

The role of TRF2 in regulating neural progenitor proliferation and survival

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Abstract

The telomere repeat binding factor 2 (TRF2) plays an important role in protecting the telomere from any unwanted recombination and DNA fusion, which could cause DNA instability and cell death through t-loop formation and inhibition of the DNA Damage Response, such as the ATM. TRF2 protein has been previously identified as being highly expressed in the hESC-derived neural progenitor cells (NPCs). In the current study, we have reduced TRF2 expression in these NPCs by RNA knockdown and investigated the role of TRF2 in cell growth under various culture conditions. We have also overexpressed TRF2 in HEK293T cells to determine whether the effects of TRF2 are cell-specific or not.

Under the routine culture condition (N2B27 + bFGF/EGF, 37°C, 5% CO₂), high TRF2 expressing NPCs showed significantly higher growth and less apoptosis ($p < 0.01$). However, under the various stress conditions (B27 withdrawal, oxidative stress and hypoxia), high TRF2 expressing cells seemed to exhibit more cell death than low TRF2 expressing cells within 1 hour of the treatment, indicating the involvement of TRF2 in the DNA damage response. Under the standard culture conditions, the expressions of ATM-dependant DDR factors were significantly lower in the high TRF2 expressing NPCs. However, the comet assay analysis showed that the high TRF2 expressing cells has a higher DNA Double Strand Breaks (DDSB), which is contradictory to the protein expressions of the DNA damage markers. The difference in proliferation and apoptosis were also observed in the TRF2 over-expressing 293T cells as compared to the control, which shows the function of TRF2 is not cell specific.

Our results indicate that TRF2 might also be involved in cellular survival and/or cell death in addition to its regulation on telomeres. Under standard culture conditions, the higher TRF2 expression seems to help in terms of proliferation or protecting the cells against apoptosis, which suggest that TRF2 may have a protective function in cells. However, in the stress conditions, TRF2 seems to have an adverse effect on the cells, which could be due to the involvement of TRF2 with the DNA damage response pathway. These results might contribute to further understand the function of TRF2 in the DDR and in cell survival.

Statement of originality

All the experiments in this thesis were performed by me, Siti Aminah Muhammad Imran, unless otherwise stated in the text.

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Dedicated to

My Mother

My world

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Abbreviations

APS	Ammonium Persulfate
AT	Ataxia telangiectasia
ATM	Ataxia telangiectasia-mutated
ATR	Ataxia telangiectasia and Rad3-related
BCA	Bicinchoninic Acid Assay
bFGF	Basic Fibroblast Growth Factor
BMP	Bone morphogenetic protein
BSA	Bovine Serum Albumin
CaCl ₂	Calcium Chloride
CDK	Cyclin dependant kinase
cDNA	complimentary DNA
Chk	Checkpoint kinase
CNS	Central nervous system
DAPI	4',6 diamidino-2-phenylindole
DDR	DNA damage response
DEPC	Diethyl pyrocarbonate
dH ₂ O	Distilled/deionised water
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl sulfoxide
DNA PK	DNA-dependant protein kinase
DNA PKcs	DNA PK catalytic subunit
DNA	Deoxyribonucleic acid
dNTP	Deoxyneucotide mix
DPBS	Dulbecco's Phosphate Buffered Saline
DSB	DNA Double-Stranded Break
dsDNA	Double-stranded DNA
DTT	Dithiothreitol

ECL	Enhanced chemiluminescence substrate
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
FACS	Fluorescence-activated cell sorting
FAT	Focal adhesion targeting
FBS	Heat-inactivated Foetal Bovine Serum
FGF	Fibroblast growth factor
GFAP	Glial fibrillary acidic protein
H ₂ O	Water
H ₂ O ₂	Hydrogen peroxide
HeBS	HEPES-buffered saline solution
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hESCs	Human embryonic stem cells
HR	Homologous recombination
hRap1	human Rap1
hTERT	Human TERT
IR	Ionizing radiation
KSR	Knockout Serum Replacement
LM-Agarose	Low melting agarose
MAP2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinases
MEF-CM	MEF-conditioned medium for hESCs
MEFs	Mouse Embryonic Fibroblasts
MgCl ₂	Magnesium chloride
MRN	Mre11-Rad50-Nbs1
mRNA	Messenger ribonucleic acid
Na ₃ VO ₄	Sodium orthovanadate
NaN ₃	Sodium azide

NEAA	Non-Essential Amino Acid
NeuN	Neuronal nuclei
NHEJ	Non-Homologous end joining
NP-40	Nonidet P-40
NPCs	Neural progenitor cells
NSCs	Neural stem cells
O ₂	Oxygen
OB	oligonucleotide/oligosaccharide-binding
OCT4	Octamer-binding transcription factor 4
P/S	Penicillin/Streptomycin
PAX6	Paired-Box 6
PCC	Pearson Correlation Coefficient
PFA	Paraformaldehyde
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
PIKK	Phosphoinositide-3-kinase-related protein kinase
PLL	Poly-L-lysine
PLL/L	PLL/Laminin
PMSF	Phenylmethanesulfonylfluoride
PNS	Peripheral nervous system
POT1	Protection of telomeres protein 1
PuroR	Puromycin resistance gene encoding for puromycin-N-acetyl-transferase
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real-time polymerase chain reaction
Rap1	Ras-related protein 1
REST	RE-1 silencing transcription factor
RIPA	Radio Immunoprecipitation Assay
RNA	Ribonucleic acid
ROCKi	Rho-associated protein kinase inhibitor

ROS	Reactive oxygen species
RPA	Replication Protein A
rpm	Revolutions per minute
RT	Room temperature
SCGE	Single Cell Gel Electrophoresis
SDS	Sodium dodecyl sulphate
Ser	Serine
Sox	Sex determining region Y box
SR	Serum Replacement
SSB	Single-stranded DNA break
ssDNA	Single-stranded DNA
SVZ	subventricular zone
TBE	Tris-Borate-EDTA
TBST	Tris Buffered Saline with Tween-20
TEBP- β	Telomere-binding protein beta
TEMED	Tetramethylethylenediamine
TER	Telomerase RNA
TERT	Telomerase reverse transcriptase
TGF β	Transforming growth factor- β
Thr	Threonine
TIF	Telomere Induced Foci
TIN2	TRF1-interacting nuclear factor 2
TPP1	Tripeptidyl peptidase 1
TRF	Telomere repeat binding factor
TRF2-S	TRF2-short
TRFH	TRF homology domain
Tyr	Tyrosine
UV	Ultraviolet
WRN	Werner syndrome ATP-dependent helicase

Chapter 1

Introduction

Neurological disorders affect millions of people worldwide that could either occur during the early development process, such as cerebral palsy or upon ageing in the form of neurodegenerative diseases, such as Alzheimer's and Parkinson's disease. The overall pathology of these diseases is exhibited as the loss of functional neurons in the brain (Dugger and Dickson, 2017), however, the pathogenesis of these diseases remains unclear. There is also a lack of effective treatments and preventive approaches for these diseases. Investigating the molecular mechanisms underlying the neuronal damage and discovering the neuroprotective factors involved may facilitate us to understand more of the diseases and help us to develop new treatments for them in the future. Human development and ageing are often associated by the telomeres and the function of the telomeric binding proteins (Machwe et al., 2004; Blasco, 2005; Armanios, 2013; Eitan et al., 2014).

1.1: Telomeres and Telomerase

Ageing is often associated with the telomere length (Blasco, 2007; Mather et al., 2010; Armanios, 2013; Xi et al., 2013; Aviv and Shay, 2018). Telomeres are nucleoprotein structures at chromosome ends, which function as protective caps to disguise chromosome ends from being recognized as a DNA double-strand break (DSB), thereby, preventing chromosome end-to-end fusions (de Lange, 2002). Although the telomere itself consists of non-coding DNA nucleotides, the telomere acts as a 'capping' of the chromosome that will protect the genetic information from erosion during DNA replication (Blackburn 2001). In mammals, telomeric DNA is composed of conserved TTAGGG repeats, which vary in length ranging from 2 to 30 kb (de Lange et al., 1990). It starts as double stranded DNA and ends as a single strand 3' overhang (de Lange et al., 1990; Zakian, 1996; Bodnar et al., 1998). There are various mechanisms that act to protect the telomere in order to prevent genomic instability. Dysfunctional telomeres are known to cause genomic instability through chromosome end-to-

end fusion. There is mounting evidence that telomere dysfunction driven genomic instability may lead to cancer and other age-related diseases (Armanios, 2013; Xi et al., 2013; Aviv and Shay, 2017; Hermann et al., 2018).

1.1.1: Telomere structure and function

The special structures at the chromosome ends were first discovered by Muller (1938) through his study on *Drosophila melanogaster*. Through the years, various investigations of the telomere have been made in different species, where it was found that in human, the telomere at the chromosome end consists of the TTAGGG repeats that runs from the 5'-end to the 3'-end and forms an overhang of the 3'-end (de Lange et al., 1990; Bodnar et al., 1998). Due to the unique sequence of nucleotides, the telomeres resume unusual structures *in vivo*. The G-rich 3'-end overhang forms foldback structures, which, when supercoiled, can be digested by nuclease and are hypersensitive to chemical modification and strand invasion (Budarf and Blackburn, 1987; Blackburn, 1991). This foldback formation is then later characterised as the t-loop structure (Griffith et al., 1999).

Linearized DNA faces a problem in replication due to its terminal end. Telomeres function to protect the chromosome end from loss of genetic information due to the chromosomal-end replication problem. End replication problem occurs when the RNA template for DNA polymerisation could not bind to the lagging strand, thus causing an overhang of the leading strand (Lundbland, 1997; Soudet et al., 2014; Wellinger, 2014). With the help of the telomeric binding proteins and enzymes, the telomeres are able to be replicated, thus preserving the genetic information from being lost through repetitive replication (de Lange, 2002, 2009; Stewart et al., 2012; Martinez and Blasco, 2015). Another function of the telomere is to protect the chromosome end from being recognised as a DSB (Webb et al., 2013).

Due to the structure of the telomere, it poses a problem in the chromosome end-replication. When the chromosome replicates, it becomes linearized to allow the polymerase to bind to the DNA double strand, which in turn exposing the chromosomal end to be detected as a DSB. However, the telomere has a mechanism that protects the chromosome end and preserves the chromosomal stability, such as the t-loop formation or through inhibiting the DNA damage response (DDR), which will protect the telomere from unwanted DNA break repair and non-homologous end joining (NHEJ).

Telomere length has often been associated with ageing (Blasco, 2007; Mather et al., 2011; Armanios, 2013; Xi et al., 2013; Aviv and Shay, 2018). However, the length of the telomere itself does not determine the length of a life-span, since a mice telomere is extensively longer than human (Kakuo et al., 1999; Blasco, 2005; Calado and Dumitriu, 2013), but their life-span is much shorter. Therefore, there are many other factors that determine the life-span other than the telomere length alone, such as the expression p53 (Wright and Shay, 2000).

As the cells divide and chromosome replicates, the telomere ends become shorter. A critically short telomere is prone to be detected as a DNA break as the telomeric binding proteins are not able to bind to the telomere sequence, thus, are exposed to chromosomal end fusion and genomic instability. However, the telomere is able to maintain its length throughout replication via the help of the ribonucleoprotein enzyme, telomerase (Bodnar et al., 1998).

1.1.2: Telomerase structure and function

Telomerase is a ribonucleoprotein and functions to add telomere repeats to chromosome ends and thereby is highly involved in maintaining the telomere length. The telomere is elongated by polymerization from the 5' to the 3' direction of the deoxynucleoside triphosphate substrates into the TTAGGG tandem repeats (Blackburn, 1991).

The telomerase consists of at least two core sub-units, which are the telomerase RNA (TER) and the reverse transcriptase protein family member (TERT). The telomerase RNA acts as a template for the synthesis of the G-rich sequence of the telomeric DNA strand, where it binds to the end part of the telomere, and the TERT adds more nucleotide from the 5' to 3' direction (Greider, 1996; Smogorzewska and de Lange, 2004). The dimeric structure of telomerase might hold two chromatids or chromosomes together or act on two newly replicated chromatids (Blackburn, 2001). The notable difference between the telomerase and DNA polymerase is the use of a fixed region of the RNA template in the telomerase to interact with the sequence of the telomeres (Zvereva et al., 2010).

The expression of telomerase in cells is thought to be associated with the immortality. The replicative life-span of some diploid human somatic cell lines could even be extended by the reintroduction of the telomerase activity (Bodnar et.al., 1998). Cells that are able to proliferate infinitely, such as the human embryonic stem cells (hESCs) and the majority of human cancer cells have high telomerase activities (Zeng et al., 2014). In contrasts, normal mammalian somatic cells have no telomerase activity and a limited proliferation capability, known as the Hayflick limit (Hayflick, 1965). This is attributed to the telomere shortening with cell divisions, which will trigger cell senescence when one or several telomeres reach critical short lengths. In supporting this theory, the cells are able to restore their proliferation if the telomerase activity is active through exogenously transfecting the cells with hTERT, such as in fibroblasts, epithelial or endothelial cells (Lee et al., 2004).

1.2: The Shelterin Complex

The telomere is composed of telomeric DNA repeats as well as many binding proteins, which help to stabilise and protect the structure of the telomere (de Lange, 2005a). The core telomere binding protein complex is the Shelterin complex. The Shelterin complex consists of six protein

subunits, namely telomere repeat binding factor 1 and 2 (TRF1 & TRF2), TRF1-interacting nuclear factor 2 (TIN2), Ras-related protein 1 (Rap1), tripeptidyl peptidase 1 (TPP1), and protection of telomeres protein 1 (POT1). The TRF1 and TRF2 bind to the double-stranded DNA, while POT1 binds to the single-stranded DNA of the 3'-overhang. TPP1 will bind to both POT1 and TIN2, which is bound to both TRF1 and TRF2. The last Shelterin subunit, Rap1 is bound to TRF2 (Figure 1.1). The Shelterin complex mainly functions to protect the human telomeres from being recognised as a DNA Double-Strand break by the ataxia telangiectasia-mutated (ATM) kinase pathway and maintaining the telomere length. The Shelterin complex binds specifically at the double- or single-stranded 5'-TTAGGG-3' repeats that can be abundantly found at the telomere ends (de Lange, 2005a).

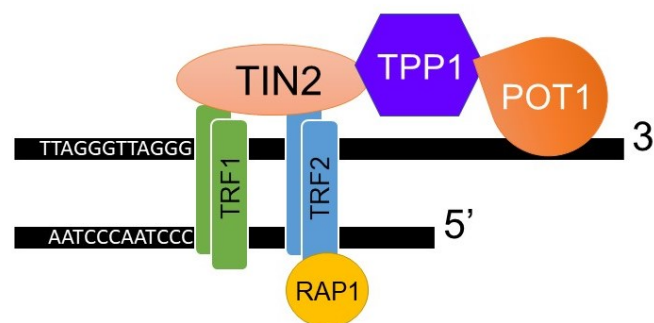


Figure 1.1: Schematic representation of the telomeric DNA and the Shelterin complex proteins. 5' to 3' overhang shows the TTAGGG sequence, while the 3' to 5' strand shows the consecutive AATCCC sequences. TRF1 and TRF2 homologues both bind to the double-stranded DNA. TIN2 associates with both TRF1 and TRF2, as well as TPP1 that helps to stabilise the complex. POT1 binds to TPP1 and can also bind to the single-stranded DNA of the 3' overhang. The last Shelterin subunit, Rap1 binds to TRF2.

1.2.1: Components and functions of The Shelterin Subunits

The 3' overhang of the telomere is synthesised by the help of the telomerase (Greider, 1996). Due to the precise arrangement of the ATC-5' in human telomeres, the regulation of telomeres elongation could be controlled, mainly by the Shelterin complex. One of the Shelterin subunits, POT1, has been found to be a negative regulator of the telomere length (Kelleher et al., 2005). POT1 regulates the maintenance of telomere length by binding to the 3' overhang of the telomere, thus, inhibiting the telomerase from elongating the telomere (Kelleher et al., 2005). A mutant POT1 has been shown to have an elongated telomere length, and overexpression of POT1 has been shown to have a shortened telomere length, due to the inability of the telomerase to elongate the telomere (Kelleher et al., 2005). POT1 was also found to bind to the G-rich strand of the single-stranded DNA (ssDNA) through the N-terminal domain (Baumann and Chech, 2001). The binding affinity of POT1 to the ssDNA is further enhanced by TPP1 (Xin et al., 2008). POT1 binding to the TIN2/TRF1 complex at the telomeres is mediated by TPP1, which is a POT1-interacting protein (Ye et al., 2004a; Liu et al., 2004a), where TPP1 interaction with POT1 will facilitate in the localisation of POT1 at the telomeres (Xin et al., 2008).

TPP1 binds to both TIN2 and POT1, which will merge the connection between the dsDNA that is bound by the TIN2/TRF1 complex as well as with the ssDNA that is bound by POT1 (Lei et al., 2003; Ye et al., 2004a, b; Liu et al., 2004a; Hockemeyer et al., 2007). TPP1 has been reported to be the human homologue of the TEBP- β in *Oxytricha nova*, which showed organisational similarities to TPP1 (Wang et al., 2007; Xin et al., 2008). In addition to binding to the C-terminal domain of POT1, TPP1 also possess an N-terminal domain that is characterised as the oligonucleotide/oligosaccharide-binding (OB) folds region that is involved in telomere maintenance and integrity (Horvath et al., 1998; Lin et al., 2004a; Xin et al., 2008).

TPP1 functions to regulate the recruitment of telomerase, which will affect the telomere elongation (Zhong et al., 2012).

TIN2 helps to tether TPP1/POT1 and TRF2/TRF1, which protects TRF1 from tankyrase as well as binding and stabilising the complex to the telomeres (Liu et al., 2004b, Ye et al., 2004b, c). The close relation between TIN2 and TRF1 was observed as depletion of TIN2 showed downregulation of telomere binding TRF1 (Ye et al., 2004b).

Rap1 protein is initially found in the budding yeast, where it predominantly binds to the multiple sites found within the TG1-3 telomeric repeats at the telomere (Klein et al., 1992). Rap1 mainly functions to regulate telomere length maintenance, as shown in studies conducted in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Giraldo and Rhodes, 1994; Krauskopf and Blackburn, 1998; Park et al., 2002). In human, Rap1 also consists of a Myb domain, similar to TRF1 and TRF2 (Li et al., 2000; Hanaoka et al., 2001; O'Connor et al., 2004). However, human Rap1 (hRap1) binds to TRF2 via its TRF2-binding domain, instead of directly binding to the telomeres as in the budding yeast (Li et al., 2000; Hanaoka et al., 2001; O'Connor et al., 2004). Rap1 in *S. pombe* is recruited to the telomeres through interaction with Taz1 (Kanoh and Ishikawa, 2001). hRap1 has structural and functional similarities to the one found in budding yeast (Li et al., 2000). Rap1 is the only Shelterin subunit that has an ortholog of the budding yeast (Li et al., 2000; Li and de Lange, 2003), which will make the investigation of its function to be more comprehensive.

Another binding protein at the telomere is the TRF1. TRF1 binds specifically to the double-stranded TTAGGG repeats of the telomere ends (Chong et al., 1995; de Lange, 2005a). TRF1 is also a homologue to the TRF2. Both have similar Myb-domain found at the C-terminus that recognises and binds specifically to the TTAGGG repeats of the telomeres (Broccoli et al., 1997). TRF1 and TRF2 also share the same TRF homology domain (TRFH) (Stewart et al., 2012). The TRFH domain of both TRF1 and TRF2 share the same architecture, which functions

in homodimerization of both proteins as well as in the recruitment of other Shelterin subunits, such as TIN2 (Fairall et al., 2001; Giraud-Panis et al., 2017). However, TRF1 and TRF2 differ substantially at the N-terminus, where TRF2 is basic while TRF1 is acidic (Broccoli et al., 1997). TRF1 and TRF2 also differ in terms of their evolving rates between different species (Broccoli et al., 1997). TRF1 functions to recruit other Shelterin subunits, such as TIN2 and POT1 to the telomeres in order to protect the chromosome end (Smogorzewska and de Lange, 2004; Ye et al., 2004a; Maestroni et al., 2016). TRF1 has been shown to regulate the telomere length (Smogorzewska et al., 2000). Although TRF1 functions to recruit other Shelterin subunits, TRF2 also has a binding domain that interacts with other Shelterin subunits, such as Rap1 that binds to TRF2 on the Rap1-binding motif (RBM) (Rai et al., 2016) and TIN2 that binds to TRF2 on the TIN2-binding motif (TBM) (Chen et al., 2008). The schematic representation of TRF2 binding domains is shown in Figure 1.2.



Figure 1.2: The structural domains of TRF2. *The domains of TRF2 from left is the N-terminal, TRFH domain, flexible linker region (Rap1 Binding Motif-RBM and TIN2 Binding Motif-TBM) and the C-terminal or the Myb domain.*

The telomeres can exist in two different states, which are ‘open’ and ‘closed’ state (Smogorzewska et al., 2000). The ‘open’ state model is when the telomere is being elongated by the telomerase and as the telomere length is further elongated, more negative regulators will bind to the repeat sequence, hence promoting the ‘closed’ state model (Smogorzewska et al., 2000). The switching between these two state models of the telomeres is regulated by both

TRF1 and TRF2. As overexpression of TRF1 or TRF2 in the cells showed shorter telomere length, it is presumed that both TRF1 and TRF2 are negative regulators of the telomere length. This is because as more TRF1 and/or TRF2 are bound at the telomeres, they will promote the ‘closed’ state model, thus, preventing the telomerase from binding to the telomere, hence shortening the telomere length (Griffith et al., 1999; Smogorzewska et al., 2000; Ohki and Ishikawa, 2004; Smogorzewska and de Lange, 2004).

1.3: TRF2 protects the telomeres from being recognised as DNA break

TRF2, which is another subunit of the Shelterin complex, plays a specific function at the telomere. The main function of TRF2 is to protect the telomere from being recognised as a DNA break site, therefore, protecting the chromosome end from any unwanted recombination and non-homologous end joining (NHEJ), which in turn will cause genomic instability (Smogorzewska and de Lange, 2002; de Lange, 2010; Karlseder et al., 2004). The TRF2 protects the telomere through two ways, which are the formation of t-loop (Figure 1.3 A) (de Lange, 2004) and inhibiting the ATM kinase activation (Figure 1.3 B) (Karlseder et al., 2004).

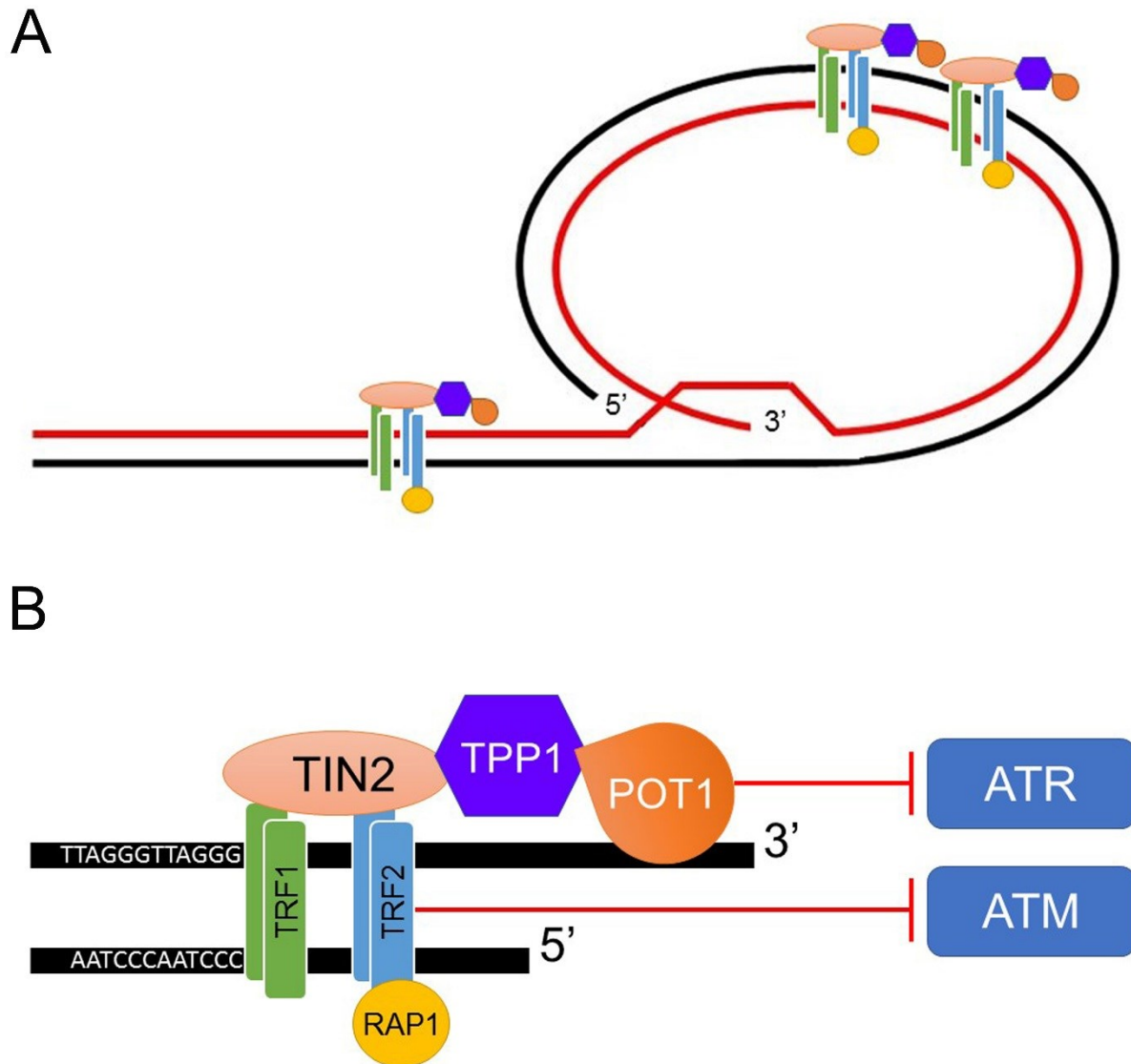


Figure 1.3: Schematic representation of the TRF2 protection at the telomeres. (A) TRF2 along with other Shelterin Complex binds to the TTAGGG repeats of the telomeres and assists in the formation of t-loop structure. (B) TRF2 directly inhibit the ATM activation, whereas POT1 inhibits the ATR activation, which will prevent the recognition of the telomeres as a DNA break by the DDR.

1.3.1: TRF2 regulates the formation of t-loop at the telomeres

One way for the TRF2 to protect the telomere is by forming the t-loop structure. The T-loop is a double-stranded DNA looped structure and was observed in mammalian telomeric DNA *in vivo* via electron microscopy (EM) (Griffith et al., 1999). The t-loop structure was formed by displacing the G-strand of the duplex telomeric repeat array, which forms the displacement loop (D-loop) (de Lange 2004, 2015). D-loop formation is homologous pairing of the double-stranded DNA and strand invasion of the single-strand overhang, where if it occurs at the telomere, it is known as t-loop (de Lange, 2004). This formation is similar to the homologous recombination that can be found throughout other parts of the chromosome during cell replication, which is known as a Holliday junction (West, 2003). Although TRF1 and TRF2 antagonise the lengthening of the telomeres, TRF1 promotes Rad 51 in mediating the D-loop formation that will facilitate telomere replication (Bower and Griffith, 2014). Interestingly, TRF2 inhibits the function of Rad 51, which will also inhibit telomeric DNA repair (Bower and Griffith, 2014). The size of t-loop is highly variable and may not be important for telomere function (de Lange, 2004). TRF2 is able to form t-loop from as low as one repeat of the 3'-TTAGGG overhangs (Stansel et al., 2001).

Loss of TRF2 was shown to cause the loss of loop formation or causing linearized chromosome-end (Griffith et al., 1999), indicating the important role of TRF2 in t-loop formation. Interestingly, although TRF1 has the same Myb-domain as TRF2 and binds to the same exact TTAGGG repeats of the telomere (de Lange, 2005a), TRF1 was not found at the t-loop region, which suggests that the t-loop is specifically formed by TRF2 (Griffith et al., 1999). Telomeres that are configured in a t-loop formation are unlikely to be detected by the Mre11, Rad50 and Nbs1 (MRN) complex, which is essential for ATM-activation (Lamarche et al., 2010; Lazzerini-Denchi and Sfeir, 2016).

1.3.2: TRF2 inhibits the ATM-dependent DNA damage response pathway at the telomeres

In addition to its function in the formation of the t-loop structure at the telomere, TRF2 has also been reported to actively inhibit the DNA damage response pathway, namely the ATM kinase from recognising the telomere as a DNA break, thus, protecting the genomic integrity (Karlseder et al., 2004). During DNA replication, the ‘closed’ state of the telomeres will be unfolded to become the ‘open’ state. Uncapped telomeres could also be recognised as a DSB when the t-loop unfolds and exposes the 3’-overhang and the 5’-end to be detected by the DNA damage response (DDR) pathway factors (Karlseder et al., 2004). The Shelterin complex, specifically TRF2 and POT1 will inhibit the ATM and ATR kinases from recognising the telomeres as a break, as ATM recognition at the telomeres was shown in TRF2 deficient cells and the repression of ATR was elevated after POT1 deletion (Lazzerini-Denchi and de Lange, 2007). TRF2 and POT1 inhibit the ATM and ATR independently. Furthermore, TRF2 protection at the telomeres is only through the inhibition of the ATM and not the ATR, as the deletion of TRF2 did not induce any activation of Chk1, which is downstream of the ATR pathway (Zhao et al., 2001). Also, ATR inhibition was shown to have no effect on the Telomere Induced Foci (TIF) formation (Lazzerini-Denchi and de Lange, 2007).

At the telomeres, if the chromosome ends became too short, it will also be prone to be recognised as a DNA break and will induce the DDR, as well as forming TIFs (Takai et al., 2003; Hewitt et al., 2012; Anderson et al., 2019). The inhibition of TRF2 will also induce the DDR and will form TIFs, as shown in previous studies (Takai et al., 2003). However, the mechanism of ATM inhibition by TRF2 at the telomeres is still unknown.

1.3.3: DNA damage response

DNA damage could occur in the cells either endogenously, through normal cellular replication and metabolism (d'Adda di Fagagna et al., 2003; Rothkamm et al., 2003; Shimizu et al., 2014; Turgeon et al., 2018), or exogenously through ultraviolet (UV), ionizing radiation (IR) or

various genotoxic compounds that could induce DNA damage (Sachs et al., 1992; Leadon 1996; Sinha and Häder, 2002; Rastogi et al., 2010). Unrepaired DNA damage could cause severe mutations and chromosomal instability, which will have detrimental effects on the cells and lead to cell death. Different stressors will cause different types of DNA damage. Normal DNA replication could induce mismatch of the nucleotides and causes mutations (Morrison et al., 1993; Brown, 2002; Boyce et al., 2015; Kunkel and Erie, 2015). Stressors such as oxidative stress will produce reactive oxygen species (ROS) from normal cellular metabolism or from external genotoxic compound, which will cause DNA breaks, either single-stranded or double-stranded (Yang et al., 2003; Coates et al., 2005; Tsolou et al., 2012). DNA breaks could cause mutations during mitosis, unwanted chromosomal fusions or during the repair through non-homologous end joining (NHEJ).

The DDR is the response mechanism which will detect any DNA damage that occurs throughout the chromosome and activates a repair cascade to the damage-site and will help the cells either to proliferate normally if the repair was successful or to activate the cellular programmed cell death if the damage was too extensive and unable to be repaired.

DNA damage that occurs at the chromosome is primarily coordinated by the phosphoinositide-3-kinase-related protein kinase (PIKK) family. The primary signalling kinases of the PIKK family that are involved in the DNA damage response are the DNA-dependent protein kinase (DNA PK), the ataxia telangiectasia mutated protein (ATM) and Rad3-related kinase (ATR) (Hiom, 2005), as shown in Figure 1.4. Each of the kinases is responsible for different types of damages that occur at the DNA.

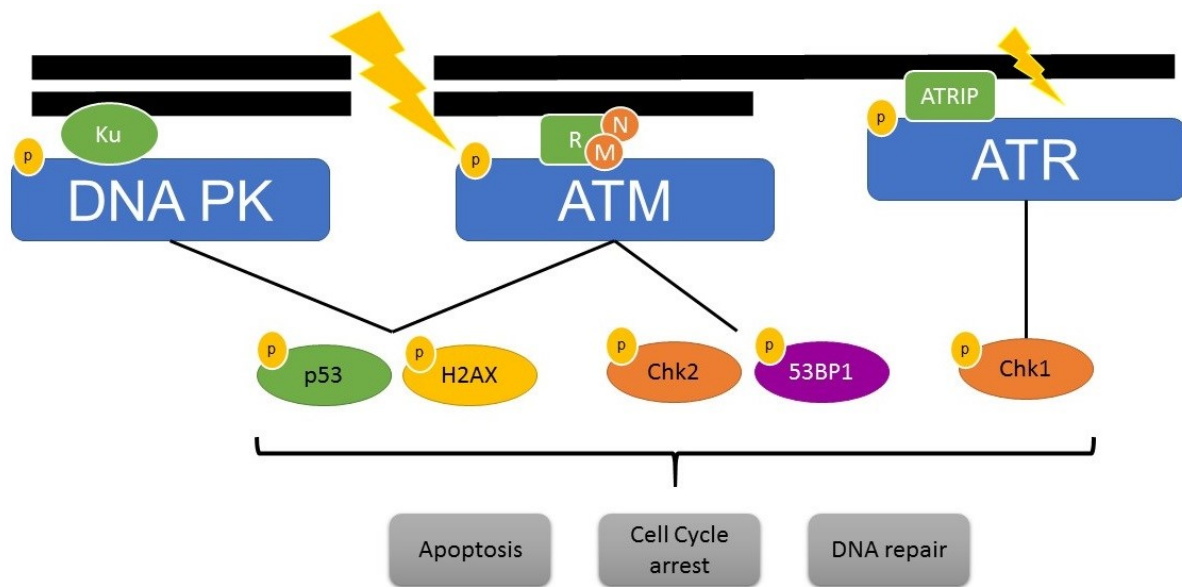


Figure 1.4: Schematic diagram of the DDR of the PIKK family. *DNA PK, activated by Ku70/80; and ATM, activated by the MRN complex both respond to the DSB. ATR/ATRIP complex binds to the single-stranded DNA break. Each of the PIKK activation will activate their downstream factors that will activate the cascade for DNA repair, cell cycle arrest or cell death.*

DNA PK

DNA-PK is a nuclear serine/threonine kinase composed of a catalytic subunit (DNA-PKcs) and a DNA-binding Ku70/80 subunit that binds specifically to the dsDNA (Walker et al., 1985; Lees-Miller et al., 1990; Dvir et al., 1992; Gottlieb and Jackson, 1993). The activity of DNA PK has been shown to be regulated in a cell cycle-dependent manner, where the activity of DNA PK was found to be high during the cell cycle checkpoints at G₀/G₁ phase and during G₂ phase (Lee et al., 1997). DNA PK has been shown to be highly involved in DSB repair, as cells that are deficient in DNA-PKcs and Ku 70/80 are unable to repair the induced DSB (Hartley et al., 1995; Yang et al., 2003; Jette and Lees-Miller, 2015). Isolation and characterisation of the catalytic subunit of DNA-PK (DNA-PKcs) showed that it also falls under the

phosphatidylinositol-3-related-kinase (PIKK) family (Hartley et al., 1995; Smith and Jackson, 1999).

DSB is recognised and bound by the Ku heterodimers, which consists of Ku70 and Ku80 subunits (Uematsu et al., 2007). DNA-PKcs recruitment to the dsDNA is assisted by Ku70/80, which in turn facilitates DNA-PK complex interaction to other DDR substrates and activates DNA-PKcs kinase activity (Gottlieb and Jackson, 1993; Calsou et al., 1999; Smith and Jackson, 1999). DNA-PKcs undergoes auto-phosphorylation upon binding at the dsDNA, which will regulate the maintenance of its association to the Ku-bound DNA ends, where it is required in the DNA end-processing and end-joining (Lees-Miller et al., 1990; Jette and Lees-Miller, 2015). The kinase activation of the DNA-PKcs is essential in the NHEJ DNA repair pathway (Mahaney et al., 2009; Wang and Lees-Miller, 2013; Davis et al., 2014), as DNA-PKcs has been found to interact with factors that are involved in NHEJ, such as X-ray-complementing Chinese hamster gene 4 (XRCC4) (Li et al., 1995; Critchlow et al., 1997), DNA ligase IV (Budman and Chu, 2005), and Artemis (Soubeyrand et al., 2006). However, DNA-PK has also been found to phosphorylate other DDR factors, such as H2AX (Stiff et al., 2004) and p53 (Kurimasa et al., 1999). However, the role that DNA-PK has in the activation of these factors are not fully understood, since H2AX and p53 can also be phosphorylated by ATM Kinase. It has also been found that DNA-PKcs kinase and ATM kinase activities are closely interrelated, where ATM hyperactivation was found when DNA-PK is inhibited (Finzel et al., 2016; Zhou et al., 2017), and ATM was found to be essential in the phosphorylation of DNA-PKcs upon IR-induced DSB (Chen et al., 2006; Zhou and Paull, 2013).

ATM

The ATM gene was first identified by Shiloh and colleagues where the gene was found to be mutated (lost or inactivated) in a human genetic disorder, known as Ataxia telangiectasia (AT) (Savitsky et al., 1995). AT is characterised by cerebellar degeneration, which leads to severe

progressive neuromotor dysfunction, immunodeficiency, genomic instability and extreme sensitivity to ionizing radiation and DSB-inducing agents. The ATM gene, which encodes a 370 kD protein, is a member of the PIKK family that has either lipid or protein kinase activity (Canman et al., 1998). ATM gene was found to be involved in the cellular responses to DNA damage and cell cycle control through its kinase activity (Keith and Schreiber, 1995).

The recruitment of ATM to the DSB site is mediated by a complex consisting of the Mre11, Rad50 and Nbs1 (MRN complex) (Uziel et al., 2003; reviewed in Lee and Paull, 2007), where MRN also plays a role in the activation of ATM (Shirata et al., 2005) and phosphorylation to the ATM-dependent downstream substrates (Buscemi et al., 2001). ATM was found to be autophosphorylated at Serine 1981, which was discovered by incubating 293T cells with radioactively labelled ATM protein and was immunoprecipitated to determine the phosphorylation site of ATM upon IR-induced DSB (Bakkenist and Kastan, 2003). The phosphorylation of ATM-Ser1981 could only occur in the presence of ATM kinase activity, where phosphorylation was not observed in cells that are deficient in ATM, such as AT cells (Bakkenist and Kastan 2003). ATM-mediated phosphorylation could either enhance or represses its downstream targets, which will affect the specific functions of its targeting proteins. It has been reported that in undamaged cells, ATM is inactive, where the kinase domain is blocked by the focal adhesion targeting (FAT) domain (Shiloh, 2003). Upon DSB induction, auto-phosphorylation of ATM at Ser 1981 removes the FAT domain that changes both domains to active monomers (Bakkenist and Kastan, 2003).

ATM activation upon DSB induction was reported to be signalled by chromatin alteration rather than direct binding at the dsDNA (Bakkenist and Kastan, 2003). However, it has also been found that a portion of the ATM was recruited at the site of DSB, where ATM and γ H2AX co-localisation was found (Andegeko et al., 2001). ATM-mediated protein activation plays a function in the cell cycle checkpoint, DNA repair, cell senescence and apoptosis. For example,

the main component in the G₁-S cell cycle checkpoint is the activation and regulation of p53 that will decide either the cells undergo cell cycle arrest or apoptosis, depending on the severity of the DNA damage (Reviewed in Shaw, 1996; Sionov and Haupt, 1999). ATM kinase will phosphorylate p53 at Ser15 that will increase the p53 activity (Banin et al., 1998; Canman et al., 1998; Khanna et al., 1998). However, p53 could also be phosphorylated at Ser15 by ATR (Tibbetts et al., 1999), Chk1 and DNA-PKcs (Shieh et al., 1999; Goudelock et al., 2003). Therefore, the mechanism to which p53 is activated in response to DNA damage is not fully understood.

Compared to its other DSB repair partner of the PIKK family, DNA-PK is responsible mainly in the NHEJ, while ATM is directly involved with homologous recombination (HR) repair as cells that are ATM-deficient resulted in HR deficiency (Morrison et al., 2000; Beucher et al., 2009; Bakr et al., 2015). However, the mechanisms by which the cells signal either NHEJ or HR repair upon DSB are not yet clear. It has been reported that upon DNA damage, the DDR pathways that are activated become ATM-dependent, although, the mechanisms on which this occur are yet to be explored (Wang et al., 2000).

p53

p53 is a transcription factor that is involved in protecting the genomic stability through its activation in the DDR, which will activate other target genes involved in the induction of cell cycle arrest, senescence and apoptosis (Reviewed in Sionov and Haupt, 1999; Prives and Hall, 1999; Vousden and Lou, 2002). In normal replicating cells, p53 is expressed at a very low level and is elevated in response to induced DNA damage, in order to mediate the repair through activation of other DDR factors, or through activation of cell cycle arrest (Donehower et al., 1992; Shaw, 1996; Sionov and Haupt, 1999). The p53-mediated cell cycle arrest will allow the cells to repair the DNA mutation and/or damage that are caused either through normal replication or exogenous genotoxic compounds (Siliciano et al., 1997; Lakin and Jackson,

1999; Caspari, 2000; Huang et al., 2014; Conti and Di Micco, 2018). The p53 protein could also induce apoptosis through the activation of genes containing p53-binding sites within their regulatory regions if the damage was unable to be repaired in order to inhibit the damaged DNA to be transferred to the daughter cells (Reviewed in Vousden and Lou, 2002). It has been reported that over 50% of cancer cells express a mutation or loss of function in p53 gene, suggesting that p53 is a tumour repressor gene (Nigro et al., 1989; Donehower et al., 1992; Hollstein et al., 1999).

In addition to p53, ATM kinase also activates Chk2, which is a cell cycle checkpoint kinase that acts as an effector upon the induction of DSB through phosphorylation of p53 at Ser 20 (Jack et al., 2004). Phosphorylation of p53 by Chk2 will interfere with the interaction between p53 and Mouse double minute 2 (MDM2) (Cheng and Chen, 2010), which is the ubiquitin ligase of p53 that will cause the degradation of p53 (Zhang and Xiong, 2001; Alarcon-Vargas and Ronai, 2002; Moll and Petrenko, 2003; Nag et al., 2013). ATM also directly phosphorylates MDM2 on Ser395 and its homologue, MDMX, which interferes with the nuclear export of the p53-MDM2 complex, and hence the degradation of p53 (Maya et al., 2001; Chen et al., 2005).

In addition, ATM has also been found to phosphorylate factors that are thought to be the sensors in DDR, such as γ H2AX and 53BP1 (Burma et al., 2001; Bekker-Jensen et al., 2005). Foci formation of γ H2AX has been found to accumulate at the site of DSB and is thought to be essential in the recruitment of other DDR factors in order to repair the DNA damage, such as the MRN complex, RAD51 and BRCA1 (Paull et al., 2000). In addition to the MRN complex, ATM has also been reported to be recruited to the DSB site by γ H2AX (Kobayashi et al., 2009).

γ H2AX

DNA forms chromatin through the folded structures of the nucleosome, consisting of the histone family, H2A, H2B, H3 and H4 (Pruss et al., 1995). H2AX is an H2A protein variant

that is not concentrated at a specific region of the DNA but is incorporated randomly into histones throughout the DNA (Ausió and Abbott, 2002). H2AX is phosphorylated at the C-terminal Ser 139 upon induction of DSB by the PIKK family member and is known as γ H2AX (Rogakou et al., 1998; Durocher and Jackson, 2001). Dephosphorylation of γ H2AX was shown to negatively regulate its function and hinder the ability to recruit other DDR to repair the damage (Cha et al., 2010). γ H2AX has been used as a DNA break marker as the foci formation of γ H2AX has been found in cells that are subjected to ionizing radiation and laser-induced DSBs and was found to be located at the DSB site (Rogakou et al., 1999; Fernandez-Capetillo et al., 2003).

However, the kinase that is responsible to phosphorylate γ H2AX, either during normal replicative DSB formation or after induction of DSB through IR and/or laser, is difficult to determine. γ H2AX has been reported to be primarily phosphorylated by ATR during replicative stress and UV radiation (Ward and Chen, 2001; Ward et al., 2004). Upon induction of DSBs, γ H2AX is phosphorylated by either the ATM (Burma et al., 2001) or DNA-PKcs, that may target γ H2AX redundantly as it was found that γ H2AX formation was not compromised in AT cell lines that are ATM-deficient (Stiff et al., 2004). However, it has also previously been shown that γ H2AX phosphorylation was highly reduced as ATM is inhibited (Burma et al., 2001; Tanaka et al., 2006), which suggests that DNA-PKcs may only play a small role in activating γ H2AX.

γ H2AX can function both upstream and downstream of the ATM pathway. In addition to recruiting ATM to the DSB site, γ H2AX has also been found to activate the phosphorylation of ATM through the interaction with the MRN complex (Kobayashi et al., 2009).

In addition to be one of the DSB markers, presence of γ H2AX has also been reported in the absence of DSB, either in serum starvation or during normal replication, where γ H2AX was

found to be involved with apoptosis (McManus and Hendzel, 2005; Lu et al., 2008; An et al., 2010). However, in this situation, the activation of γ H2AX is mediated by MAPK, DNA-PKcs and Chk2, rather than the ATM kinase pathway (Tu et al., 2013).

ATR

Along with ATM and DNA-PK, ATR is also a member of the PIKK family that functions as both sensor and transducer in the DNA damage response pathway. Most studies involving ATR are performed in budding yeast. ATR is found to be mainly involved in stalled replication forks (Byun et al., 2005), as well as UV-induced single-stranded DNA breaks (Ünsal-Kaçmaz et al., 2002; Zou and Elledge, 2003; Byun et al., 2005), where it will activate other downstream factors involved in cell cycle checkpoints and the DDR. ATR will phosphorylate its downstream substrates such as Chk1, p53, BRCA1 and Rad17, which is involved in the cell cycle checkpoint and DNA damage response (Tibbetts et al., 1999, 2000; Sanchez et al., 1999; Liu et al., 2000). ATR has also been found to co-localise with its downstream substrates at the site of DNA damage (Zhou and Elledge, 2000).

Upon DNA damage, ATR and its interacting protein (ATRIP), which is a regulatory partner of ATR (Cortez et al., 2001), will get recruited to the site of damage and will activate the DNA repair and checkpoint signalling. Although ATR shares some similarities with ATM in terms of phosphorylating the same substrates and having overlapping responses to DNA damage (Reviewed in Elledge, 1996), ATR still has a specific function, especially in the cell cycle checkpoint. While ATR is also able to detect and bind to DSB, it has been reported that its affinity in recognising DSBs is lower compared to the Replication protein A (RPA)-ssDNA complexes (Zou and Elledge, 2003). The importance of ATR in the progression of cell cycle is further demonstrated as disruption in ATR which will cause chromosomal fragmentation and developmental abnormality that will lead to embryonic lethality (Brown and Baltimore, 2000; Klein et al., 2000).

Cell cycle checkpoint

Cell proliferation involves the progression of cell division, which is known as the cell cycle. The eukaryotic cell cycle is divided into four phases, which are; G1, S (DNA synthesis), G2, M (mitosis) phases (Reviewed in Alberts et al., 2002). G1, S and G2 phases are also known as the interphase, which will take up around ~23 hours in normal human cell proliferation, while mitosis will take about ~1 hour. The time taken for each phase varies depending on the proliferation capabilities of each cell type (Smith and Martin, 1973; Shedden and Cooper, 2002; Cooper and Shedden, 2003). There are various checkpoints through the cell cycle progression, as shown in Figure 1.5, which will help to ensure no mutation will be passed to the daughter cells.

Chk1 and Chk2 are two of the cell cycle checkpoint kinases that function as effectors to detect any abnormalities in the cell throughout cell proliferation (Elledge, 1996; Castedo et al., 2004). The checkpoint kinases will activate the regulatory substrates, such as the cyclin-dependent kinases (Cdks), BRCA1 and p53, which will induce cell cycle arrest (Lee et al., 2000; Falc et al., 2001; O'Neill et al., 2002; Zhang et al., 2003).

The activation of Chk1 and Chk2 could either be induced by DSB or DNA damaged from genotoxic compounds, as well as by damage from normal replication, such as stalled replication fork that will generate ssDNA breaks (Zou and Elledge, 2003; Lukas et al., 2003; Lambert and Carr, 2005; Tourrière and Pasero, 2007). As mentioned earlier, both Chk1 and Chk2 are regulated by other upstream factors, namely the PIKK family.

Chk1 activation and modification are mediated by ATR that is shown to cause cell cycle arrest at the G2/M phase in the presence of DNA damage (Sanchez et al., 1999; Zhao et al., 2001). Chk1 has been shown to be important in the cell cycle phase transition, which Chk1 mutant mouse embryonic cells showed to interrupt G2 arrest upon DNA damage induction (Liu et al.,

2000). This report suggests the importance of Chk1 in the early embryonic development, where Chk1 mutants resulted in embryonic lethality due to the disruption in the cell cycle checkpoint (Liu et al., 2000; Takai et al., 2000; Zhao et al., 2002).

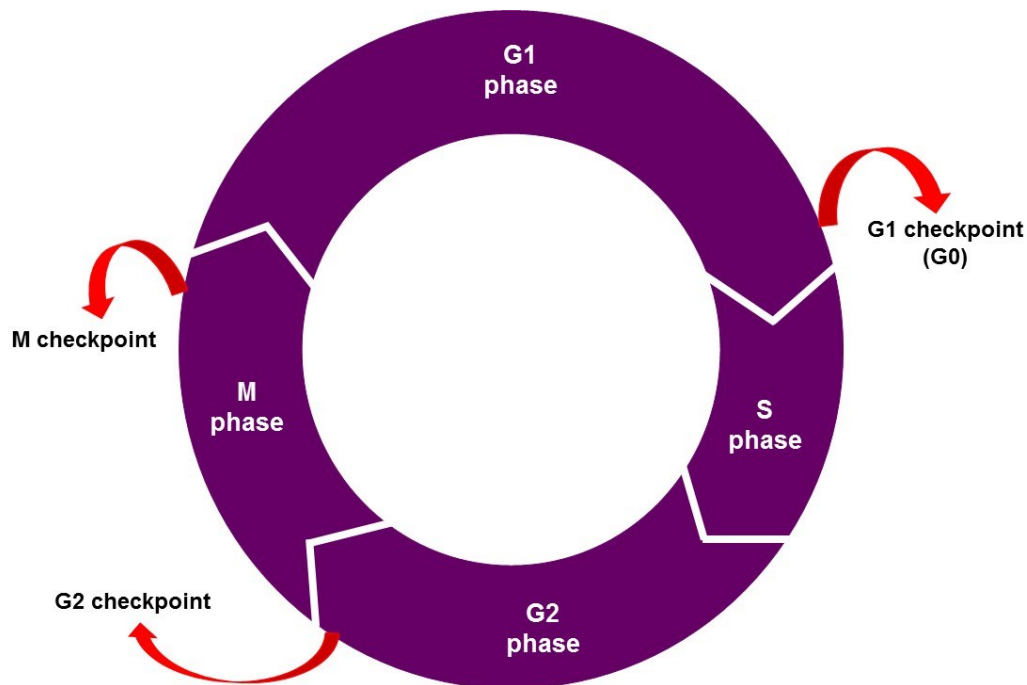


Figure 1.5: Illustration of the cell cycle phases and checkpoints. *G1 and G2 phases represent cell growth, S phase represents DNA synthesis and M phase represents mitosis, which consists of prophase, metaphase, anaphase and telophase. G1 checkpoint indicates cell growth checkpoint that will rest at G0 if unrepaired. G2 checkpoint is DNA synthesis checkpoint, and M checkpoint is mitosis checkpoint.*

Chk2 is a cell cycle checkpoint kinase that is downstream of the ATM kinase pathway. In normal replication, DNA damage could still occur due to stalled replication fork (Rothkamm et al., 2003; Mehta and Haber, 2014; Syeda et al., 2014). DNA damage that occurs in normal cellular replication will activate Chk2 (Castedo et al., 2004). This will inhibit the activation of the cyclin-dependent kinase (Cdk1) and Cyclin B1 complex, which will lead to cell cycle arrest in order for the cells to either repair the damage, or go through apoptosis (Nigg, 2001). ATM-activated Chk2 will phosphorylate Cdc25, which will inhibit the activation of the cyclin-dependent kinases and cell cycle progression (Matsuoka et al., 1998; Chaturvedi et al., 1999).

Chk2 inhibition will cause detrimental effects, as the cells will continue to proliferate even if mutations occur due to induced DNA damage, which then will cause genome instability and cell death (Castedo et al., 2004). Chk2 has also been found to be expressed in all cell cycle phases and can be activated upon DNA damage, even in non-proliferation and quiescent cells, which is different than G2/M phase dependent Chk1 (Lukas et al., 2001).

1.4: Extra-telomeric functions of TRF2

Other than its role at the telomeres, TRF2 has also been found to localise at extra-telomeric regions of the chromosome (Bradshaw et al., 2005; Huda et al., 2012). The Myb-domain of TRF2 is highly specific to recognise and bind to the G-rich site of the telomeres (Hanaoka et al., 2009). However, the other binding domain of TRF2, which is the N terminal, plays central roles in recruiting TRF2 to non-telomeric regions (Jung et al., 2004; Bradshaw et al., 2005; Fouch  et al., 2006). It has been shown that TRF2 was found to be located at other chromosomal sites that are outside of the telomeres (Simonet et al., 2011; Yang et al., 2011). At the telomere, TRF2 has been shown to be a protein hub that is able to either directly bind or indirectly affect and recruit other proteins to protect the telomere (Chen et al., 2008; Kim et al., 2009; Feuerhahn et al., 2015). In addition, TRF2 has also been found to be associated with other proteins outside of the telomeres (Karlseder et al., 2004; Roy et al., 2018). The role of TRF2 as a protein hub showed a lot of potential functions that TRF2 may play outside of the telomeres. Table 1 shows the proteins that have been reported to either directly or indirectly interact with TRF2, both in telomeric and non-telomeric regions.

Table 1: List of proteins that interact (directly or indirectly) with TRF2 at the telomeres and outside of the telomeres.

Proteins	Function	Reference (s)
TRF1	Telomere protection Telomere length regulation Recruit other Shelterin subunits	de Lange, 2005; Smogorzewska et al., 2000; Ohki and Ishikawa, 2004; Chen et al., 2008; Maestroni et al., 2016;
Rap1	Telomere protection Telomere length regulation Assists in TRF2 recognition of the telomeric DNA Stabilise the inhibition effect of TRF2 towards the ATM activation	de Lange, 2005; O'Connor et al., 2004; Rai et al., 2015; Li and de Lange, 2003; Janouskova et al., 2015; Li et al., 2015;
TIN2	Stabilise TRF1/TRF2 complex at the telomere Telomere length regulation	de Lange, 2005; Ye et al., 2004 (a) Ye et al., 2004 (b);
POT1	Telomere protection Binds to ssDNA of the telomere Negatively regulate telomerase activity <i>in vitro</i>	de Lange, 2005; Baumann and Chech, 2001; Lei et al., 2003; Ye et al., 2004 (c); Kelleher et al., 2005;

Proteins	Function	Reference (s)
TPP1	Telomere protection Recruitment of telomerase Regulation of telomerase activity Recruitment of TIN2 and POT1 to the telomeric DNA	de Lange, 2005; Hockemeyer et al., 2007; Zhong et al., 2012; Wang et al., 2007; Xin et al., 2008;
TERRA	Telomere length regulation Facilitates heterochromatin formation	Wang et al., 2015; Deng et al., 2009; Poulet et al., 2011;
TERT	Telomere length regulation	Kim et al., 2016; Mukherjee et al., 2018;
Core histones	Telomere stabilisation and protection	Konishi et al., 2016;
ATM	Recognition of telomere as DNA break Regulate telomerase recruitment DNA damage response	Denchi& de Lange, 2006; Buscemi et al., 2009; Tong et al., 2015; Elledge, 1996; Bradhsaw et al., 2005; Kastan and Lim, 2005; Huda et al., 2009; Kitagawa and Kastan, 2015;
Ku70/80	DNA damage response and repair	Ribes-Zamora et al., 2013; Celli, Denchi and de Lange, 2006;
p38	DNA damage response Cell cycle checkpoint control	Wood et al., 2009; Roy et al., 2018; Phong et al., 2010;

1.4.1: TRF2 is required for neural differentiation in hESCs

Previously, our lab has shown that TRF2 protein expression is highly and specifically elevated in hESCs-derived neural progenitor cells (NPCs), but not in other hESC-derived cell types (Ovando-Roche et al., 2015). These results suggest that there may be an underlying role of TRF2 in NPC formation, as well as in brain development and function.

Human embryonic stem cells (hESCs)

Embryonic stem cells were first isolated from mice inner cell mass (ICM) from pre-implantation mouse blastocysts *in vitro* (Evans and Kaufman, 1981; Martin, 1981). These cells, termed mESCs, could self-renew indefinitely in culture, whilst being able to be differentiated into cells derived from all three germ layers, except for placenta. The pluripotency ability of these cells was evident when injected into blastocysts, where they were able to produce chimaeras, contributing to the formation of all tissues, including germ cells and thus, generating entire embryos via germline transmission (Nagy et al., 1990 and 1993). ESCs self-renewal and pluripotency is governed by key intrinsic and extrinsic factors. The most studied intrinsic factors, OCT4, SOX2 and Nanog are highly expressed in ESCs and downregulate as the cells differentiate (Rogers et al., 1991; Reubinoff et al., 2000; Chambers et al., 2003; Fong et al., 2008). The significant role of these factors in maintaining pluripotency has been further shown in their ability to reprogram somatic cells into induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007; Yu et al., 2007; Yamanaka et al., 2007; Nakagawa et al., 2008). The first hESCs line was also derived from the ICM of pre-implantation embryos (Thomson et al., 1998). Similar to the mESCs, hESCs can be differentiated into three different germ layers, which are ectoderm, mesoderm and endoderm through embryoid body formation (Cai et al., 2006; Itskovitz-Eldor et al., 2000). Each germ layer will be able to further differentiate into different types of cells.

Although there may be some similarities between mESCs and hESCs, as they are both derived from ICM, they exhibit some phenotypical difference. Developmentally, mESCs represents a more naïve pre-implantation state, whereas hESCs showed a primed post-implantation state (Nichols and Smith, 2009) that are similar to mouse epiblast-derived stem cells (EpiSCs) (Brons et al., 2007; Tesar et al., 2007). These differences require different extrinsic factors to maintain the pluripotency of the cells.

Extrinsic factors, such as growth factor signalling pathways, are also very important for regulating self-renewal of ESCs. However, these factors may differ between mouse and human ESCs. For example, maintenance and propagation of mESCs properties require leukaemia inhibitory factor (LIF) (Smith et al., 1988; Williams et al., 1988), while hESCs self-renewal has been shown to require the presence of FGF2/bFGF (Reubinoff et al., 2000). This was shown due to lack of LIF receptor (LIFR)/gp130 complexes in hESCs to maintain its pluripotency (Brandenberger et al., 2004). 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). FGF has been shown to be essential in maintaining cell proliferation and pluripotency in hESCs (Lanner and Rossan, 2010; Coutu and Galipeau, 2011).

Neural development

Similar to maintaining hESCs in culture, *in vitro* differentiation of hESCs to specific lineages also requires specific growth factors and inhibitors, according to what is known in regard to embryonic development. One of the signalling molecules thought to inhibit hESCs towards neural lineage is the bone morphogenetic protein (BMP), which is an epidermal inducer (Hawley et al., 1995). Inhibiting the BMP signalling, specifically BMP4 and BMP2, will induce neural differentiation, which has been shown in *Xenopus* and mice (Xu et al., 1995; Reichert et al., 2013). Although in *Xenopus*, inhibition of BMP has shown to be able to induce

neural differentiation, it is more complex in other vertebrates, where in addition to the BMP signalling inhibition, neural induction is also thought to be regulated by other signalling pathways, such as FGF and Wnt (Zhang et al., 2001; Linker and Stern, 2004; Marchal et al., 2009). However, hESCs are similar to *Xenopus* embryos, where hESC neural differentiation is also critically driven by the inhibition of BMP signalling (Gerrard et al., 2005). Combination of both BMP and TGF β signalling is reported to enhance the speed and efficiency of neural differentiation, such as the use of SB431542 and dorsomorphin as inhibitors for both signalling pathways (Chambers et al., 2009; Bhinge et al., 2014).

In vivo, the first isolation of the neural stem cells (NSCs) is from the embryonic mouse central nervous system (CNS) and the peripheral nervous system (PNS) (Temple, 1989; Stemple and Anderson, 1992; Cattaneo and McKay, 1990). After which, it has been discovered that NSCs can also be isolated from a specific region of the adult brain, the hippocampus and the subventricular zone (SVZ) (Reynolds and Weiss, 1992; Lois and Alvarez-Buylla, 1992). The specific characteristics of these isolated cells were their ability to differentiate into neurons, astrocytes, radial-glia and oligodendrocytes.

In vitro, hESCs-derived NPCs can be characterised using specific molecular biomarkers, such as SOX1 (Aubert et al., 2003), PAX6 (Wen et al., 2008; Zhang et al., in review), SOX2 (Avilion et al., 2002; Rizzino, 2009), as well as Nestin (Fukuda et al., 2003; Mignone et al., 2004; Park et al., 2010). These biomarkers play specific roles from the self-renewal ability of the NPCs and to further differentiate into other neuronal lineages. The different expression levels of these molecular biomarkers made it possible to characterise the different stages of the cells in neural differentiation: such as Tuj1 as a marker for immature neurons; NeuN and MAP2 as a marker for mature neurons; and GFAP as the astroglial marker (Ambrogini et al., 2004; Park et al., 2012; Zhang and Jaio, 2015). It has been reported that different stages of NPCs have different preferential in neuronal differentiation, for example, early embryonic NPCs are more prone to

be differentiated into neurons, while late embryonic NPCs prefer gliogenesis (reviewed in Alvarez-Buylla et al., 2001; Temple, 2001; Zhang and Jaio, 2015).

In addition to the role that TRF2 plays in neural development, TRF2 has also been found in neuroglial cytoplasm, where it induces neurite-like processes (Jung et al., 2004). During neural differentiation, TRF2 mRNA in rat was found to generate a short TRF2 protein isoform known as TRF2-short (TRF2-S). It was shown to be able to elevate the silencing of neuronal genes by REST, which will induce neural differentiation (Grammatikakis et al., 2016b). Both TRF2-S and TRF2 are able to bind to REST and form TRF2-REST4 complex, which will suppress the function of REST and enhance the neuronal gene transcription, as shown in Figure 1.6 (Ovando-Roche, et al., 2015; Grammatikakis et al., 2016).

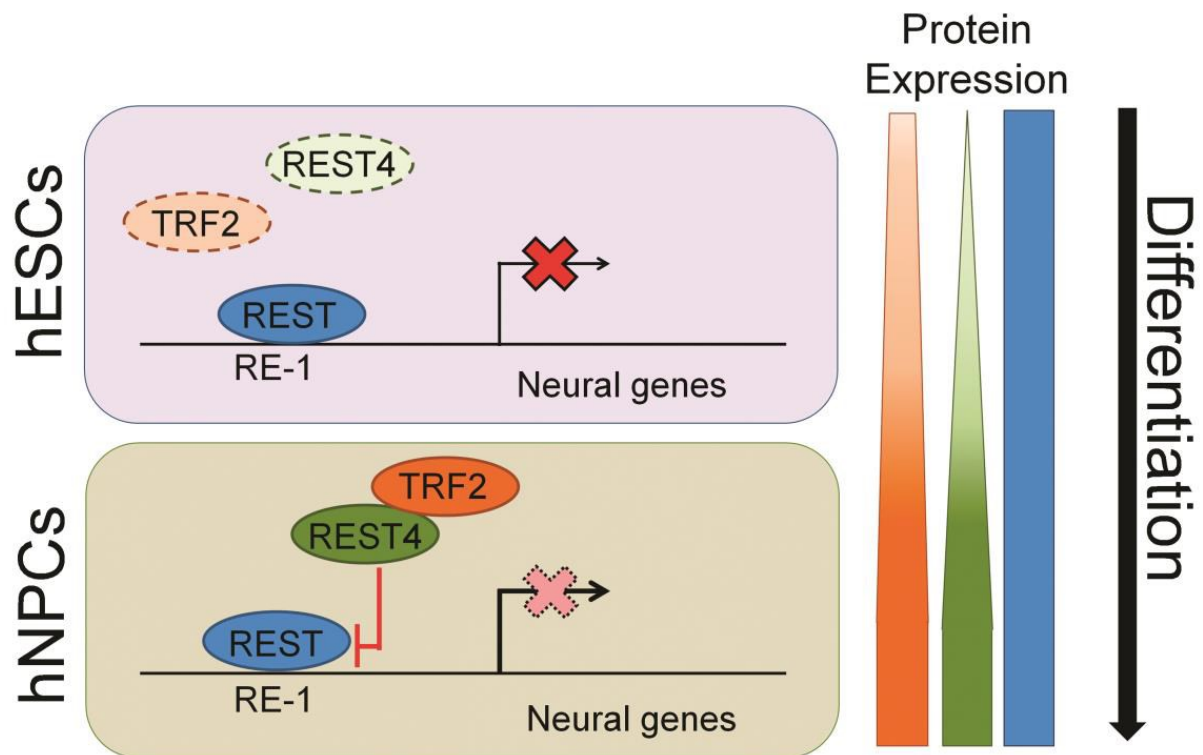


Figure 1.6: Schematic model for the role of TRF2, REST and REST4 in regulating neural differentiation. Oval cartoons with straight lines indicate the high abundance of the indicated proteins, while the dotted oval indicates the low expression of the proteins. The arrow shows transcription of the gene and the red cross indicates inhibition of the transcription, while the pink cross indicates an activation of some transcription. The model shows that TRF2 stabilisation towards REST4, thus enhancing its expression that will elevate the inhibition of REST towards the neural genes, leading to the induction of neural differentiation. Image adapted from Ovando-Roche et al., 2015.

A recent report has also shown that in human developing brain, the telomerase expression is downregulated (Ishaq et al., 2016). In contrast, another report has also shown that an increase in TRF2 expression does not affect the telomerase expression as well as its activity (Smogorzewska et al., 2000). This contradiction could be due to the different types of cells used in these two studies, as TRF2 is only endogenously highly expressed in the developing brain as compared to other tissue types (Jung et al., 2004). As TRF2 protein expression also seemed to decrease in terminally differentiated neurons, it suggests that TRF2 function is specific to the NPCs (Ovando-Roche et al., 2015).

1.4.2: TRF2 is involved in the DNA damage response outside of the telomere

The function of TRF2 in the DNA damage response has been mainly investigated at the telomeres. Very little is known regarding the involvement of TRF2 in the DNA damage response outside of the telomeres. Previous reports have shown that TRF2 is localised at the site of the laser-induced DNA break outside of the telomeres within seconds upon the DSB induction (Bradshaw et al., 2005; Huda et al., 2009). The recruitment of TRF2 at the DNA break site is transient, where within seconds, TRF2 was shown to localise at the site of DSB, but the expression gradually declines over time, and the recruitment is decreased after multiple exposures to induce DSBs (Bradshaw et al., 2005; Huda et al., 2012). This result suggests that there are other factors that are regulating the recruitment of TRF2 at the DSB site.

One of the factors that are regulating the recruitment of TRF2 to the extra-telomeric DSBs is through the phosphorylation of TRF2 that is initially thought to be activated by the ATM kinase (Tanaka et al., 2005). However, it has also been shown that TRF2 phosphorylation and recruitment to the DSBs were observed in ATM-deficient, AT-primary fibroblast cells, which suggests that ATM is not necessarily required for the phosphorylation and recruitment of TRF2 to the DSB site. A similar effect was again observed in DNA-PKcs-deficient, human glioma cell lines, which suggests that DNA-PKcs was also not essential in the TRF2 DSBs recruitment (Lieberman, 2006; Huda et al., 2012). Further investigation is needed in order to address the mechanism of TRF2 recruitment to the DSB site and to explore the function that TRF2 actually play as it is bound to the DSB.

Recent studies have found that rapid phosphorylation of ATM is important in the NHEJ repair of DSBs (Huda et al., 2009; Kong et al., 2018). There has also been reported that TRF2 depletion will inhibit HR and delayed Rad51 foci formation upon γ -irradiation and overexpression of TRF2 will stimulate HR and inhibit NHEJ (Mao et al., 2007). Nevertheless, the mechanism of TRF2 involvement in DNA repair is still unknown.

It was also shown that TRF2 recruitment to the DSBs is dependent of the activation of another DNA damage sensor, PARP1, as well as the binding domains of TRF2 (Kong et al., 2018). Another report also showed the direct interaction of TRF2 with the p38 MAPK upon the induction of DNA damage (Roy et al., 2018). These reports show that there are many possibilities that TRF2 may interact with the DDR, where TRF2 will further recruit other factors that will repair the damaged DNA.

1.5: TRF2 is important in understanding human diseases

TRF2 has been shown to have a role in human development and diseases. The main role of TRF2 in the telomere should not be minimised. TRF2 is involved in the protection of the telomeres from end-to-end chromosome fusion and suppress checkpoint activation that will lead to senescence or apoptosis (de Lange, 2002). The telomeres have been associated with many ageing and degenerative diseases (Reviewed in Armanios, 2013). The role of TRF2 at the telomeres could help to further explain telomere attrition in degenerative diseases, which will assist in protecting the telomere ends from early degradation. Degenerative diseases and ageing related diseases, such as Werner Syndrome could be reduced by protecting the telomeres from degradation. TRF2 has been found to directly bind with Werner syndrome ATP-dependent helicase (WRN) and mediates WRN exonuclease activity, which is important for protecting the telomere, hence the cells, against premature senescence and maintaining genomic stability (Machwe et al., 2004).

TRF2 is also involved in many neurodegenerative diseases, such as Alzheimer's and Parkinson's, as TRF2 has been found to be essential in cell survival and protects the cells from senescence (Schipper, 1996; Zhang et al., 2006, 2007). Overexpression of TRF2 might also cause telomere attrition, which may lead to short telomeres in the cells that will develop many

detrimental diseases, such as Dyskeratosis Congenita (Mitchell et al., 1999; Armanios and Blackburn, 2012; Mangaonkar et al., 2018).

Outside of the telomeres, TRF2 is mainly involved in neuronal development (Zhang et al., 2008) and DNA damage response (Bradshaw et al., 2005). Overexpression of TRF2 has also been shown to cause shorter telomeres, where it is one of the main causes of degenerative diseases, cancer and ageing (Muñoz et al., 2006). TRF2 is highly expressed in the NPCs, which is highly abundant in the developing brain (Ovando-Roche et al., 2015). It has been shown that there is a high prevalence of brain tumour in children (The 12 ICCG Diagnostic Groups, 2006-2008), which suggests that the developing brain is more prone to develop into cancer (Johnson et al., 2014).

The high expression of TRF2 in various cancer cells also poses therapeutic possibilities, where knocking out TRF2 will cause massive cell death (Biroccio et al., 2013). The role of TRF2 in DNA damage response could further help us to understand the mechanism in cancer development and could also help in reducing its progression.

1.6: Aims and objectives

Given the telomeric and extra-telomeric functions of TRF2 in the DDR and DNA repair (de Lange, 2005b; Karlseder et al., 2004; Bradshaw et al., 2005), as well as the high expression of TRF2 in the hESCs-derived NPCs (OvandoRosche et al., 2015), I thought that there would be an underlying protective function that the TRF2 may have in the NPCs. Therefore, this study aims to assess this hypothesis through the following objectives:

1) To determine the effects of TRF2 on cell proliferation and survival in NPCs and HEK293T cells under standard culture conditions

A gain- and loss- of function experiment have been applied to help characterise the function of TRF2 in cell proliferation and survival in these two cell types

2) To explore the effects of TRF2 on cell survival in the NPCs and HEK293T cells under stress conditions

Different culture conditions to induce stress in these cell types were performed in order to further explore TRF2 functions in cell survival

3) To further investigate TRF2 relationship with the DNA damage response pathway in NPCs and HEK293T cells

Explore the interactions that TRF2 has with the ATM-dependent DNA damage response to understand the effect that TRF2 has in cell survival

Chapter 2

Materials and Methods

2.1 Cell culture

2.1.1: Materials

2.1.1.1: Cell Lines

Cell Line	Source
H1 hESC (WA-01)	Male human ES cell line isolated by Thomson et al., 1998, distributed by WiCell under MTA licence.
HEK293T	Human embryonic kidney cell line obtained from ATCC, cat # ATCC® CRL-3216™.
Mouse Embryonic Fibroblasts (MEFs)	Wild-type MEFs isolated from E13.5 CD-1 mouse.
HT1080	Fibrosarcoma cell line, obtained from ATCC, cat # ATCC® CCL-121™.
BJ-5ta	Fibroblast cells immortalised with hTERT, obtained from ATCC, cat # ATCC® CRL-4001™.
TRF2 control NPCs	Derived from hESCs and transduced with lentivirus of GFP-shRNA (Ovando-Roche et al., 2014).
TRF2 knockdown NPCs	Derived from hESCs and transduced with lentivirus of TRF2-shRNAs (Ovando-Roche et al., 2014).

2.1.1.2: Growth Factors and Inhibitors

The working stock of all growth factors were reconstituted in Dulbecco's Phosphate Buffered Saline (DPBS) supplemented with 0.2% BSA as carrier to the indicated concentration. All inhibitors were reconstituted in DMSO with the exception of puromycin and ROCKi, which were reconstituted in tissue culture grade H₂O.

Reagent	Supplier	Cat#	Working Concentration
Basic Fibroblast Growth Factor (bFGF)	Peprtech	100-18B-500	10ng/ml
Epidermal Growth Factor (EGF)	Peprtech	AF-100-15	20ng/ml
SB431542	R&D/Tocris	1614/10	10μM
Dorsomorphin	R&D/Tocris	3093/10	1μM
Puromycin	Sigma-Aldrich	P8833	1~2μg/ml
Rho-Kinase Inhibitor (ROCKi)	Sigma-Aldrich	1254	10μM

2.1.1.3: Culture Media Components

Component	Supplier	Cat#
200 mM L-Glutamine	Sigma-Aldrich	G7513
20% Bovine Serum Albumin (BSA)	Sigma-Aldrich	A9576
50X B27 Supplement	Life Technologies	17504-044
100X N2 Supplement	Life Technologies	17502-048
Dulbecco's Modified Eagle AQ Media (DMEM-AQ)	Sigma-Aldrich	D0819
Heat-inactivated Foetal Bovine Serum (FBS)	Sigma-Aldrich	10500064
Knockout Serum Replacement (KSR)	Sigma-Aldrich	10828-028
DMEM:F12 (1:1)	Life Technologies	21331020
KO-DMEM	Life Technologies	10829-018
β-mercaptoethanol (55 mM)	Life Technologies	31350-010
Non-essential Amino Acid (NEAA)	Sigma-Aldrich	M7145
Neurobasal	Life Technologies	21103-049
100X Penicillin/Streptomycin (P/S)	Life Technologies	P0781

2.1.1.4: Cell Culture Media and Stock Solutions

All media and stocks were filtered, sterilised and stored at 4 °C.

Knockout Serum Replacement Medium (KSR-M)	KO-DMEM supplemented with: 20% KSR 1 mM L-glutamine 1x NEAA 0.1 M β -mercaptoethanol for TC 1x Penicillin-Streptomycin
MEF-conditioned medium for hESCs (MEF-CM)	MEF conditioned KSR-M supplemented with: 1 mM L-glutamine 10 ng/ml bFGF
N2/B27 medium	DMEM/F12 /Neurobasal medium (1:1) supplemented with: 0.5 x B27 0.5 x N2 1 mM L-glutamine
N2 medium	DMEM/F12 /Neurobasal medium (1:1) supplemented with: 0.5 x B27 1 mM L-glutamine
D10 medium	DMEM-AQ supplemented with: 10% FBS 1x NEAA 1x Penicillin-Streptomycin

2.1.1.5: Disassociation Enzymes

Enzyme	Supplier	Cat#
TrypLE Express	Thermo Fisher	12604013
Collagenase IV	Thermo Fisher	17104-019
Trypsin-EDTA	Sigma-Aldrich	T3924

2.1.1.6: Chemicals, Kits and Coating Reagents

Reagent	Supplier	Cat #
2% Gelatine in PBS	Sigma-Aldrich	G1393
Calcium Phosphate Transfection Kit	Sigma-Aldrich	CAPHOS-1KT
Laminin	Sigma-Aldrich	L2020
0.01% Poly-L-lysine (PLL)	Sigma-Aldrich	P4707
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich	D2650
Dulbecco's Phosphate Buffered Saline (DPBS)	Sigma-Aldrich	D8537
Matrigel® Matrix Growth Factor Reduced	BD Bioscience	354230
Site-directed Quickchange mutagenesis kit	Agilent	200519
30% H ₂ O ₂	Sigma-Aldrich	H1009
Water Tissue Culture Grade (TC-H ₂ O)	Sigma-Aldrich	W3500
Trypan blue solution, 0.4%	Thermo Fisher	15250061
EVE cell counting chamber slides	Cambridge bioscience	EVS-1000-50

2.1.2: Methods

2.1.2.1: Culture and propagation of hESCs

i. 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 5 grays (Gy) (IBL 637 Cell irradiator), and seeded with MEF medium at ~250,000 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 MEFs-conditioned media (CM) was collected into sterile 150 ml collection bottles (Corning) and stored at -80 °C 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 to use for hESCs cell culture.

ii. Preparation of matrigel coated plates

Growth factor-reduced matrigel was slowly thawed at 4 °C overnight and diluted 1:2 with ice cold KO-DMEM. The diluted matrigel-KO-DMEM solution was aliquoted in 1 ml volumes and stored in -20 °C until further use. To prepare the matrigel coated plates, frozen stock matrigel solution was slowly thawed at 4 °C overnight and then diluted 1:20 with chilled KO-DMEM which was then plated into cell culture plates (1 ml per well for 6 well plates) and stored overnight at 4 °C. The plates can be left stored at 4 °C for 2 weeks. In emergency cases, the matrigel coated plates can be incubated at room temperature for an hour.

iii. Freezing and thawing of hESCs

Confluent monolayer cells were collected similar to passaging but using Serum Replacement (SR) medium plus 100 μ L of dimethylsulfoxide (DMSO) instead of MEF-CM. The cells were collected inside the cryotube and put in the Mr. Froster for overnight incubation in -80°C before transferred in the liquid nitrogen for long term storage. Cells were recovered from long term freezing by thawing rapidly in 37°C water bath. When an ice pellet is visible, the cells were quickly removed from the cryotube and MEF-CM supplemented with 8 ng/ml of bFGF were added. Pipette up and down a few times to break the colony and put the cells in a new matrigel-coated plate.

iv. Routine culture and maintenance of hESCs

H1 (passage numbers 60 to 80) cells were grown and maintained in an undifferentiated state on matrigel-coated plates with MEF-CM supplemented with 8 ng/ml of bFGF. They were routinely passaged in 6-well plates (Corning) at 1:3 ratio by incubating for 2-5 minutes with 200 U/ml collagenase IV at 37°C . The collagenase was then washed off from the cells with PBS and the cells were scrapped with a 5 ml strippette (Corning) to generate small clumps of cells that were subsequently plated onto new matrigel-coated wells.

2.1.2.2: Neural differentiation of hESCs

Confluent monolayer hESCs were passaged 1:3 onto a new matrigel-coated plate with MEF-CM supplemented with 8 ng/ml of bFGF for one day. Neural differentiation was initiated by changing the media with the N2B27 medium supplemented with 10 ng/ml of Dorsomorphin and 10 μ M/ml of SB43542 (Bhinge et al., 2014, 2016). Colonies were allowed to differentiate for 8 days with changing the media every day. Once the neuroectodermal colonies were observed, the cells were dissociated using TrypLE into single cells onto matrigel-coated plates. At this stage, the Dorsomorphin and SB43542 were changed to 10 ng/ml of bFGF and EGF. Rock inhibitor was used in the first two passages to protect cells from dying.

2.1.2.3: Culture and propagation of NPCs

i. Preparation of Poly-L-Lysine/Laminin (PLL/L) coated plates

For culturing neural progenitor cells and neuron, PLL/L coated plates were used. The previously aliquoted 120 μ l Laminin (0.1 mg/ml) were slowly thawed in 4 °C overnight. On the next day, the PLL was diluted in 1:6 ratio with PBS. 1mL of the solution was placed onto a 6-well culture plate and left to incubate for at least 1 hour in the culture hood (room temperature). Dilute the thawed 120 μ l aliquot of Laminin solution with 6mL PBS making the final concentration to 20 μ g/ml. Aspirate the PLL solution from the well and place 1mL of the Laminin solution onto the well. For emergency, the PLL/L coated plates can be incubated in room temperature for 1 hour before use. The PLL/L coated plates may be stored in 4 °C for a month.

ii. Routine culture and maintenance of NPCs

The cells were taken out from the liquid nitrogen tank and thawed in the 37 °C water bath. The cells were quickly removed from the cryotube and plated on a 6 well culture plate (1 well per tube) coated with PLL/L and added 2 mL of N2B27 media supplemented with 10 ng/mL of EGF and bFGF. The medium was changed the next day to remove the residue DMSO. The cells were passaged (1:3) with TrypLE after it reached a 100% confluency. The culture condition used (37 °C, 5% CO₂ and 20% O₂ in N2B27 media, supplemented with 1 µg/mL of EGF and bFGF) is referred to as the standard culture condition henceforth.

2.1.2.4: Culture and propagation of HEK293T and BJ-5ta

HEK293T and BJ-5ta cells were routinely cultured in D10 medium in T75 flasks or 6 well culture plates. Once reaching ~100% confluency, they were split out in a ratio from 1:3 to 1:10 using Trypsin-EDTA. For long-term storage, the cells were typically frozen at 1×10^6 per cryovial in fresh growth media supplemented with 10% DMSO.

2.1.2.5: TRF2-control and knockdown NPCs

The TRF2-control and knockdown NPCs were generated previously in the lab (Ovando-Roche et al., 2014) and were cultured routinely in NPCs culture condition. Both cells contain cDNA encoding puromycin resistance (PuroR) protein, which allows the transduced cells to survive in puromycin selection. The cells used were polyclonal cells (pooled cells) and not monoclonal cells.

2.1.2.6: TRF2 expression vector

The original plasmids were obtained from Addgene unless indicated and all restriction enzymes as well as the modification enzymes were purchased from New England Biolabs. Lentiviral vectors for overexpressing TRF2 derived from pLVTHM (Addgene 12247, Wiznerowocz and Trono, 2003). The stop codon of PURO cDNA was mutated in pPUR (Clontech) to enable the insertion of a 2A foot and mouth disease virus sequence PCR fragment downstream of the 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. This expression vector was generated by Ovando-Roche et al. (2014).

2.1.2.7: Calcium phosphatase transfection

$\sim 1 \times 10^6$ HEK293T cells/10 cm plate were cultured in 10 ml DMEM AQ media (Sigma) + 10% FBS supplemented with Pen/Strep. The next day, the media was changed with 6 ml of DMEM for 10 cm plate prior to transfection. Transfection mix containing 2X HeBS (in tube A) and DNA with CaCl_2 (in Tube B) were prepared. Up to 15 to 20 μg DNA can be used for one 10 cm plate. Tube A and tube B were mixed by slowly adding the DNA and CaCl_2 mix (drop-wise), while aerating the 2 x HeBS (tube B) using a 1 ml pipet. The mixture was placed in room temperature for 20 minutes before added evenly into the culture media. The media was removed after 12 to 16 hours after transfection and 10 ml of pre-warmed fresh media was added to the plate. The HEK293T transfected cells used were polyclonal (pooled cells) and not monoclonal cells.

2.1.2.8: Treatment conditions

i. B27 withdrawal

5 x 10⁵ of NPCs were seeded and left in culture with the N2B27 culture medium supplemented 10 ng/mL of bFGF and EGF (standard culture conditions). After overnight culture with the routine conditions, the culture media was changed to N2 medium supplemented with 10 ng/mL of bFGF and EGF for 1 hour. The culture media of the control cells were changed with N2B27 media supplemented with 10 ng/mL of bFGF and EGF. The cells were collected and counted for further analysis.

ii. Oxidative stress treatment

The oxidative stress was induced by culturing cells in their proper growth media containing 100 μ M hydrogen peroxide (H₂O₂). The 30 % H₂O₂ solution was dissolved in water to 1 mM as a working stock solution before adding to the growth media prior to treatment. The non-treated cells were cultured in their respective growth media, added with the same volume of water as the H₂O₂ in the treated cells. The stock and dissolved solutions of the H₂O₂ were kept in 4 °C, avoiding light.

iii. Hypoxia treatment

The hypoxic condition was induced by incubating the cells in the hypoxic chamber (Whitley H35 hypoxystation). The oxygen level was set to 0.5% for the hypoxia condition. In order to determine the difference between cell culture oxygen condition and physiological oxygen level, the cells were incubated for overnight in 20% and 5% oxygen level, respectively. The cells were collected and counted for further analysis after 1 hour in the hypoxic condition. The non-treated cells were cultured in the routine conditions.

2.1.2.9: Cell count and cell viability assay

During routine culture and treatments, the cells were counted using the automated cell counter machine to get the total, live, dead cell number and viability. The cells were collected from the culture plate and re-suspended in media, depending on the cell concentration. 10 μ L of the cell suspension were collected and added to 10 μ L of Trypan blue to stain the dead cells. The cells were put in the cell counter slide chamber (Cambridge Bioscience) and counted using Countess™ Automated Cell Counter (Life Technologies).

2.2 Molecular Biology – Protein

2.2.1: Materials

2.2.1.1: Reagents, Chemicals, and Kits

Reagent	Supplier
30% Acrylamide/Bis	Sigma
4',6 diamidino-2-phenylindole (DAPI)	Sigma
Ammonium Persulfate (APS)	Sigma
Bicinchoninic Acid Assay (BCA) Protein Quantification Kit	Thermo Fisher
Bromophenol blue	Sigma
Bovine Serum Albumin (BSA)	Sigma
CL-Xposure film	Scientific Labs
Dithiothreitol (DTT)	Sigma
Donkey Serum	Sigma
Enhanced chemiluminescence substrate (ECL)	Thermo Fisher
Glass Coverslips	VWR
Glass Slides	VWR
Glycerol	Sigma
Goat serum	Sigma
Immobilon® polyvinylidene fluoride (PVDF) membrane	Millipore
Luminata Forte Enhanced ECL substrate	Millipore
Methanol	VWR
Mowiol 4-88	Sigma
Nonidet P-40 (NP-40)	Anachem
PageRuler Plus Protein Ladder	Thermo Fisher

Paraformaldehyde	NEB
Phenylmethanesulfonylfluoride (PMSF)	Sigma
Phosphatase Inhibitor Cocktail 2	Sigma
Propidium Iodide	Sigma
Protease Inhibitor Cocktail	Sigma
RNAse A	Sigma
Skim milk powder	Sigma
Sodium azide (NaN ₃)	Sigma
Sodium deoxycholate	Sigma
Sodium dodecyl sulphate (SDS)	Sigma
Sodium orthovanadate (Na ₃ VO ₄)	Sigma
Tetramethylethylenediamine (TEMED)	Sigma
Triton X-100	Sigma
Tween-20	Sigma
β-mercaptoethanol	Sigma

2.2.1.2: Buffers and Solutions

Immunoblot

Radio Immunoprecipitation Assay Buffer (RIPA)	25 ml 1 M Tris-HCl pH 8.0 (50 mM) 15 ml 5 M NaCl (150 mM) 5 ml NP-40 (1% v/v) 2.5 g Sodium deoxycholate (0.5% w/v) 0.5 g SDS (0.1% w/v) Autoclaved water to 500 ml
5X Sample buffer	1.25 ml 0.5 M Tris-HCl pH 6.8 (50 mM) 2.5 ml glycerol (25 % v/v) 2 ml 10% SDS (2% w/v) 400 µl 0.5% bromophenol blue (0.02 % w/v) 500 µl β-mercaptoethanol (715 mM) Autoclaved water to 10 ml
8% Polyacrylamide Running Gel	2.5 ml 0.5 M Tris-HCl pH 6.8 (125 mM) 2.7 ml 30% Acrylamide/Bis (6% v/v) 100 µl 10% SDS (0.1 % w/v) 75 µl 12% APS (0.075 % w/v) 18 µl TEMED (0.18 % v/v) Autoclaved water to 10 ml
10X SDS Running Buffer	30.3 g Tris base (250 mM) 144.2 g Glycine (1.9 M) 50 ml 20% SDS (1%) Autoclaved water to 1 L
Transfer Buffer	5.82 g Tris base (48 mM) 2.93 g Glycine (39 mM) 3.75 ml 10% SDS (0.04% w/v) 200 ml methanol (20% v/v) Autoclaved water to 1 L
Tris Buffered Saline with Tween-20 (TBS-T)	20 ml Tris-HCl pH 7.6 (20 mM) 26 ml NaCl (130 mM) 1 ml Tween-20 (0.1 % v/v) Autoclaved water to 1 L

Blocking buffer	5 g BSA or Skim milk (5% w/v) 1 L 1X TBS-T
Stripping buffer	10 ml Tris-HCl pH 7.5 (20 mM) 286.6 g GnHCl (6 M) 1 ml NP-40 (0.2% v/v) 3.5 ml β -mercaptoethanol (0.1 M) Autoclaved water to 500 ml
SDS lysis buffer (2X)	5 ml 0.5M Tris-HCL (6.5 pH) 2.5 ml 20 % SDS 12.5 ml autoclaved water 5 ml Glycerol
Immunostaining	
Fixation buffer	4 g paraformaldehyde dissolved in 90 ml autoclaved water at 60 °C 10 M NaOH added dropwise till solution clears 10 ml 10X DPBS Adjust pH to 7.2 with 9.61 M HCl acid Adjust volume to 100 ml with autoclaved water
Blocking/permeabilising buffer	1 ml goat serum or donkey serum (10% v/v) 0.25 g BSA (2.5% w/v) 30 μ l Triton X-100 (0.3% v/v) DPBS to 10 ml
Mowiol mounting solution	12 ml 200 mM Tris-HCl pH 8.5 (135 mM) 6 g glycerol 2.4 g Mowiol 4-88 6 ml autoclaved water Heat tubes at 60 °C to dissolve Mowiol

2.2.1.3: Primary and Secondary Antibodies

Primary antibodies	Type	Company	Catalogue Number	IF Dilution	WB Dilution
β -Actin	mouse monoclonal	Sigma-Aldrich	A5441	1:5000	1:5000-10,000
Caspase 3	rabbit polyclonal	Cell Signalling	9662	-	1:1000
Cleaved-Caspase 3 (D175)	rabbit polyclonal	Cell Signalling	9661	1:400	1:1000
Nestin	mouse monoclonal	R&D	MAB1259	1:200	1:500
OCT4	rabbit polyclonal	Abcam	ab19857	1:200	1:500
OCT4	mouse monoclonal	Santa Cruz	sc-5279	1:250	1:1000
phospho-ATM (Ser1981)	mouse monoclonal	Cell Signalling	4526	-	1:1000
phospho-ATM (Ser1981)	Rabbit polyclonal	Abcam	ab79891	1:300	
phospho-Histone 3 (Ser10)	mouse monoclonal	Cell Signalling	9706	1:400	1:1000
phospho-P53 (Ser15)	mouse monoclonal	Cell Signalling	9286	1:400	1:1000
P53	rabbit polyclonal	St. John's Lab	STJ94890	-	1:500-2000
phospho-Chk2 (Thr68)	rabbit polyclonal	Cell Signalling	2661	1:100	1:1000
Sox2	rabbit polyclonal	Abcam	ab97959	1:500	1:1000
Sox2	goat polyclonal	R&D System	AF2018	1:400	-
TRF1	mouse monoclonal	Millipore	04-638	1:100	1:1000
TRF2	rabbit polyclonal	Cell Signalling	13136	1:100	1:1000
TRF2	mouse monoclonal	Millipore	05-521	1:100	1:1000
γ -H2A.X (Ser139)	rabbit polyclonal	Cell Signalling	9718	1:400	1:1000

Secondary antibodies	Company	Catalogue Number	IF Dilution	WB Dilution
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	-
Donkey Anti-Mouse IgG Alexa Fluor 488	Life Technologies		1:400	-
Donkey Anti-Rabbit IgG Alexa Fluor 568	Life Technologies		1:400	-
Goat anti-mouse Light chain specific	Strattech	211-032-174	-	1:25000
Mouse anti-rabbit Light chain specific	Strattech	211-032-171	-	1:25000

Tissues	Company	Catalogue Number
Human Brain Whole Tissue Lysate (Adult Whole Normal)	NovusBio	NB820-59177
Human Kidney Tissue Lysate (Adult Normal)	NovusBio	NBP2-29078
Human Skin Whole Tissue Lysate (Adult Whole Normal)	NovusBio	NB820-59254

2.2.2: Methods

2.2.2.1: Protein extraction and quantification

The cells from the culture plate were lysed using cold radio immunoprecipitation 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. The protein extract was further run through a 21 G syringe to destroy any DNA and the cell membrane in order to retrieve the protein. After the protein has been extracted, bicinchoninic acid (BCA) protein assay kit was used to determine the protein concentration. The concentrations of the protein were calculated using Microsoft Excel.

2.2.2.2: Immunoblotting

For immunoblotting, 20 µg of the total protein from each extract were separated using 8-12% SDS-polyacrylamide gel and electroblotted onto polycylnidene fluoride (PVDF) membranes. The membranes were incubated with primary antibodies diluted in TBS at 4°C overnight and then reacted with secondary antibodies for 1 hour at room temperature after washing. Finally, the membranes were subjected to ECL substrate reaction and developed onto films to observe the protein bands.

2.2.2.3: Immunocytochemistry

Confluent cells were split and cultured onto coverslips coated with either matrigel for hESCs or PLL/L for NPCs until 90 % confluent. The medium was aspirated, and the cells were washed twice with PBS, then fixed with 4 % paraformaldehyde (PFA) at room temperature for at least 10 minutes. The fixed cells on coverslips could be stored in PBS at 4 °C for up to a few months before further analysis. To continue with the immunocytochemistry, the cells were washed with PBS three times at 10 minutes intervals. This process is done in a 4-well chamber slide or the 24-well plates. After the wash, the cells were incubated in PBS containing 10 % appropriate serum and 0.1 % Triton x-100 for 1 hour with mild agitation (0.5 ml/well for 4-well chamber slide or 24-well plates) to block non-specific binding of antibodies. The cells were then incubated in primary antibody at appropriate dilution in PBS containing 10 % serum for 1 hour at RT or overnight at 4 °C. After the incubation, the cells were washed again with PBS three times at 10 minutes intervals before incubated with the secondary antibody, diluted in PBS for 30 minutes in the dark. After another two times washing process in the dark, DAPI was incorporated in the third wash for nuclear staining. The coverslips were then mounted on a glass slip using mowiol.

2.2.2.4 Propidium Iodide (PI) Staining

For adherent cells, the cells were disassociated with TrypLe or Trypsin and centrifuged at 12,000 rpm. The cells pellets were washed with PBS and centrifuged again. The cell pellet was then resuspended in 300 μ l PBS before fixed with 70% of cold ethanol that were added dropwise to the cell suspension, while vortexing gently. The cells were left on ice for 1 hour before pelleted again with centrifugation. Cold PBS containing 0.25% Triton X-100 were added to the cell pellet to permeabilise the cells for staining. After the sample was permeabilised on ice for 15 minutes, they were centrifuged as above and then re-suspended and incubated with 0.2 mg/ml of RNase A at 37 °C for 30 minutes to remove RNAs. The cells were centrifuged, and the supernatant were removed carefully. The cells were then pelleted and re-suspended with 350 μ l of PBS containing 50 μ g/ml PI and kept in the dark at room temperature for 30 minutes before analysed by flow cytometry using the FACScalibur flow cytometer (BD Biosciences) with CellQuest software.

2.3 Molecular Biology – DNA/RNA

2.3.1: Materials

2.3.1.1: Reagents and Kits

Reagents and Kits	Supplier	Cat#
Chloroform:Isoamyl Alcohol 24:1	Sigma-Aldrich	P2069
Comet SCGE Assay Kit	Enzo Life Sciences	ADI-900-166
Deoxyneucleotide mix (dNTP)	Sigma-Aldrich	D7295
Ethanol	VWR	20820.327
Isopropanol	Thermo-Fisher	BP2618212
Magnesium chloride (MgCl ₂), anhydrous	Sigma-Aldrich	M8266
DEPC-treated water	Invitrogen	750024
Murine RNase Inhibitor	NEB	M0314S
Oligo (dT) ₁₂₋₁₈	Thermo-Fisher	10753741
ProtoScript II Reverse Transcriptase	NEB	M0368S
SYBR® Green Jumpstart™ Taq Ready Mix	Sigma-Aldrich	S4438
Tri Reagent®	Sigma-Aldrich	T9424

2.3.1.2: Buffers and solutions

Buffers	
10X Tris-Borate-EDTA Buffer (TBE)	108 g Tris Base 55 g Boric Acid 9.3 g EDTA Autoclaved water to 1 L

2.3.1.3: Primers for qRT-PCR

Primer	Forward strand	Reverse strand
Brachyury	TGCTTCCCTGAGACCCAGTT	GATCACTTCTTTCCTTTGCATCAA G
OCT4	TCGAGAACCGAGTGAGAGGC	CACACTCGGACCACATCCTTC
PAX6	TCCGTTGGAAGTGGAGT	GTTGGTATCCGGGGACTTC
SOX2	GCCGAGTGGAACTTTTGTCG	GCAGCGTGTACTTATCCTTCTT
TRF2	GACCTTCCAGCAGAAGATGC	GTTGGAGGATTCCGTAGCT
β -Actin	TGTCTGGCGGCACCACCATG	AGGATGGAGCCGCCGATCCA

2.3.2: Methods

2.3.2.1: RNA Isolation, cDNA Synthesis and Quantitative Real-time PCR (qRT-PCR)

Total RNA from hESCs, NPCs and glial cells were isolated from 6-well plates using 1ml TRI Reagent (Sigma) per well. RNA was prepared using chloroform extraction followed by isopropanol precipitation, 75% ethanol wash before final re-suspension in diethylpyrocarbonate (DEPC)-treated water. cDNA was prepared using Superscript II First Strand synthesis reverse transcriptase (Life Technologies) using 2 µg of RNA template. Target cDNA sequences were amplified using specific primers mixed with SYBR® Green Jumpstart™ Taq Ready Mix (Sigma) which were added to the cDNA samples. Real-time PCR was performed on a DNA Engine Opticon 2 RT-PCR system (BioRad) which measured SYBR® Green fluorescence in real-time during PCR.

2.3.2.2: Neutral Comet Assay

The Neutral Comet Assay was performed using the Comet SCGE Assay Kit from Enzo Life Sciences following the manufacturer's instructions. Adherent cells were dissociated using Tryp-Le (for NPCs) or Trypsin (for HEK293T cells) and counted. Cells were centrifuged and re-suspended in cold PBS at 1×10^5 /mL. LM-Agarose were melted in a beaker of boiling water for 5 minutes, with the cap loosened, and then cooled in a 37 °C water bath for at least 20 minutes prior to use. The cells were combined with the molten LM-Agarose (at 37 °C) at a ratio of 1:10 (v/v) and 75 µl were immediately pipette onto a Comet Slide. After incubating the slides for at least 10 minutes at 4 °C in the dark, the slides were immersed in pre-chilled Lysis Solution and leave at 4 °C for 30 minutes. Afterwards, the slides were washed with 1X TBE buffer before electrophoresis using a horizontal electrophoresis apparatus and applied the power supply (1 volt/cm) for 10 minutes. The slides were then rinsed with deionized water and immersed in 70% ethanol for 5 minutes. The slides were left to dry overnight at room temperature, with desiccant. The slides were stained with 100 µl of the diluted CYGREEN® Dye solution for 30 minutes and were allowed to dry completely at 37 °C. The slides were viewed using the epifluorescence microscopy using the FITC filter (excitation/emission; 489/515 nm). The tail moment of the comet assay was quantified using the Open Comet software.

2.4 Softwares

Adobe Photoshop CS5

Leica LAS AF Lite

Microsoft Office 2013 (Word, Excel, Powerpoint)

Opticon Monitor 3

ImageJ or Fiji win64

2.5 Statistical analysis

2.5.1: Student's t-test

Student's t-test was used to compare between two groups. P-value of less than 0.05 (<0.05) is considered as statistically significant, while p-value of equal to or more than 0.05 (≥ 0.05) is not significant. The data presented will be normalised to the control, except otherwise stated in the text.

2.5.2: One-way ANOVA

One-way ANOVA was used to compare between more than two groups. In this study, it was used to compare between TRF2 expression in hESCs, early passage and late passage NPCs. The data presented was normalised to the hESCs.

2.5.3: Standard deviation (SD)

The standard deviation used in this study was the standard error of the mean (SEM).

Chapter 3

Results

**High TRF2 expression promotes cell proliferation and
inhibits apoptosis in standard growth cultures**

3.1 Introduction

TRF2, a key component of the Shelterin complex, plays a vital role in retaining and protecting the telomere length and integrity, which ultimately maintains the genome stability (Bradshaw et al., 2005; de Lange, 2005). In addition to this, TRF2 has been found to function outside the telomeres, in the repair of intra-chromosomal regions (Bradshaw et al., 2005; Huda et al., 2012). Apart from its interaction with the DNA damage response factors, TRF2 has also been shown to be highly expressed in certain types of cancer cells (Bai et al., 2014; Chen et al., 2017), which indicates an extra-telomeric function of TRF2. Furthermore, TRF2 was reported to have a role during embryonic development, especially in the central nerve system. The levels of TRF2 proteins were highly elevated upon differentiating hESCs to the neural lineages, although its mRNA levels remained unchanged (Ovando-Roche et al., 2014). Similarly, TRF2 expression was reported to be abundant in the human brain in comparison with other tissues (Jung et al., 2004). Gain-and-Loss of function studies on TRF2 revealed its function in neural differentiation: Overexpressing TRF2 in the hESCs promoted the cells to differentiate to the neural lineages, whereas disruption of TRF2 expression hindered both the neural differentiation of the hESCs and maintenance of NPCs properties (Ovando-Roche et al., 2014). However, it is unclear whether this high level of TRF2 expression in the NPCs and brain has any effect on neural cell proliferation and apoptosis, given that it plays a crucial role in DNA damage response and repair.

The aim of this chapter is to determine whether TRF2 has any effect on cell proliferation and apoptosis. The levels of TRF2 in the cells were knocked-down in NPCs, whereas overexpressed in HEK293T and BJ-5ta cells through genetic modification. In addition, I also characterised the expression of TRF2 during the neural differentiation of hESCs to ascertain its abundance and specificity to this lineage and *in vivo*.

3.2 Results

3.2.1 TRF2 protein level is increased significantly after neural induction from hESCs

In order to explore the role of TRF2 in human neural cells, initial experiments were designed to identify the dynamic TRF2 expression during hESC neural differentiation. Previously, TRF2 protein level was shown to be elevated upon differentiating hESCs to the neural lineage (Ovando-Roche et al., 2014). In order to confirm this findings, H1 hESCs were subjected to neural differentiation using the established differentiation protocol (Bhinge et al., 2014, 2016), by changing hESCs culture from MEF-conditioned medium to N2B27 medium, supplemented with Dorsomorphin and SB43542, to suppress BMP and TGF β signalling pathways, respectively (Chambers et al., 2009; Patani et al., 2009; Zhou et al., 2010; Morizane et al., 2011; Bhinge et al., 2016). The process of neural differentiation was divided into three stages, according to the changes in cell morphology (Figure 3.1 A). Following the formation of neural rosettes, the Dorsomorphin and SB43542 were replaced with bFGF and EGF to maintain NPCs proliferation/self-renewal, where they can be maintained in culture over several months. After neural rosette formation, the early passage NPCs appeared to mainly generate neurons, then gradually changed to favour more glial differentiation with the increasing cell passaged in culture (Gerrard et al., 2005).

These changes in differentiation preference reflect, to a certain extent, the embryonic neurodevelopment in which neuronal differentiation occurs prior to glial generation. In our differentiation, before passage 10 (early passages) the NPCs were more neuronal and after passage 15 (late passages), they favour glial identity (Noctor et al., 2008). The different morphologies of hESCs and NPCs were evident through light microscopy as shown in Figure 3.1 B, while there were no distinct, albeit subtle difference observed between the early and late passage NPCs.

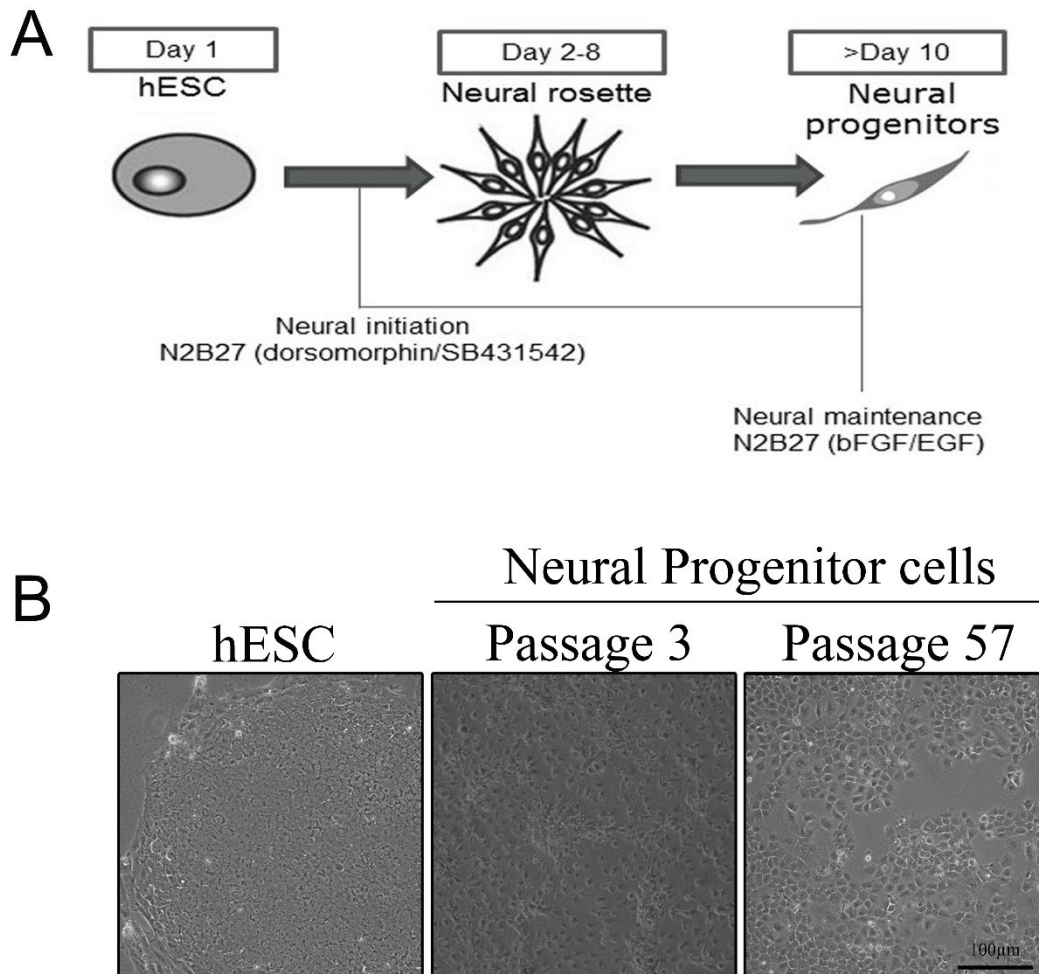


Figure 3.1: Neural differentiation from hESCs. (A) Schematic diagram of neural induction from hESCs to neural progenitors using Dorsomorphin and SB431542 supplemented in N2B27 culture media and the maintenance of NPCs in N2B27 supplemented with bFGF/EGF. (B) Phase contrast microscopy representative image of hESCs and hESCs-derived NPCs depicting morphological changes upon neural differentiation. Scale bar = 100µm.

During the neural differentiation process, characterisation of the cells was performed through qRT-PCR analysis to analyse the mRNA levels of the pluripotent marker, OCT4, neuronal marker PAX6, and endoderm marker, Brachyury, as well as TRF2 mRNA level. The analysis showed downregulation of OCT4 and Brachyury, and upregulation of PAX6, after neural induction from hESCs (Figure 3.2), which suggests that the cells indeed have successfully differentiated to the neural lineage. As shown in the previous study, TRF2 mRNA level showed no clear change upon neural differentiation from hESCs (Figure 3.2) (Ovando-Roche et al., 2014).

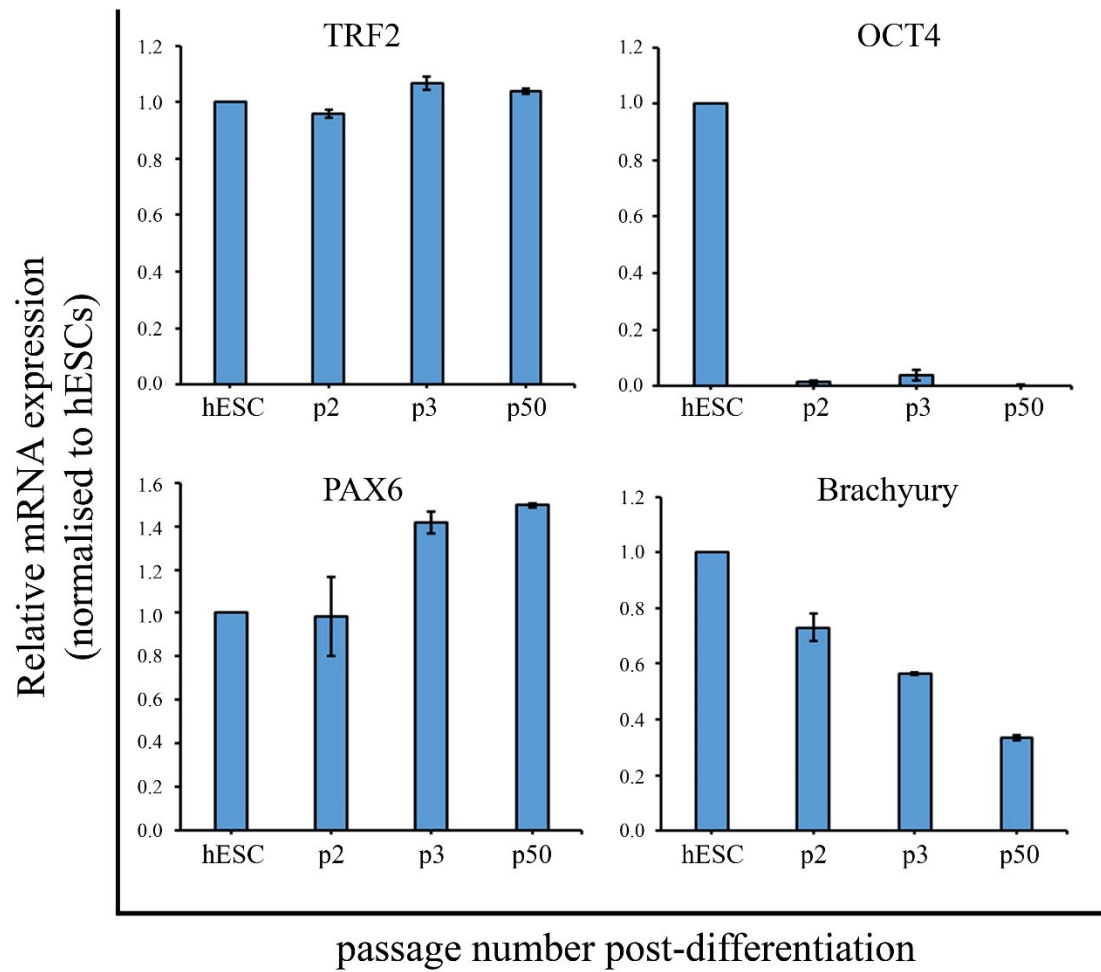


Figure 3.2: mRNA expression upon neural differentiation. *mRNA expression of TRF2, OCT4, PAX6 and Brachyury was analysed by qRT-PCR during neural differentiation from H1 hESCs at indicated time points. Data is calculated as fold changes relative to undifferentiated hESCs and presented as mean $\pm$ SD from two independent experiments. B-actin was used as the housekeeping gene.*

However, TRF2 protein level exhibited a significant increase after hESCs differentiated to NPCs, in both the early and late passages ($p < 0.001$, Figure 3.3 A). Meanwhile, OCT4 protein level became undetectable, which was consistent with its dramatic downregulation at the mRNA level (Figure 3.2). As the NPCs can be maintained in culture over several months, TRF2 protein level was compared between the early ($< p10$) and late passages ($> p30$) of the cells. TRF2 protein level showed no obvious difference between the early and late passages. Immunostaining of TRF2 also showed an increase in expression after differentiation from hESCs, and a reduction in the pluripotent marker, OCT4 (Figure 3.3 B).

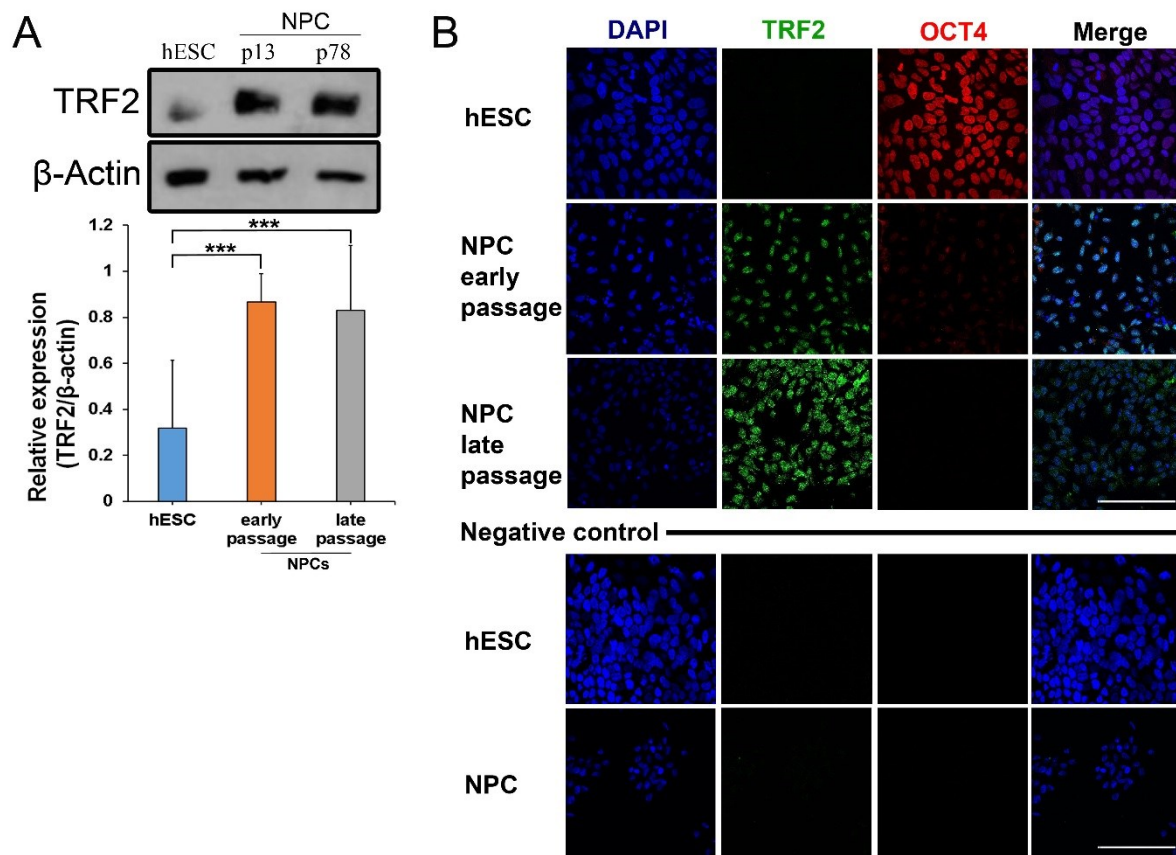


Figure 3.3: TRF2 protein level after neural differentiation. (A) Immunoblot to show indicated protein levels in neural differentiation from hESCs, the NPCs were taken from the early (<p10) and late (>p30) passages. Top: representative immunoblot images from three different experiments. Bottom: Densitometry analysis of three different immunoblots. TRF2 expression were calculated against the β -actin and presented as mean $\pm$ SD from four independent experiments. ***P-value < 0.001, n=4 using one-way ANOVA. (B) Immunostaining of TRF2 (green) and OCT4 (red) after neural differentiation from hESCs. The NPCs were taken from early and late passages. Dapi staining (blue) indicates the nucleus. Cells stained without the primary antibodies were used as the negative control. The scale bar represents 100 μ m.

3.2.2 High TRF2 protein level may exist specifically in NPCs and human brain

Next, this high level of TRF2 specific to NPCs is to be confirmed, as it was previously shown that differentiation of hESCs to other lineages do not induce any increase of TRF2 protein level (Ovando-Roche et al., 2014). However, it is unclear whether this specificity also occurs *in vivo*. One study showed that TRF2 was expressed at higher levels in the human brain than the other tissues (Jung et al., 2004). Due to the difficulties in obtaining normal fetal brain tissues, TRF2 protein level was analysed in three human adult tissue types, namely adult brain, kidney and skin. The skin tissue was chosen due to their high proliferative capabilities in normal human body (Takeo et al., 2015), while kidney was chosen to represent tissues that derived from a different germ layer (Gilbert, 2000; Lodish et al., 2000). These tissues were obtained from NovusBio. TRF2 protein level was shown to be high in the normal adult brain tissue while no TRF2 was observed in skin and kidney tissues (Figure 3.4 A), confirming that TRF2 level is specifically high in the brain tissues, at least in the three tissues tested.

Additionally, TRF2 protein level was also analysed in different non-tumorigenic human cell lines, hESCs, BJ-5ta and HEK293T. BJ-5ta is derived from normal foreskin fibroblast that has been immortalised by over-expressing hTERT (Hahn et al., 1999). TRF2 protein level is shown to be much higher only in the NPCs than the other cell lines, where the lowest TRF2 expression was observed in the BJ-5ta cells (Figure 3.4 B). The high TRF2 protein level in both NPCs and brain tissue suggests that there is an underlying function of TRF2 in the brain.

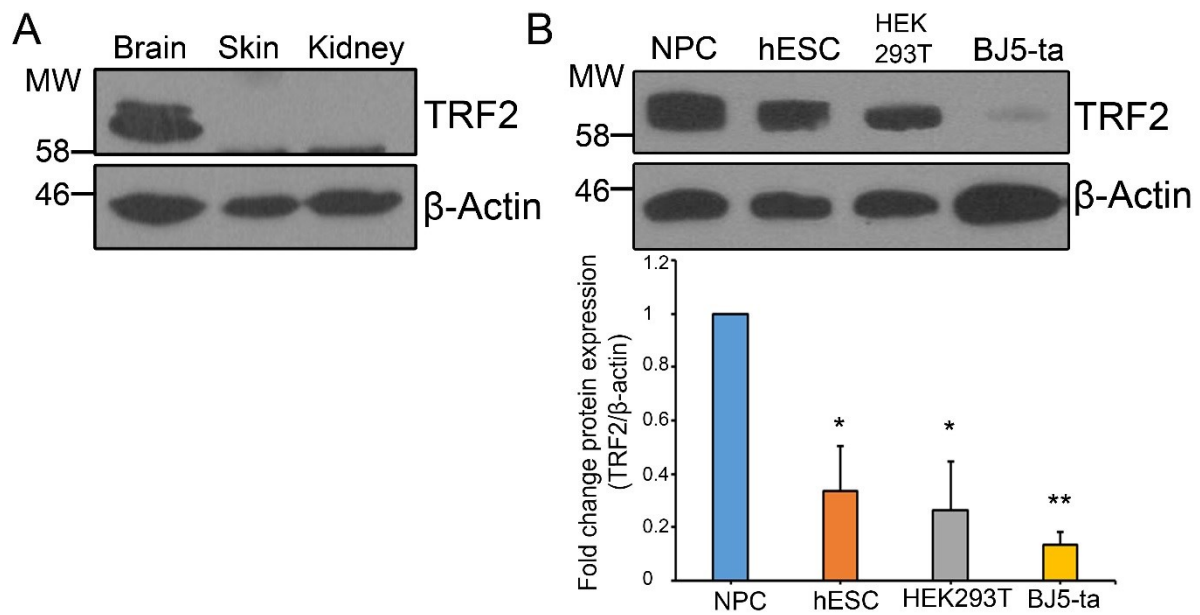


Figure 3.4: TRF2 protein level in different cell types and tissues. (A) Immunoblot image of TRF2 protein level in cell lysates isolated from different adult human tissues (brain, skin and kidney). (B) Top: representative immunoblot image of TRF2 protein level in different cell types (NPCs, hESCs, HEK293T cells, BJ-5ta cells). Bottom: Densitometry analysis (normalised to NPC) of the immunoblot, * $P < 0.05$, ** $P < 0.01$ using one-way ANOVA, $n = 3$.

3.2.3 High levels of TRF2 in NPCs promotes Cell Growth

In order to further investigate the function of this high level of TRF2 in NPCs, the gain and loss of function study were performed using hESC-derived NPCs with either TRF2 overexpression or shRNA-knockdown that had been previously generated in the lab. However, TRF2-overexpressing NPCs did not grow as much and was insufficient to obtain meaningful data. Therefore, only TRF2-knockdown (TRF2-KD) cells were cultured for further analysis. NPCs that were generated by ectopic expression of scrambled shRNA were used as controls (Ovando-Roche et al., 2014).

Knockdown of TRF2 in NPCs was confirmed by immunoblotting and immunostaining analysis, which shows more than 50% reduction of TRF2 protein level, as compared to the control, (Figure 3.5 A). Immunostaining of TRF2 in the NPCs control showed that the high TRF2 protein level was localised throughout the nucleus, as what has been reported (Ovando-Roche et al., 2014).

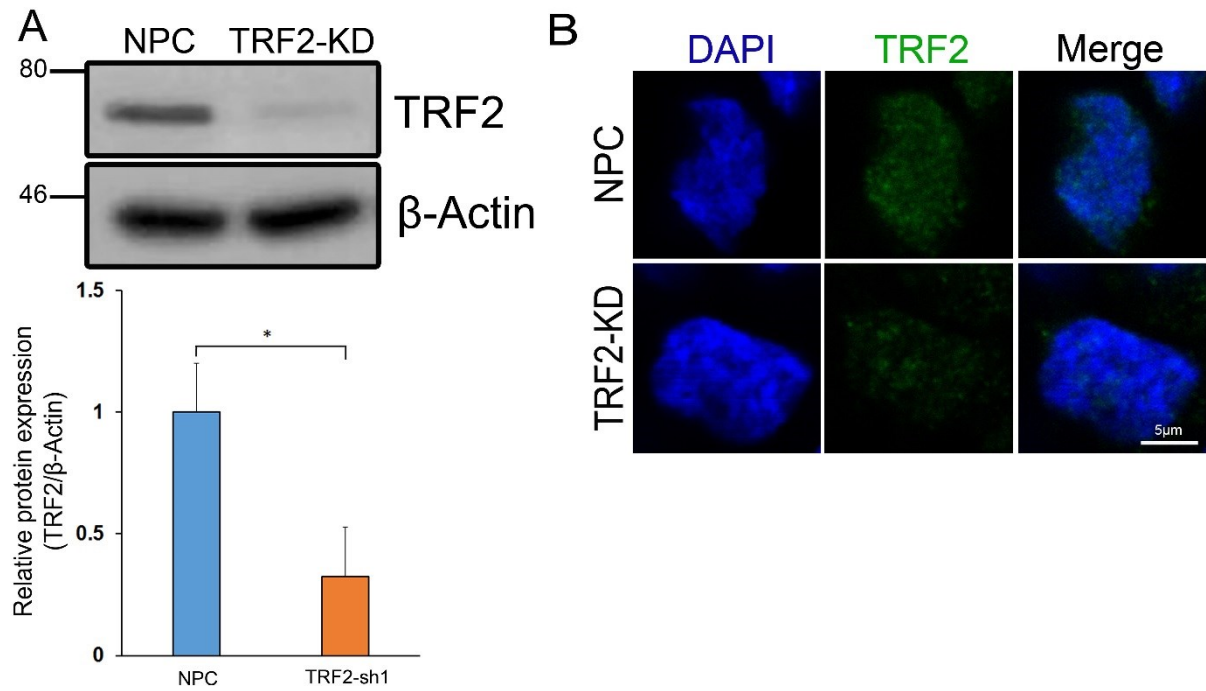


Figure 3.5: TRF2 protein level in NPCs. (A) Top: representative immunoblot of TRF2 protein level in TRF2 control and knockdown NPCs. Bottom: Densitometry analysis of the immunoblot. * $P < 0.05$, $n = 3$ using Student's t -test. (B) Immunostaining of TRF2 (green) in TRF2 control and knockdown NPCs. DAPI staining (blue) indicates the nucleus. The scale bar represents 5 μ m.

Through routinely culturing the control and TRF2-KD NPCs, it was observed that there was a difference in growth rate between the cells. The control cells with higher expression of TRF2 seemed to have a higher growth rate than TRF2-KD cells. To validate this observation, we monitored the control and TRF2-KD NPCs in routine culture condition. The cells were counted every 3 days, from day 0 until day 6. The growth curve showed that the control higher TRF2 expressing cells have a significantly higher growth compared to TRF2-KD cells (Figure 3.6 A). The live cell counts also confirmed that the control higher TRF2 expressing cells have a significantly higher number of live cells compared to TRF2-KD cells, where TRF2-KD NPCs has a 20% lower number of live cells (Figure 3.6 B). The reduced TRF2 protein level in these cells, however, did not affect the cell morphology and both cell types maintained their neural morphology (Figure 3.6 C). These data demonstrate that higher levels of TRF2 promote cell growth in NPCs.

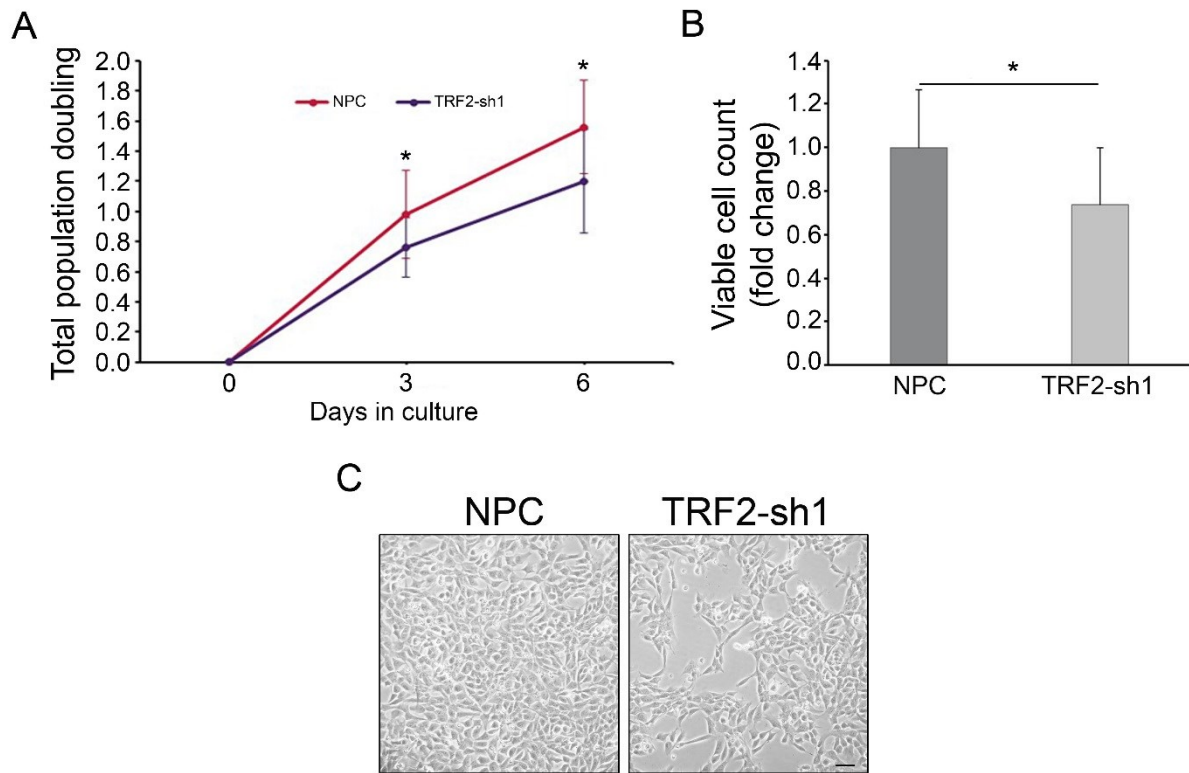


Figure 3.6: TRF2-KD in NPCs resulted in reduced cell growth. (A) Growth curves of TRF2 control and knockdown NPCs in standard culture condition. (B) The viable cell count between TRF2 control and knockdown NPCs after overnight culture, normalised to control NPCs. Cells were counted using the Countess™ Automated Cell Counter by Life Technologies. The figure shows the cell count as mean $\pm$ SD from four independent experiments. (C) Phase contrast images of TRF2 control and knockdown NPCs. The scale bar represents 50 μ m. *P-value < 0.05, n=4.

3.2.4 TRF2 promotes proliferation and inhibits apoptosis

In order to address whether the difference in cell growth observed was due to high proliferation or low apoptosis in these cells, the mitotic marker, phospho-Histone 3 (pH3), and the caspase-dependent apoptotic marker, cleaved caspase 3 (Cl-casp3), were examined by immunoblotting and immunostaining. A significantly higher level of pH3 protein level ($p < 0.05$) was observed in the control NPCs with high TRF2 expression than in TRF2-KD NPCs (Figure 3.7 A, B). The pH3 protein shown only stained the actively proliferating cells that are in the mitotic phase (Figure 3.7 B). In contrast, the apoptotic marker, Cl-casp3, showed significantly lower expression in the control than in TRF2-KD NPCs ($p < 0.05$) (Figure 3.7 C, D), and the immunostaining showed that the signal was located in the nucleus of the cells (Figure 3.7 D), indicating that the cells were in an apoptotic state (Brassenot et al., 2009).

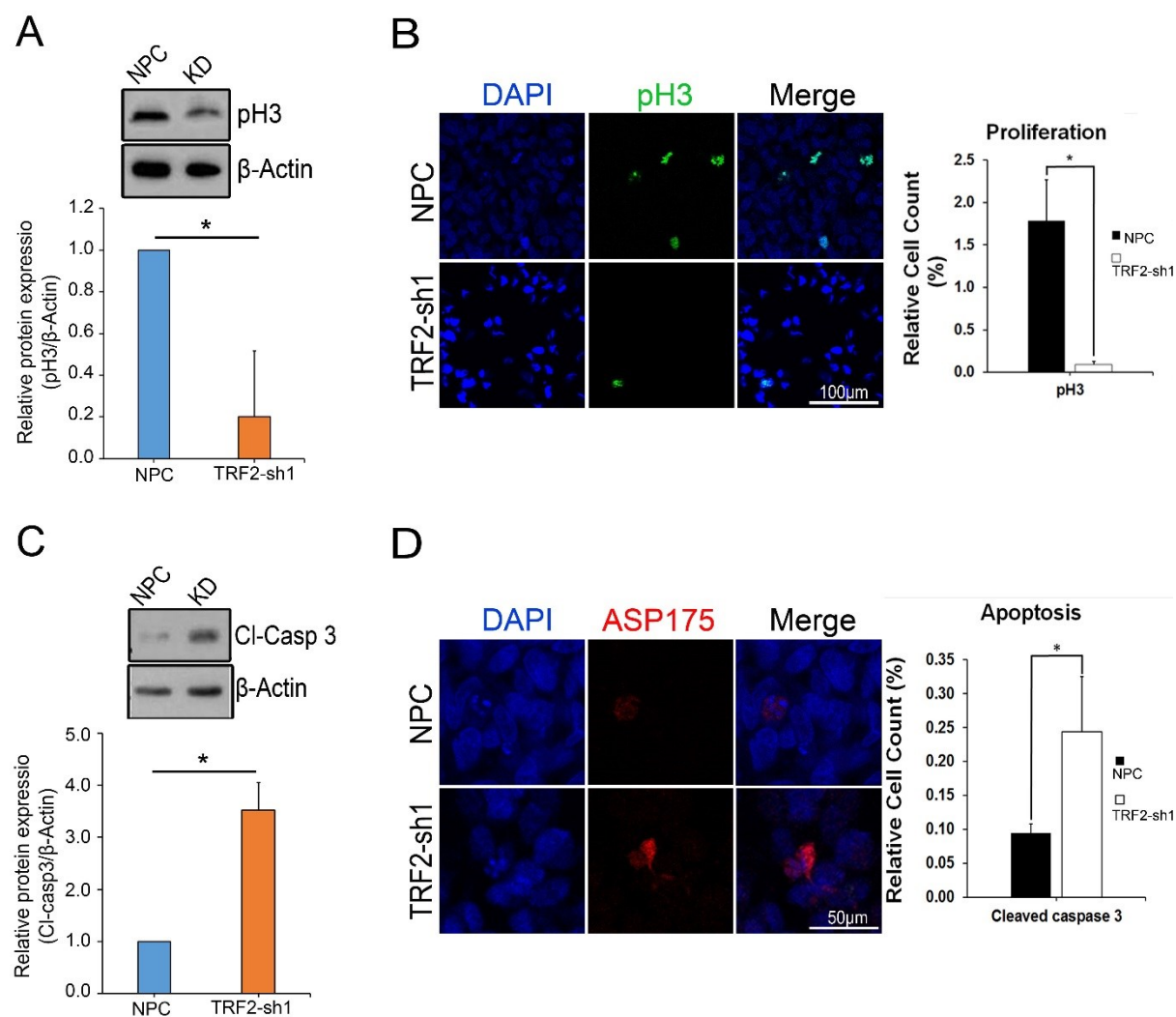


Figure 3.7: Reduced proliferation and increased apoptosis in TRF2-KD NPCs. (A) Top panel: Representative blot of the pH3 protein level in TRF2 control and knockdown NPCs. Bottom panel: Densitometry analysis of the antibody of interest against β -actin, normalised to the control. The figure shows the analysis as mean $\pm$ SD from four independent experiments. *P-value < 0.05, n=4. (B) Left panel: Representative image of pH3 immunostaining (green). DAPI stain (blue) indicates the nucleus. The scale bar represents 100 μ m. Right panel: Representative image from 3 independent biological samples. The scale bar represents 100 μ m. Percentage of the positive antibody staining against the total number of cells counted (Dapi/blue staining). Total cells counted were 1728 and 1280 cells for TRF2 control and knockdown, respectively. (C) Top panel: Representative blot of the Cl-casp3 protein level in TRF2 control and knockdown NPCs. Bottom panel: Densitometry analysis of the antibody of interest against β -actin, normalised to the control. The figure shows the analysis as mean $\pm$ SD from four independent experiments. *P-value < 0.05, n=4. (D) Left panel: Representative image of Cl-casp3 immunostaining (red). DAPI stain (blue) indicates the nucleus. The scale bar represents 50 μ m. Right panel: Representative image from 3 independent biological samples. The scale bar represents 100 μ m. Percentage of the positive antibody staining against the total number of cells counted (Dapi/blue staining). Total cells counted were 1728 and 1280 cells for TRF2 control and knockdown, respectively.

In addition, I also compared cell cycle status in these two cell types by flow cytometry analysis of propidium iodide (PI) stained cells. The figure includes the representative graph for TRF2 control NPCs (Figure 3.8 A) and TRF2-knockdown NPCs (Figure 3.8 B). The results showed that both cell types contained a high proportion of G0/G1 phase cells with much less percentage of S phase and G2/M phase cells and that no significant difference in cell cycle was detected between the control and TRF2-KD NPCs (Figure 3.8 C). Although the Cl-Casp3 protein level was significantly lower in the control than TRF2-KD NPCs, there was no significant difference between the two cell types in the sub-G0/G1 population in the cell cycle analysis that reflect apoptotic cells (Figure 3.8 C). This discrepancy could be due to the lower sensitivity of the cell cycle analysis as it is unable to differentiate between the dying or dead cells and senescent cells. The results indicate that TRF2 has no effect on the cell cycle in NPCs. Both results demonstrated that TRF2 promotes proliferation and protects the cells from apoptosis. However, the mechanism underlying this phenotype is not yet known.

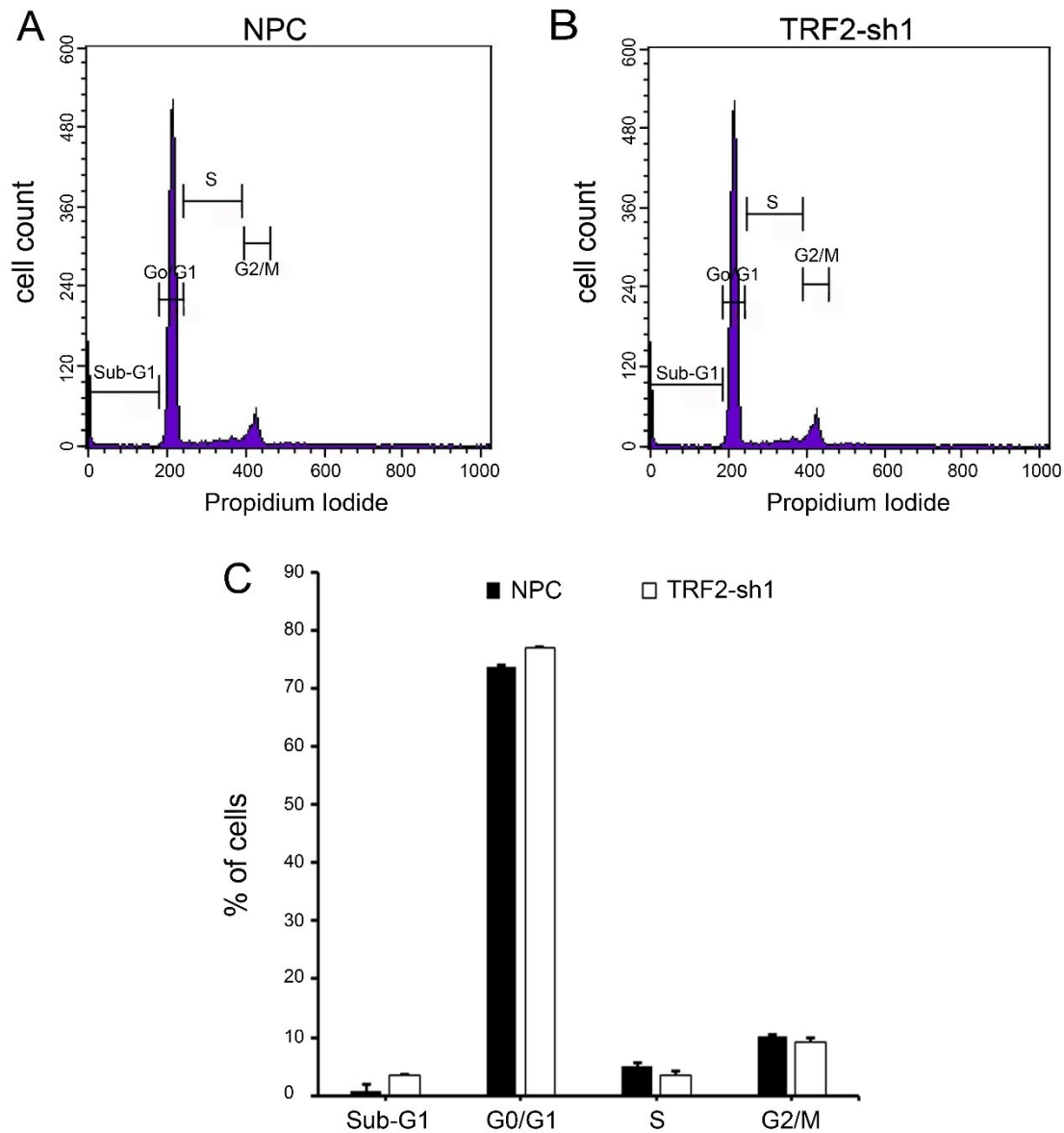


Figure 3.8: No difference in cell cycle between TRF2 control and knockdown NPCs. Representative graph of the flow cytometry analysis with the PI stained cells for the (A) Control NPCs and (B) TRF2-KD NPCs. (C) Analysis of each cell cycle stage. Not significant, P -value > 0.05 using Student's t -test, $n=4$.

3.2.5 The role of TRF2 in promoting cell growth is not cell type-specific

It has been shown above in the NPCs that high levels of TRF2 promoted cell growth by increasing cell proliferation and preventing cells from apoptosis. Upon knockdown of TRF2, the cell growth was significantly reduced. However, it is unclear whether this function of TRF2 is cell type-specific. Therefore, the experiments were designed and carried out in other cell lines, such as HEK293T and BJ-5ta cells.

To examine the effect of TRF2 in cell growth, overexpressing TRF2 in HEK293T and BJ-5ta cells was done due to the low endogenous expression of TRF2 in these two cell types that are similar to that of TRF2-KD NPCs. The expression vector was generated previously in the lab (Ovando-Roche et al., 2014), in which PuroR (puromycin resistant gene) and TRF2 cDNAs were linked with a 2A foot and mouth disease virus sequence and under the control of EF1 α promoter (Figure 3.9 A). 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. The vector containing PuroR cDNA only was used as the control.

The HEK293T were transfected with control or TRF2 overexpressing (TRF2-OE) plasmids and selected with puromycin in HEK293T culture conditions. The surviving cells were confirmed by immunoblotting analysis for the overexpression of TRF2 (Figure 3.9 B). In addition, TRF2 levels in these cells were also compared with TRF2 levels in the NPCs. The result shows that TRF2 protein level in TRF2-OE HEK293T is slightly higher than the control NPCs, although it was not statistically significant. However, it could be used to compare between the high and low TRF2 expressing HEK293T cells as the difference between the two was highly significant ($P < 0.01$, higher significance than the NPCs). Moreover, TRF2 protein levels in HEK293T control cells were similar to that in TRF2-KD NPCs (Figure 3.9 B).

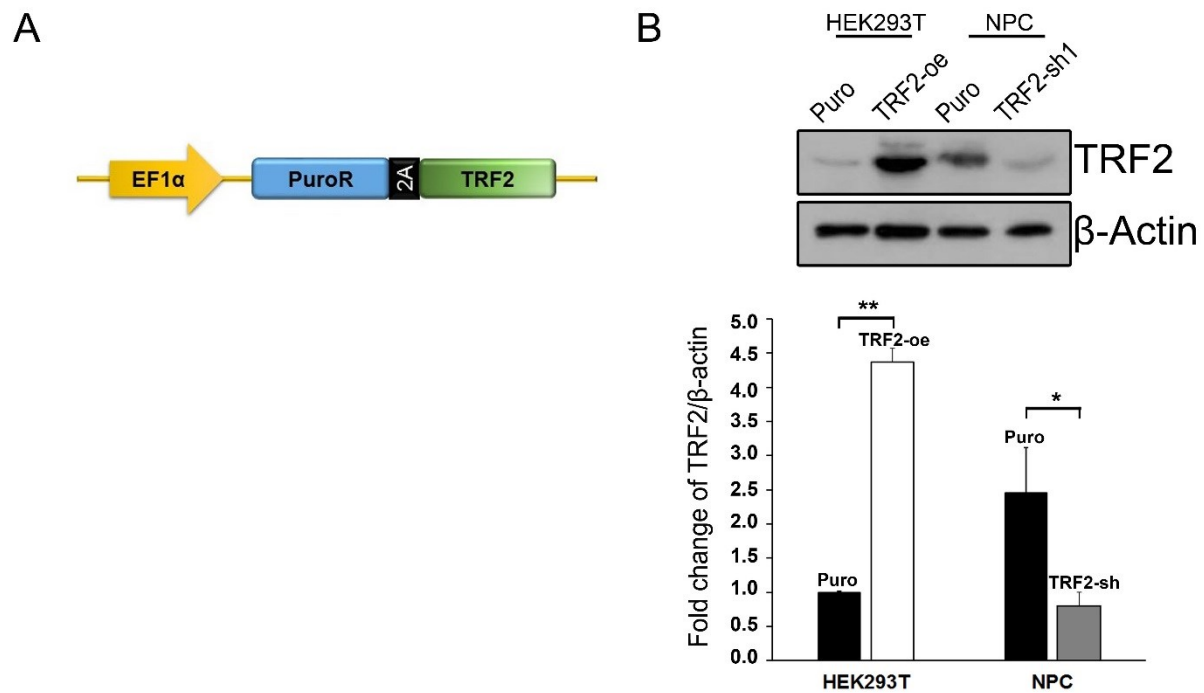


Figure 3.9: Higher cell growth in TRF2 overexpressing HEK293T cells. (A) Schematic map of TRF2-expressing lentiviral vector. EF1 α , EF1 α promoter; PuroR, puromycin resistant gene; 2A, 2A foot and mouth disease virus sequence; TRF2, TRF2 cDNA. This vector construct was prepared by Ovando-Roche et al. (2014). (B) Top panel: Representative blot of TRF2 protein level in control (puro) and TRF2-OE HEK293T cells together with TRF2-control (puro) and knockdown NPCs in standard culture conditions. Bottom panel: Densitometry analysis of the Western blot normalised to the HEK293T puro (control) to show the fold change in TRF2 expression level. The figure shows TRF2 expression level as mean $\pm$ SD. *P-value < 0.05, **P-value < 0.01, using Student's t-test, n=4.

Like in NPCs, it was again observed that TRF2-OE HEK293T cells reached confluency faster than the low TRF2 HEK293T controls after seeding the same number of cells (Figure 3.9 C). The growth curve analysis showed that the high TRF2-OE HEK293T cells have a significantly higher population doubling than the controls (Figure 3.10 A) The cell count of the cells after overnight culture also shows that the high TRF2 expressing cells have a higher number of viable cells compared to the control (Figure 3.10 B).

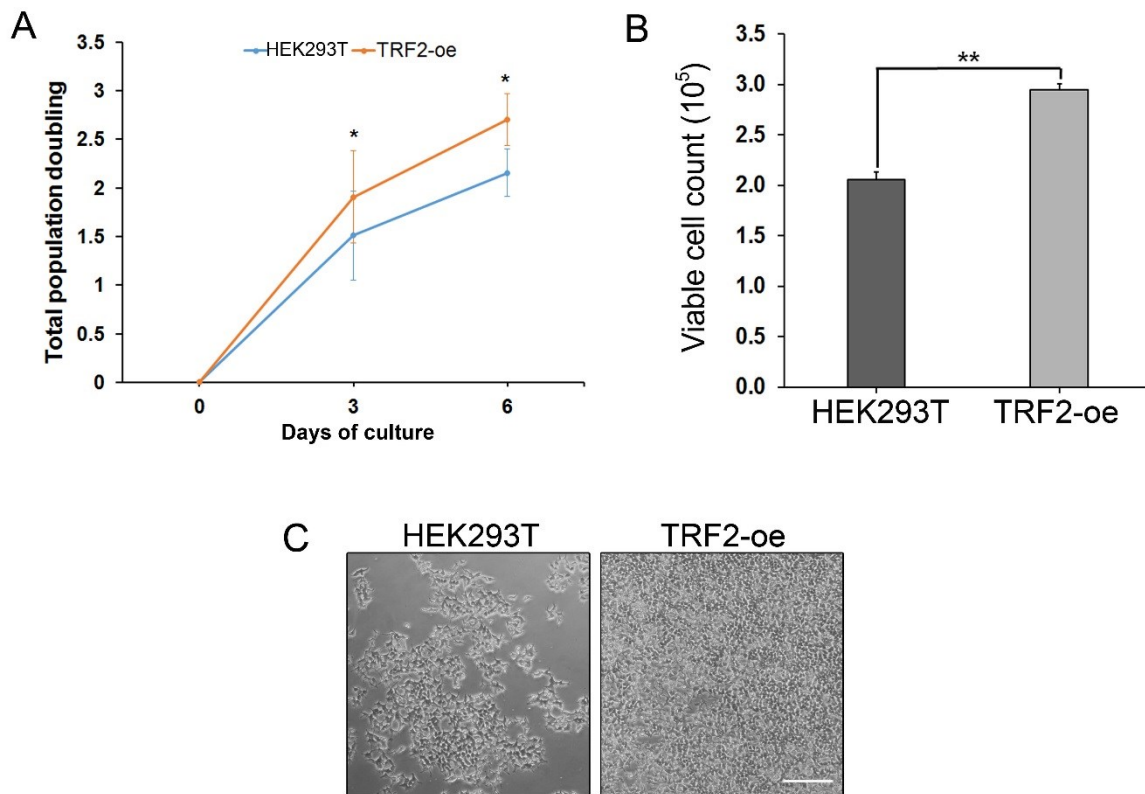


Figure 3.10: TRF2-oe in HEK293T promotes cell growth. (A) Growth curves of the control and TRF2-OE HEK293T cells in standard culture conditions. **P*-value < 0.05, using Student's *t*-test, *n*=3 (B) Difference in viable cell number between the control and TRF2-OE cells after overnight culture in standard culture conditions. Cells were counted using the Countess™ Automated Cell Counter by Life Technologies. The figure shows the cell count as mean ± SD from four independent experiments. ***P*-value < 0.01, using Student's *t*-test, *n*=4. (C) Phase contrast images of the control and TRF2-OE HEK293T cells in standard culture conditions. The scale bar represents 100 μm.

Similar to NPCs, the difference in cell growth between the control and TRF2-OE HEK293T cells was again demonstrated to be due to high proliferation, as shown in the high pH3 protein levels in TRF2-OE cells compared to the control (Figure 3.11 A). Furthermore, there was also lower apoptosis, as shown in the Cl-casp3 protein level in TRF2-OE cells compared to the control (Figure 3.11 B). These findings confirm that the high levels of TRF2 also promotes cell proliferation and protects the cell from apoptosis in HEK293T cells under the routine culture condition.

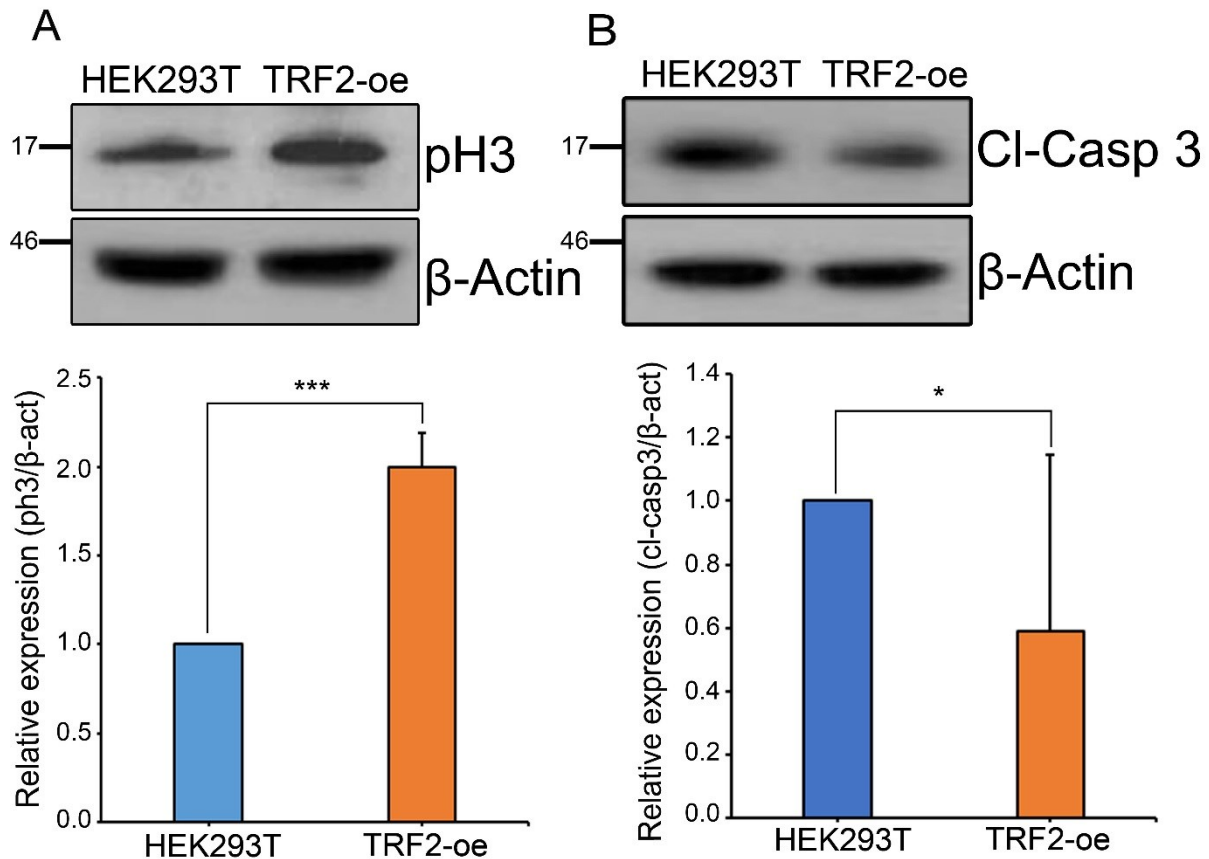


Figure 3.11: TRF2 overexpressing HEK293T cells promotes proliferation and protects from apoptosis. (A) pH3 protein level in TRF2 control and overexpressing HEK293T cells, representative blot (upper panel) and densitometry analysis (lower panel). *** $P < 0.001$ using Student's *t*-test, $n=8$ (B) Cl-casp3 protein level in TRF2 control and overexpressing HEK293T cells, representative blot (upper panel) and densitometry analysis (lower panel). * $P \leq 0.05$ using Student's *t*-test, $n=5$. The figure shows the analysis as mean $\pm$ SD.

Similar to the NPCs, the cell cycle analysis in these HEK293T cells also revealed that there was no significant difference between the control and TRF2-OE HEK293T cells in all of the cell cycle phases (Figure 3.12).

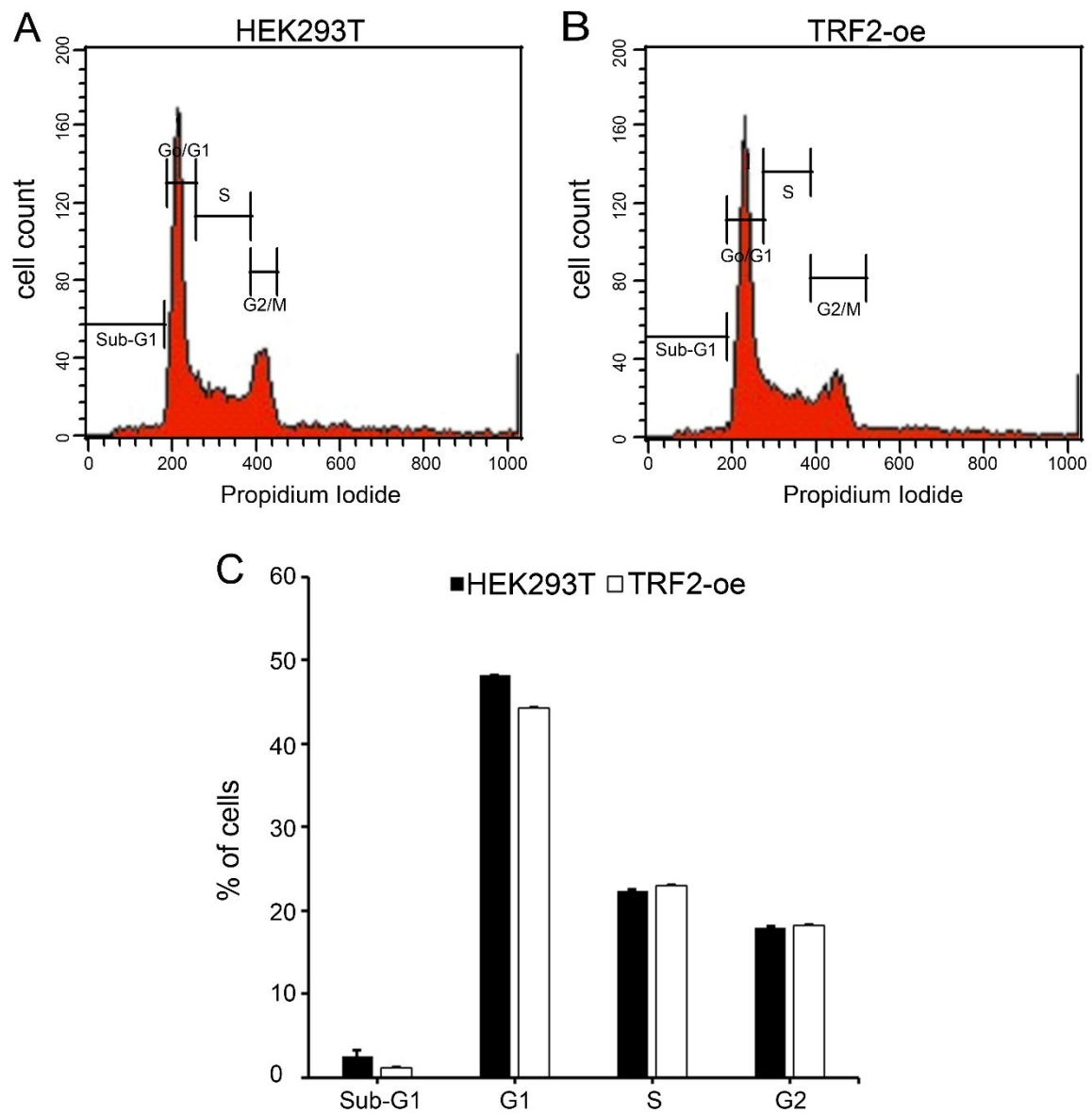


Figure 3.12: No difference in cell cycle between TRF2 control and overexpressing HEK293T. Representative graph of the PI stain for the (A) Control and (B) TRF2-overexpressing cells. (C) Analysis of each cell cycle stage. Not significant, P -value > 0.05 using Student's t -test, $n=4$.

TRF2 was also overexpressed in another cell line, BJ-5ta, as it was done in HEK293T cells (Figure 3.13 A). Cell growth was investigated using the growth curve analysis and showed that TRF2-OE BJ-5ta cells grew significantly faster than the controls (Figure 3.13 B) and more viable cells were identified by trypan blue staining in TRF2-OE cultures than the controls after overnight culture (Figure 3.13 C). Similarly, the quick cell growth in TRF2-OE cells was due to high proliferation, as shown by the pH3 protein level (Figure 3.13 D) and low apoptosis, as shown by the Cl-casp3 protein level (Figure 3.13 E). However, TRF2-OE BJ-5ta cells could not continue to grow in culture after a couple of passages, which limited further investigation. Nonetheless, these results suggest that the role of TRF2 in promoting cell growth is not cell type-specific.

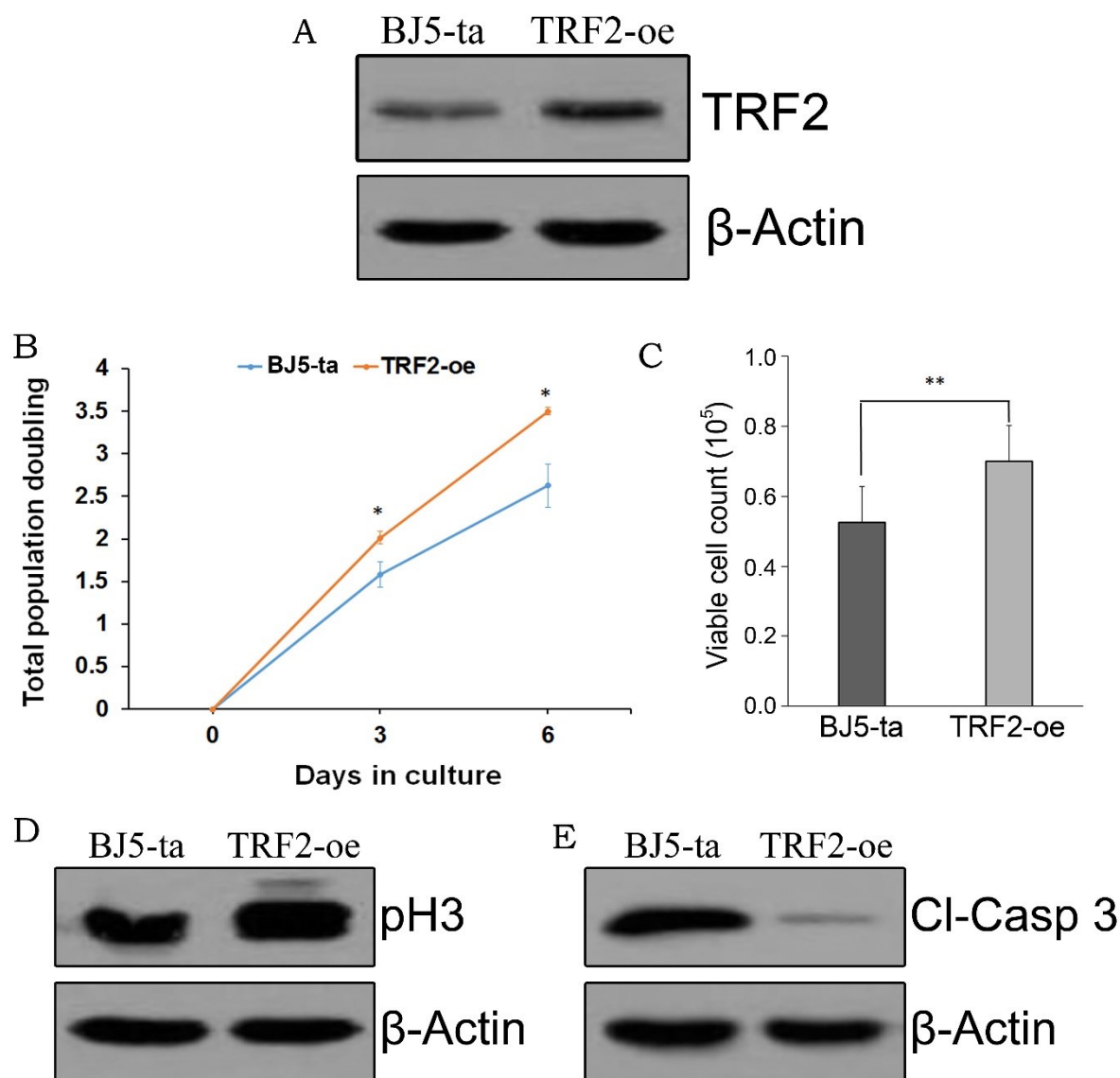


Figure 3.13: Higher cell growth in TRF2 overexpressing BJ-5ta cells. (A) TRF2 protein level in TRF2 control and overexpressing BJ-5ta cells. (B) Growth curves of the control and TRF2-OE BJ-5ta fibroblasts cells in routine culture condition. (C) Difference in the viable cell number between the control and TRF2-OE cells after culturing overnight. Cells were counted using the Countess™ Automated Cell Counter by Life Technologies. The figure shows the cell count as mean $\pm$ SD from four independent experiments. *P-value < 0.05, **P-value < 0.01, n=3. (D) Immunoblot of pH3 and (E) Cl-casp3 protein level in TRF2 control and overexpressing BJ-5ta cells in routine condition. The immunoblot experiment was only done once due to the cells inability to grow in culture.

3.3 Discussion

In this chapter, the expression of TRF2 was characterised in hESCs, NPCs, HEK293T and BJ-5ta cells, as well as in tissues, namely brain, kidney and skin (Figure 3.4). It was found that TRF2 protein level was significantly elevated upon differentiating hESCs to the neural lineage and was maintained at a high level in NPCs (Figure 3.3). This increase in TRF2 protein level in the hESCs-derived NPCs has been reported previously to have an importance in neural differentiation and development (Ovando-Roche et al., 2014; Grammatikakis et al., 2016a). The high level of TRF2 protein level appeared to be restricted in the NPCs and brain, but not in the other cell types and tissues. The lowest expression of TRF2 was found in BJ-5ta cells, which could be due to the high expression of hTERT in these cells that could downregulate the expression of TRF2 (Escoffier et al., 2005; Campbell et al., 2006). This high expression of TRF2 in the NPCs suggests that there is an underlying function specific to this cell type.

Given the abundant TRF2 protein in the NPCs, it was observed that TRF2 was not restricted to be localised only at the telomeres. Instead, it was spread throughout the nucleus (Ovando-Roche et al., 2014). In addition to the localisation of TRF2 throughout the nucleus, it has also been reported previously that TRF2 can be found in the cytoplasm of terminally differentiated neural cells (Jung et al., 2004). A later report has shown that a splice variant of TRF2, termed TRF2-S (TRF2 short), was expressed in the cytoplasm of mature neurons (Zhang et al., 2011; Grammatikakis et al., 2016b), suggesting that there might be an extra-telomeric function of TRF2.

In this study, it was found that the high TRF2 expressing cells showed higher cell growth as compared to the low TRF2 expressing cells (Figure 3.6, Figure 3.10 and Figure 3.13). Furthermore, this cell growth difference might be attributed to the increase in proliferation in the high TRF2 expressing cells. This is consistent with the findings that TRF2 is highly expressed in cells with high proliferation, such as keratinocytes and cancer cells (Minty et al.,

2008; Zhang et al., 2009; El Maï et al., 2014) and that the disruption of TRF2 may significantly impair the proliferation capacity of the cells (Martinez et al., 2010; Bai et al., 2014; Pal et al., 2015). The increase in proliferation in the high TRF2 expressing cells was shown by the changes in the expression of pH3 protein (Figure 3.7, Figure 3.11 and Figure 3.13), which is a mitotic marker. pH3 is expressed when the cells are in the mitosis phase and are actively proliferating, as the Histone-H3 is phosphorylated at Serine 10 (Ser 10) can only be observed at the entry of mitosis and not at G2 phase of the cell cycle (Gurley, Walters and Toby, 1974; Gurley et al., 1978). Although the high TRF2 expressing cells show a higher number of cells that are in the mitotic phase, cell cycle analysis shows that there is no significant difference in the number of cells in the G1, S and G2 phases between the high and low TRF2 expressing cells (Figure 3.8 and Figure 3.12). The difference in proliferation observed could be due to the changes in the cell cycle rate, rather than the proportion of each cell cycle phases (Matson and Cook, 2017).

In addition to its function in promoting proliferation, high TRF2 protein level was also shown to protect the cells from apoptosis, as the high TRF2 expressing cells expressed a lower level of cleaved caspase 3 (Figure 3.7, Figure 3.11 and Figure 3.13). It has also been reported previously that the inhibition or elimination of TRF2 will induce apoptosis (Multani et al., 2000; Biroccio et al., 2006). This phenotype was observed not only in NPCs but also in HEK293T and BJ-5ta cells, which means that the function TRF2 plays is not cell type-specific. These results suggest that TRF2 may help cell survival.

The gain of function experiment through overexpressing TRF2 in the NPCs could not be completed due to the inability of the cells to continue growing in culture. TRF2 overexpressing NPCs were only able to be maintained in culture for a couple of passages, which made it difficult to be investigated further. This could be due to the adverse effects of TRF2 overexpression in NPCs as the endogenous expression of TRF2 in these cells is already very

high. Exogenously overexpressing TRF2 could have an effect in shortening the telomere length, which has been reported previously (Smogorzewska, 2000; Blanco et al., 2007; Muñoz, Blanco and Blasco, 2006). Although the endogenous TRF2 expression in the NPCs is higher than other cell types, it does not interfere with the telomere length as there are many other regulatory factors that help to maintain the telomere length in the NPCs, such as the high expression of telomerase (Chen et al., 2007; Cheung et al., 2014; Liu et al., 2018). The high expression of TRF2 in these NPCs was also reported to not have any effect in the telomere length, as knocking down TRF2 did not increase the telomere length significantly (Ovando-Roche et al., 2014).

The inability of NPCs with TRF2 overexpression to continue growing in culture could also be due to the function of TRF2 in the formation of t-loop structure at the telomere, where in addition to protecting the telomere ends from being recognised as a DNA double-strand break, it also inhibits the telomerase from being recruited at the telomere to help in elongating the telomere length (Hockemeyer and Collins, 2015). Therefore, exogenous overexpression of TRF2 could hinder the binding of telomerase at the telomere, thus making the telomeres to critically shorten and become senescent.

The overexpression of TRF2 in the BJ-5ta cells also could not be further explored as the cells were not able to continue growing in culture. As shown in the results, BJ-5ta cells express the least amount of TRF2 protein compared to any other cell types. According to previous studies, hTERT immortalisation in cells was able to elongate the short telomere, but also shorten a long telomere (Ouellette et al., 2000; Zheng et al., 2014). However, it is able to stably maintain the short telomere at an optimal length in order to continue proliferation (Ouellette et al., 2000; Zheng et al., 2014). Although the telomere length was able to be maintained, thus making the cells continue to grow in culture infinitely, the telomere length was kept at a sub-senescent average size (Ouellette et al., 2000). Overexpressing TRF2 in these cell types has been shown

to further shortens the telomeres, thus exposing the telomere ends to be recognised as a DNA break and induce senescence or cell death (Karlseder et al., 2002; Richter et al., 2007). Therefore, the inability of TRF2 overexpressing BJ-5ta cells to continue growing in culture could be due to the negative regulation of TRF2 with the telomere length.

These problems in TRF2 overexpressing cells were not observed in the HEK293T cells, where TRF2 overexpressed HEK293T cells were able to grow for a long period of time. However, it could be due to the expression of the mutant SV40 large T antigen in this cell type where it will deregulate the retinoblastoma protein (pRb) and p53 pathways that are highly important in cell cycle regulation. Therefore, HEK293T cells have tumorigenic potential, especially in cells that have been passaged for more than 60 times (Shen et al., 2008; Stepanenko and Dmitrenko, 2015). However, as HEK293T cells are easily transfected, this cell type is chosen to study the function of TRF2 in other cell types that endogenously express a low level of TRF2.

In conclusion, this chapter has shown that TRF2 is highly expressed, specifically in the brain and NPCs, where they promote cell proliferation and protect the cells from apoptosis in the standard culture conditions. The function of TRF2 in promoting proliferation and inhibiting apoptosis are not cell-type specific. These phenotypes suggest that TRF2 may have a protective function.

Chapter 4

Results

**High TRF2 expression increases cell apoptosis under
stress culture conditions**

4.1 Introduction

In Chapter 3, the results indicate that TRF2 promotes cell proliferation and protects the cells from apoptosis under the standard culture conditions, where high TRF2 expressing cells have a significantly higher cell growth compared to the low TRF2 expressing cells. These observations were seen in both NPCs and HEK293T cells, suggesting that TRF2 functions are not cell type-specific.

However, to study the role of TRF2 in cell survival, it is also important to investigate the ability of TRF2 to protect the cells from stress-induced cell death. Upon exposure to external stress, the cells would normally undergo many changes in order to adapt and protect themselves via activation of the cellular stress response (Fulda et al., 2010).

Different types of stress conditions, such as oxidative stress and hypoxia, activate different cellular stress responses to regulate cell survival. The cellular stress response can activate cell repair mechanisms that will in turn trigger multiple factors, which help to repair any damage induced by the stress. The cells return to their normal state after the damage has been repaired successfully. The cellular stress response can also result in a temporary adaptation to the stress, which is known as a pre-apoptotic cell stress response (Nipic et al., 2010). This state of response is only temporary and is usually encountered after mild stress towards the cells, where the cells revert to their normal state in the absence of further stress (Nipic et al., 2010; Banic et al., 2011). The cellular stress response can lead to the induction of autophagy and cell death in cases where the cells are unable to tolerate the stress (Milisav, 2011). Apoptosis could either be caspase-dependent or caspase-independent (Norberg et al., 2010; Milisav, 2011).

Oxidative stress is due to the accumulation of DNA damage in the cells, which is caused by reactive oxygen species (ROS) (Trachootham et al., 2008). Oxidative stress is considered as the hallmark of the many pathological conditions, such as neurodegenerative diseases, ageing

and cancer. Hypoxia is the condition in which there is a low amount of oxygen available to the cells and it could lead to oxidative stress, and DNA damage, ultimately resulting in many other diseases, such as cerebral palsy (Liu et al., 2008). The mechanisms underlying cell protection and survival when exposed to these stress conditions vary and involve multiple response pathways. Due to the ability of TRF2 to interact with many factors in the telomeric and extra-telomeric protection, there may be a relationship between the TRF2 and other cellular response pathway factors, associated with protecting the cells when exposed to stress conditions.

The aim of this chapter is to further explore the functional role of TRF2 under the standard conditions and assess the similarities/differences to that in stress conditions. Different stress conditions, which are B27 withdrawal, induction of oxidative stress through H₂O₂ treatment and hypoxia were chosen in order to fully understand the role that TRF2 plays in NPCs.

4.2 Results

4.2.1 High TRF2 in NPCs increases cell apoptosis after B27 withdrawal

Given that TRF2 promotes proliferation and protects the cells from apoptosis in NPCs under the standard culture conditions, TRF2 may also have a protective role in NPCs under stress conditions. In order to assess this, the B27 supplement was first withdrawn from the serum-free NPC cultures. B27 is a culture supplement that contains various protective reagents, such as superoxide dismutase and glutathione, which act as anti-oxidants. It also contains several types of vitamins, proteins and hormones that aided in cell growth *in vitro*; which has been shown to increase neural cell survival (Brewer et al., 1993). Neural cells are typically cultured in a serum-free condition, and for this reason, additional supplementation that is provided by B27 is needed. Therefore, it is believed that B27 withdrawal from the culture medium will induce chemical stress onto the cells.

The cells were first seeded and incubated overnight in the standard culture condition. The next day, the B27 was withdrawn by changing the culture medium to N2 only media for the treated cells, whilst the media of the non-treated cells (control) were left unchanged. The cells remained in culture for 1 hour (acute response). After 1 hour, the treated and non-treated cells were collected, and the live cells were counted in both the high TRF2 expressing NPCs and the TRF2 knockdown NPCs. To further study the chronic response, some of the cells were incubated in N2 media for another 24 hours, and the cells were counted on the next day. The cell counts of the treated cells (N2 1 hour, N2 o/n) were normalised to the control of each cell type. The cell count showed that there was a higher cell number in the TRF2 knockdown NPCs compared to the high TRF2 expressing cells (Figure 4.1 A, $p < 0.01$), which contrasts the results from standard culture condition. After the overnight culture with N2 media, there was no significant difference in terms of cell number, although there was a slightly higher number in the lower TRF2 expressing cells (Figure 4.1 A). The difference in cell number was further

investigated and it was found that there was significantly higher cell death in the high TRF2 expressing cells compared to the knockdown (Figure 4.1 B, $p < 0.05$).

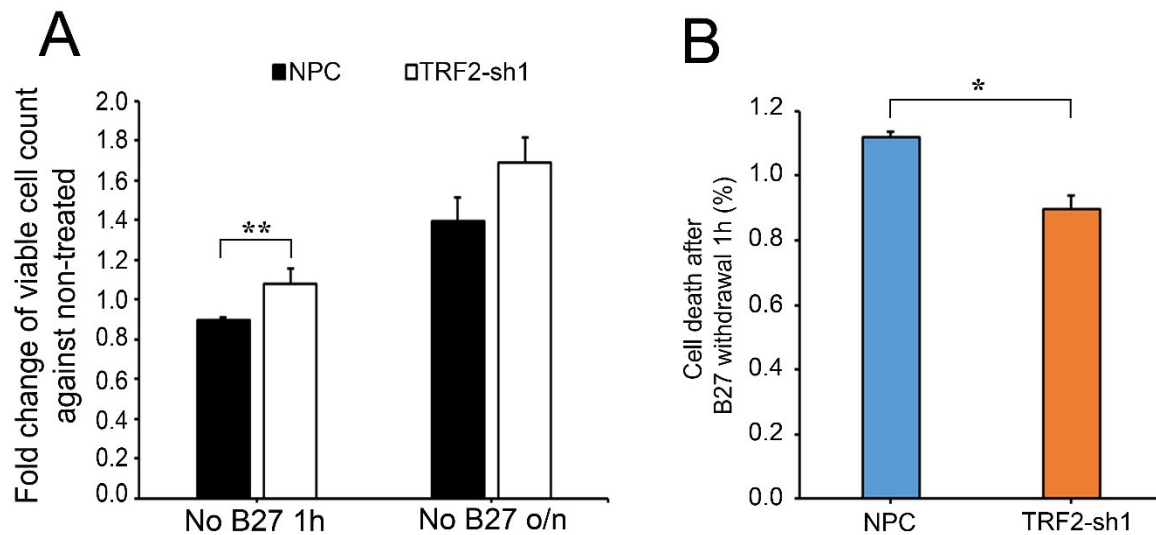


Figure 4.1: Reduced cell number in TRF2-control, but not the TRF2-knockdown NPCs.

(A) Fold change in viable cell number between the TRF2-control and knockdown after 1 hour and overnight withdrawal of B27 reagent in the culture medium. The cell count was normalised to the untreated cells of the respective cell types. **(B)** The percentage of cell death (dead cells/total number of cells $\times$ 100) after 1-hour withdrawal of B27 reagent in the culture medium. Cells were counted using the Countess™ Automated Cell Counter by Life Technologies. The figure shows the cell count as mean $\pm$ SD from four independent experiments. * P -value < 0.05 , ** P -value < 0.01 using Student's t -test, $n=4$.

In order to determine whether this change in cell number in the high TRF2 expressing cells was due to reduced proliferation or increase in apoptosis, apoptotic marker, Cl-casp 3 and the proliferation marker, pH3 were analysed in these cells. In the acute response experiment, there were no differences in the pH3 protein level. Due to the short period of treatment time, there would not be any proliferation expected. Therefore, the high TRF2 expressing cells still express a significantly higher pH3 compared to the TRF2 knockdown NPCs (Figure 4.2 A, $p < 0.05$). However, after overnight culture in the N2 media, there were no significant differences in the pH3 protein level between the two cell types (Figure 4.2 A).

During stress condition, the important thing to note is the apoptosis level, as the cell count showed that there was a significantly higher cell death after B27 withdrawal in the high TRF2 expressing cells compared to the knockdown. The active caspase protein level also showed to be significantly higher in the high TRF2 expressing cells after 1-hour of B27 withdrawal (Figure 4.2 B, $p < 0.05$). After overnight culture in the N2 media, there were no significant differences in the Cl-casp 3 protein level, even though the high TRF2 expressing cells showed a slightly elevated expression compared to the knockdown (Figure 4.2 B). The lack of differences in terms of proliferation and apoptosis after overnight culture in the N2 media correlates to the comparable cell number in Figure 4.1 A.

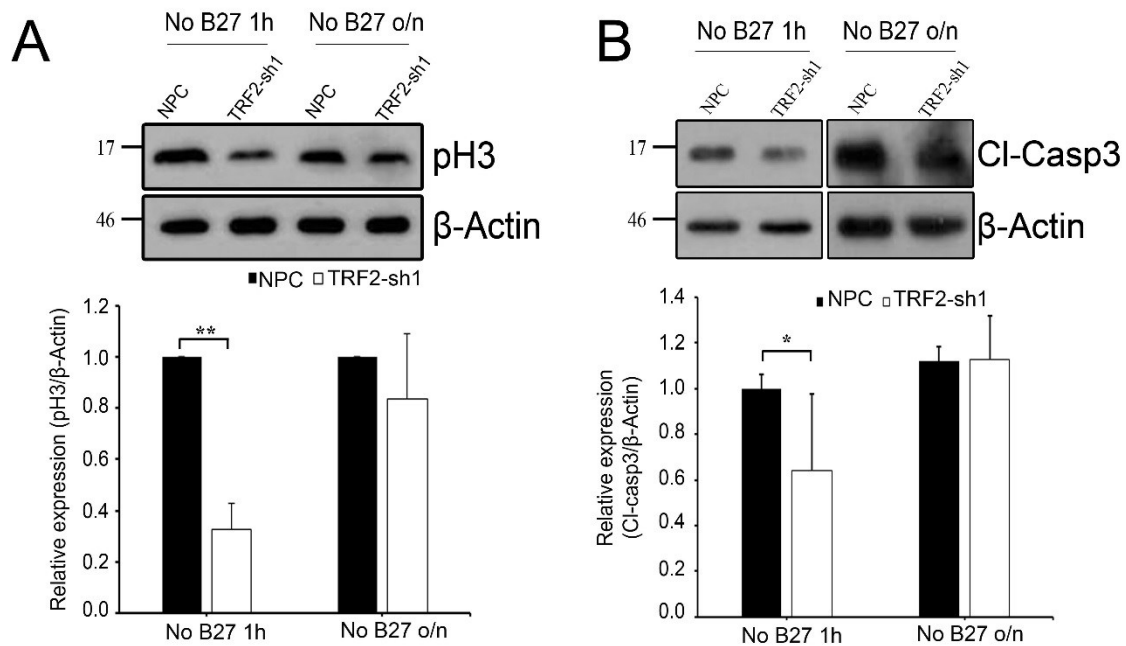


Figure 4.2: Reduced apoptosis in TRF2-knockdown after B27 withdrawal, but no change in pH3 level. (A) pH3 protein level in TRF2-control and knockdown NPCs after withdrawal of B27 from the culture medium. Top: Representative blot from three independent experiments. Bottom: Densitometry analysis from three independent immunoblots. The analysis was done by measuring the band intensity via imageJ software and the fold change was calculated (N2 overnight/N2 1h). (B) Cl-casp 3 protein level in TRF2-control and knockdown NPCs after withdrawal of B27 from the culture medium (1 hour and overnight culture). Top: immunoblot image of Cl-casp3 protein. The blot for the B27 withdrawal overnight does not represent the graph. Bottom: Densitometry analysis from three independent immunoblots. The analysis was done by measuring the band intensity via imageJ software and the fold change was calculated (N2 overnight/N2 1h). The figure shows the cell count as mean $\pm$ SD from four independent experiments. *P-value < 0.05 using Student's t-test, n=4.

4.2.2 High TRF2 increases apoptosis in oxidative stress condition

A different stress condition was used in order to further investigate the increase in cell death in the high TRF2 expressing cells that were observed previously. As TRF2 is highly expressed in many highly proliferative cancer cells, there may be a relation between the TRF2 and carcinogenicity, other than to promote proliferation. One of the causes of cancer is due to the presence of reactive oxygen species (ROS) that will induce damage to the DNA, hence causing a mutation leading to cancer. It has been reported that hydrogen peroxide can induce oxidative stress in cells that are in culture (Miguel et al., 2008; Chen et al., 2016; Ismail et al., 2016), therefore 100 μ M of H₂O₂ treatment for 1 hour was used as an oxidative stress model. The 100 μ M of H₂O₂ was chosen based on previous reports that showed induction of oxidative stress and DNA damage (Driessens et al., 2009; Su et al., 2016; Chen et al. 2016), and the 1-hour treatment time was chosen in order to observe the acute response in the different TRF2 expressing cells.

After the induction of oxidative stress through culturing for 1 hour in H₂O₂, the cell count data indicated that there was a 20% reduction of cell number in the high TRF2 expressing cells, but there was less than 10% reduction in the TRF2 knockdown cells (Figure 4.3 A, $p < 0.01$). The percentage of cells in the TRF2 knockdown was also significantly higher compared to the high TRF2 expressing cells. This was due to the significantly higher number of cell death in the high TRF2 expressing cells compared to the knockdown (Figure 4.3 B, $p < 0.05$). The high cell death was further confirmed by the expression of Cl-casp 3 protein. The active caspase 3 protein level showed a significant elevation after culture in oxidative stress condition in the high TRF2 expressing cells compared to the TRF2 knockdown NPCs (Figure 4.3 C, right panel, $p < 0.05$).

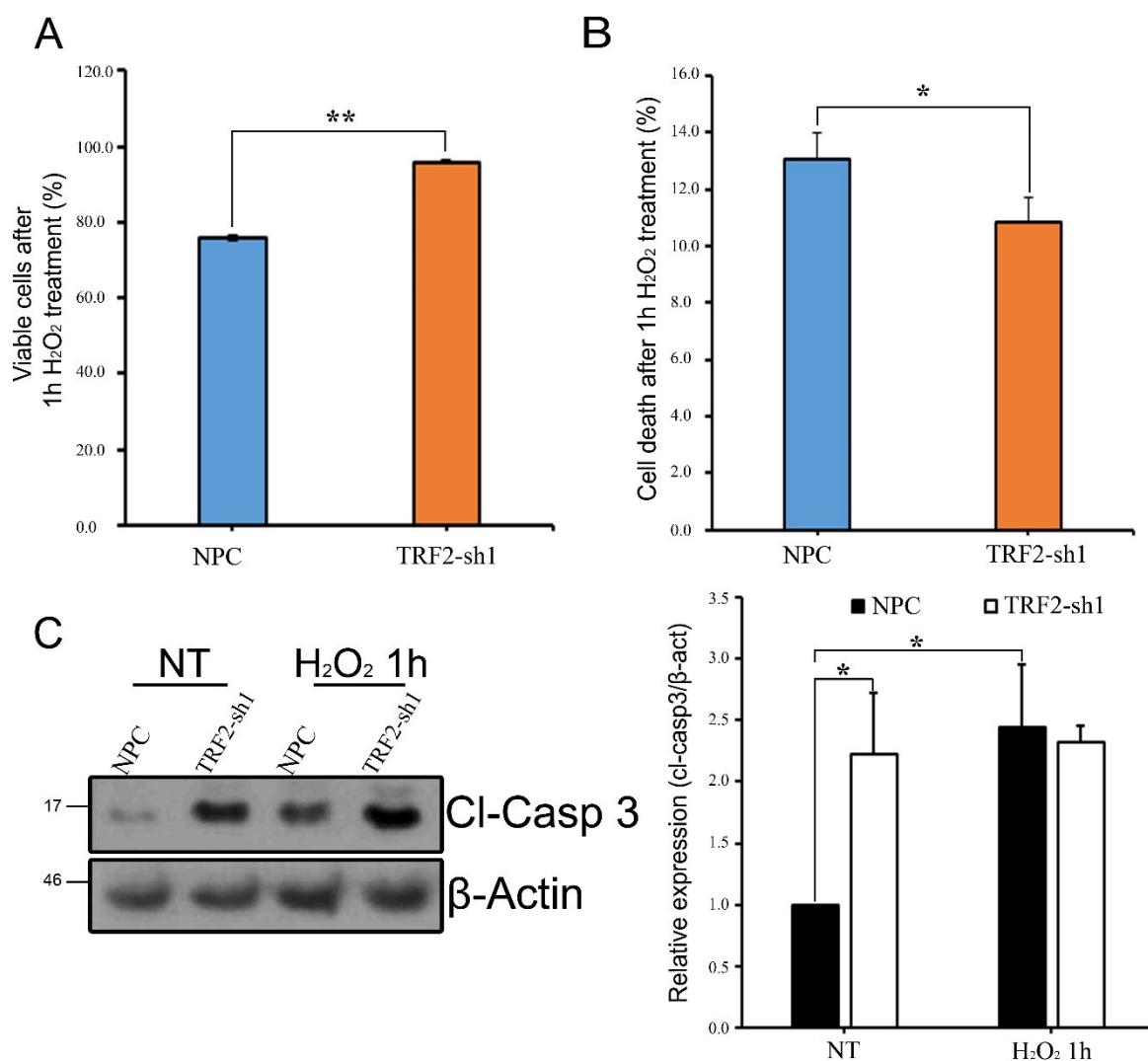


Figure 4.3: High TRF2 induce more cell death after oxidative stress in NPCs. (A) The percentage of viable cells of the TRF2-control and knockdown NPCs after 1-hour culture in 100 μ M H₂O₂. Cells were counted using the Countess™ Automated Cell Counter by Life Technologies. ***P*-value < 0.01 using Student's *t*-test, *n*=7. (B) The percentage of cell death in TRF2-control and knockdown NPCs after 1-hour culture in 100 μ M H₂O₂. The figure shows the cell count as mean $\pm$ SD from four independent experiments. Cells were counted using the Countess™ Automated Cell Counter by Life Technologies. **P*-value < 0.05 using Student's *t*-test, *n*=7. (C) The Cl-casp 3 protein level in TRF2-control and knockdown NPCs after 1-hour culture in 100 μ M H₂O₂. Left: immunoblot image of the Cl-casp 3 expression and β -Actin. The image is not a representative of the graph. Right: Densitometry analysis of the Cl-casp 3 protein level relative to the β -Actin. ****P*-value < 0.001 using Student's *t*-test, *n*=4.

The oxidative stress experiment was repeated using HEK293T cells in order to determine whether the effect observed in the NPCs would be similar in other cell types. The cell count indicated that there was more than 30% reduction of cell number in the high TRF2 expressing cells after 1-hour treatment with 100 μ M of H₂O₂, but there was around 10% reduction of cell number in the low TRF2 expressing cells (Figure 4.4 A, $p < 0.05$). The percentage of live cells in the low TRF2 expressing cells was also significantly higher compared to the high TRF2 expressing cells, after 1-hour treatment with 100 μ M of H₂O₂ (Figure 4.4 A, $p < 0.05$). This was due to the significantly higher number of cell death in the high TRF2 expressing cells compared to the low TRF2 expressing cells (Figure 4.4 B, $p < 0.05$). The high cell death was further confirmed by the expression of Cl-casp 3 protein. The active caspase 3 protein level showed a significant elevation in both cell types under oxidative stress condition (Figure 4.4 C). However, there was a significant increase of Cl-casp 3 expression in the high TRF2 expressing HEK293T cells compared to the control (Figure 4.4 C, right panel, $p < 0.001$), and almost no change in the low TRF2 expressing cells, which coincides with the number of cell death (Figure 4.4 B, $p < 0.05$).

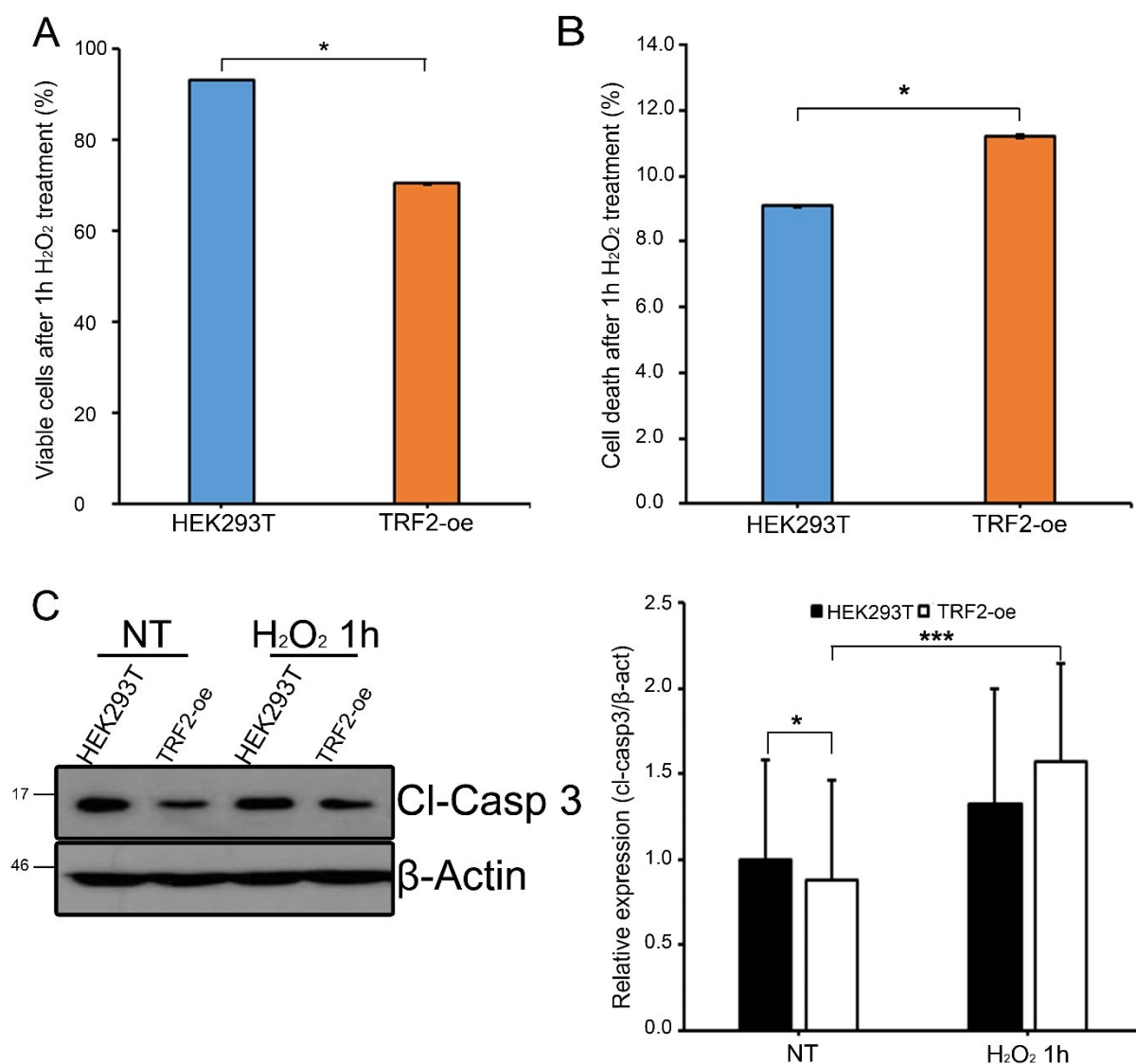


Figure 4.4: High TRF2 induce more cell death after oxidative stress in HEK293T cells. (A) The percentage of viable cells of the TRF2-control and overexpressing HEK293T cells after 1-hour culture in 100 μ M H₂O₂. Cells were counted using the Countess™ Automated Cell Counter by Life Technologies. **P*-value < 0.05 using Student's *t*-test, *n*=7. (B) The percentage of cell death in TRF2-control and overexpressing HEK293T cells after 1-hour culture in 100 μ M H₂O₂. The figure shows the cell count as mean $\pm$ SD from four independent experiments. Cells were counted using the Countess™ Automated Cell Counter by Life Technologies. **P*-value < 0.05 using Student's *t*-test, *n*=7. (C) The Cl-casp 3 protein level in TRF2-control and overexpressing HEK293T cells after 1-hour culture in 100 μ M H₂O₂. Left: immunoblot image of the Cl-casp 3 expression and β -Actin. The image is not a representative of the graph. Right: Densitometry analysis of the Cl-casp 3 protein level relative to the β -Actin. **P*<0.05, ****P*-value < 0.001 using Student's *t*-test, *n*=5.

4.2.3 High TRF2 increases apoptosis in hypoxic condition

The adverse effect of TRF2 in stress conditions could explain the developmental impairment of the brain in the presence of stress during gestation. For example, a fetus exposed to hypoxic conditions in utero may impair brain development leading to cerebral palsy. In order to further investigate this, I cultured the cells under hypoxia. The hypoxic condition was achieved by culturing the cells in 0.5% O₂ incubator (hypoxic chamber).

A report previously suggested that there was a difference in the rate of cell proliferation when they were cultured in the culture condition of 20 % O₂ and the physiological oxygen level 5 % O₂ (Csete, 2005). In order to assess whether there is actually any difference between these two oxygen levels, the cells were seeded with the same number of cells ($\sim 5 \times 10^5$) and were cultured overnight. The cell count data from the following day showed that there is no difference in cell number between the two oxygen level, where the high TRF2 expressing cells still had a significantly higher number of cells compared to the knockdown (Figure 4.5 A, $p < 0.05$). Therefore, the standard culture condition oxygen level (20 % O₂) can be used as the control against the hypoxic condition (0.5 % O₂).

In the hypoxic condition, there was about 10% reduction of cell number in the high TRF2 expressing cells. However, the TRF2 knockdown cells showed a 20% increase in cell number, but it did not reach statistical significance. The percentage of cells after hypoxia showed a significant difference between the high TRF2 expressing cells compared to the knockdown (Figure 4.5 B, $p < 0.05$). This result could indicate that TRF2 promotes cell death in hypoxia condition. In addition to the difference in cell number, the Cl-casp 3 protein level was examined to determine the level of apoptosis in both cell types after 1 hour of culture under hypoxic condition. The result showed that the Cl-casp 3 protein level is increased in the high TRF2 expressing cells under hypoxic condition. However, the low TRF2 expressing cells seemed to slightly downregulate the Cl-casp 3 protein level, although it was not statistically significant

(Figure 4.5 C, right panel). This result could explain the increase in cell number in the low TRF2 expressing cells under hypoxia compared to the high TRF2 expressing cells, which is due to reduced apoptosis.

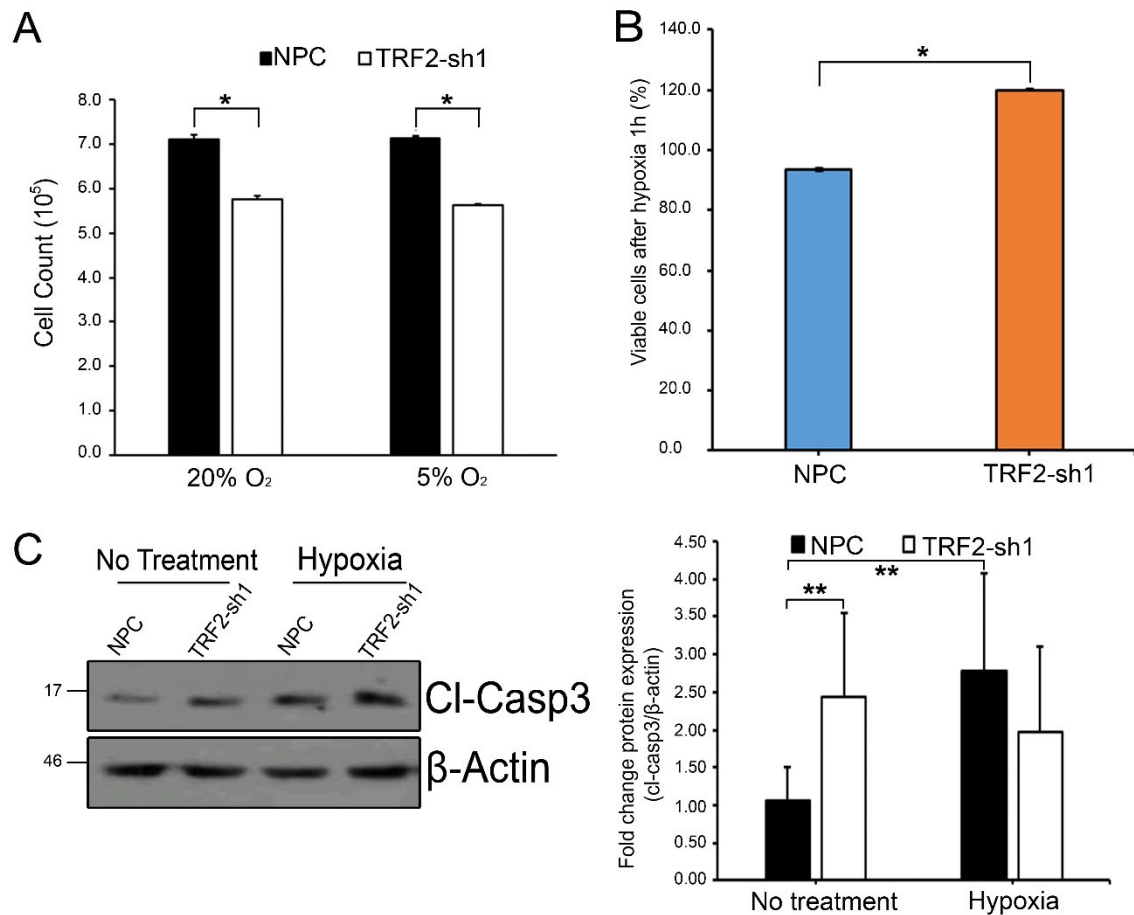


Figure 4.5: No difference in cell number and viable cells after culture in Hypoxic condition. (A) The TRF2-control and knockdown NPCs live cell number after culturing in standard oxygen level condition (20 % O₂) and physiological oxygen level (5 % O₂). (B) The percentage of viable cells in TRF2-control and knockdown NPCs after culturing in standard oxygen level condition (20 % O₂) overnight, then changing to the hypoxic condition (0.5 % O₂) for 1 hour. The hypoxic condition was obtained by culturing the cells in a hypoxic chamber. Cells were counted using the Countess™ Automated Cell Counter by Life Technologies. The figure shows the cell count as mean ± SD from four independent experiments. *P-value < 0.05 using Student's t-test, n=3. (C) The Cl-casp 3 protein level in TRF2-control and knockdown NPCs after 1-hour culture in 100 μM H₂O₂. Left: immunoblot image of the Cl-casp 3 expression and β-Actin. The image is not a representative of the graph. Right: Densitometry analysis of the Cl-casp 3 protein level relative to the β-Actin. **P-value < 0.001 using Student's t-test, n=3.

Similar to the oxidative stress experiments, the hypoxic condition was repeated using HEK293T cells in order to determine whether the effects observed in NPCs can be observed in a different cell type. When HEK293T cells were cultured under hypoxic condition, there was almost 40% reduction of cell number in the high TRF2 expressing cells. However, the low TRF2 expressing cells showed a 5% increase in cell number, although it was not significant. This phenotype was similar to the one observed in the NPCs, however, there was a higher reduction in the high TRF2 expressing HEK293T cell number compared to the NPCs in the hypoxic condition. The percentage of cells after hypoxia showed a significant difference between the high TRF2 expressing HEK293T cells as compared to the low TRF2 expressing HEK293T cells (Figure 4.6 A, $p < 0.01$). The result could indicate that TRF2 promotes cell death under hypoxic condition, which was further proven by the significantly higher number of cell death in the high TRF2 expressing HEK293T cells compared to the low TRF2 expressing HEK293T cells (Figure 4.6 B, $p < 0.05$). Further to the difference in cell number, the Cl-casp 3 protein level was examined in order to determine the level of apoptosis in both cell types after 1 hour of culture under hypoxic condition. The result showed that the Cl-casp 3 protein level was increased in the high TRF2 expressing cells under hypoxic condition, however, the low TRF2 expressing cells seemed to significantly downregulate the Cl-casp 3 protein level (Figure 4.6 C, $p < 0.05$). This result could explain the increase in cell number in the low TRF2 expressing cells under hypoxia, compared to the high TRF2 expressing cells, which is due to lower apoptosis.

The results of the HEK293T cells showed similarity with the NPCs, which suggest that the effects observed due to the different levels of TRF2 are not cell type-specific.

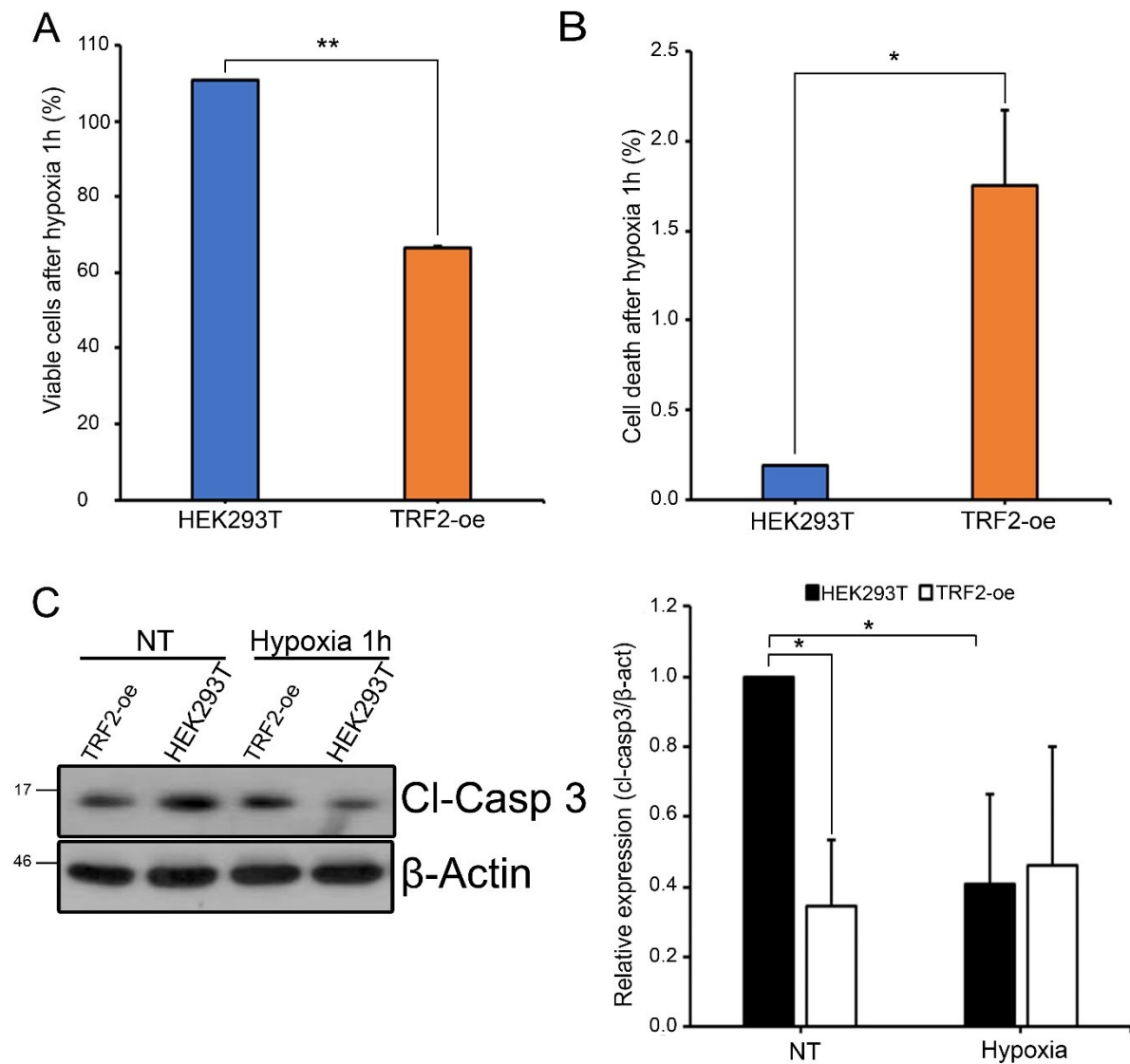


Figure 4.6: High TRF2 induce more cell death after hypoxia in HEK293T cells. (A) The percentage of viable cells of the TRF2-control and overexpressing HEK293T cells after 1-hour culture in 0.5% O_2 condition. Cells were counted using the Countess™ Automated Cell Counter by Life Technologies. ** P -value < 0.01, $n=4$. (B) The number of cell death in TRF2-control and overexpressing HEK293T cells after 1-hour culture in 0.5% O_2 condition. The figure shows the cell count as mean $\pm$ SD from four independent experiments. Cells were counted using the Countess™ Automated Cell Counter by Life Technologies. * P -value < 0.05 using Student's t -test. (C) The Cl-casp 3 protein level in TRF2-control and overexpressing HEK293T cells after 1-hour culture in 0.5% O_2 condition. Left: immunoblot image of the Cl-casp 3 expression and β -Actin. The image is not a representative of the graph. Right: Densitometry analysis of the Cl-casp 3 protein level relative to the β -Actin. * P -value < 0.05 using Student's t -test, $n=3$.

4.3 Discussion

Since the TRF2 appeared to protect the cells in standard culture condition, it was essential to further explore the protective role of TRF2 in stress conditions. In the standard culture conditions, the internal and external environments were set to a relatively optimal level in order to promote cell growth and survival. However, under stress conditions, the cells are required to adapt to the physiological and pathological changes in order to maintain homeostasis and survive (Lockshin and Zakeri, 2001). The cellular adaptation, which is also known as the cellular stress response, could either be protecting the cells by helping the cells to resist the stress, through specific molecular repair and protection mechanism, or causing cell death (Fulda et al., 2010). In this chapter, the role of TRF2 under three different stress conditions, withdrawal of B27, oxidative stress and hypoxia, were addressed. The results showed that in stress condition, either when there is a reduction in nutrients (B27 withdrawal), oxidative stress and hypoxia induction, cells with higher expression of TRF2 have more cell death as compared to cells with lower TRF2 expression. The cell death in these cells is shown in both the cell count and through the increase in the Cl-casp 3 protein level.

4.3.1 The differential role of TRF2 in nutrient-deprived condition

Given that B27 contains multiple protective supplements in order to help and maintain cell survival in culture (Brewer and Cotman, 1989; Brewer et al., 1993; Chen et al., 2008; Bardy et al., 2015), removing it from the media will deprive the cells of those protective elements, hence will cause detrimental effect towards the cells (Young, Martinez and Spada, 2009), as shown in the results. Interestingly, those damaging effects seemed to be more evident in the high TRF2 expressing cells, giving rise to a significantly higher number of cell death. This indicates that TRF2 might promote acute cell death in response to the stress by the withdrawal of B27.

Although withdrawal of B27 from the media could induce cellular stress, which could cause damage to the cells, it is considered to be mild stress and the cells seemed to be able to adapt

to this stress and to proliferate after a longer culture (Figure 4.1 A). This was shown by the pH3 expression and the increase in cell number of both cell types after overnight culture without B27. The high proliferation capacity of the high TRF2 expressing cells remained unchanged even after withdrawal of B27, either for 1 hour or overnight. Therefore, the difference in cell number was mainly due to the difference in apoptosis level and not due to proliferation.

4.3.2 H₂O₂-induced Oxidative stress causes more cell death in high TRF2 expressing cells

In addition to the function of TRF2 observed after withdrawal of B27 in the culture media, it was important to explore other types of stress conditions. Another cellular stress response is oxidative stress. Oxidative stress occurs when there is a reduction in antioxidants and when there is an accumulation of ROS-induced DNA damage in the cells, which includes H₂O₂ (Trachootham et al., 2010). H₂O₂-induced oxidative stress could lead to lipid peroxidation and DNA lesion, which are highly associated with cancer development (Frei, 1994; Wijeratne, Cuppett and Schlegel, 2005; Miguel, Augusto and Gurgueira, 2008). Since there are many cancer cells that also express a high level of TRF2 (Muñoz, Blanco and Blasco, 2006; Diehl et al., 2011; El Maï et al., 2014; Ozden et al., 2014; Chen et al., 2017; Saha et al., 2017), it is important to explore the function of TRF2 under this condition.

Similar to the effects of high TRF2 after withdrawal of B27, high TRF2 expressing cells under oxidative stress also showed more reduction in cell number and more cell death as compared to the lower TRF2 expressing cells. The results indicate consistency of TRF2 in promoting cell death under stress conditions. Interestingly, there was not much difference in the Cl-casp 3 expression in the lower TRF2 expressing cells after H₂O₂-induced oxidative stress as compared to the high TRF2 expressing cells. This suggests that the reduction of cell number is not caused by caspase-dependent apoptosis when there is a low level of TRF2, where the cells may be put through the caspase-independent cell death (CICD) after oxidative stress induction (Tait and

Green, 2008). It could also be due to the insignificant difference in the reduction of cell number in the low TRF2 expressing cells after H₂O₂-induced oxidative stress, therefore, the expression level of Cl-casp 3 is almost similar in the low TRF2 expressing cells. The result further confirmed that high TRF2 in the cells induced apoptosis when cultured under stress condition, and lower TRF2 seems to protect the cells from the programmed cell death.

4.3.3 Hypoxia-induced stress in high TRF2 expressing cells induces apoptosis

Oxygen level is essential in maintaining the equilibrium in cell homeostasis, as well as maintaining the cell survival for long-term *in vitro* culture. In the standard cell culture condition, the oxygen level used was 20-21%, which is close to the atmospheric oxygen level (21%). This oxygen level is also known as normoxia (Mckeown, 2014). However, the oxygen level *in vivo* has been found to be different in different tissue types, such as; lung is 14.5% (Mckeown, 2014), arterial blood is ~12% (Csete, 2005; Brahimi-Horn and Pouyssegur, 2007), with kidney and brain as low as ~1-2% (Chow et al., 2001; Erecińska and Silver, 2001; Simon and Keith, 2008). Due to the oxygen level in most tissues are at the range of 5-10%, a lot more studies have been conducted by using that range of oxygen level, which is also known as the physiological normoxia (Csete, 2005; Mckeown, 2014; Mohyeldin, Garzón-Muvdi and Quiñones-Hinojosa, 2010). However, the result showed that there is no difference in terms of cell number when the cells are cultured in normoxia and physiological normoxia. Therefore, the 20% oxygen level could be used as the control standard condition.

The hypoxia oxygen level was set at 0.5%, as the oxygen level found in brain tissues is ~1-5% (Erecińska and Silver, 2001). Although the brain has the lowest amount of oxygen as compared to other organs (Tsaroucha et al., 2017), 0.5% was chosen as the cut-off, as the experiment was also performed in HEK293T cells, which are kidney cells. Therefore, 0.5% oxygen level was chosen as the hypoxia condition in this study.

Csete (2005) also reported that there was a higher proliferation rate in cells that are cultured in a lower oxygen level, which could explain the increase in cell number and reduction in active caspase 3 protein level in the low TRF2 expressing cells under hypoxic condition, although the oxygen level that was cultured in the study was in the range of physiological normoxia. This could mean that when TRF2 is low, the cells are not only able to withstand the stress induced by hypoxia, but it could also help the cells to further proliferate. This correlates with many other studies that reported culturing the cells in a lower oxygen level (2-10%), aids proliferation as compared to the atmospheric normoxia (20%), where the cells used expressed low levels of TRF2 (Krieger, 1996; Csete, 2005; Mohyeldin, Garzón-Muvdi and Quiñones-Hinojosa, 2010).

In contrast, the high expressing TRF2 cells showed a significant reduction of cell number after cultured in hypoxic condition, with a significant increase in cell death and Cl-casp 3 expression. Physiologically, as the part of the human body that expresses a high level of TRF2 is the developing brain, suggests that when the body is exposed to low levels of oxygen, the part that will be most affected is the brain. This could be seen in pathological conditions, such as cerebral palsy, where the development of the brain is impaired when the fetus experienced hypoxia in utero.

From these results, it can be concluded that TRF2 has a differential role when the cells are under stress conditions, where high TRF2 seems to promote apoptosis. Lower levels of TRF2 expression in the cells not only help to withstand the induction of stress, but it also seems to promote cell survival, especially in hypoxic condition, where the cells even increase in cell number. The increase in cell number in low TRF2 expressing cells of both HEK293T cells and NPCs suggests that cells, other than NPCs not only could withstand low levels of oxygen in culture, it also proliferates more. Since NPCs are the only cells that endogenously express high levels of TRF2, hypoxic conditions are detrimental to the cells. The effects of TRF2 observed could be replicated in different types of cells, suggests that TRF2 function is not cell specific.

Chapter 5

Results

**Exploring the possible mechanisms underlying the
differential effects of TRF2 in different culture conditions**

5.1 Introduction

The different phenotypes described in the previous chapters suggested that TRF2 may have a differential role in different culture conditions. It has been well-studied that TRF2 plays a role in protecting telomeres from recognition as double-stranded DNA breaks and preventing telomeres from activating DNA damage response (Karlseder et al., 2004). One of the mechanisms by which TRF2 is thought to have this function is when TRF2 binds to ATM and inhibits the activation of ATM kinase pathway at the telomere in order to protect the 3'-overhang from being recognised as a DNA break (Karlseder et al., 2004). ATM kinase is a member of the PIKK family that is rapidly activated in response to DNA damage. ATM kinase phosphorylates multiple proteins that will function in the recruitment of other factors to the DNA damage site to repair the DNA break, including γ H2AX (Kobayashi et al., 2009). TRF2 has also been found at the sites of DNA breaks outside of the telomeres and was co-localised with the DNA damage response factor, γ H2AX (Bradshaw et al., 2005; Huda et al., 2012). The recruitments of TRF2 to the DNA damage sites occur rapidly and transiently where it was localised at the DNA break sites ~2s after laser induction, which is faster than other DNA damage response, such as 53BP1, ATM and γ H2AX that are reported to be recruited to the DNA break sites within minutes (Rogakou et al., 1998; Kobayashi et al., 2004; Falck et al., 2005; Podhorecka et al., 2010). However, TRF2 localisation at the DNA damage site gradually declines (Bradshaw et al., 2005). These studies suggest that there is a direct association between TRF2 and the DNA damage response, either in assisting the DNA damage response to repair the DNA break or in protecting the telomere ends from being recognised as a break.

TRF2 has been shown to function as a protein hub, being able to interact with proteins associated within and outside of the telomeres (Kim et al., 2009; Benarroch-Popivker et al., 2016). As such, it is anticipated that TRF2 may directly or indirectly affect other proteins that are associated with DNA damage response and repair in the cellular stress response mechanism

in protecting cell survival. For example, TRF2 directly binds to ATM and inhibit ATM auto-phosphorylation at the telomeres in order to protect the telomere ends from being recognised as a DNA double-stranded break (DSB) (Karlseder et al., 2004). Furthermore, TRF2 was also found to directly bind with p38 of the mitogen-activated protein kinases (MAPK) at the extra-telomeric DNA break regions (Roy et al., 2018).

Therefore, this chapter aimed to explore whether there are any differences in the DNA damage response marker activation between high TRF2 expressing control and TRF2-KD NPCs. This chapter also aimed to investigate the changes in the activation of the ATM-dependent DNA damage response, such as pChk2, γ H2AX and pp53 in the standard culture condition and oxidative stress condition.

5.2 Results

5.2.1 TRF2 suppresses ATM-dependent DNA damage response

As TRF2 has been shown to inhibit ATM kinase at the telomere, any changes in the ATM kinase pathway after TRF2 has been knocked-down in the NPCs would be investigated under standard growth culture conditions. Markers that are downstream of the ATM kinase were chosen, namely phosphorylation of Chk2 at Threonine 68, phosphorylation of p53 at Serine 15, and phosphorylation of H2AX at Serine 139. The total protein of all the factors was also observed as a control. β -Actin was used as the loading control. The results showed that, in TRF2-KD NPCs, there was a significant increase in the activation of Chk2, p53 and H2AX as shown in the levels of pChk2 ($p < 0.001$), pp53 ($p < 0.01$) and γ H2AX ($p < 0.01$) (Figure 5.1). This is consistent with other studies that TRF2 inhibits the ATM kinase activation, thus suppressing the phosphorylation of its downstream targets, as the markers shown in Figure 5.1 are activated by the ATM kinase due to DSBs (Banin et al., 1998; Canman et al., 1998; Khanna et al., 1998; Matsuoka et al., 2000; Burma et al., 2001; Karlseder et al., 2004; Lavin and Koslov. 2007). However, γ H2AX and pp53 could also be phosphorylated at the same Serine 139 and Serine 15 sites, respectively by DNA PK (Shieh et al., 1997; Rogakou et al., 1998; Woo et al., 1998). Therefore, the result shows that as the expression of TRF2 decreases, there was increased activation of ATM kinase and its downstream targets, namely in Chk2, p53 and H2AX. This also suggests that TRF2 suppresses ATM activity.

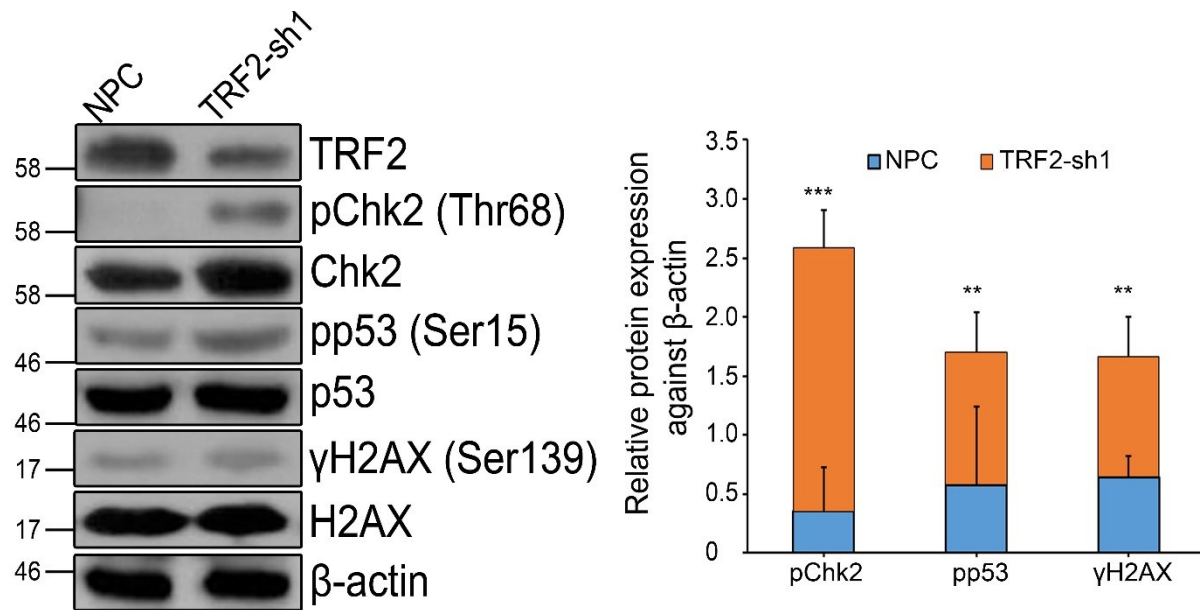


Figure 5.1: TRF2 inhibits DNA damage response markers in the NPCs standard culture condition. *Left panel: Immunoblot representation of DNA-damage response markers, downstream of the ATM kinase pathway as indicated in TRF2-control and -knockdown NPCs. Samples were from cell lysates of TRF2-control and -knockdown NPCs cultured in standard culture conditions. Right panel: Statistical analysis of the DNA damage response markers relative to β-actin. The figure shows the analysis as mean ± SD from four independent experiments. ** $p < 0.01$, *** $p < 0.001$ using One-way ANOVA.*

5.2.2 DNA damage response in NPCs is not restricted at the telomere

To further understand the mechanism of TRF2 in the DNA damage response, it is important to investigate whether the function of TRF2 observed is restricted at the telomere or at other parts of the chromosome as well. Co-immunostaining of TRF1 with TRF2 and γ H2AX was done in order to investigate whether the localisation of TRF2 and γ H2AX are at the telomere or at other chromosomal regions. TRF1 has the same architectural structure and similar C-terminal Myb-binding domain as TRF2 (Broccoli et al., 1997). TRF1 also binds to the same TTAGGG repeats as TRF2 and is conserved at the telomere, where it also functions to protect the telomere ends (de Lange, 2005). Therefore, TRF1 can be used as a marker for the telomere.

The result showed that in the high TRF2 expressing control NPCs, TRF2 not only co-localised with TRF1 but also in other areas throughout the nucleus, which is consistent to our previous staining in the same cells (Ovando-Roche et al., 2014). The same finding was also reported in another study where the overexpressing TRF2 cells showed the localisation of TRF2 throughout the nucleus and not only conserved at the telomeres (Smogorzewska et al., 2000). However, in TRF2-KD NPCs, most of TRF2 is co-localised with TRF1, where the Pearson Correlation Coefficient (PCC) showed a significantly higher correlation between TRF1 and TRF2 ($p < 0.001$) (Figure 5.2). This indicates that as TRF2 is highly expressed, TRF2 will not only be localised at the telomeric region but also in other parts of the chromosome. In contrast, when the cells have a lower expression of TRF2, most of TRF2 is located at the telomere.

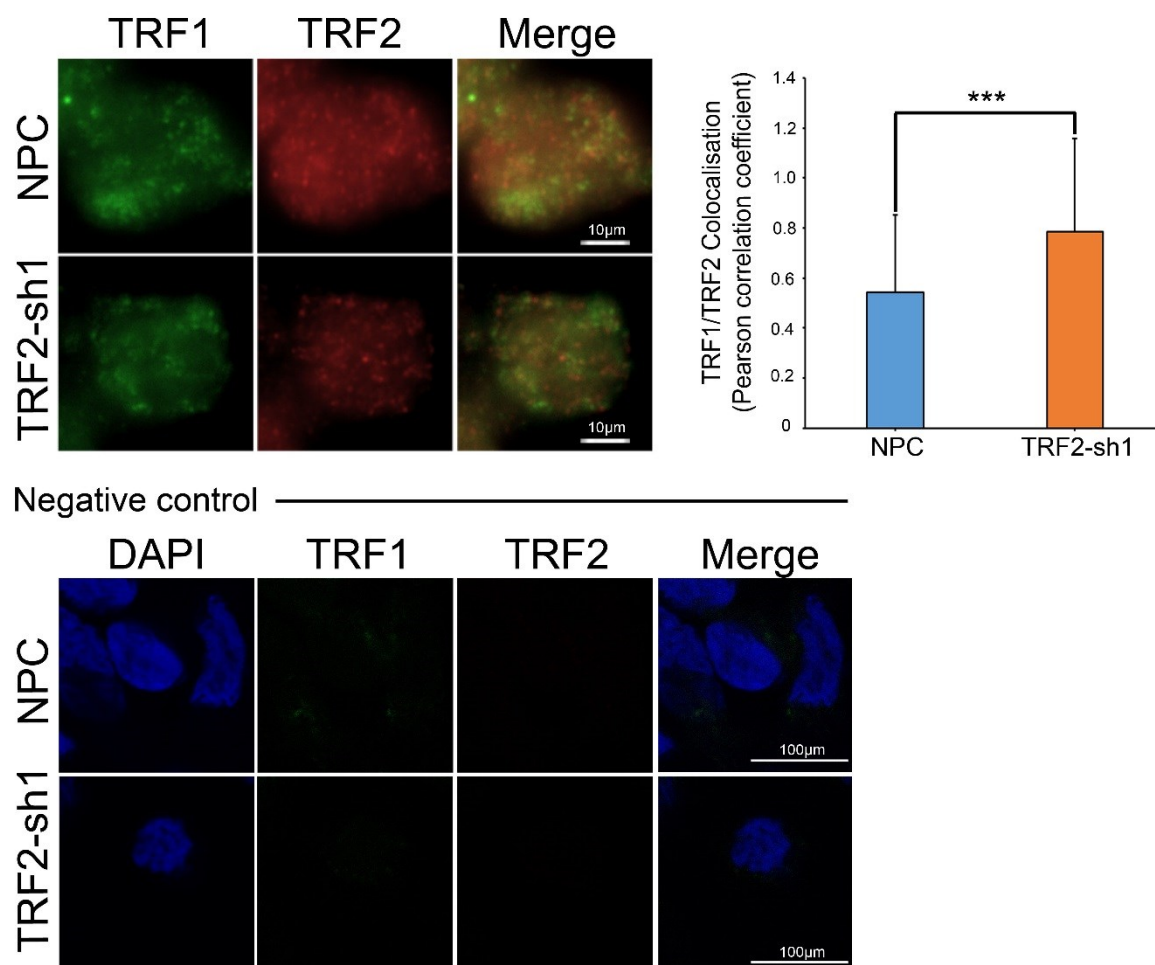


Figure 5.2: Localisation of TRF2 is not restricted at the telomeres in high TRF2 control NPCs under standard culture conditions. *Left panel: Immunostaining image representative of TRF1 (green) and TRF2 (red) to show localisation of TRF2 at the telomere (represented by TRF1) in the knockdown and high TRF2 expressing NPCs. Scale bar = 10 μ m. Immunostaining of the cells without the primary antibodies was used as negative control. Scale bar = 100 μ m. DAPI stain (blue) indicates the nucleus. Right panel: Statistical analysis of the Pearson Correlation Coefficient (PCC) to show the co-localisation between TRF1 and TRF2 in TRF2 control and knockdown NPCs. Analysis was performed using Co-loc2 software, Fiji plugin. The figure shows the analysis as mean $\pm$ SD. *** p < 0.001 using Student's t-test, n = 30.*

To confirm that the elevation of DNA damage response in TRF2-KD NPCs as shown in Figure 5.1 was not due to telomeric break recognition, co-immunostaining of TRF1 and γ H2AX was performed. The result showed that in the high TRF2 expressing and knockdown NPCs, most of the γ H2AX were not co-localised with TRF1, where there was no significant difference ($p>0.05$) in the co-localisation of γ H2AX and TRF1 between both cell types (Figure 5.3). The result indicates that the DNA damage response observed in both the high TRF2 expressing and knockdown NPCs are not telomeric as the PCC of TRF1 and γ H2AX shown was less than 0.2 in both cell types. In addition, a previous study reported that knocking down TRF2 in the NPCs did not induce any telomere fusion and genomic instability (Ovando-Roche et al., 2014). Given the function of TRF2 in the protection of the telomere ends from being recognised as the DNA break, even though there is an increase in the DNA damage response activation in TRF2-KD cells, it would be expected that the telomere remains intact and protected, as long as there is TRF2 expressed in the cells (van Steensel et al., 1998; de Lange 2005). As TRF2 is conserved at the telomere when the expression is low, TRF2 is still able to protect the telomere from being recognised as the DNA break site.

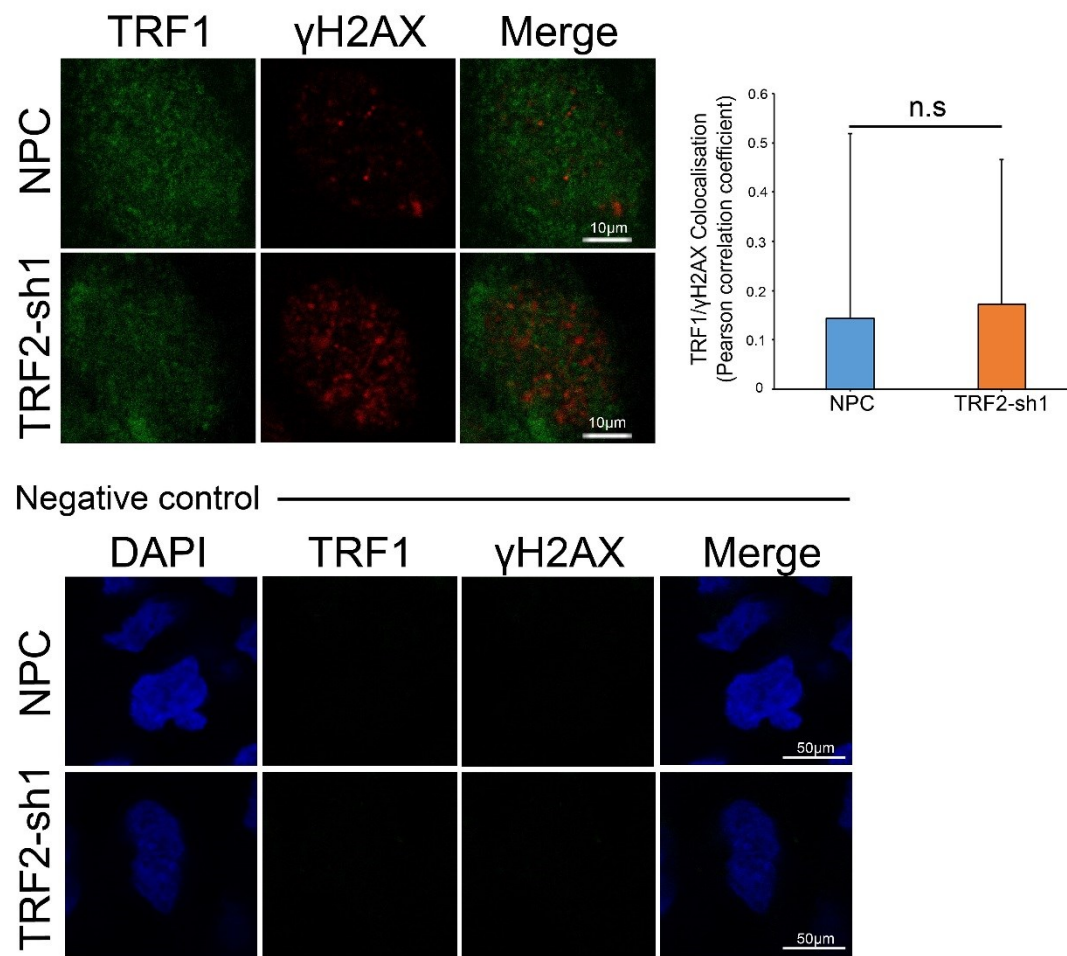


Figure 5.3: Localisation of γ H2AX is not restricted at the telomeres under standard culture conditions. Left panel: Immunostaining image representative of TRF1 (green) and γ H2AX (red) to show localisation of γ H2AX at the telomere (represented by TRF1) in the knockdown and high TRF2 expressing NPCs. Scale bar = 10 μm. Immunostaining of the cells without the primary antibodies was used as negative control. Scale bar = 50 μm. DAPI stain (blue) indicates the nucleus. Right panel: Statistical analysis of the Pearson Correlation Coefficient (PCC) to show the co-localisation between TRF1 and γ H2AX in TRF2 control and knockdown NPCs. Analysis was performed using Co-loc2 software, Fiji plugin. The figure shows the analysis as mean $\pm$ SD. Non-significant (n.s) $p > 0.05$, using Student's *t*-test, $n = 20$.

5.2.3 TRF2 does not impede DNA damage response in oxidative stress condition

As shown above, in the standard culture condition, TRF2 appeared to inhibit the activation of the ATM kinase and reduce the phosphorylation of downstream factors in the high TRF2 expressing cells. Next, this inhibition was further investigated whether it would occur differently in the H₂O₂ treated cultures, which could explain the different phenotype that was observed in Chapter 4. Both the high TRF2 expressing controls and TRF2-KD NPCs were treated with 100 μ M H₂O₂ for one hour, as in Chapter 4.2.2. The γ H2AX signals were detected by immunostaining and compared to the untreated cells. The results revealed that there was a significant increase in the γ H2AX foci formation in both cell types after H₂O₂ treatment (Figure 5.4 A, right panel, $p < 0.05$). Interestingly, although a significantly higher γ H2AX positive cells were detected in TRF2-KD cells than the controls under standard culture conditions, there was no significant difference between the two cell types 1 hour after H₂O₂ treatment despite a slightly higher γ H2AX foci formation in TRF2-KD NPCs than the high TRF2 expressing control NPCs (Figure 5.4 A, right panel).

Since the phosphorylation of ATM could not be successfully detected by immunoblot analysis in this study, immunostaining was used instead to show the activation of ATM by autophosphorylation in the untreated and treated NPCs. It showed that the activated ATM was significantly higher in TRF2-KD NPCs than the high TRF2 expressing control NPCs under the standard culture conditions (Figure 5.4 B, right panel, $p < 0.05$). These results are in agreement with the results shown in Figure 5.1 A and Figure 5.4 A on the ATM-downstream DNA damage response. Furthermore, similar to the data on γ H2AX, upon H₂O₂ treatment, the activation of ATM was significantly increased in both cell types under oxidative stress conditions ($p < 0.05$). However, the difference between the two cell-types under the oxidative stress conditions were not significant, even though there are slightly higher pATM-positive cells in the controls (Figure 5.4 B,). Moreover, upon oxidative stress, the foci formation of both γ H2AX and pATM

are located throughout the nucleus (Figure 5.4). Due to the high amount of dispersion of the foci, it was difficult to determine the co-localisation of the markers with TRF1 and TRF2. However, it could be speculated that the DNA damage response markers are located at various sites of DNA damage within the chromosome, and not necessarily at the telomeres, due to the high intensity of stress induced by the H₂O₂ treatment. The results indicate that even with the initial inhibition of the DNA damage response by TRF2 in the standard culture conditions, the DDR was still able to be activated, possibly to similar levels.

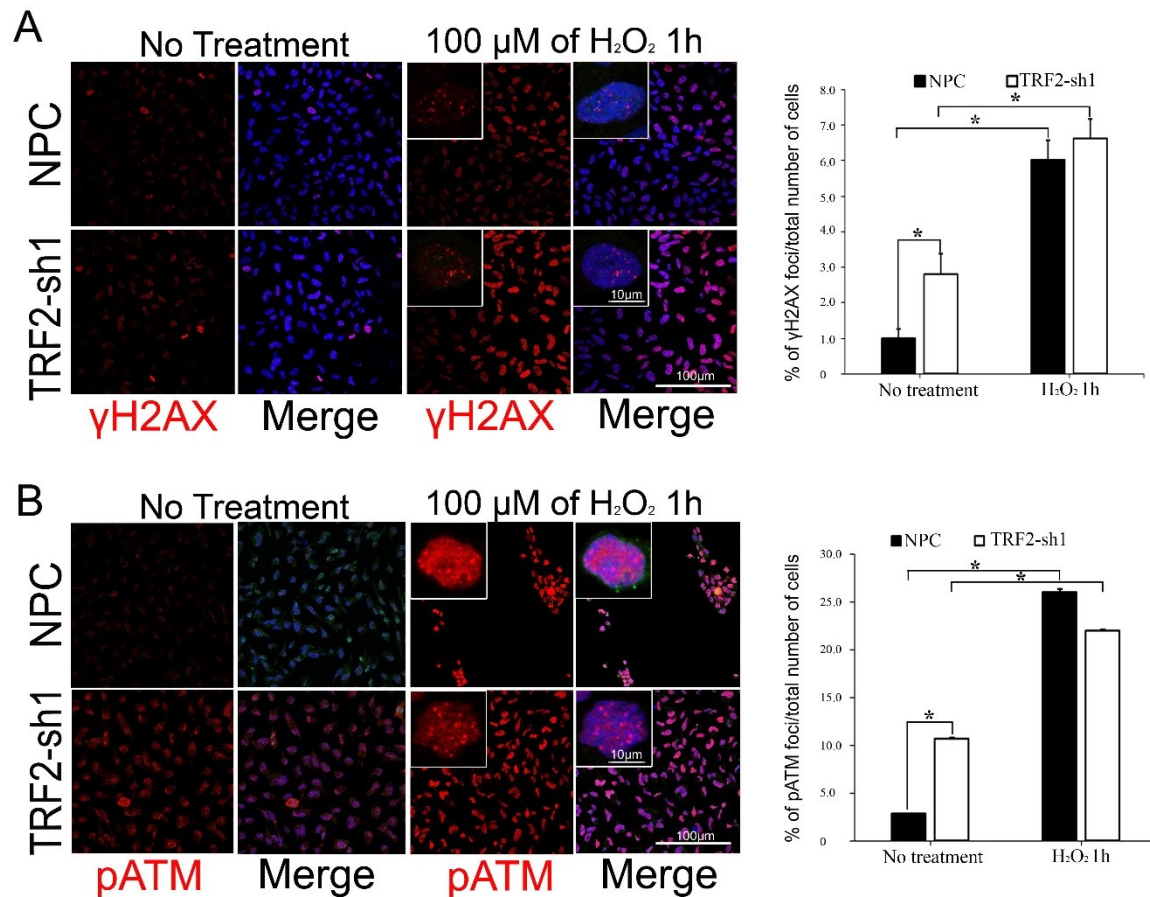


Figure 5.4: TRF2 suppresses γ H2AX formation under standard culture conditions. (A) Left panel: The immunostaining representative images of γ H2AX expression in TRF2 control and knockdown NPCs, before and after treatment with 100 μ M of H_2O_2 . Right panel: Statistical analysis of the % of γ H2AX foci formation. The figure shows the analysis as mean $\pm$ SD from three independent experiments. * $p < 0.05$ using Student's *t*-test. (B) Left panel: The immunostaining representative images of pATM expression in TRF2 control and knockdown NPCs, before and after treatment with 100 μ M of H_2O_2 . Right panel: Statistical analysis of the % of pATM foci formation. The figure shows the analysis as mean $\pm$ SD from three independent experiments. * $p < 0.05$ using Student's *t*-test.

5.2.4 Increase in the DNA damage response does not coincide with the increase in DNA damage/break

As previous results have shown, TRF2 affects the DNA damage response by suppressing the activation of the ATM-dependent response, such as pChk2, pp53 and γ H2AX under standard culture conditions. It has also been shown that TRF2 affects with the DNA damage response is not restricted at the telomere, as the localisation of TRF2 and γ H2AX was not conserved at the telomeres. In the standard culture conditions, TRF2 appeared to suppress the activation of the DNA damage response markers. However, the question remained whether the lower levels of the DNA damage response markers in the high TRF2 expressing control NPCs indeed reflected the less DNA damage/breaks in these cells. Therefore, comet assay was performed to explore the amount of double-stranded DNA break (DSB) in the high TRF2 expressing control and TRF2-KD NPCs. The Neutral Single Cell Gene Electrophoresis (SCGE) Comet assay detects double-stranded DNA breaks (DSB), which will be useful in order to quantify the amount of DNA break in these cells, specifically the DSB and eliminate the Single-stranded DNA break (SSB). The amount of DSB in the cells was measured through analysing the tail moment using the OpenComet software analysis, which calculates the tail length x the tail DNA %, which represents the amount of DNA fragments occurred due to DSB (Gyori et al., 2014).

The results showed that in the standard culture condition, the high TRF2 expressing cells displayed a significantly higher amount of DSB compared to the low TRF2 expressing NPCs (Figure 5.5 A). However, upon administration of oxidative stress, both cell types showed a significantly higher amount of DSB than before the treatment (Figure 5.5 A, left panel). It is to be expected as H_2O_2 has been shown to induce DSB (Driessens et al., 2009). Therefore, there should be more DSB in the treated cells compared to the untreated cells. In parallel to the results shown, there was a significantly higher amount of DSB in TRF2-knockdown NPCs ($p < 0.001$)

than in the high TRF2 expressing control NPCs ($p < 0.05$) after the induction of oxidative stress (Figure 5.5 A, right panel).

The difference in the amount of DSB between the two cell types in the absence of the H_2O_2 oxidative stress suggests that TRF2 may induce DNA break in the cells as there is a significantly higher amount of DSB in the high TRF2 expressing cells compared to the knockdown. However, this might not be true, as high proliferation in the high TRF2 expressing NPCs might generate more DNA fragments during replication, thus leading to the high DSB signals (Thompson and Limoli, 2000; Rothkamm et al., 2003). In order to assess this hypothesis, the cells were cultured in two different densities (90% and >100% confluency) to induce cell-cell contact inhibition, with the purpose of reducing the proliferation capacity of the cells and therefore, eliminating the significant difference in terms of cell growth between the cells. After seeding, the cells were left in the standard culture condition overnight. The next day, the Neutral SCGE Comet assay was performed. The result showed that there was a significant difference between the seeding density, where the amount of DSB was reduced by half in the high TRF2 expressing cells (Figure 5.5 B, right panel, $p < 0.001$). In the representative image, the high seeding density showed almost no comet tail formation in both cell types (Figure 5.5 B, left panel). Therefore, it can be concluded that the amount of DSB in the untreated cells was not due to the induction of DSB by TRF2, but due to the DNA fragmentation that was caused during proliferation. Consequently, due to its high proliferation capacity, the high TRF2 expressing cells also exhibited a high amount of DSB.

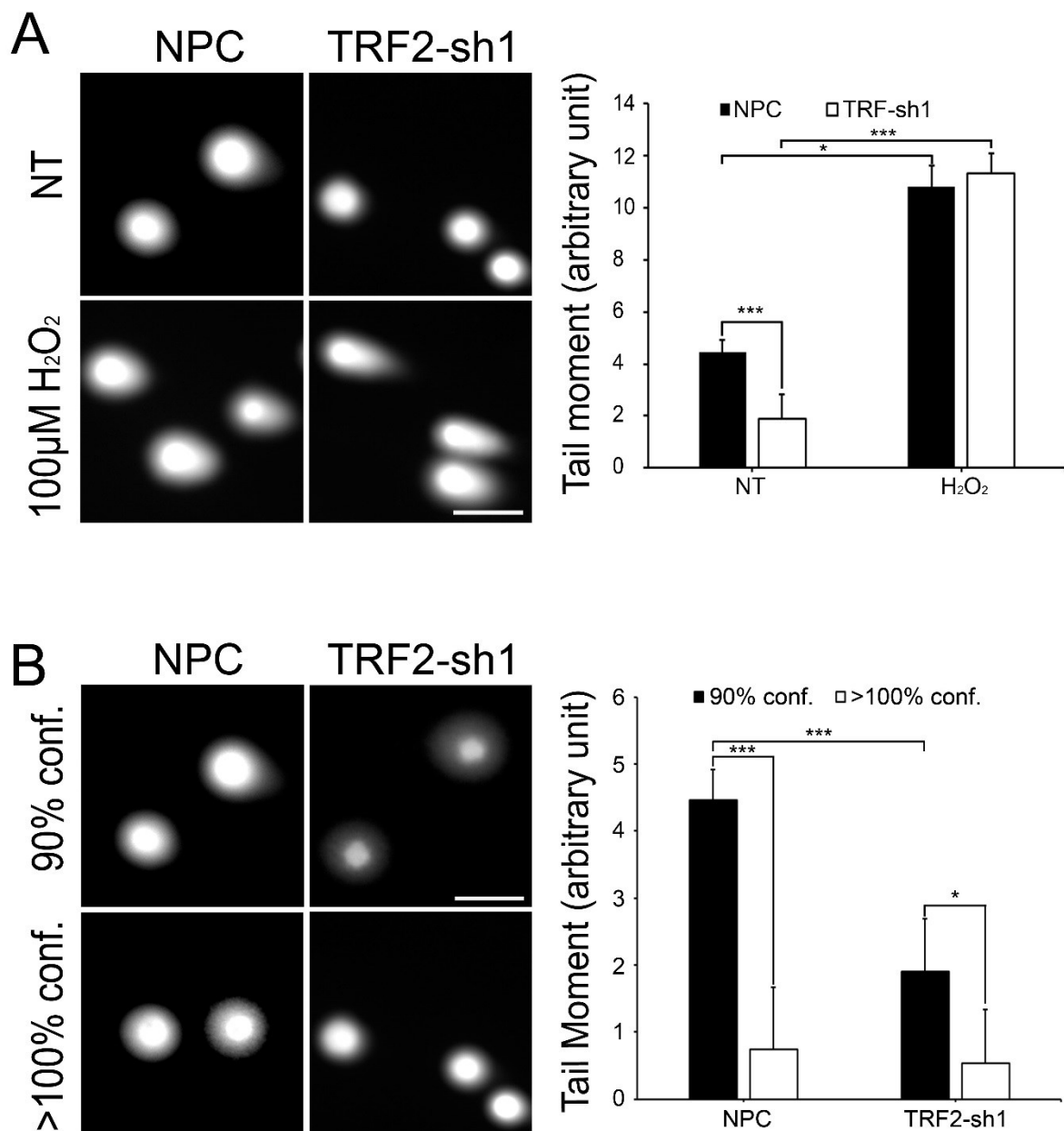


Figure 5.5: Comet assay analysis of the dsDNA break in NPCs. (A) Left panel: Representative image of the comet assay from the high TRF2 and knockdown NPCs, in standard and oxidative stress conditions. Scale bar = 10μm. Right panel: Tail moment analysis of the comet assay, * $P < 0.05$, *** $P < 0.001$ using Student's *t*-test, $n = 16$ cells. (B) Left panel: Representative image of the comet assay from the high TRF2 and knockdown NPCs, in different culture density. Scale bar = 10μm. Right panel: Tail moment analysis of the comet assay, * $P < 0.05$, *** $P < 0.001$ using Student's *t*-test, $n = 160$ cells. The analysis was done using Open Comet software with ImageJ. The tail moment represents the tail length against the DNA % (Gyori et al., 2013).

5.2.5 The effect of TRF2 on the DNA damage response is not cell-type specific

Similar to Chapters 3 and 4, the experiments performed in the NPCs were all repeated in the HEK293T cells in order to determine whether the effect of TRF2 that was observed in NPCs is cell specific or otherwise. In the standard culture condition, TRF2-overexpressing HEK293T cells showed more than 50% downregulation of the ATM-dependent DNA damage response markers, γ H2AX and pp53 (Figure 5.6).

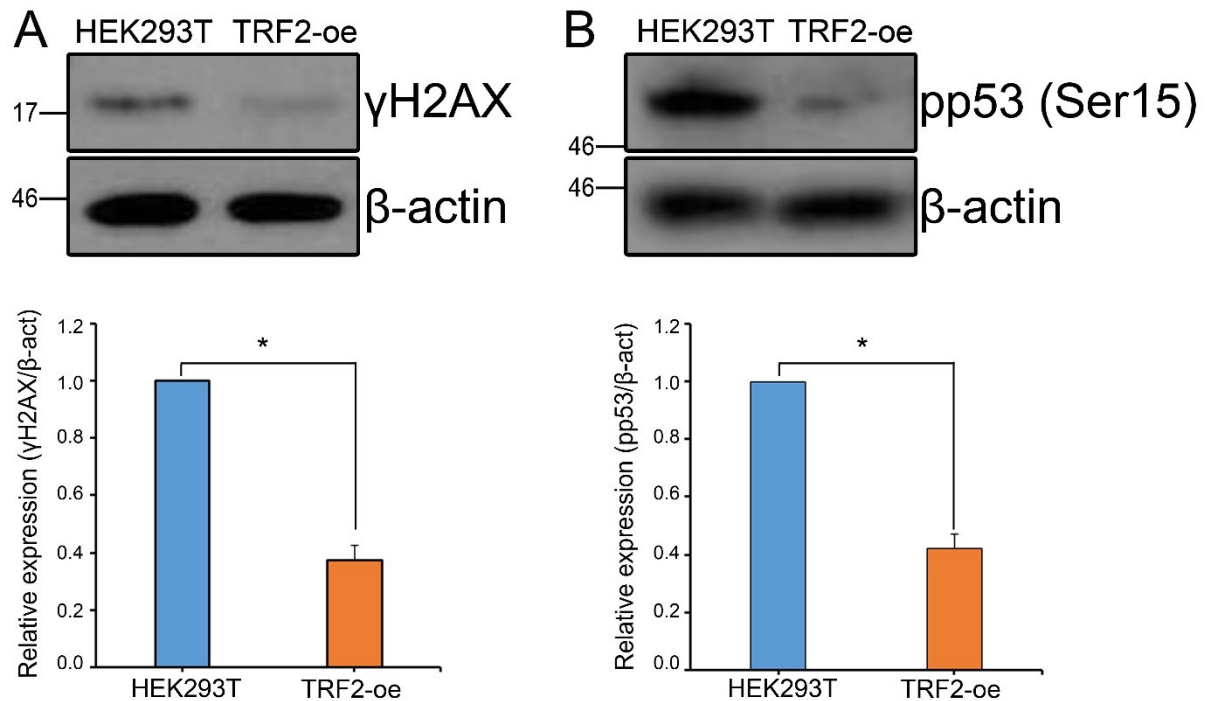


Figure 5.6: ATM-dependent DNA damage response markers protein level in HEK293T cells. (A) Top panel: Representative blot of the γ H2AX from the control and TRF2-overexpressing HEK293T cells, cultured in the standard conditions. Bottom panel: Statistical analysis of the γ H2AX protein level, normalised to β -actin. The figure shows the analysis as mean $\pm$ SD from three independent experiments. * $p < 0.05$. (B) Top panel: Representative blot of the pp53 (Ser15) from the control and TRF2-overexpressing HEK293T cells, cultured in the standard conditions. Bottom panel: Statistical analysis of the pp53 (Ser15) protein level, normalised to β -actin. The figure shows the analysis as mean $\pm$ SD from three independent experiments. * $p < 0.05$.

The amount of DSB in HEK293T cells was further analysed in order to assess whether the downregulation of the ATM-dependent DNA damage response markers was due to lower DNA damage in the cells or due to the inhibition by TRF2. The SCGE Comet assay analysis showed that TRF2-overexpressing cells have more DSB compared to the lower TRF2 control HEK293T cells in the standard culture conditions, which are similar to the NPCs results (Figure 5.7, $p < 0.05$). After the induction of oxidative stress, both cell types showed a significant increase in the DSB. However, TRF2 control HEK293T cells showed a more significant increase ($p < 0.001$) compared to TRF2-overexpressing HEK293T cells ($p < 0.01$) (Figure 5.7).

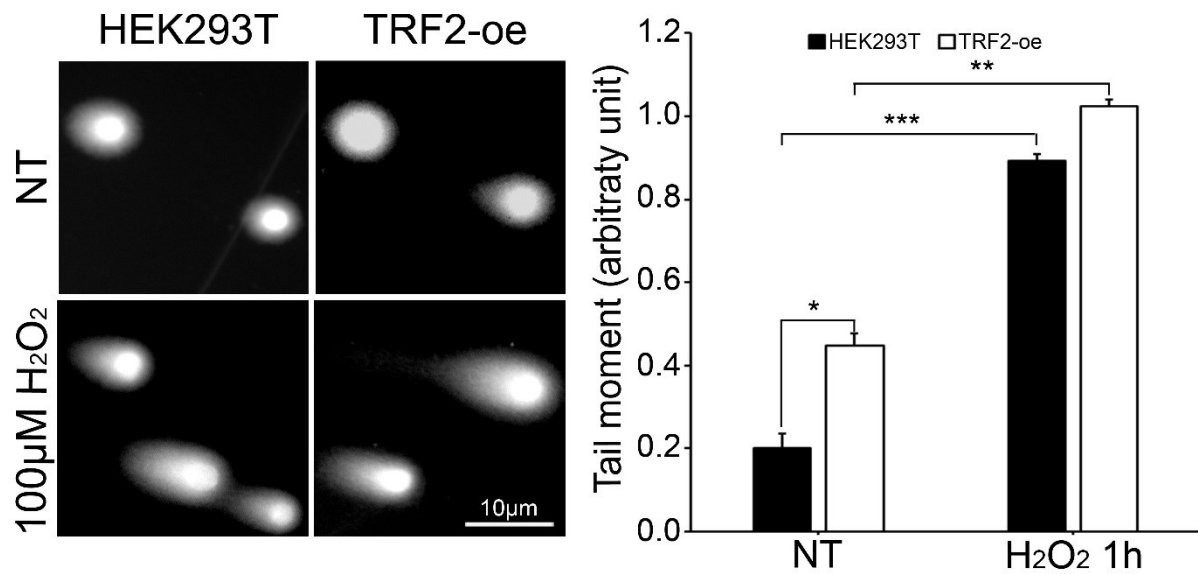


Figure 5.7: Comet assay analysis of the dsDNA break in HEK293T cells. *Left panel: Representative image of the comet assay from the control and TRF2-overexpressing HEK293T cells, in the standard and oxidative stress conditions. Scale bar = 10µm. Right panel: Tail moment analysis of the comet assay, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using Student's t -test, $n = 68$ cells. The analysis was done using Open Comet software with ImageJ. The tail moment represents the tail length against the percentage of DNA at the tail (Gyori et al., 2013).*

5.2.6 TRF2 also affects other DNA damage response

In addition to the direct interaction that TRF2 has with the ATM kinase pathway, TRF2 has also been found to have an effect with other DNA damage responses, namely p38 of the MAPK (Roy et al., 2018). Due to the inhibition of TRF2 with the ATM kinase pathway, it is worth to look at other DNA damage response in order to further understand the function that TRF2 play in the DNA damage response. Another DNA damage response factor that will be recruited at the site of DNA damage is the p53 binding protein, 53BP1. Although many studies have reported that 53BP1 is downstream of the ATM kinase (Anderson, Henderson and Adachi, 2001; Rappold et al., 2001; Ward et al., 2003), it has also been reported that 53BP1 recruitment to the DSB is independent of the ATM kinase. 53BP1 is not only considered as upstream of ATM, it could also induce the auto-phosphorylation of ATM (Mochan et al., 2003, 2004; Lee et al., 2010; Rocha et al., 2016) and was recruited to the site of DNA break within a short period of time (Anderson, Henderson and Adachi, 2001; Iwabuchi et al., 2003). The result showed that in the high TRF2 expressing NPCs, 53BP1 protein level is higher compared to the TRF2-KD cells (Figure 5.8), which corresponds with the amount of DNA break in the cells (Figure 5.5 A).

Another DNA damage response marker that was observed was the phosphorylation of p38 (Thr180/Tyr182). P38 is part of the MAPK pathway, where it is activated in response to stress (Thornton and Rincon, 2009). It has been previously reported that physiological stress increases the activation of p38 along with the expression of TRF2 (Ludlow et al., 2017) and that p38 modulates the expression of TRF2 (Spallarossa et al., 2009). The activation of p38 has also been associated with the DNA damage response, which will induce the pp38 nuclear translocation upon the induction of DNA damage (Wood et al., 2009). Due to the recent finding that showed TRF2 to be co-localised with pp38 (Roy et al., 2018), the protein level of pp38 in the TRF2 control and knockdown NPCs was further investigated. Similar to 53BP1 protein

level, the result showed that activation of p38 is higher in the high TRF2 expressing NPCs compared to the TRF2-KD cells (Figure 5.8). Total p38 was used as the control and β -actin was used as the loading control. As TRF2 has been shown to directly bind to p38 upon DNA damage (Roy et al., 2018), it is expected that a significantly high level of p38 (Thr180/Tyr182) is expressed in the high TRF2 expressing NPCs, as compared to the TRF2-KD cells. The result confirms that the high DNA damage response marker expressions that were observed in Figure 5.1 is due to the inhibition of TRF2 towards the ATM kinase and the ATM-downstream DNA damage response.

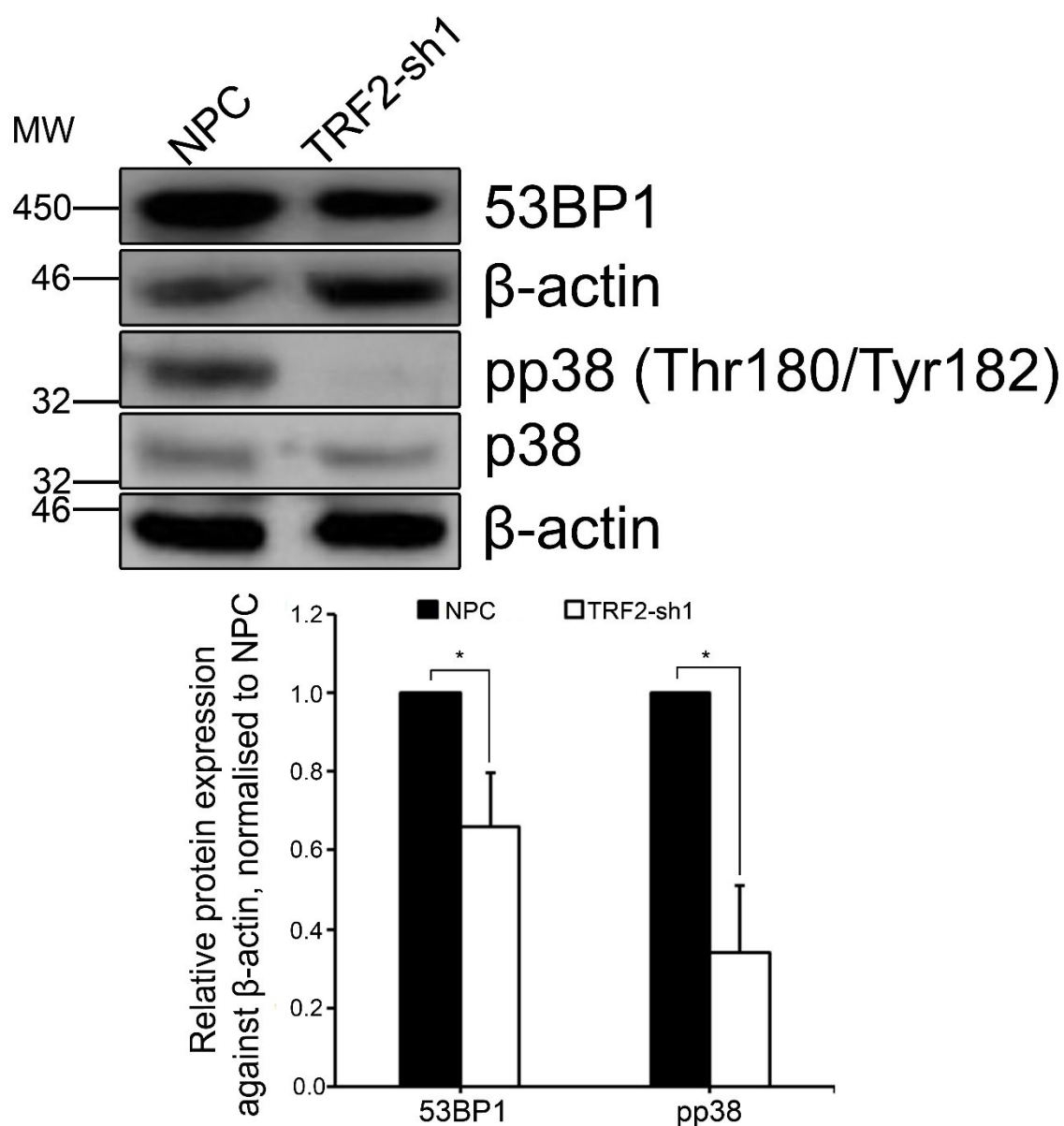


Figure 5.8: Immunoblot of other DDRs that are affected by TRF2 under standard culture conditions. *Top panel: Blot representative of 53BP1 and phospho-p38 (Thr180/Tyr182) in high TRF2 and knockdown NPCs. Total p38 was used as a control and β -Actin was used as the loading control. Bottom panel: Statistical analysis of the 53BP1 and pp38 protein level in high TRF2 and knockdown NPCs. The figure shows the analysis as mean $\pm$ SD from three independent experiments. * $p < 0.05$ using the Student's t-test.*

5.3 Discussion

In this chapter, the relationship between TRF2 and the DNA damage response pathway was described. Under standard conditions, TRF2 was shown to inhibit the activation of the ATM kinase pathway and its downstream effectors, as TRF2-KD in NPCs revealed a downregulation of the activated markers of the DNA damage response pathway. This is similar to what has been reported previously (Karlseder et al., 2004). Although, most of the reports showed that the inhibition of the DNA damage response by TRF2 is mainly conserved at the telomeres and not in other regions of the chromosome (Karlseder et al., 2004; Buscemi et al., 2009), the TRF2 expression in NPCs has been shown to be much higher than in other cell types and not restricted to the telomere region. Therefore, it is likely that the effect of TRF2 is not limited to the telomere regions. This was further assessed by looking at the co-localisation of the DNA damage response markers, namely γ H2AX, in order to determine whether it is telomeric or non-telomeric.

TRF1 antibody was used to show the location of the telomere since TRF1 specifically binds to the TTAGGG repeats of the telomere (de Lange, 2005). As the result shows that TRF2 is not restricted at the telomere when it is highly expressed, it indicates that there is an extra-telomeric function of TRF2. γ H2AX was also shown to not localise at the telomere in the standard culture condition, as it does not co-localise with TRF1.

The γ H2AX foci formation in the untreated cells raised questions whether it was due to the background of the staining or that γ H2AX foci could still be formed in standard culture condition without any stress-induced DNA damage. It has been shown that even in the standard culture condition, there is still some foci formation of γ H2AX. γ H2AX foci formation may also occur in the absence of DNA damage or during normal replication due to its involvement in cell cycle regulation (Tu et al., 2013). H2AX can be phosphorylated in mitotic cells, where more γ H2AX foci were found in cells that are in the cell cycle stage G2/M phase (McManus

and Hendzel, 2005). Therefore, the γ H2AX foci shown in the untreated cells may not necessarily be the recognition of a DNA break. A few reports have shown that lowering the level of TRF2 could expose the telomeres to be recognised as a DNA break (Cesare and Karlseder, 2012; D'Alcontres et al., 2007; Cesare et al., 2013). However, the results showed that the TRF2 levels are higher in NPCs than most of the other cell types. It is also shown to be at similar levels to that of TRF2 in other cells. Furthermore, previous reports also showed that knocking down the TRF2 in the NPCs did not increase any NHEJ and telomere fusion (Ovando-Roche et al., 2014; Cesare et al., 2009). Therefore, it is believed that the telomere protection in the TRF2-KD NPCs remains intact. Consequently, the increase of DNA damage response in the TRF2-KD NPCs could be due to the lower inhibition of the response by TRF2 in non-telomere regions. While not altering the telomere integrity, TRF2 has the priority to bind to telomeres (Karlseder et al., 2004). In addition, to confirm whether the γ H2AX foci shown in the untreated cells was due to proliferation, double staining with Ki67 should be made in order to distinguish the proliferating cells from the non-proliferating cells.

Interestingly, the different level of DDR activation did not coincide with the amount of DSB in the cells. The comet assay analysis showed that although the high TRF2 expressing cells has lower DNA damage response activation, the cells exhibit a higher amount of DSB. However, DNA breaks can sometimes occur during normal cellular replication, either through a stalled or collapsed replication fork (George et al., 2007; Cannan and Pederson, 2016; Syeda et al., 2014), or when the t-loop structure unwinds, thus exposing the 3'- and 5'-end of the telomere to be recognised as a DNA break (Sarek et al., 2015; Higa et al., 2017). This suggests that the increase in the DDR activation does not necessarily mean an increase in the DNA break. The low DDR activation shown could be due to the inhibition by TRF2. The result also indicates that the amount of DSB found in the untreated cells could be due to replication DNA break, as the cells cultured in a higher density displayed a lower amount of DSB.

The significant increase of ATM activation in the stress condition, also known as hyperphosphorylation, could be due to the initial inhibition by TRF2 in the standard culture condition. Hyperphosphorylation of ATM was reported when there is a deficiency in Rad18 after treatment with H₂O₂ (Yang et al., 2013). Therefore, inhibition by TRF2 in the standard culture conditions could also cause the hyperphosphorylation of ATM after H₂O₂ treatment. However, it was not seen in the activation of γ H2AX under stress conditions. There was still a higher expression of γ H2AX in the TRF2-KD NPCs after oxidative stress compared to the high TRF2 expressing NPCs. This showed that both cells have an almost similar increment of γ H2AX expression upon oxidative stress, which indicates that the ability to activate H2AX may not be affected by the different levels of TRF2 expression in the cells. This indicates that the activation of H2AX in the stress condition that was observed could be due to other DNA damage response pathways involved, such as DNA PK and ATR kinase, in addition to the ATM.

In addition to the ATM kinase, γ H2AX is also regulated by other DNA damage response pathways, namely DNA-PKcs (An et al., 2010), ATR (Lobrich et al., 2010) and p38 MAPK pathway (Lu et al., 2008; Dong et al., 2014). However, the ATR-dependent γ H2AX activation is through the recognition of SSB (Zou and Elledge, 2003). Due to other factors in the DNA damage response pathway that may interact with TRF2, exploring other DNA damage response markers would help to further explore the role of TRF2 in the DNA damage response.

53BP1 was chosen due to its function as a detector in the DNA damage response pathway, where 53BP1 was found to localise at the site of DNA damage within a short period of time where the damage occurred (Iwabuchi et al., 2003). It can recruit other DNA damage repair (Mochan et al., 2003, 2004; Panier and Boulton, 2014), which will repair the DNA break (Zimmerman and de Lange, 2014; Lottersberger et al., 2015; Rocha et al., 2016). In addition to its function in the DNA damage response, 53BP1 has also been found to localise at the

kinetochores of cells during mitosis, which suggests its function in cell cycle and proliferation (Jullien et al., 2002). The active form of p38 MAPK was also chosen due to recent findings that showed the direct interaction between pp38 and TRF2 (Roy et al., 2018). The involvement of these two factors in the cellular stress response and DNA damage response mechanism might help to further understand the function that TRF2 has in cell survival.

In conclusion, TRF2 seem to inhibit the ATM-dependent DNA damage response under the standard culture conditions. The increase in DDR activation in the TRF2-KD NPCs and low expressing TRF2 in HEK293T cells does not coincide with the amount of DSB in the cells. The results showed that TRF2-KD NPCs and low expressing TRF2 HEK293T cells have a lower amount of DSB as compared to the high expressing TRF2 NPCs and HEK23T cells, which suggests the increase in DDR activation is due to the lower TRF2 expression. These results suggest that there is more involvement of TRF2 with the DDR that needs to be explored further.

Chapter 6

General Discussion

This study has shown that the expression of TRF2 is specifically expressed at a high level in the hESC-derived NPCs and in the brain tissues. By genetic manipulation approaches, it was revealed that high expression of TRF2 functions to promote cell proliferation and to inhibit apoptosis, where it may serve to protect the cells. However, when the cells were subjected to different types of stress, high levels of TRF2 resulted in an increase in cell apoptosis, which indicates that TRF2 has a differential role in different cell culture conditions. The different culture conditions seemed to mainly affect the apoptosis level in the cells, as in B27 withdrawal, where the proliferation in these cells was similar. These results suggest that the differential roles of TRF2 may be due to the interaction that TRF2 has with the DDR, where TRF2 protects the telomeres by inhibiting the activation of DDR, which was shown by the lower DDR activation in TRF2-knockdown cells under the standard culture conditions. However, upon the induction of DNA damage, TRF2 was recruited at the DSB site and directly affected other DDR factors that either repair the damage or push the cells into senescence or apoptosis. The activation of DDR was increased in both TRF2 control and knockdown cells after DNA damage induction.

6.2 Specific high TRF2 expression in hESC-derived NPCs may reflect the status *in vivo*

The study on TRF2 has been gathering a lot more attention over the years, especially in terms of understanding its function and role in the biological processes. The functions that TRF2 plays in the telomeres and outside of the telomeres poses many therapeutic possibilities that can be explored and manipulated in order to remedy cancer-related or degenerative diseases. However, since most studies on TRF2 mainly involved cancer-type or immortalised cell lines, it has been difficult to hypothesise how TRF2 would function in normal human cells. As the telomeric structure and its binding proteins are different between mice and human, *in vivo* study has also been an obstacle in exploring the function of TRF2. However, this study has shown

the different expressions of TRF2 in different types of human tissues, which could establish a foundation to further explore the function that TRF2 has in human, in addition to its already known importance in the telomeres and in the DNA damage responses (Karlseder et al., 2004; de Lange, 2005). Kidney and skin tissue were chosen to represent other tissue types in the human body.

The brain tissue sample that was used in this study was the whole brain tissue that would include NPC-rich regions, such as the SVZ of the lateral ventricles or the SGZ of the dentate gyrus in the hippocampus (Reynold and Weiss, 1992; Lois and Alvarez-Buylla, 1992). Previously reported study that showed the high expression of TRF2 in the brain also did not mention the specific region of the brain, which was assumed to be the whole brain as well (Jung et al., 2004). The brain is among the organ that was found to have a short telomere length, along with skin and liver (Dlouha et al., 2014). The skin and liver are among the highly proliferative tissues in the human body, where it will regenerate in a short period of time, namely around 28 days and 6 weeks, respectively (Gurtner et al., 2008; Takeo et al., 2015; Alison and Lin, 2016). Therefore, the telomere length of these two tissues is expected to be short, as the high proliferation will cause telomere attrition and shortening. However, the brain does not replicate as much as these two tissues/organs, where most of the regeneration is done by specific cell type, such as the NPCs (Martino et al., 2011). Therefore, the short telomere observed in the brain could be due to the high expression of TRF2. In conclusion, the *in vivo* results showed that TRF2 is highly expressed in the brain compared to other tissue types, which indicate that there may be a specific function that TRF2 play in the brain.

One of the interesting findings was that the different TRF2 level in the cells does not correlate with the telomere length, where it has previously reported (Ovando-Roche et al., 2014). The high TRF2 expression in the NPCs did not shorten the telomeres in these cells and was neither

shorter compared to the lower expressing TRF2 cells, such as the hESCs, as in previous report (Ovando-Roche et al., 2014).

6.3 TRF2 holds a differential function in cell growth depending on the culture conditions

This study also showed that TRF2 promotes proliferation, not only in NPCs but also in other cell types. This finding is consistent with other studies that showed high expression of TRF2 promotes the cells to proliferate. High TRF2 expression has also been found in many highly proliferative cancer cells (Muñoz et al., 2006; Diehl et al., 2011; Chen et al., 2017).

Interestingly, within the same cell type, after reducing TRF2 protein level through shRNA manipulation, there was a significant difference observed in terms of proliferation and apoptosis in these cells. However, these differences did not affect the telomere length of the cells as shown in a previous study (Ovando-Roche et al., 2014). Even though TRF2 is highly expressed in the control NPCs, the telomeres are not shorter compared to the TRF2 knockdown NPCs, which indicates that TRF2 did not cause telomere attrition in the NPCs (Ovando-Roche et al., 2014). However, other studies did report that TRF2 affects the telomere length, where overexpression of TRF2 in human cancer cell lines and human fibroblasts will cause the telomere to be shorter (Smogorzewska et al., 2000; Richter et al., 2007; Nera et al., 2015). This difference could be due to the cell types that were used. In contrast to the NPC, which endogenously expressed high levels of TRF2, the fibroblasts express low levels of TRF2. Therefore, the exogenous overexpression of TRF2 could cause the telomere to shorten due to the negative regulation by TRF2. The high increase in TRF2 in the other cell types will affect the binding of other regulatory proteins to the telomeres that help to regulate the telomere length, such as the TERT (Bandaria et al., 2016). It could also be that the telomere length was

not affected by the high TRF2 expression in the NPCs due to the high TERT expression and high telomerase activity (TA) that helped to maintain the telomere length in these cells, as reported in previous studies (Klapper et al., 2001; Cheng et al., 2007). Although TERT has also been reported to be highly expressed in the adult mouse brain, the TA is reduced in adult neurons (Ishaq et al., 2016).

In addition to the telomere length, the high proliferation observed in the high TRF2 expressing cells did not affect the cell cycle, where an almost similar number of cells were found in every cell cycle stage in these two cell types. The high proliferative cells have a similar number of cells in each cell cycle compared to the slow proliferating cells. However, the rate of each cell cycle may be different. The highly proliferative cells could have a shorter period during each cell cycle stages (Alberts et al., 2002; Matson and Cook, 2017), as shown in the pH3 staining that there are more cells that are positively stained with pH3 in the highly proliferative cells. The pH3 only stains the actively dividing cells during the mitosis stage and will not stain cells that are senescent (Tsuta et al., 2011; Kim et al., 2017; İlhan et al., 2018). Therefore, the findings indicate that high TRF2 promotes proliferation by increasing the rate of cell division and does not affect the cell cycle. The low proliferation rate observed in the TRF2 knockdown cells may indicate that lack of TRF2 hindered the replication process. Low TRF2 has higher DDR factor levels, such as γ H2AX. This could be due to stalled replication fork, hence activating the ATR, which will then phosphorylate H2AX. In addition to the role of TRF2 in promoting proliferation, TRF2 was also shown to help protect the cells from apoptosis. However, the mechanism involved is not yet known.

From this study, it has been found that although in the standard culture condition, TRF2 seems to have a protective role in the cells. However, it has an opposite effect under stress conditions, where TRF2 seems to promote apoptosis. This differential effect of TRF2 in different culture conditions indicates that TRF2 was not only unable to protect the cells from stress, but also

seemed to promote the cells to die. However, the mechanisms underlying this phenotype is yet to be explored (see next section for further discussion).

The role of TRF2 in cells that undergo stress conditions is scarcely investigated. However, there are some studies that have reported the effects that TRF2 has on cells under stress conditions, where TRF2 was shown to localise at the nucleolus, which has been reported to be important in ribosomal RNA transcription, processing, modification, and ribosomes assembly (Zhang et al., 2004; Sommerville et al., 2005; Olsen and Dundr, 2005). Overexpression of TRF2 was able to rescue the rRNA inhibition under stress conditions, as well as able to promote cell-cycle arrest in hepatocarcinoma cells (Yuan et al., 2018). Nevertheless, most studies on TRF2 in cells under stress conditions would still either focus on the role that TRF2 has at the telomeres (Bakkenist et al., 2004; Ye et al., 2010; Hewitt et al., 2012) or the effect of TRF2 inhibition together with drug-treatments to induce apoptosis in cancer cells (Roy et al., 2018). Previous studies on the effect of TRF2 in normal cells under stress conditions mainly focused on its function at the telomere or through eliminating (knockout) TRF2 from the cells (Blanco et al., 2007; Richter et al., 2007; El Maï et al., 2014). Knocking out TRF2 from the cells would cause cell death. Therefore, any increment of apoptosis observed due to stress or drug treatments in the TRF2-knockout cells can be inconclusive.

One interesting study that reports on similar findings to this current research showed that REST-dependent TRF2 in non-neuronal cancer cells to be resistant to oxidative damage (Kwon et al., 2013). Since TRF2 is expressed at a low level in non-neuronal cells, the report suggests that in a low TRF2 expressing cells, TRF2 protects the cells from oxidative stress, which is comparable to what has been shown in this current study. However, this study did not address whether the TRF2 in the NPCs and HEK293T that were used was REST-dependent or not.

6.4 The differential effect of TRF2 is due to its interaction with the DNA damage response pathway

TRF2 has been shown to be a protein hub that interacts with different proteins, either at the telomeres (van Steensel, Smogorzewska and de Lange 1998; Karlseder et al., 1999; de Lange et al., 2005) to protect the chromosome end, or outside of the telomeres (Roy et al., 2018). Little is known on the extra-telomeric functions of TRF2, in addition to its interaction with REST, which promotes neural differentiation (Zhang et al., 2008; Ovando-Roche et al., 2014); as well as the effect that TRF2 has with the DNA damage response pathway (Bradshaw et al., 2005; Huda et al., 2009, 2012).

At the telomere, TRF2 is known to bind to the ATM kinase and will inactivate ATM-dependent DNA damage response in order to protect the telomeres from being recognised as a DNA break site (Karlseder et al., 2004). This is similar to what has been shown in this study, in the standard culture condition, TRF2 inhibits the activation of ATM-dependent DNA damage response, where a decrease in TRF2 was shown to increase the activation of ATM-dependent DDR factors. However, the inhibition of TRF2 towards the ATM-dependent DDR could either be conserved at the telomeres or also be involved with other parts of the chromosome. This was due to the localisation of γ H2AX shown in the results was not only at the telomeres but also at other chromosomal regions. Nevertheless, it could be theorised that in the standard culture condition, most of the DNA damage response inhibition by the TRF2 is at the telomeres, as there will be little amount of DSB occurring during normal replication that will activate the DNA damage response.

TRF2 has also been found to be localised at the site of DNA damage within the short period of time upon the induction of DNA break (Bradshaw et al., 2005). The localisation of TRF2 at the site of the DSB was shown to be transient and dynamic (Bradshaw et al., 2005). This

transient localisation of TRF2 is thought to be due to the phosphorylation of TRF2 at Thr188 by the ATM Kinase, which enables it to bind to the DNA damage site (Tanaka et al., 2005; Huda et al., 2012). Phosphorylated-TRF2 was also found to localise at the DNA break site within 30 minutes of post-irradiation and was gone after 2 hours (Tanaka et al., 2005). The mechanisms on how TRF2 was recruited to the DNA damage site and the function that TRF2 plays in the DNA damage response pathway is still unknown.

Figure 6 shows the proposed diagram on the effect that TRF2 has with the ATM-dependent DNA damage response pathway in the cells under standard conditions and under stress conditions. In standard conditions, it is proposed that the TRF2 inhibition of the ATM kinase pathway is mainly at the telomeres, as what has been reported previously (Karlseder et al., 2004). In stress conditions, the ATM affects the recruitment of TRF2 to the DNA break site. 53BP1 and γ H2AX have other factors, such as the DNA-PKcs that could also recruit them to the DNA break site (Zimmermann and de Lange, 2014; Podhorecka et al., 2010).

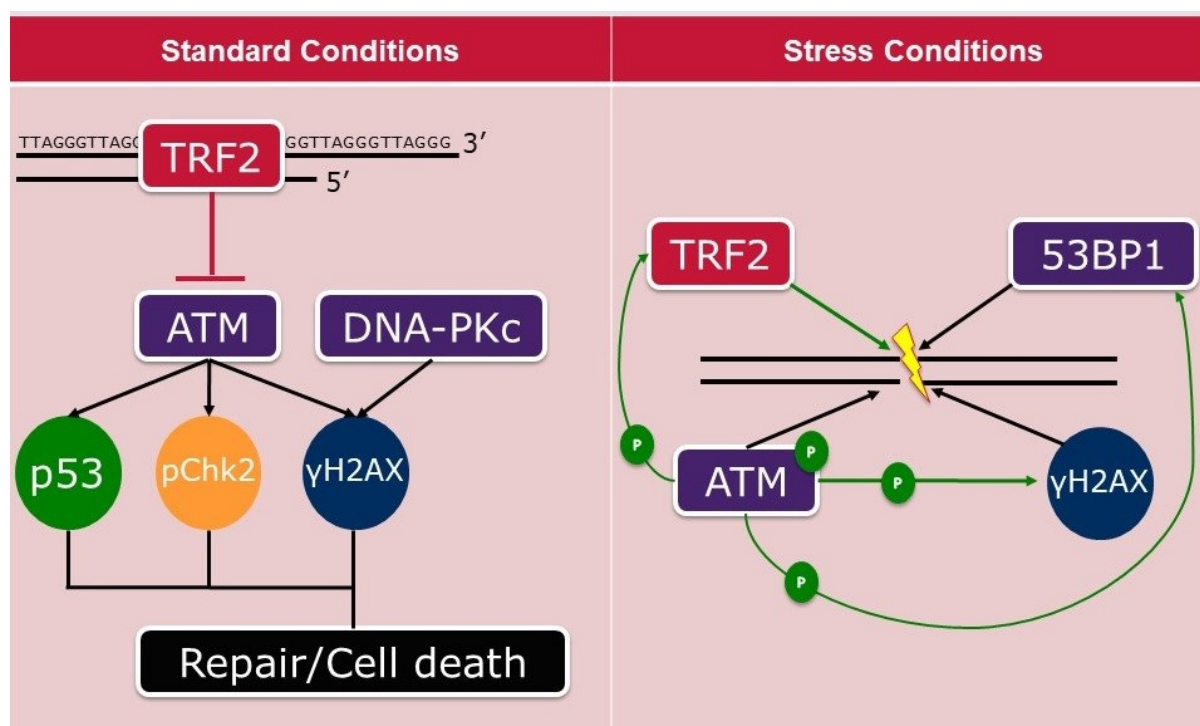


Figure 6: Proposed diagram for TRF2 effects on the DNA damage response under standard conditions and stress conditions.

Other than the ATM-dependent DNA damage response, TRF2 has also been found to bind with p38 MAPK, as well as to affect 53BP1, where high TRF2 expressing cells will also express a high expression of 53BP1 as shown in the results in Chapter 5. This indicates that TRF2 also directly or indirectly affects other DNA damage response pathways. In addition to the direct interaction that TRF2 has with the p38 MAPK (Roy et al., 2018), the mechanisms on how TRF2 is affecting the other DNA damage responses, namely the 53BP1 is still unknown.

6.5 Future Work

There are many limitations to this study. However, this research helped to lay out the foundation in further understanding of the role and function that TRF2 has in human. One of the future works that can be done in relation to the function of TRF2 in human cells is the mechanism of TRF2 in regulating cell proliferation. Since the cells that express a high level of TRF2 will have a significantly higher proliferation, there must be a role that TRF2 has in promoting the cells to highly proliferate. It could be due to the role that TRF2 plays as a protein

hub, which helps TRF2 to interact with many different proteins, such as RTEL1 that is recruited to the telomeres by TRF2 to help the unwinding of t-loop for DNA replication during S-phase (Sarek et al., 2015). TRF2 was also found to bind with WT1 and activates the PDGFR β promoter to promote proliferation and regulates angiogenesis in tumour cells (El Maï et al., 2014). However, the study on the interactions of TRF2 with the cell proliferation factors are still limited. TRF2 may directly or indirectly be involved in promoting cell proliferation.

Another important part to further investigate is the mechanisms on how TRF2 is affecting the DNA damage response pathway, specifically with the ATM kinase pathway upon induction of DNA break. At the telomeres, Karlseder et al. (2004) showed that TRF2 directly interacts with the ATM and inhibits the autophosphorylation of ATM at Serine 1981. However, this inhibition by the TRF2 has not been explored at other parts of the chromosome. As this study has shown that the γ H2AX expression under standard condition is not only conserved at the telomeres, it is worth to explore whether TRF2 will also inhibit ATM kinase in other parts of the chromosome and to investigate the mechanisms involved.

In addition to the effects TRF2 has on ATM-dependent DNA damage response, TRF2 has also been reported to be recruited at the site of the DNA break site. There are multiple theories as to how TRF2 is recruited and the binding sites of TRF2 at the DNA break site (Huda et al., 2009; Tanaka et al., 2005) as well as the role TRF2 plays in DDR (Mao et al., 2007; Roy et al., 2018). Therefore, to further show how TRF2 affects DDR, mutation analysis on the TRF2 control and knockdown cells in the standard culture conditions and after the induction of DSB, would be able to help in understanding the role of TRF2. DSB can be induced at a specific region through transient expression of I-SceI Endonuclease and the site could be monitored for repair either through NHEJ or HR (Bellaïche et al., 1999; Seluanov et al., 2004).

In order to ascertain whether TRF2 may have an effect in the DDR mechanism, further experiment is needed to test the difference in repair kinetics after DNA damage induction between high and low TRF2 expressing cells. This could be done through analysing the comet assay of the cells post-treatment at different time points. It is hypothesised that cells with higher TRF2 level would have the ability to repair the DNA damage faster than cells with lower TRF2 level. Therefore, the high TRF2 expressing cells might have a shorter comet tail compared to the low TRF2 expressing cells, after a certain period of time post-treatment.

6.6 Implications on development and disease

TRF2 is involved in the protection of the telomeres from end-to-end chromosome fusion and suppress checkpoint activation that will lead to senescence or apoptosis (de Lange, 2002). The role of TRF2 at the telomeres could help to further explore the telomere attrition causes in degenerative diseases, which will assist in protecting the telomere end from early degradation. Therefore, TRF2 will also protect from senescence and aging related diseases, such as Werner Syndrome, where TRF2 was found to directly bind with WRN and mediates WRN exonuclease activity, which is important for protecting against premature senescence and maintaining genomic stability (Machwe et al., 2004). Overexpression of TRF2 has also been shown to cause shorter telomeres, which is one of the main causes of degenerative diseases, cancer and aging (Muñoz et al., 2006).

The high expression of TRF2 in various cancer cells also poses therapeutic possibilities, where knocking out TRF2 will cause massive cell death (Biroccio et al., 2013). The role of TRF2 in DNA damage response could further help to understand the mechanism in cancer development and could also help in reducing its progression.

From this study, it has been confirmed that TRF2 is specifically higher in the NPCs, which are abundant in developing brain, rather than in adult brain. The effect that was observed in this

study, where high TRF2 promotes proliferation, suggests that there may be a higher chance of tumour development in developing brain compared to adult brain. Previous report concurs that childhood brain cancer was found to be the most common type of paediatric solid tumour (Johnson et al., 2014). The high prevalence of brain cancer among children could be due to the high TRF2 expression in the brain, specifically in the developing brain.

In addition to the role that TRF2 plays in promoting proliferation, it has also been found that TRF2 protects the cells from apoptosis in the standard culture condition and promotes apoptosis in the stress conditions. This differential role of TRF2 could explain the pathology of some neurodevelopmental diseases that occurred when stress was induced during gestation that causes severe damage to the brain before causing any further damage to other organs, such as cerebral palsy. Furthermore, in understanding the function of TRF2 in cell survival and its involvement in DDR could have a potential in therapeutically reducing the impact of such neurodevelopment impairment or in preventing it. It could also have a potential in drug treatments or gene targeting in treating diseases that may involve TRF2.

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