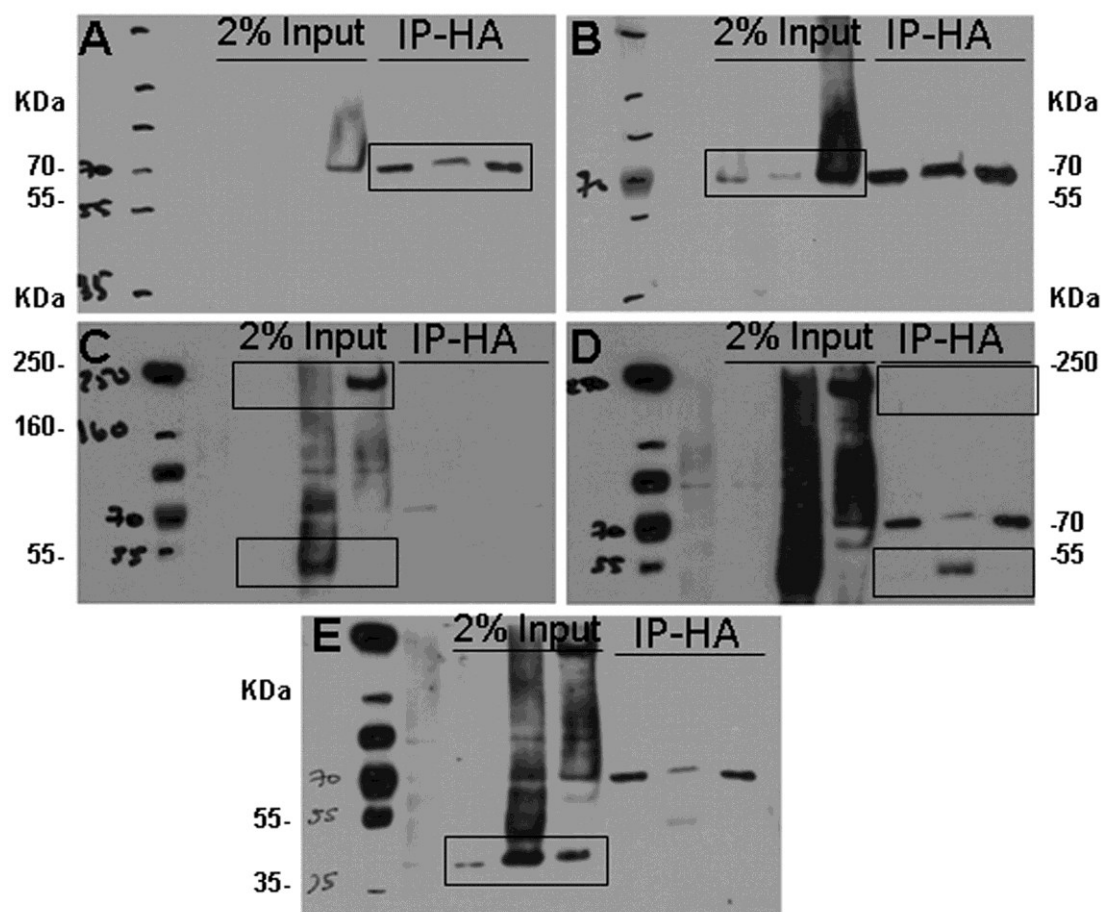


Figure A8. Full immunoblots corresponding to Figure 6.3 C.

(A) Full HA immunoblot. (B) Full HA immunoblot upon long exposure. (C) Full Myc immunoblot. (D) Full Myc immunoblot upon long exposure. (E) Full β -Actin immunoblot. Black rectangles indicate area selected for figure 6.3 C.