Electron microscopy and biochemical analysis of native telomeric chromatin

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**NANYANG
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**ELECTRON MICROSCOPY AND BIOCHEMICAL
ANALYSIS OF NATIVE TELOMERIC CHROMATIN**

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SCHOOL OF BIOLOGICAL SCIENCES

2019

ELECTRON MICROSCOPY AND BIOCHEMICAL ANALYSIS OF NATIVE TELOMERIC CHROMATIN

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SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2019

Statement of Originality

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Date: January 25, 2019

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Abbreviations

ALT:	Alternate Lengthening of Telomeres
APBs:	ALT-associated Promyelocytic leukemia Bodies
bp:	base pair
CAS9:	CRISPR associated protein 9
CEMOVIS:	Cryo Electron Microscopy Of Vitreous Sections
CLEM:	Correlative Light and Electron Microscopy
CRISPER:	Clustered Regularly-Interspaced Short Palindromic Repeats
Cryo-EM:	Electron Cryo- Microscopy
DAB:	Diaminobenzidine
DTT:	Dithiothreitol
EDTA:	Ethylenediaminetetraacetic acid
eGFP:	Enhanced Green Fluorescence Protein
EtBr:	Ethidium Bromide
kDa:	kilo Daltons
MNase:	Micrococcal Nuclease
PML:	Promyelocytic Leukemia
POT1:	Protection Of Telomeres 1
Rap1:	Repressor/Activator Protein 1

SCE:	Sister Chromatid Exchange
SDS-PAGE:	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
STORM:	Super-resolution fluorescence imaging by Stochastic Optical Reconstruction Microscopy
T-SCE:	Telomere Sister Chromatid Exchange
TBST:	Tris-Buffered Saline and Tween 20
TER:	Telomerase RNA component
TERRA:	Telomeric End Repeat-containing RNA
TERT:	Telomerase Reverse Transcriptase
TIN2:	TRF1- Interacting Nuclear Protein 2
TPP1	TINT1/PTOP1/PIP1
TRF1:	Telomere Repeat-binding Factor 1
TRF2:	Telomere Repeat-binding Factor 2

Summary

Human telomeres are G-rich sequence repeats of DNA that span the terminal ends of chromosomes with an average length of about 10,000 base pairs. Telomeric DNA acts as a platform for the recruitment of a six protein-complex called shelterin. The shelterin complex protects chromosome ends from fusion and genome instability. Telomeric DNA is progressively shortened during each round of cell division, which leads to replicative senescence in somatic cells. For indefinite proliferation, approximately 85% of human cancer cells maintain their telomere length through reactivation of an enzyme called telomerase. The other 15% of human cancer cells use the Alternative Lengthening of Telomeres (ALT) pathway. Telomeres contain nucleosomes and a wealth of information exists on the functional role of telomere-binding proteins that regulate telomere length. However, little is known about the higher-order structure of telomeric chromatin in mammalian cells. Understanding the structure of telomeres will give insights into the interplay between nucleosomes and shelterin in the assembly of telomeric chromatin and regulating access to telomeric DNA, which ultimately regulates telomere length.

The aim of this study is to obtain insight into the structural organization of mammalian telomeric chromatin using electron microscopy (EM). To achieve this, three approaches were employed. First, the isolation of native telomeric chromatin from human and mouse cells. Second, *in-vitro* reconstitution of telomeric DNA with a shelterin protein called Telomeric Repeat-binding Factor 1 (TRF1). The presented data show that it is challenging to prepare samples suitable for EM analysis using these two approaches, due to protein loss during isolation or aggregation of the reconstituted material. The third approach used in this study and most successful was to apply correlative light and electron microscopy (CLEM) to visualize the ultrastructure of telomeres *in-situ*. For this experiment, I used a GFP-APEX2 tandem probe to selectively label TRF1 and telomeric chromatin in ALT cancer cells (human osteosarcoma U2OS cell line). CLEM and electron tomographic analysis shows that the telomeres closely associated with non-telomeric heterochromatin near the nuclear envelope. Inspection of electron micrographs reveals that ALT telomeres have a mean diameter of 345 nm and irregular shape. Furthermore, image analysis reveals that each telomere is composed of a complex network of interconnected fibers with a diameter that ranges from 10 nm to 35 nm.

1 Introduction

1.1 Telomeric chromatin

The ends of eukaryotic linear chromosomes are called telomeres or telomeric chromatin. Telomeres are composed of G-rich DNA (1, 2), histones, shelterin proteins (3) and a non-coding RNA transcript called telomeric repeat-containing RNA (TERRA) (4). Telomeric DNA consists of 'TTAGGG' repeat sequences that end with a G-rich single-stranded overhang (the G-overhang). Telomere length varies among organisms and cell types. In humans, the length of the double-stranded region range from 2,000-20,000 base pairs (bp's) and the single-stranded region between 50-500 nucleotides (5, 6) (Figure 1).

Figure 1: Schematic illustration of human telomeric DNA. Human telomeres are composed of tandem 'TTAGGG' repeats sequences. The double-stranded region is 2,000-20,000 base pairs long. The single-stranded G-overhang between 50-500 nucleotides (7).

Telomeres undergo progressive shortening of DNA during DNA-replication, which is termed the 'end-replication problem'. Telomeres neutralize this problem by recruiting telomerase, a reverse transcriptase that adds telomeric DNA to telomeres (Figure 2). Telomeres also offer a solution against 'end-protection problem' by protecting chromosomal termini from being recognized as double-strand breaks and activation of DNA repair mechanisms (8) (Figure 2).

Figure 2: Schematic illustration of chromosome-end protection and replication problems. Identification of chromosome-ends as DNA damage foci is termed as the end-protection problem (left-hand side). During DNA replication, gradual loss of DNA at chromosome-ends is called end-replication problem (right-hand side) (9). Reprinted by permission from Springer Nature.

1.2 End-replication problem

Semiconservative DNA replication cannot completely copy the extreme ends of the linear chromosomes (10). As a result, telomeric DNA is progressively lost during each round of cell division. Cells undergo replicative senescence when telomeres reach a critically short length. This limit, called the Hayflick limit, occurs after 40 to 60 rounds of cell division in human somatic cells. Hence, telomere length serves as an ageing clock that prevents indefinite division (11). Elizabeth Blackburn discovered an enzyme, called telomerase, that can add telomeric DNA repeats to maintain telomere length (12). Telomerase prevents replication-dependent loss of telomeric DNA and cellular senescence in constitutively dividing germ-line and embryonic stem cells (31). Telomerase is not expressed in most human somatic cells. Cancer cells manage to escape senescence by acquiring a mechanism to maintain their telomere length. In 85-90 % of cases, cancer cells acquire the ability of indefinite proliferation by telomerase

reactivation (13). Thus, telomerase is active in embryonic stem cells, inactive in somatic cells and reactivated in cancer cells. Some cancer types use an alternative to telomerase called Alternative Lengthening of Telomeres (ALT) pathway for telomere maintenance.

1.2.1.1 Alternative lengthening of telomeres

Alternative lengthening of telomeres (ALT) was discovered in yeast by Lundblad and Elizabeth Blackburn 1993 who noticed that some telomerase-null yeast cells were able to maintain their telomere length without telomerase. This phenomenon was observed to be dependent on the RAD52 gene, which encodes a homologous recombination protein (14). Subsequent studies demonstrated that such a telomerase-independent mechanism of telomere maintenance also exists in mammalian cells (15, 16). Although an abrupt increase or decrease in the telomere length had indicated events of recombination in telomerase-negative cells (17), the first direct evidence was provided by Roger Reddel who reported that a DNA tag inserted into a single telomere of telomerase-negative cells could be copied to other telomeres (18). This demonstrated that telomere elongation in ALT cells involves DNA recombination (19). Homologous-recombination dependent 'unequal telomere sister chromatid exchange' and 'homologous recombination-dependent telomeric DNA-replication' are two models that have been proposed for ALT. The first model relies on 'Sister Chromatid Exchange' (SCE), a process by which equivalent segment of chromosomes are exchanged between sister chromatids through homologous recombination. Telomere Sister Chromatid Exchange (T-SCE) is several orders of magnitude more prevalent in telomerase-negative cells (20). During 'unequal telomere sister chromatid exchange', as the name suggests, a small segment of one telomere is exchanged

for a significantly larger segment of the other telomere. The resultant chromosome has unequal telomere length, one of which is inherited by each daughter cell. The cells with long telomeres would undergo more replications and eventually outcompete cells with short telomeres (21) provided long telomere are segregated into one daughter and every shortened telomere into the other. However, no such mechanism has been observed to date and this hypothetical model awaits experimental validation (22).

The homologous recombination-dependent telomeric DNA-replication model has gained broader acceptance in the field due to its agreement with experimental observations (18). According to this model, a single-stranded telomere end invades the double-stranded region and uses it as a template for DNA replication to extend telomere length. The alternative lengthening of telomere does not necessitate the involvement of a telomere from another chromosome (23). This mechanism also allows extrachromosomal telomeric DNA, that constitutes ~15% of total telomeric DNA in ALT cells, to be used as a template (19, 24, 25).

Figure 3: Schematic illustration of two models for alternate lengthening of telomeres (ALT). **a:** Unequal telomere sister chromatid exchange model where a small segment of one telomere is exchanged for its larger counterpart of the other telomere. **b:** Homologous recombination-dependent telomeric DNA-replication model, where the single-stranded telomeric G-overhang is used as a template and invades the double-stranded region of telomeric DNA. Figure originally published in (19) is reprinted by permission from Springer Nature.

The size of telomeres in ALT cells ranges from undetectable to > 50 kb in length (24). A subset of ALT chromosomes ($\sim 7\%$) does not appear to contain telomeric DNA at all. These telomere-free chromosomes have been proposed to be a by-

product of DNA recombination (24, 26). On the other hand, it has been observed that a subpopulation of telomeres in ALT-cells forms telomere clusters (27) which likely mediate telomere-telomere recombination and drive targeted telomere insertions in the chromosome (28). Another striking character of ALT-cells is the presence of extrachromosomal DNA in the form of single or double-stranded DNA circles called 'c-circles' and 't-circles', respectively. These DNA molecules vary in size ranging from 0.7- 57 kb in length and are proposed to serve as a substrate for homologous recombination-dependent telomere elongation (24, 29, 30). Although the extra-chromosomal telomeric DNA has also been reported in yeast, plants, vertebrates and even in telomerase positive cell lines (31-33), its high abundance in ALT cells is generally considered as a hallmark of telomerase-negative cancers. ALT cells also contain ALT-associated promyelocytic leukemia bodies (APBs). These doughnuts shaped nuclear structures vary in number (10-30/cell) and size (0.1-0.5 μm). APBs are known to contain PML protein (ProMyelocytic Leukemia protein), telomeric DNA, telomeric proteins hTRF1 and hTRF2 and a number of proteins involved in DNA synthesis and recombination (19, 34, 35).

While telomerase-dependent telomere maintenance is generally adopted by epithelial-origin carcinomas, cancers of mesenchymal origin more frequently activate ALT (36, 37). Why ALT is activated in mesenchymal cancers is unclear. Since mesenchymal stem cells express low amounts of telomerase (38), it is proposed that constitutive repression of telomerase may predispose cells from this lineage to adopt the ALT mechanism (39).

The highest incidence (59%) of ALT cancer is observed in Osteosarcomas (bone cancers). Other soft tissue sarcomas, diffuse malignant pleural mesothelioma,

astrocytoma gastric carcinoma are also examples of cancers with a high frequency of ALT characteristics (reviewed in (40)). The U2OS and SaOS-2 (human osteosarcoma), WI-38 VA13/2RA (SV40-immortalized human lung fibroblast), GM00847 (human fibroblasts), LB188 (rhabdomyosarcoma) cell lines are commonly used in the research laboratories to study alternate lengthening of telomeres (24, 41-43).

1.3 End-protection problem

Eukaryotic linear chromosome ends must be distinguished from broken DNA that requires repair. DNA damage activates ATM (Ataxia Telangiectasia Mutated) and ATR (Ataxia Telangiectasia Rad3 related) protein kinases. ATM and ATR recognize double and single-stranded DNA breaks, respectively and recruit other DNA damage response proteins to DNA damage foci. Telomeres repress ATM and ATR kinase signaling and protect chromosome ends from non-homologous end joining (NHEJ) and homology-directed repair (HDR) (7). Unprotected chromosome ends are considered as broken DNA and repaired by NHEJ and HDR DNA repair pathways. NHEJ results in chromosome end-to-end fusions (44) whereas activation of HDR results in variable and unstable telomeres leading to genome instability (7). Cytological and genetic data show that dysfunctional telomeres associate with DNA damage proteins such as 53BP1, γ -H2AX, Rad17 and ATM (45). Under physiological conditions, activation of DNA damage responses, which is a hallmark of telomere dysfunction, leads to replicative senescence. In this case, the telomere attrition progresses to the level where its length is critically short and insufficient to recruit the minimum number of shelterin proteins necessary to uphold end protection. The resultant unprotected telomere

gives rise to constitutive overactivation of DNA damage response that could neither add telomeric DNA repeats nor recruit additional shelterin complexes. This persistent DNA damage response – through the ATM pathway – halts cellular proliferation through p53 tumor suppressor and regulating transcription factors. What follows this series of cellular events is irrevocable cell cycle withdrawal – a hallmark of cellular senescence (46). Conversely, inactivation of the p53 allows telomere attrition to continue thereby circumventing senescence, which results in telomere-telomere fusions (47).

Shelterin proteins play a fundamental role in protecting telomeres from being recognized as damaged DNA foci. The role of shelterin in protection against the activation of DNA damage pathways was first reported by Titia de Lange group in 2002. They detected upregulation of ATM kinases upon telomere-repeat binding factor 2 (TRF2; a shelterin protein) deletion in human lung IMR90 fibroblasts (44). Subsequent studies showed that RNAi-mediated inhibition of protection of telomeres 1 (a shelterin protein) upregulates ATR kinases in HeLa cells, indicating that POT1 suppress this second major checkpoint activator (48). Given the close interaction and structural interdependence among shelterin proteins, it is challenging to delineate the exclusive outcome upon individual protein depletion. A comprehensive study conducted on mouse embryonic fibroblast cells (MEFs) has shown that a double knockout of TRF1/TRF2 results in shelterin-free telomeres. Experiments using ligase IV (NHEJ repressor), Ku80 (HDR repressor) and 53BP1 (DSB repair) deficient MEFs showed that removal of the shelterin proteins activates as much as six different DNA damage responses; ATM and ATR signaling, classical-NHEJ, alt-NHEJ, homologous recombination, and 5' - resection (8). Therefore, shelterin in general and TRF1

and TRF2 specifically are essential for protecting telomeres from being misrecognized as damaged DNA foci.

1.4 Shelterin

Titia de Lange's group primarily identified a number of telomere-specific proteins that form a complex named 'shelterin' (3). The composition, structure and function of shelterin have diverged across species (49). Despite some divergence in protein composition, the role of shelterin in telomere protection is conserved. Different organisms have different proteins that bind to the double-stranded telomeric repeats and to the single-stranded G-overhangs. In mammals, the shelterin proteins include Telomere repeat binding factor 1 and 2 (TRF1, TRF2), TRF1- interacting nuclear protein 2 (TIN2), Repressor/activator protein 1 (Rap1), TINT1/PTOP1/PIP1 (TPP1) and Protection of telomeres 1 (POT1). Shelterin-related complexes are also found in other eukaryotes. For instance, telomeres of *Schizosaccharomyces pombe* contain Taz1 (a TRF homologue), Rap1, Pot1, Tpz1, Poz1 and Ccq1. Telomeres of *Saccharomyces cerevisiae* contain chromosome end-binding proteins such as Rap1, Cdc13, Stn1 and Ten1 in lieu of shelterin complex (50).

TRF1 and TRF2 bind to the double-stranded region of telomeric-DNA (51). Rap1 lacks direct DNA binding capacity and localizes to telomeres through its binding to TRF2 (Figure 4). TIN2 binds to TRF1, TRF2 and binds TPP1 (52). POT1 binds to the single-stranded G-overhang and interacts with TPP1 (53) (Figure 4). Quantitative immunoblotting experiments have shown that TRF2 is slightly more abundant than TRF1 in HeLa cells. TRF2 and Rap1 were present at 1:1 stoichiometry and there were sufficient amounts of TIN2 to bridge TRF1 and

TRF2. TPP1 and POT1 were present at 1:1 stoichiometry, however, 2-10 fold less abundant compared to the other shelterin proteins (54).

Figure 4: Schematic illustration of telomere structure. Telomeres are the ends of linear chromosomes. Telomeric DNA acts as a platform for the recruitment of six shelterin proteins. TRF1 and TRF2 bind to the double-stranded region of telomeric DNA. Rap1 interacts with TRF2. POT1 binds to the single-stranded G-overhang in complex with TPP1. TIN2 interacts with TRF1, TRF2 and TPP1.

1.4.1.1 Double-stranded telomeric-DNA binders: TRF1 and TRF2-Rap1 complex

TRF1 was identified based on its *in-vitro* specificity for telomeric tandem repeats (55, 56). TRF2 was identified as TRF1 paralog in bioinformatics based data search (51). TRF1 and TRF2 are homodimeric proteins that interact with TIN2, which links them together with TPP1-POT1 (Figure 4). Simultaneous depletion of

TRF1/TRF2 results in telomeres completely devoid of shelterin proteins resulting in the activation of DNA damage responses (8). Structural and biochemical studies have provided the evidence that conditional deletion of TRF2 in MEFs triggers DNA damage response and leads to chromosome fusion (Figure 5). TRF2 deletion is also shown to abrogate 'T-loop' formation (57); a lasso-like structure proposed to be adopted by telomeres (ref. section 1.7.1.2, Figure 11).

Figure 5: Metaphase spreads of chromosomes in the presence **(A)** and absence **(B)** of TRF2. Green: telomeric FISH with a FITC-conjugated peptide nucleic acid [CCCTAA]³ probe. Red: DNA stained with DAPI (57). Reprinted with permission from Elsevier.

TRF1 and TRF2 contain C-terminal DNA-binding domains (SANT/Myb or homeodomains) and a dimerization domain called the TRF homology domain (TRFH). Depending on the protein, both domains are joined by a 100-200 amino acid long flexible hinge region (58). Figure 6 shows interactions and X-ray crystallographic structures of TRFH and the homeodomains of TRF1 and TRF2 bound to DNA published by Daniela Rhodes' group (59, 60). Whilst the two TRFs have the same architecture, the N-terminus of TRF2 has basic Gly/Arg-rich (GAR) domain whereas TRF1 contains an acidic domain at its N-terminus.

Human Rap1, unlike its counterpart in yeast, does not bind DNA and localizes to telomeric chromatin through TRF2 (61). Rap1 knockout results in differential expression of a number of genes in human cells, which suggest its role in transcriptional regulation (62). The absence of Rap1 does not induce telomere fusion, telomere length changes and other telomere defects (63). Rap1 contains an N-terminal BRCT domain, a C-terminal TRF2-interacting domain and centrally positioned Myb domain. Figure 6 shows known interactions and structures of C-terminal TRF2 interacting domain and Myb domain that were solved by X-ray crystallography (64) and NMR (65), respectively.

1.4.1.2 Single-stranded telomeric DNA binder: POT1

POT1 is the most well-conserved of the shelterin proteins. It was identified by sequence homology search against telomere end-binding proteins (TEBP) in unicellular organisms (66). POT1 interacts with TPP1 by its C-terminal region. *In-vitro* studies have demonstrated that TPP1-POT1 complex increases the activity and processivity of the human telomerase core enzyme by a factor of 2-3 (67). POT1 binds to single-stranded telomeric DNA with two N-terminal oligosaccharide /oligonucleotide binding (OB) domains. Figure 6 shows domain interactions of POT1 and structure of its OB domains bound to single-stranded telomeric DNA as determined by X-ray crystallography (68).

1.4.1.3 Bridging proteins: TIN2, TPP1

TIN2 bridges TRF1 and TRF2 and recruits TPP1 to telomeres. Deletion of TIN2 abolishes localization of other shelterin components and up-regulates ATR and ATM kinases (52). Deletion of TIN2 in mouse embryonic fibroblast cells compromises TPP1/POT1 binding to the telomere. In the absence of TIN2, TPP1/POT1 fails to block ATR activation. TIN2 mutations, such as substitution of

lysine 280 with glutamic acid have been reported to associate with Dyskeratosis congenital, a premature ageing disorder with impaired telomerase function (69).

The N-terminal region of TIN2 interacts with TRF2 (70) and TPP1 (52), whereas its C-terminal FXLXP motif interacts with the TRFH domain of TRF1. Figure 6 shows the TIN2 domain interactions. The structure of TIN2 peptide (amino acid 256- 276) in-complexed with the TRFH domain of TRF1 was determined by X-ray crystallography (Figure 6B) (67).

TPP1, a mammalian counterpart of telomere end-binding protein (TEBP) in unicellular organisms, was discovered from the database searches of TIN2 interacting proteins (71). TPP1 plays a crucial role in telomerase recruitment at telomeres. *In-vitro* studies have provided the evidence that telomerase acts preferentially on the telomeric DNA that is coated with TPP1/POT1 (72). TPP1 contains an N-terminal OB domain, a C-terminal TIN2 binding domain and a centrally located POT1 binding domain. N-terminal OB domain of TPP1 directly associates with the TERT subunit of telomerase during its recruitment at telomeres. Deletion of TPP1 OB domain showed impaired telomere elongation in HTC75 cell lines (73). Figure 6 shows the domain interactions of TPP1 and the structure of its OB domain that was determined by X-ray crystallography (74).

Figure 6: Published structures, domain organization and interactions of the shelterin proteins. **A:** The interacting domains and DNA binding sites are indicated by double-arrow links. Daniela Rhodes' group kindly provided the interaction map. **B:** The X-ray crystal structures of the shelterin proteins. The structures were rendered using the Chimera software from UCSF. PDB IDs used (from left to right): 1FEX (65), 3K6G (64), 1H6P (59), 1W0U (60), 1H6O (59), 1W0T (60), 1XJV (68), 2I46 (67), 3BQO (74).

1.5 TERRA

In 2007, Joachim Lingner's group discovered that telomeres (the G-rich strand) are transcribed into noncoding RNA termed TERRA, which remains associated with telomeric heterochromatin (4). TERRA has been implicated to play a crucial role in telomere length regulation, telomere replication, telomeric DNA damage response, telomeric chromatin composition changes, and telomere mobility [reviewed in (75)]. However, the molecular mechanisms by which TERRA carry

out these functions at a given stage of cell cycle remains to be understood (75, 76).

1.6 Chromatin context of telomeres

Whilst research focus has been directed at identifying shelterin components and their functional role, it has been known for almost three decades that telomere contains nucleosomes (77). Nucleosomes are the basic structural unit of chromatin in eukaryotes. The nucleosome core contains 146 base pairs of two-turn superhelix DNA that tightly wraps around a histone octamer composed of two copies of each histone protein, H2A, H2B, H3 and H4 (Figure 7A). Nucleosomes are connected by short linker DNA into arrays that give them 'beads-on-a-string' organization in an electron microscope (Figure 7B) (78). Nucleosomal array appears as 10 nm in diameter in low ionic strength, hence termed as '10 nm fiber'. Linker histone H1 binds to nucleosome cores and linker DNA and induces compaction of chromatin fibers. The nucleosomal array can be folded into '30 nm fiber', whose formation is dependent on the presence of linker histone. The *in-vivo* existence of 30 nm fiber is debated [reviewed in (79)]. Electron microscopic work by Aaron Klug (80, 81) and Woodcock (82) on chromatin proposed ever since competing 'solenoid' and 'zigzag' models of chromatin compaction. According to solenoid or 'one-start' helix model consecutive nucleosomes interact with each other and follow a helical trajectory with bending of linker DNA (77). The zig-zag or 'two-start' helix model states that alternate nucleosomes with relatively straight linker DNA interact with each other to form a two-start helix (82, 83). Schematic illustration of both models is shown in Figure 7C.

Figure 7: Nucleosomes and higher-order levels of chromatin organization. **A:** Crystal structure of the nucleosome core particle PDB: 1AOI. Ribbon traces of DNA (tan) and eight histone proteins (cyan: H3, orange: H4, yellow: H2A, green: H2B). **B:** Negatively stained electron micrograph of chromatin isolated from rat thymus at low salt (78). **C:** 30nm fiber chromatin models (79). The image in inset B and C are reprinted with permission from AAAS and Springer Nature, respectively.

Telomeres are a heterochromatin entity of the genome. They contain HP1 (heterochromatin protein 1) protein, which is a characteristic feature of heterochromatin. They also carry heterochromatin-specific histone marks including histone 3 and 4 trimethylation at lysine 9 (H3K9me3) and 20 (H4K20me3), respectively (84). A common approach to isolate chromatin for EM analysis is to first cleave it into shorter fragments using micrococcal nuclease (MNase) digestion of DNA. MNase preferentially cleaves linker DNA between nucleosomes. Nucleosomal organization at telomeres has been mapped by MNase digestion and subsequent detection of telomeric DNA by Southern

blotting. The analyses suggest that telomeric DNA in rat and *Nicotiana tabacum* (tobacco) is packaged in nucleosomes with an unusual repeat length of 157 bp, which is 40 bp shorter than bulk chromatin in these cells (77, 85). Similarly, a short telomeric nucleosome repeat length (150-165 bp's) has been reported in human cell lines (86). The observation that linker histone (H1) is about four-fold less prevalent at telomeres than non-telomeric heterochromatin, is in agreement with their short nucleosome repeat length (87).

Telomeric nucleosome organization is believed to differ from non-telomeric chromatin. *In-vitro* studies, where free energies of nucleosome formation were measured by competitive reconstitution, demonstrate that telomeric sequences have least affinities ($\Delta\Delta G$ TTAGGG₃₀ = 1.35 versus ~0 kcal/mol for average mono-nucleosomal DNA) for a histone octamer (88). However, MNase studies have shown that telomeric DNA wraps around the histone octamer with the expected periodicity of ~10.2 bp (89). Photobleaching experiments demonstrate that telomere-histone octamer association is more stable than TRF1 and TRF2, which exchange rapidly at telomeres. It remains unknown whether histones and shelterin proteins cooperate or compete in binding telomeric DNA [reviewed in (90)]. It has been suggested that shelterin protein bind telomeric DNA between nucleosomes (91).

1.7 Telomere architecture

A number of studies have investigated the structure of non-telomeric chromatin using EM. The elegant work by Aaron Klug (81), John Finch, Roger Kornberg (92), Cristopher Woodcock (82) and Pierre Chambon (93) have reported the structure of native chromatin that ranges from mono-nucleosome level to the snapshot of whole nuclear chromatin while spilling out of the lysed nucleus.

However, very little is known about the structure of telomeric chromatin at physiological conditions. EM studies by Woodcock has reported that decondensed telomeric chromatin from chicken erythrocytes exhibit 'beads-on-a-string' conformation (94). The authors of the study have discussed the 'beads-on-a-string' morphology of telomeres but could not interpret the results in relation to the shelterin components.

Figure 8: Telomeric and non-telomeric chromatin. **A:** Dark-field electron microscopy of chicken liver chromatin protruding from a lysed nucleus (93). **B:** Nucleosomal array of rat liver chromatin obtained after MNase digestion (81). The sample was prepared in a buffer that did not contain mono/divalent salt. **C:** Rat liver mono/di/tri and tetra nucleosomes obtained after MNase digestion (92). **D:** Phosphotungstic acid stained telomeric chromatin from chicken erythrocytes in 5 mM NaCl originally published in (94). Telomeric chromatin was isolated after selective nuclease digestion of non-telomeric chromatin. The image in **A** is Reprinted with permission from Elsevier.

In summary, the higher-order structure of human telomeric chromatin is unknown. The findings that human telomeres strongly attach to nuclear components and precipitate with the insoluble 'nuclear matrix' fraction (95, 96) has prevented intact telomeres to be isolated. The salt-insoluble fraction of the nucleus is generally referred to as the nuclear matrix (97, 98). Telomere attachment was observed in several cell types and during various stages of the cell cycle. Nature and stability of telomere attachment was extensively investigated using 0.1 % Triton X10, 2 M NaCl, 10 mM β -mercaptoethanol, 10 mM o-phenanthroline, 10 mM EDTA, 10 mM EGTA, 20 μ g RNase A/ml of specimen, 50 μ g proteinase K/ml of specimen, 1 % SDS and 1 % Triton-X100 followed by 1 M NaCl. With the exception of SDS solubilization and proteolytic degradation by proteinase K, none of the said treatments could 'release'/solubilize telomeres (Figure 9A). Another study reported that telomeres from a number of human cell lines remained attached to the nuclear matrix even after degrading into as small as 1 kb DNA fragments. The authors interpreted the result as telomeres attach to the nuclear matrix along their entire length through an array of attachment-sites (Figure 9B).

Figure 9: Biochemical characterization of the solubility of isolated telomere fractions from HeLa cells. **A:** Nuclear preparation was incubated with the mentioned chemical agents and subsequently centrifuged to separate soluble telomeres (fraction S) from the precipitated (fraction P2). Fractions were analyzed for the presence of telomeric DNA by the northern blotting. Lanes 1 and 2: Control. Lanes 3 and 4: RNase. Lanes 5 and 6: Proteinase K. Lanes 7 and 8: SDS. Lanes 9 and 10: 1 % Triton X-100. Lanes 11 and 12: incubation in 1 % Triton X-100 followed by 1 M NaCl resulted in tertiary pellet P3 and supernatant S3 (66). **B:** Southern blot analysis of nuclear matrix-bound telomeric DNA in HeLa-S cells. The sample was digested with the increasing amount of DNase I (0.25 - 500 ng/ml) and centrifuged to separate precipitated (P) from soluble (S) fraction. The data was originally published in (96)

The unique single-stranded G-rich 3' overhang DNA of telomeres is the basis 'G-quadruplex model' and 'T-loop model' of telomere structure (Figure 10).

Figure 10: Schematic illustration of a G-quadruplex and the T-loop model. **A:** Telomeric DNA **B:** G-quadruplex structure **C:** T-loop model where is where the G-overhang overhang loops back into the double-stranded regions of telomeric DNA (9). Reprinted with permission from Springer Nature.

1.7.1.1 G-quadruplex model

Single-stranded G-rich DNA sequences, such as the telomeric ends can fold into G-quadruplex DNA by the formation of G-tetrads through Hoogsteen base pairing G- (Figure 10A-B). A wealth of structural information has shown the chemical properties of such structures and their topological repertoire (99, 100). In 2001, immunostaining using a G-quadruplex specific antibody demonstrated that G-quadruplexes are present inside cells and are cell cycle regulated (101). G-quadruplexes can inhibit the activity of the telomerase enzyme (102). The propensity for telomeric DNA to form G-quadruplexes has been suggested to play an important role in telomere length maintenance (103).

1.7.1.2 T-loop model

T-loop model was proposed by Titia de Lange group in 1999 after their electron microscopic observation of lariat structures present in purified crosslinked telomeric DNA from human and mouse cells (Figure 11A) (104). According to this model, single-stranded G-rich 3' overhang DNA folds back and displaces upstream duplex telomeric DNA (displacement loop or D-loop) to form a closed-circle structure called T-loop (Figure 10A, C). Experimental results suggest that TRF2 is required for T-loop formation as such structure is thermodynamically unfavourable (105). In 2004, Woodcock reported similar lariat structures adopted by purified and chemically fixed telomeres from avian erythrocytes and quiescent mouse lymphocytes (Figure 11B). Compared to the T-loops reported by Titia de Lange, which represented purified telomeric-DNA, T-loops by Woodcock represented telomeric-DNA with associated proteins which resemble nucleosome-like beads. However, not all of the telomere-specific proteins were associated with telomeric-DNA at the time of telomere isolation. The authors mentioned (did not provide data) that mouse TRF1 and its avian homolog were not detected by western blotting and purified TRF1 was added to the telomere preparation before imaging. With the exception of exogenous biotinylated-TRF1, which was traced by a streptavidin-gold marker in EM images, none of the telomere-specific protein or nucleosomes could be resolved in telomeric T-loops or linear chromatin fiber (Figure 11B). Notably, only 25 % of telomeres were estimated to be in t-loop configuration (94). Consistent with this, a recent study has reported a similar percentage of telomeres in T-loop configuration in mouse embryonic fibroblasts cells (Figure 11C). In this study super-resolution fluorescence imaging by stochastic optical reconstruction microscopy (STORM)

was used to visualize crosslinked telomeric DNA. Cre-mediated conditional deletion of individual shelterin proteins showed that TRF2 was required for the formation of T-loops whereas other shelterin protein had no effect on the frequency of T-loops occurrence (57).

Figure 11: Structure of Telomere. **A:** Electron microscopic images of telomeric DNA from HeLa cells in T-loop configuration (104). **B:** Electron microscopic image of T-loop structure adopted by telomere-enriched chromatin from chicken erythrocytes originally published in (94). **C:** STORM image of telomere from mouse fibroblast cells. Telomeric DNA was hybridized with an Alexa-647-labeled [CCCTAA]³ peptide nucleic acid probe (57). The image in inset A and C are reprinted with permission from Elsevier.

1.8 Correlative light and electron microscopy

The light microscope is a powerful instrument to obtain spatiotemporal information about macromolecules inside cells and tissue samples. However, its spatial resolution is not sufficient to directly visualize the structure of proteins and nucleosomes, which typically range from 0.1 to 10 nm in size (106). In contrast, an electron microscope can provide high-resolution spatial information of biomolecules, but it cannot provide temporal information in living cells (106-108). In order to get a better understanding of the complex and dynamic organization of cellular components, there was an increasing demand to study the same samples on the scale of a micrometre to nanometers. Correlative light and

electron microscopy unify the capabilities of light and electron microscopy and allows one to pinpoint cellular structures in whole cell images with a light microscope and subsequently obtain high-resolution information of the same in an electron microscope (107-109). Since the earliest concept of correlative light and electron microscopy in mid-nineties of the 20th century (110, 111), a series of labelling techniques have been developed to visualize and pinpoint cellular structures of interest. The most commonly used tags for CLEM are genetically encoded fluorescence proteins, for instance, GFP. Such fluorescent tags serve to pinpoint cellular structures in a fluorescence microscope and subsequently detect the same structures in electron micrographs via immuno-gold labelling (112). Other markers such as mini singlet oxygen generator (MiniSOG) (113, 114), fluorescent recombinant electron-dense ferritin (FerriTag) (115), self-labeling SNAP/CLIP-tags (116), quantum dots, lipid droplets, and fluorescently labelled gold particles have served well for the dual light and EM examination of cellular components by CLEM (107, 117, 118).

Sequential light and electron microscopy contrast can also be obtained by osmiophilic diaminobenzidine (DAB) polymers. The DAB polymerization can be induced by photo-oxidation, or by peroxidases (106, 108, 109). In 2012, Alice Yen-Ping Ting reported the development of 'APEX', a genetically encodable electron microscopic tag, which does not require photo-activation to generate contrast. This 28 kDa enzyme was developed by modifying a plant cytosolic heme-dependent ascorbate peroxidase (APX) resulting in enhanced APX (APEX). Owing to its peroxidase activity, APEX catalyzes the polymerization of 3,3'-diaminobenzidine (DAB) in the presence of hydrogen peroxide and produces an osmiophilic precipitate, which can be stained with Osmium tetroxide to obtain

contrast in electron micrographs. In contrast to conventional tags, APEX is catalytically active in all cellular compartments, withstands strong chemical fixation, and remarkable in preserving the ultrastructural details of the specimen (119). Since over-expression of APEX tag causes cellular toxicity, the same research group developed a derivative of APEX called APEX2. This modified version features higher DAB-conversion capability even in lower concentrations without detectable cellular toxicity (120).

There are three well-characterized CLEM sample-processing methods. Firstly, pre-embedding CLEM where the sample is imaged in light microscope before embedding into plastic resin, which is finally used for electron microscopic examination. Secondly, post-embedding CLEM where the sample is imaged in light microscope after embedding into plastic resin followed by electron microscopic examination. Thirdly, the Tokoyasu method where sample is prepared by cryofixation and light microscopic examination is done before electron microscopic examination. All of the above-mentioned methods use fluorescent markers to identify cellular targets through light microscope, which are subsequently traced in electron microscope for ultra-structural details.

CLEM using pre-embedding is most practical and commonly used approach due to its ability to register specific cellular events at different time points and proceed with electron microscopy to seek ultrastructural details of the same with excellent preservation of cellular morphology. In this approach, once the cellular event of interest is recoded by fluorescence signal, the specimen is fixed by using fixatives. Alternatively, the cells are fixed prior imaging if a cellular event of non-dynamic nature is under observation. The location of the cell is marked using a

Matek gridded Petri dish (121), chemically treated, embedded into epoxy resin and finally ultra-thin sections are obtained by cell-sectioning.

The disadvantage of pre-embedding CLEM is that it can only be applied to monolayer cells or very thin specimens that are visible in light microscope so that the area of interest could be marked. In addition, potential changes in the specimen due to fixation and non-correlation in the Z-axis between light and electron microscope images are main caveat of this approach (122). Moreover, in genetically encoded approaches, the target protein is tagged and overexpressed, which may cause artefacts. Most of the genetically coded tags are ~25 kDa in size, which could potentially perturb the function of the protein. In addition, obtaining accurate distribution of staining using photo-oxidation tags such as DAB is technically challenging (123). Efforts to minimize such artefacts are ongoing, such as by improving their photophysical properties, thus requiring fewer labelled molecules to visualize targets as shown with APEX2 improvement over APEX in cells (120).

Despite the above-mentioned caveats, CLEM has provided important insight into the assembly and structure of macromolecules inside cells. For example, understanding the curvature of clathrin-coated pits (124), birth of the Golgi apparatus (125), trafficking of Golgi intermediates (126), nuclear pore complexes (127), sub-diffraction-limited connections in neuronal networks (128) host-pathogen interactions (129, 130), tracking viral proteins (131), imaging HIV-1 during early stages of infection (132), detection of molecular structures such as lipopolysaccharide and its association with fibrin fibers in Parkinson's Disease, Alzheimer's Disease and Type 2 Diabetes Mellitus (133) and numerous other cellular and sub-cellular structures (106-109, 118, 134-146).

Another worth mentioning example is a recent CLEM study published by Ou *et al* (147). In this study, the authors have used a DNA intercalator to generate singlet oxygen through DAB mediated photo-oxidation followed by osmium tetroxide staining for *in-situ* visualization of interphase and mitotic chromatin in SAECs and U2SO cells. Followed by the DAB reaction and osmium tetroxide staining, the cells were fixed, resin embedded, sectioned and finally electron tomography data was collected for three-dimensional visualization of chromatin, a similar approach to that of used herein.

Biochemical, genetic and cytological studies have not only elucidated the biological roles of telomeres but also dissected the function of its specific components. However, very little is known about the high-order structure of human telomeric-chromatin. The reason is that thus far human telomeres could neither be purified due to their insoluble nature nor successfully reconstituted for structural studies. Except for the X-ray crystallographic or NMR structures of shelterin domains, not a single full-length protein structure of shelterin proteins is available to date. These components are insufficient to build a composite structural model of telomeres and do not address the relationship to the underlying chromatin structure. The T-loop model represents only a subpopulation of telomeres from chicken and mouse origin and may not reflect the dominant population of mammalian telomeres. It should also be noted that the presence of nucleosomes at telomeres has been largely ignored. Most electron microscopy and super-resolution studies of telomeres to date were conducted after removing histones and shelterin proteins.

Despite the efforts by electron microscopic and STORM based studies in elucidating telomere structure, we are still far from a clear understanding of how

telomeric components are spatially organized. How telomeric chromatin differs from non-telomeric heterochromatin and what is the higher order structure of entire telomere in its native *in-situ* state largely remains unanswered. These questions of fundamental importance cannot be addressed without three-dimensional structural information of telomeres. In this context, the work presented here focuses to obtain the three-dimensional structure of mammalian telomeres using electron tomography. To try to achieve this, three approaches were employed. Firstly, isolation of intact telomeres from human and mouse cells for EM analysis. Secondly, reconstitution of telomeric DNA with TRF1. Thirdly, correlative light and electron microscopy analysis of telomeres in ALT cells.

2 MATERIAL AND METHODS

2.1 Materials

Chemicals and reagents used in this study were purchased from Sigma (Singapore), Promega Corp. (Singapore), Roche Diagnostics (Mannheim, Germany), VWR International (USA), Bio-Rad Laboratories (Hercules, CA, USA), Becton-Dickinson (USA) or Vivantis (Malaysia) as specified. Electron microscopy grade reagents and support grids were obtained from Electron Microscopy Sciences (Hatfield, USA) or Agar Scientific Ltd (United Kingdom). pET-31b(+) vector (EMB Biosciences, Darmstadt, Germany) was provided by Daniela Rhodes laboratory (NTU Institute of Structural Biology, NTU) and its modified version containing eGFP-APEX2 tagged hTRF1 was generated and kindly provided by Eric von Otter, a PhD student in Sara Sandin's laboratory (SBS, Nanyang Technological University, Singapore). pUC 57 vector containing 150 bp of telomeric DNA was purchased from Genescript (NJ, USA). Purified recombinant hTRF1 was kindly provided by Vinod Kumar Vogirala, a Post-doctorate fellow in Sara Sandin's laboratory (SBS, Nanyang Technological University, Singapore).

Specimens of spleen, heart and kidney from C57BL/6 mice were kindly provided by Pazhanichamy Kalalingam, a research fellow in Thirumaran Thanabalu laboratory (SBS, Nanyang Technological University, Singapore). The mice were bred and maintained in Animal Research Facility (SBS, Nanyang Technological University, Singapore). Information about primary antibodies against telomerase, TRF1, TRF2, Rap1, TIN2, TPP1, POT1 and secondary anti-mouse/rabbit antibodies is tabulated in Table 1. HT1080 cell line was kindly provided by Alexander Ludwig (SBS, Nanyang Technological University, Singapore). hESCs

(047-XX Genea Biocells, Sydney, Australia) and U2OS cell line (Human bone osteosarcoma epithelial cells), respectively, were kindly provided by Peter Dröge and Dr Sze Siu Kwa (SBS, Nanyang Technological University, Singapore). Electron microscopy data was collected in NISB cryo-EM facility at Nanyang Technological University, Singapore.

Table 1: List of antibodies used in this study

Antibodies	Origin	Manufacturer	Catalogue #
Telomerase	Rabbit	Santa Cruz	sc-393013
TRF1	monoclonal Mouse	Biotechnology Inc. (CA, USA) Abcam (Cambridge, UK)	ab10679
TRF2	monoclonal Mouse	Novus Biologicals (Co, USA)	IMG-124A
Rap1	monoclonal Rabbit polyclonal	Bethyl Laboratories, Inc. (Texas, USA)	A300-306A
TIN2	Rabbit	Abcam (Cambridge, UK)	ab197894
TPP1	monoclonal Rabbit polyclonal	Abcam (Cambridge, UK)	Ab112050
POT1	Rabbit	Abcam (Cambridge, UK)	ab124784
Anti-mouse IgG	monoclonal Goat polyclonal (HRP conjugate)	Thermo Scientific (USA)	A16072
Anti-rabbit IgG	Goat polyclonal (HRP conjugate)	Thermo Scientific (USA)	A16104

2.2 Cell culture

HT1080 and U2OS cells were grown in 150 x 25 mm petri dish (Corning Inc., USA) as a monolayer in DMEM GlutaMax culture medium (Life technologies,

Singapore) containing 10 % fetal calf serum, 1 % penicillin and streptomycin (Life technologies, Singapore) at 37°C and 5% CO₂ in humidified condition.

hESCs were plated at 1.25×10^4 cells per each 10 cm collagen-coated culture plate (Corning® BioCoat™ Collagen I) with 12 ml of M2 medium (Genea Biocells, Sydney, Australia) that contained penicillin and streptomycin at a concentration of 50 U/ml and 50 µg/ml, respectively. The culture medium and plates were pre-warmed at 37°C before use and cells were grown at 37°C in 5 % CO₂, 5 % O₂ and 90 % humidity. Culture medium was changed every 48 hours and the cells were grown until they reached near confluency. To harvest, cells were washed once with PBS and trypsinized with Passaging Solution (Genea Biocells, Sydney, Australia), which was subsequently neutralized with Neutralizing Solution (Genea Biocells, Sydney, Australia). All solutions were warmed at 37°C prior to use.

2.3 Synchronization of HT1080 cells

HT1080 cells were grown as a monolayer as described in section 2.2. Cell-cycle synchronization at metaphase was carried out using the protocol developed by Fuku *et al* 2008 (148) with some modifications. Briefly, at ~60 % confluency, cells were placed at 4°C for 60 minutes followed by addition of Colcemid (Sigma, Singapore), to a final concentration of 0.01 µg/ml. After overnight incubation at 37°C, cells were isolated by gently tapping the sides of the tissue culture dish.

2.4 Restriction enzyme digestion of chromatin

Restriction enzyme digestion of chromatin was carried out using the protocol published by Woodcock *et al* 2004 (94) with some modifications. Nuclear chromatin/mitotic chromosomes were digested with CfoI, HaeIII, HinfI, MspI and RsaI (Promega Corp., Singapore) at a concentration of 10 U/µg of DNA in buffer

B (Promega Corp., Singapore) and bovine serum albumin at a final concentration of 0.1 µg/ml. The protease inhibitor cocktail (Roche) was present during all steps of restriction enzyme digestion. The reaction was carried out at 37°C for 4 hours. Restriction enzyme digested chromatin was dialyzed overnight against 2 litres of a buffer that contained 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 0.3 mM EDTA, 14 mM β-mercaptoethanol and 0.5 mM PMSF using SnakeSkin (Thermo Scientific, USA) dialysis tubing of 3.5K molecular weight cut-off. For the second round of restriction enzyme digestion, BamHI and EcoRI (NEB, Singapore) at a concentration of 10 U/µg of DNA were added into the reaction tube in a buffer with the final concentrations of 10 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.3 mM MgCl₂, 1 mM DTT. A change in total volume of the reaction mixture dropped the final concentration of bovine serum albumin, which was adjusted to 0.1 µg/ml to maintain optimal enzymes' activity. The second round of the restriction enzyme digestion was carried out for 2 hours at 37°C. Finally, the reaction was stopped by adding 1 mM EDTA and 0.2 mM EGTA and the tube was kept on ice for 30 minutes. To avoid chromatin aggregation and ensure that cleaved chromatin fragments are liberated in solution, monovalent ion concentration in the buffer was increased. This step is termed as 'chromatin-release' in the following text (Figure 17, step 4). Equal volume of sample was divided into different tubes and chromatin-release was carried out at 4°C for 2 hours using a range of pH (7.5 - 9), concentrations of NaCl (75-2000 mM), EDTA (1-10 mM), EGTA (0.2-2 mM), NP-40 (1%) and DTT (1-5 mM). Finally, the samples were centrifuged at 800 g for 6 minutes to separate soluble supernatant containing digested chromatin. These samples were subsequently used for Southern blot analysis with or without fractionation by 5-40 % sucrose gradient.

2.5 Purification of mitotic chromosomes

Cell pellets containing 1.5×10^7 cells were washed with 1X PBS to remove residual culture medium. Cell membranes were lysed to release chromosomes by using 1.5 ml of a hypotonic buffer that contained 10 mM HEPES pH 8 and 1 % Triton X-100. The cell suspension was kept on ice for 15-20 minutes. The samples were passed twice through a 25-gauge needle. Cellular morphology and lysis were observed at each step under Olympus ix70 inverted fluorescence & phase contrast microscope (Olympus, Japan). The lysates were centrifuged at 200 g for 3 minutes at 4°C. The supernatant containing mitotic chromosomes were centrifuged further at 1800 g for 30 min to collect the chromosome as a pellet. Chromosomes were resuspended in 1 ml of resuspension buffer that contained 15 mM Tris HCl pH 7.4, 80 mM KCl, 20 mM NaCl, 2 mM EDTA and laid over 10 ml of resuspension buffer containing 70 % glycerol. The preparations were centrifuged at 1750 g for 20 min at 4°C and chromosomes were collected from the interphase of the two solutions. Finally, the purified chromosomes were washed in restriction enzyme buffer that contained 10 mM Tris HCl pH 7.5, 50 mM NaCl and 6 mM MgCl_2 . The restriction enzyme digestion was carried out as described in section 2.4 except RNase A (Sigma, Singapore) at a final concentration of 0.1 mg/ml was included in both rounds of restriction enzyme digestion. RNase A was added to cleave RNA of ribosomal and other ribonucleoproteins, which adhere to mitotic chromosomes (149) and could hinder the access of restriction enzymes to the cleavage sites. Chromatin release step was carried out at 4°C for 2 hours using a range of pH (8/9), concentrations of NaCl (100-2000 mM), EDTA (1/10 mM), EGTA (0.2/2 mM), NP-40 (0.1/1%) and DTT (1/5 mM). 1X protease inhibitor cocktail (Roche Diagnostics, Germany) and

freshly prepared 1 mM DTT (Sigma, Singapore) was present during every step of chromosome isolation.

2.6 Isolation of hESCs nuclei

hESCs were grown as described in section 2.2. Cells ($\sim 2 \times 10^7$) were lysed in 1 ml of a buffer that contained 10 mM HEPES pH 8, 10 mM NaCl, 1 mM CaCl_2 , 5 % sucrose, 0.5 % NP-40 and 0.5 % Sodium Deoxycholate. Cell lysis was assisted by passing the cell suspension through a 25-gauge needle. Cellular morphology and lysis were observed at each step under Olympus ix70 inverted fluorescence & phase contrast microscope (Olympus, Japan) after staining the nuclei with Hoechst stain (Sigma, Singapore). Nuclei were pelleted by centrifugation at 1860 g for 10 minutes and washed with a buffer that contained 20 mM Tris-HCl pH 7.4, 60 mM NaCl, and 0.3 mM MgCl_2 . The first round of restriction enzyme digestion was carried out as described in 2.4 except that a total amount of 20 units of RNase H (NEB, Singapore) were added to digest RNA from RNA-DNA heteroduplexes. After the first round of restriction enzyme digestion, the nuclear suspension was passed through a needle of 31-gauge to ensure nuclear lysis. The second round of restriction enzyme digestion was carried out as mentioned in section 2.4. Chromatin release step was carried out at 4°C for 2 hours using a buffer containing a range of NaCl (200-2000 mM), 10 mM EDTA, 2 mM EGTA, 1% NP-40 and 5 mM DTT at pH 9. 1X protease inhibitor cocktail (Roche Diagnostics, Germany) and freshly prepared 1 mM DTT (Sigma, Singapore) was present during every step of nuclei isolation.

2.7 Isolation of mouse spleen lymphocytes

Spleens were obtained from 4-6 weeks old C57BL/6 mice (18/23/26/40 mice in independent experiments) were provided by Pazhanichamy Kalalingam, a

research fellow in Thirumaran Thanabalu laboratory (SBS, Nanyang Technological University, Singapore). Spleens were immediately placed in ice-cold DMEM GlutaMax culture medium containing 10 % fetal calf serum (Life technologies, Singapore). Spleens were cut into 3-4 mm pieces with sterile scissors and placed into 70 cell strainers. Spleen pieces were passed through a cell strainer using the plunger end of a syringe to obtain a homogenous cell-suspension. Cells were washed with ice-cold DMEM GlutaMax culture medium containing 10 % fetal calf serum (Life technologies, Singapore) and pelleted by centrifugation at 200 g for 10 minutes. Red blood cells were lysed by resuspending and incubating the cells for 15 minutes at room temperature in RBC lysis buffer that contained 155 mM NH_4Cl , 12 mM NaHCO_3 and 0.1 mM EDTA. Cells were washed 1X in the RBC lysis buffer to clear lysed fragments of red blood cells followed by centrifugation at 200g for 10 minutes to obtain a cell pellet. Cells were washed 2X with 50 ml of ice-cold resuspension buffer that contained 10 mM Tris HCl pH 6, 60 mM KCl, 12 mM NaCl, 340 mM Sucrose, 0.15 mM Spermine, 0.5 mM Spermidine, 15 mM β -mercaptoethanol, 2 mM EDTA and centrifuged at 1164 g for 10 minutes at 4°C using Eppendorf centrifuge 5804r (Eppendorf). Cells were lysed in 50 ml of buffer (0.5 % Triton X-100 containing resuspension buffer) and nuclei were released by on and off gentle agitation for 3-5 minutes. Gelatinous material containing spleen connective tissue and splenocytes were removed by centrifugation at 200 g for 10 minutes. Purified lymphocytic nuclei were recovered from the soluble suspension by centrifugation at 2620 g for 10 minutes at 4°C and restriction enzyme digestion was carried out as described in section 2.4. The chromatin release step was carried out at 4°C for 2 hours using Tris HCl pH 7.5, 75 mM NaCl, 1 mM EDTA, 0.2 mM EGTA and

0.1% NP-40. Finally, samples were fractioned using 5-40% sucrose gradient and analyzed by Southern and Western blot analysis. 1X protease inhibitor cocktail (Roche Diagnostics, Germany) and freshly prepared 1 mM DTT (Sigma, Singapore) was present throughout the sample preparation.

2.8 Isolation of mouse kidney and heart cells

A total number of four kidneys and four hearts were obtained from 4-6 weeks old C57BL/6 mice as described in section 2.7. The organs were cut into 3-4 mm pieces with sterile scissors and washed with ice-cold PBS. The organ-pieces were placed into 10 ml of DMEM medium with 10% FBS, 1 mg/ml of Type I Collagenase (Life technologies, Singapore) and incubated for 1 hour at 37°C. Samples were constantly mixed during the incubation. Single-cell suspensions were obtained by passing the samples through an 18-gauge needle followed by a filtration step using 40 um cell strainers (Corning®, USA). From this point, the isolation of nuclei from heart and kidney cells were carried out as described in section 2.7. Restriction enzyme digestion of these samples was carried out as described in section 2.4. Equal volume of samples was divided into different tubes and chromatin-release was carried out at 4°C for 2 hours in a buffer that contained 10 mM Tris HCl pH 7.5, a range of NaCl concentration (100 – 2000 mM), 10 mM EDTA, 2 mM EGTA, 1 % NP-40, 5 mM DTT and 1X protease inhibitor cocktail.

2.9 DNA purification

To reduce protein contamination, DNA was purified after treating samples with proteinase K (Life technologies, Singapore) in the presence of 20 mM Tris HCl pH 8, 1 mM EDTA and 0.5 % SDS. The reaction was carried out at 55°C for 4

hours followed by DNA purification using standard phenol-chloroform Isoamyl alcohol (PCI) protocol (150).

2.10 Southern blot detection of telomeric DNA

Telomeric DNA was detected by Southern blot analysis using TeloTAGGG Telomere Length Assay (Roche Diagnostics, Germany). Briefly, 2-4 µg of purified DNA was separated by electrophoresis in 0.8% agarose gel containing 0.02 µg/ml Ethidium Bromide (EtBr) using 1X TAE buffer (40 mM Tris HCl pH 8, 20 mM Sodium Acetate and 1 mM EDTA). Gels were imaged using Gel Doc™ EZ Imager (Bio-Rad Laboratories, USA) and DNA fragments larger than 5 kb were depurinated with 0.25 M HCl for 8 minutes to facilitate efficient blotting. Denaturation step was performed in 0.5 M NaOH and 1.5 M NaCl for 2 x 15 minutes, followed by neutralization in 0.5 M Tris HCl and 1.5 M NaCl for 2 x 15 minutes while gentle shaking. DNA was transferred to a Hybond-N+ nylon membrane (GE Healthcare) by overnight capillary transfer using 20x SSC (3 M NaCl, 0.3 M Sodium Citrate pH 7). DNA was cross-linked to the membrane using a UV dose of 120 mJoule/cm² for 30 seconds in HL-2000 Hybrilinker UV crosslinker (UVP laboratory products, UK). From this point, all steps were performed in sealed roller bottle in a ProBlot™ hybridization oven (Labnet International Inc., USA) using the chemical provided by Roche Diagnostics (Germany). Pre-hybridization was performed at 42°C for 60 minutes using the DIG Easy Hyb whereas hybridization was carried out at 42°C overnight using a telomeric DNA probe. The membrane was washed in 2 x SSC and 0.1 % SDS at room temperature for 2 x 5 minutes followed by 0.2 SSC and 0.1 % SDS at 52°C for 2 x 20 minutes to remove unspecific signal. All subsequent steps were carried out at room temperature. The membrane was incubated with freshly prepared

blocking solution for 30 minutes before incubating in Anti DIG-AP solution for another 30 minutes. Finally, the membrane was washed in 1x washing buffer for 15 minutes, equilibrated in detection buffer for 5 minutes, floated with substrate solution for 5 minutes and exposed to X-ray film (Carestream Health Inc., USA) for 5 min. The X-ray film was developed on Kodak X-OMAT 2000 processor (Carestream Health Inc., USA).

2.11 Density gradient ultra-centrifugation

Sucrose gradients were prepared from 5 and 40 % sucrose solutions in a buffer that contained 5 mM HEPES, pH 7.5, 25 mM NaCl and 0.2 mM EDTA. The gradients were prepared using Gradient Station_{ip} (Biocomp Instruments, Canada). The samples were added on top of the sucrose gradient and centrifuged either at 32,000 rpm in SW41Ti rotor (Beckman Instruments) or 37,000 rpm in an SW6Ti rotor (Beckman Instruments) for 75 min at 4°C. Fractions of 400 µl or 800 µl were collected manually.

2.12 Western blot analysis

Protein samples were separated by 12 % SDS-PAGE and transferred to a polyvinylidene difluoride membranes (Immobilon®, Millipore™, USA) in a freshly prepared buffer that contained 25 mM Tris HCl, 192 mM Glycine and 20 % methanol using Blotting System (Bio-Rad Laboratories, USA) according to the manufacturer's instructions. To prevent non-specific binding of the antibodies, membranes were blocked with 5% skimmed milk in TBST buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) and incubated with primary antibodies for overnight at 4°C. The membranes were washed three times for 10 minutes with TBST buffer and probed with horseradish peroxidase-conjugated anti-mouse/rabbit antibodies for 45 minutes at room temperature. The membranes

were washed again (3X 10 minutes) with TBST buffer. Finally, chemiluminescence generated by horseradish peroxidase was detected by Immobilon Western Chemiluminescent HRP Substrate (Immobilon®, Millipore™, USA) according to the manufacturer's instructions.

2.13 Negative stain electron microscopy

Purified chromosomes/chromatin were prepared for electron microscopic analysis by negative stain following a published protocol (151). 5 µl of sample was added to a freshly glow-discharged continuous carbon-coated 300 mesh copper grids. The procedure of carbon coating is described in section 2.14. After 2 minutes, excess fluid was removed with Whatman® (GE Healthcare Life Sciences, England) blotting paper and stained with 1% Uranyl Acetate (Sigma, Singapore) for 1 minute. Excess stain was blotted, and the grids were air dried. Electron micrographs with an object pixel size of 3.6 Å /pixel were recorded on a 4k x 4k Eagle (FEI Company) CCD camera using a Tecnai T12 (FEI) transmission electron microscope operated at 120 kV. A defocus of -2.5 µm was applied while recording images.

2.14 Electron cryo-microscopy of telomeric chromatin

Telomeric chromatin fraction were dialyzed overnight at 4°C against 2 liters of a buffer that contained Tris HCl pH 7.5, 5 mM NaCl, 1 mM EDTA, 0.2 mM EGTA, 0.1% NP-40, 1 mM DTT and 0.5 mM PMSF. The samples were concentrated using 100 kDa cut off concentrators (Amicon Inc., USA) and used to prepare cryo-EM grids. In some experiments, 50 µl of the samples were dialyzed against a buffer with specified concentrations of NaCl followed by cryo-EM grid preparation. Samples were not concentrated in these cases. For this small-scale dialysis, 50 µl of each sample was placed into the chamber of Dialysis buttons (Hampton

Research, USA) and sealed by 3.5 kDa molecular weight cut-off SnakeSkin (Thermo Scientific, USA) dialysis tubing. Samples were dialyzed overnight at 4°C against 500 ml of buffer that contained Tris HCl pH 7.5, variable concentration of NaCl (5-75 mM), 1 mM EDTA, 0.2 mM EGTA, 0.1% NP-40, 1 mM DTT and 0.5 mM PMSF. Dialyzed samples were used for cryo-EM grid preparation using holy grids, carbon or Graphene oxide coated grids specified in Table 2.

Graphene oxide coated grids were prepared following a published protocol (152). Graphene oxide (Sigma, Singapore) was diluted in deionized water to a final concentration of 0.2 mg/ml and its aggregates were removed by centrifugation of tube at 300 g for 30 seconds. Grids were glow discharged for 1 min and floated atop 3 µl of freshly prepared graphene oxide solution. After 1 minute, grids were gently blotted using Whatman® (GE Healthcare Life Sciences, England) blotting paper. Grids were immediately washed three times by floating atop 10 µl droplets of deionized water, air dried and used to plunge-freezing sample without any additional treatment.

For carbon coating of grids, first mica sheets were coated with 12/16 nm carbon using Leica EM ACE600 (Leica, Germany) carbon coater operated at 120 W, 3 x 10⁻⁶ mbar and -180°C. To stabilize the carbon-film, mica sheets were kept in a dry box with a controlled humidity of 25% for 2-4 weeks. Grids were coated with carbon by floating off the carbon-film from mica sheet on water at an angle of ~45°. Water was carefully taken out by 5cc syringe while guiding the carbon film to fall on grids. Grids were air dried and kept at room temperature until used.

Grids (except Graphene oxide coated grids) were glow discharged for 1 minute and transferred to Vitrobot™ Mark IV (FEI) chamber maintained at 4°C and 90 %

humidity. A total volume of 4 μ l was applied on the grid. The excess sample was blotted using a blot force and time specified in Table 2. After 30 seconds, grids were plunged into liquid ethane. For manual blotting, grids were accessed using sample window of Vitrobot™ (FEI) and excess sample was blotted using a small piece of filter paper held with tweezers. Plunged-freeze grids were clipped using c-clip rings and transferred to Tecnai Arctica (FEI) transmission electron microscope using a cryo-shuttle under cryogenic temperature. The microscope was operated at 200 kV and images were recorded on Flacon III (FEI) direct electron detector.

Table 2: Different conditions tested for cryo-EM grid preparation of telomeric chromatin

NaCl (mM)	Grid Type	Carbon (nm)	Graphene Oxide	Blotting
5/25/75	Quantifoil R2/1 (holy grids)	X	x	Blot force 1, Blot time 2sec
5/25/75	Quantifoil R2/1 (holy grids)	12	x	Blot force 1, Blot time 1sec
25/75	Lacy-200-Cu	12	x	Blot force 1, Blot time 1sec
25	Lacy-200-Cu	X	✓	Blot force 1, Blot time 1sec
25	Lacy-200-Cu	X	✓	Manual blotting
25	Holy carbon-200 (variable hole)	X	✓	Blot force 1, Blot time 1sec
25	Holy carbon-200 (variable hole)	X	✓	Blot force 1 *Back blotting (8sec)
25	Holy carbon-200 (variable hole)	X	✓	Blot force 1 *Back blotting (3sec)
25	Quantifoil R2/1 (holy grids)	16	x	Manual blotting
25	Quantifoil R2/1 (holy grids)	X	✓	Blot force 1, Blot time 1sec
25	Quantifoil R2/1 (holy grids)	X	✓	Manual blotting
25	Lacy-200-Cu	16	x	Manual blotting
25	Holy carbon-200 (variable hole)	16	x	Manual blotting

*Teflon was placed at the sample front and blotting filter paper at the backside of the grid.

2.15 Purification of telomeric DNA

The pUC 57 plasmid containing 150 bp of telomeric DNA was transformed in *E. coli* Sure2 cells (Stratagene, USA) and grown at 16°C overnight in LB medium containing 100 µg/ml of Ampicillin (Sigma, Singapore). Plasmid DNA from different colonies was amplified, extracted using Qiagen plasmid DNA purification kit (Qiagen, Singapore) and digested with SmaI and PstI (NEB, Singapore) restriction enzymes. The DNA sequence of the clones (C1, C3, and C4) that gave expected restriction enzyme digested product of 150 bp was confirmed by DNA sequencing (Axil Scientific Gene Sequencing, Singapore).

Figure 12: Restriction enzyme digestion of pUC57 plasmid containing 150 bp insert of telomeric DNA. Plasmid DNA was digested using SmaI and PstI and analyzed in 0.8% agarose gel after staining with ethidium bromide. DNA ladder of 1 kb and 100 bp (NEB, Singapore) are labelled from the left to right, respectively.

For large-scale plasmid DNA extraction, cells were grown at 16°C overnight in Terrific broth (Sigma, Singapore) and plasmid extraction was carried out using

alkaline lysis protocol (153). Cells were harvested by centrifugation at 8,000 g for 10 minutes at 20°C and the pellet was re-suspended in alkaline lysis buffer I (25 mM Tris pH 8.0, 10 mM EDTA, 50 mM glucose). Alkaline lysis buffer II (1% SDS, 0.2 M NaOH) was added to the cell suspension, mixed vigorously for 2 minutes and kept on ice for 20 minutes. The tubes were vigorously mixed every 5 minutes during this time. Cold alkaline lysis buffer III (4 M K-Acetate, 2 M Acetic acid) was added and mixed immediately by inverting the tubes. After 2 minutes of gentle mixing, the suspensions were kept on ice for 20 minutes. The tubes were gently inverted every 5 minutes during this time. The tubes were centrifuged at 10,000 g for 20 minutes at 4°C and the supernatant was filtered through sterile gauze. The supernatant volume was measured, 0.52 volume of isopropanol was added to the suspension and kept at room temperature for 30 minutes.

The tubes were centrifuged at 16,000 g for 30 minutes at 20°C and the pellets were air-dried for ~1 hour. The pellets were dissolved in TE buffer (10 mM Tris HCl pH 8, 1 mM EDTA) and treated with RNase A (Sigma, Singapore) for overnight in a 37°C shaker. The tubes were centrifuged at 10,000 g at 20°C for 30 minutes to remove insoluble material and the supernatants were transferred into fresh tubes. To every 20 ml of supernatant, 10 ml of phenol was added to remove contaminant proteins. The tubes were vortexed for 1 minute and centrifuged at 27,000 g for 20 minutes at 20°C. The upper aqueous phase was carefully removed, and the abovementioned step was repeated. To remove phenol, the aqueous phase was extracted with 10 ml of CIA (chloroform-isoamyl alcohol, 24:1) using Teflon tubes (Nalgene, USA) and centrifuged at 12,000 g for 10 min at 20°C. NaCl and PEG (Sigma, Singapore) were added to the supernatant to get a final concentration of 0.5 M NaCl and 10% PEG and the

tubes were incubated at 37°C. After 5 minutes, the tubes were kept on ice for 30 minutes followed by 10 minutes of centrifugation at 27,000 g at 4°C. The supernatants were carefully removed, and the pellets were air-dried and re-suspended in TE buffer. Contaminating PEG was removed by two times extraction with CIA as described above. Sodium acetate was added to the aqueous supernatant at a final concentration of 300 mM. Finally, plasmid DNA was purified by ice-cold ethanol precipitation.

Plasmid DNA was digested overnight with SmaI (NEB, Singapore) at 25°C, followed by a second round of restriction enzyme digestion with PstI (NEB, Singapore) at 37°C. Restriction enzyme digested telomeric DNA fragment was purified using PEG precipitation.

2.16 DNA-hTRF1 binding assay

Purified hTRF1 at specified molar concentrations was incubated with 0.25 mM telomeric DNA in a reaction buffer that contained 20 mM HEPES pH 7.5 and variable salt concentrations ranging from 10 mM NaCl through 150 mM NaCl. The reaction tubes were kept on ice for 15 minutes to ensure complete binding. Samples were immediately applied on Quantifoil® R2/1 cryo-EM support grids coated with 2 nm continuous carbon (Electron Microscopy Sciences, USA). Cryo-EM grid preparation and imaging were carried out as described in section 2.14. For fixation, Glutaraldehyde (Electron Microscopy Sciences, USA) was added to a final concentration of 0.2% and the tubes were kept on ice for 5 minutes. Finally, the reaction was quenched by the addition of 10 mM Tris HCl pH 7.5.

2.17 Confocal microscopy

Confocal microscopic imaging was carried out using a published protocol (154). CorrSight confocal spinning disk microscope (FEI) equipped with an Orca R2 CCD camera (Hamamatsu). Images were recorded using a 40x oil objective (NA 1.3, EC Plan Neofluar M27, Zeiss) lens. Fluorescence confocal images were acquired utilizing the 488 nm laser line (65 mW; iChrome MLE-LFA) and a standard GFP filter. Colour and contrast adjustments, image format conversion and other steps of image-processing were carried out in ImageJ or Fiji (155, 156) and Photoshop CS6.

2.18 Transfection of U2OS cells

pET-31b (+) vector (EMB Biosciences) containing eGFP-APEX2 tagged hTRF1 insert was transformed into NEB® 10-beta competent *E. coli* cells (NEB, Singapore) and grown at 16°C overnight in LB medium containing 50 µg/ml of Kanamycin (Sigma, Singapore). Plasmid DNA was extracted using Qiagen plasmid DNA purification kit (Qiagen, Singapore). DNA was digested with EcoR1 and BamH1 (NEB, Singapore) restriction enzymes and analyzed on 0.8% agarose gel for expected size of restriction enzyme digestion product (Figure 13). The DNA sequence was further confirmed by DNA sequencing (Axil Scientific Gene Sequencing, Singapore).

Figure 13: Restriction enzyme digestion of pET-31b (+) vector containing eGFP-APEX2 tagged hTRF1 insert. Plasmid DNA was digested using EcoRI or EcoRI + BamHI and analyzed on 0.8% agarose gel after staining with ethidium bromide. DNA fragments of expected size are labelled.

A screening experiment was performed to find conditions best optimized for transient transfection. Cells were grown in 22.1 mm Petri dishes (Corning Inc., USA) as a monolayer in antibiotic-free DMEM GlutaMax culture medium (Life technologies, Singapore) containing 10 % fetal calf serum (Life technologies, Singapore). Upon reaching 70-80% confluency, cells were washed with PBS and antibiotic and serum-free DMEM GlutaMax medium was added into the Petri dishes. Variable quantities of plasmid DNA and Lipofectamine 2000 (Thermo Fisher, USA) tested in this screen are tabulated in Table 3. Lipofectamine 2000 and plasmid DNA was priory mixed in serum-free DMEM medium and dropwise added into the medium, mixed and incubated for 4 hours at 37 °C. Cells were washed with pre-warmed PBS and incubated again for 16 hours in antibiotic-free

DMEM GlutaMax medium containing 10 % fetal calf serum. Finally, cells were fixed in 4% Paraformaldehyde and the percentage of cells expressing eGFP-APEX2 tagged hTRF1 was calculated by counting transfected cells per 1000 untransfected cells in confocal fluorescent light microscope images. The data was summarised in tabulated form using Microsoft Word version 16.16.10. Confocal fluorescence images were acquired as described in section 2.17. A concentration of 800 ng of DNA and 0.5 μ l of Lipofectamine was observed to give a maximum transfection efficiency of 10 % for the cells grown in 22.1 mm Petri dishes.

Table 3: Transfection efficiency screen

		Plasmid DNA (ng)					
		100	200	400	600	800*	1000
Lipofectamine (μ l)							
0.25	Transfected cells /1000 cells	16 (1.6%)	18 (1.8%)	33 (3.3%)	70 (7%)	72 (7.2%)	73 (7.3%)
0.5		34 (3.4%)	35 (3.5%)	44 (4.4%)	75 (7.5%)	100* (10%)	97 (9.7%)
1		22 (2.2%)	36 (3.6%)	39 (3.9%)	65 (6.5%)	88 (8.8%)	90 (9.0%)
2		26 (2.6%)	24 (2.4%)	33 (3.3%)	56 (5.6%)	66 (6.6%)	64 (6.4%)
3		17 (1.7%)	19 (1.9%)	22 (2.2%)	36 (3.6%)	35 (3.5%)	33 (3.3%)

* Maximum transfection efficiency

2.19 Correlative Light and Electron Microscopy

U2OS cells were grown as a monolayer in 35 mM MatTek (Corning Inc., USA) glass-bottom dishes in antibiotic-free DMEM GlutaMax culture medium containing 10 % fetal calf serum. Cells were transiently transfected using a ratio

of Lipofectamine 2000 to plasmid-DNA that was optimized in the screening experiment described in section 2.18. After 16 hours of incubation, cells were fixed with 2% Glutaraldehyde (Electron Microscopy Sciences, Hatfield, USA) for 1 minute at room temperature in CB buffer (0.1 M Sodium Cacodylate and 2 mM CaCl_2 pH 7.4). Fixation was continued after adding fresh fixative and incubation on the ice for 1 hour. From this point, all steps were carried out on ice unless stated. Cells were washed 3 x 2 minutes with chilled CB buffer. Unreacted aldehydes, which can increase nonspecific fluorescence, were blocked by rinsing cells 6 x 5 minutes in CB buffer containing 0.2% glycine. Cells were washed again 5 x 2 minutes with chilled CB buffer. Bright field and Confocal Z-stack images in 3x3 tiles of the areas of interest were recorded as described in section 2.17. Cells were treated with filtered DAB solution; 0.14 mM Diaminobenzidine (10X stock prepared in 0.1 M HCl) in CB buffer, to which 0.03% H_2O_2 was added just before use. After 100 seconds, DAB solution was aspirated, and the cells were rinsed 5 x 2 minutes with chilled CB buffer. Bright field images in 3x3 tiles of the areas of interest were recorded again as described in section 2.17. These post DAB reaction images were used to trace the areas of interest during sectioning and subsequent electron microscopic screening. Next, cells were treated with CB buffer containing 1% osmium tetroxide (Sigma, Singapore). After 1 hour, osmium tetroxide solution was aspirated, and its traces were removed by washing the cells with chilled MilliQ water for 5 x 2 minutes. Cells were stained for 1 hour with a filtered solution of 1 % uranyl acetate prepared in MilliQ water. The stain was aspirated, and cells were washed 5 x 2 minutes with MilliQ water.

Figure 14: APEX2 mediated DAB polymerization reaction. **A:** Schematic illustration of APEX2 mediated DAB polymerization reaction in the presence of H_2O_2 . The Osmiophilic polymer generated in the first step reacts with OsO_4 to generate electron dense signal at its localization. **B:** Pre (top) and post (bottom) images of APEX2 mediated DAB polymerization showing the generation of localized electron dense signal. White circles highlight the localized signal in both images.

A dehydration step was carried out by treating the cells with cold graded ethanol series (20%, 50%, 70%, 90%, 2 X 100%) 2 minutes each and finally with anhydrous ethanol 2 x 3 minutes at room temperature. This point forward, all steps were carried out at room temperature unless otherwise stated. Cells were infiltrated with Durcupan ACM resin (Electron Microscopy Sciences, Hatfield, USA) using 1:3, 1:1 and 3:1 (v/v) resin to anhydrous ethanol ratios for 45 minutes each and overnight with 100% resin. Following day, infiltration was continued by 6 changes, 1 hour each of freshly prepared resin. The Durcupan resin used in all of the infiltration steps was prepared by mixing component A, B, C and D as 11.4, 10, 0.3 and 0.1 grams respectively. Finally, cells in 100 % resin were incubated at 60 °C for 48 hours in a hot air oven (Model 652, Thermo Scientific, USA). The

consistency of resin was changed from a viscous fluid to hard due to resin polymerization after this step. Areas of interest were traced by comparing the fluorescence and post DAB reaction images and marked atop resin using a permanent marker whilst observing in the light microscope. The dishes were secured on a vice to excise ~5 x 5 mm of the marked areas with a jeweller's hacksaw. The excised plastic regions were mounted face-down on a dummy block using cyanoacrylic glue and left undisturbed for overnight at room temperature. Blocks were mounted on Leica Ultracut UC7 (Leica, Germany) ultramicrotome and trimmed using a razor blade. To assist in determining the orientation, block face was cut in a trapezoid shape. Sections of 70 and 150 nm thickness were cut using an Ultra 35° diamond knife (DiATOME). Sections were floated on water, fished out on to a 100 square mesh copper grids (Electron Microscopy Sciences, Hatfield, USA) and dried overnight at room temperature.

To stabilize the cell section, front and back sides of the grids were coated with 2 nm layer of amorphous carbon using a High Vacuum Sputter Coated (EM ACE600, Leica, Germany). The carbon coating was carried out under 3×10^{-6} mbar vacuum using 120 V current, 20.6° as sample tilt and 88° as coating angle with a rotational speed of 5 units. Carbon thickness was measured by a side-mounted quartz crystal. For better alignment procedures during tomogram reconstruction, 10 nm gold particles (BB International, UK) were used as a fiducial marker on the front and back side of the sections. The gold solution was prepared by mixing 900 µl of 10 nm Gold solution with 1400 µl 0.1% bovine serum albumin and filtered through a 0.2 µm Acrodisc Syringe Filter (Pall Corporation, USA). Carbon is inherently hydrophobic, which is undesirable for gold coating. To circumvent this problem, each side of the grids was stepwise glow discharged for

60 seconds at 5 mA under 10×10^{-4} mbar vacuum using High Vacuum Carbon Coater (208c, Cressington Scientific Instrument Ltd., Watford, UK) followed by submerging in gold fiducial solution for one minute. Finally, the grids were dried overnight at room temperature.

2.20 Telomere counting and spatial distribution analysis of telomeres

The number of telomeres in confocal Z-stack images was counted in Fiji (155, 156) using '3D Object Counter' v2.0. The fluorescence signal dots of eGFP in each cell were selected by setting a threshold value and telomere counts were obtained by selecting the 'Objects', 'Statistics' and 'Summary' options. For telomere counting, statistical analysis and graphical representation of the raw data as bar graph was obtained using Microsoft Excel version 16.16.10.

Telomere-center to nuclear envelop distance was measured in two steps. In the first step, the nuclear boundary of DAPI stained cells was marked with the help of the fluorescence signal of DAPI. Nuclear boundaries were manually traced in all the z-stacked images of the cells and nuclear masks were generated using the threshold option. In the second step, fluorescence signal dots of eGFP in each cell were selected by using a threshold option to generate a map of 'Centroids'. Next, '3D Segmentation' of the nuclear mask and telomeric centroid was generated using '3D manager' of '3D' plugin. Nuclear mask and telomeric centroid were added to "ROI manager" (Region of interest manager) and telomere to nuclear surface distance were measured by selecting 'Distance to surface (unit)' and measure 'Distances' option. The output table containing measurements values was imported to Microsoft Excel for the graphical representation of data.

2.21 Electron Microscopy of plastic embedded cell-sections

Electron micrographs of plastic embedded cell-sections were recorded as described in section 2.13. To minimize shrinking and distortion artefacts, grid squares to be imaged were first exposed to an electron beam at a dose rate of $10 \text{ e}/\text{\AA}^2\text{s}$ for 15 minutes, a process referred to here as 'baking'. Cells of interest were identified using the post-DAB confocal image as a reference. Electron micrographs with object pixel sizes of 8.78 or 2.27 nm/pixel were recorded using 2-second exposure, a range of defocus (2-5 μm) and at a dose rate of 5-10 $\text{e}/\text{\AA}^2\text{s}$.

2.22 Correlation between light and electron microscopic images

The low magnification electron microscopic images were superimposed by their corresponding pre-DAB fluorescent images to correlate fluorescent signal of eGFP with the electron dense signal of DAB polymer generated by APEX2 reaction. To compensate for the cell-section shrinking and distortion artefacts, 'warp' feature of the Photoshop CS6 (Version 13) was used to fine-tune the correlation. After correlation, electron micrographs for 2D analysis of telomeres were recorded with an object pixel size of 9.6 and 3.6 $\text{\AA}$ /pixel as described in section 2.13.

2.23 Two-dimensional image analysis of telomeres

For two-dimensional analysis of telomeres, TEM images with an object pixel size of 3.6 $\text{\AA}$ /pixel were converted into 8-bit files after adjusting the contrast in Fiji (155, 156). Telomere densities in these images were traced and extracted in image processing software Amira (Thermo Fisher Scientific, USA). Density tracing-threshold was manually adjusted while inspecting discernible features of telomeres in raw images. Telomere shape and size were measured using extracted densities, which helped in the demarcation of telomere boundary and

allowed the best approximation of measurements. Telomeres of heterogeneous and elongated morphology remained a challenge to analyses, where diameter represents the closest approximation of long and axis of the extracted densities. Statistical analysis (mean and standard deviation), inferential statistics (confidence intervals) and graphical representation for telomere size and shape as boxplots were obtained using PlotsOfData (157). The data was summarized in tabulated form using Microsoft Word version 16.16.10.

2.24 Tomography

The 150 nm thick cell-sections selected for tomography data collection were baked for an additional 10 minutes (ref. section 2.21) and coated with 10 nm gold particles for the second time (ref. section 2.212.19). Tomography data was recorded on a 200kV Tecnai Arctica (FEI) transmission electron microscope equipped with a Flacon III direct electron detector. Tomographic tilt series were recorded by tilting the sample $\pm 65^\circ$ and recording an image at every degree using the TEM Tomo Software (FEI). The parameters used for data acquisition are tableted in Table 4. The object pixel size in the tilt-series was 2.78 Å/pixel. For dual-axis tilt series, grids were manually rotated to 90° and reinserted into the electron microscope.

Table 4: TEM Tomography settings for automatic data acquisition

Search		Eucentric height		Autofocus	
Camera	BM-Falcon	Camera	BM-Falcon	Camera	BM-Falcon
Binning	2	Binning	2	Binning	2
Readout	Half	Readout	Half	Readout	Half
Exposure time	0.8 sec	Exposure time	0.2 sec	Exposure time	0.2 sec
Mode	Linear	Mode	Linear	Mode	Linear
Probe	Nanoprobe	Probe	Nanoprobe	Probe	Nanoprobe
Magnification	16000x	Magnification	39000x	Magnification	39000x
Defocus	-20 μm	Spot size	6	Spot size	6
Spot size	6	Intensity	0.47	Intensity	0.47
Intensity	0.5				
Tracking		Drift		Data acquisition	
Camera	BM-Falcon	Camera	BM-Falcon	Camera	BM-Falcon
Binning	2	Binning	1	Binning	1
Readout	Half	Readout	Half	Readout	Full
Exposure time	0.2 sec	Exposure time	1 sec	Exposure time	1 sec
Mode	Linear	Mode	Linear	Mode	Linear
Probe	Nanoprobe	Probe	Nanoprobe	Probe	Nanoprobe
Magnification	39000x	Magnification	39000x	Magnification	39000x
Defocus	-2 μm	Spot size	6	Defocus	-2 μm
Spot size	6	Intensity	0.3	Spot size	6
Intensity	0.47			Intensity	0.47
Tilt series (Autofocus)		Tilt series (Tracking)		Tilt series (Acquisition)	
Check autofocus before acquisition	Yes	Track after acquisition	Yes	Start angle	0
High tilt	1	Track before acquisition	Yes	Max Negative angle	-65°
Low tilt	1			Max Positive angle	+65°
Switch angle	30°	High tilt	1	Stage Relaxation	5 sec
		Low tilt	1	Low tilt step	1°
		Switch angle	30°	High tilt step	1°

2.25 Tomogram reconstruction

Tomograms were reconstructed into 3D volumes using IMOD eTomo software (Boulder Laboratory for 3-D Electron Microscopy of Cells, Colorado). Gold particles on both sides of the grid were used as fiducials markers during alignment procedures. The tomograms were trimming in the final step of generating the 3D volume binned to the factor of 2, giving a final tomographic pixel size of 0.556 nm.

2.26 Tomogram analysis

Tomography data was pre-processed in Fiji software (155, 156), where tomograms were low pass filtered to 20-40 Å using 'Gaussian blur 3D' option. The contrast was adjusted by 'Auto' contrast option and the data was converted into 8 bits. This step improved the signal-noise-ratio in the reconstructed tomograms (Figure 15).

Figure 15: Contrast enhancement of reconstructed tomograms. Representative unfiltered (left) and 20 Å filtered tomogram.

Reconstructed tomograms were cropped into a square shape using UCSF Chimera (158) V. 1.10.2. Two-dimensional Z-stacked averages of 6, 10, 20 or 40 nm were obtained by binning the data along Z-axis using Fiji. A tomogram, for instance, if contains 150 raw images, upon binning along z-axis would yield 15 Z-stack averages of 10 nm.

2.27 Measurement of telomeric chromatin

Tomogram volumes were dissected into Z-stacked images of 10 nm to measure the thickness of telomeric chromatin fibers in raw image stacks. These measurements were performed in Fiji (9, 10). Thickness of telomeric chromatin fibers and fiber connections in three-dimensional volumes were obtained using image processing software Amira (Thermo Fisher Scientific, USA). Statistical analysis (mean and standard deviation), inferential statistics (confidence intervals) and graphical representation for fiber measurement and fiber connection as violin plots were obtained using PlotsOfData (157). The data was summarized in tabulated form using Microsoft Word version 16.16.10.

Acknowledgements: Wee Mei Ling, a project officer in Sara Sandin's laboratory helped in sectioning of plastic embedded cell sections.

Figure 17: Schematic illustration of telomere isolation for electron tomographic analysis.

3.1.1.1 Isolation of telomeres from HT1080 metaphase cells

Telomere isolation from metaphase chromosomes was attempted considering three factors. First, my experimental observation and published results (95) show that human telomeres are attached to an insoluble nuclear matrix during G_1 , S and G_2 phase, however, it remains unknown whether this is also the case during

metaphase. Second, during interphase, telomeres also associated with nuclear A-type lamins (A/C) filaments (160), which constitute a prime component of the nuclear matrix (96, 161). At the onset of mitosis, cyclin B/Cdk1 protein kinase-dependent phosphorylation causes depolymerization of A-type lamins, thereby making them soluble throughout mitosis (162). Third, nuclear envelope-associated proteins, which constitute nuclear matrix are absent during mitosis. I purified metaphase chromosomes from synchronized HT1080 cells and their iconic X shape morphology was verified by electron microscopy (Figure 18A). As expected, the purified chromosomes could be fragmented by restriction enzyme digestion and clearly distinguishable from electron microscopy images of intact chromosomes (Figure 18B).

Figure 18: Representative electron micrographs showing the morphology of an undigested chromosome (**A**) and digested chromosome fragments (**B**).

3.1.1.2 Telomere isolation under the variable salt concentration

After establishing the protocol for chromosome purification and restriction enzyme digestion, the next step was to isolate telomeres. To identify the salt concentration required to solubilize the digested chromatin, a range of NaCl

concentrations were analyzed. This 'chromatin-release' step (described in section 2.4 and illustrated in Figure 17, step 4) was carried out using Tris HCl pH 8, 100-350mM NaCl, 1 mM EDTA, 0.2 mM EGTA, 0.1% NP-40 and 1 mM DTT. Subsequently, the samples were centrifuged to separate the insoluble pellet from soluble chromatin (supernatant). The top panel in Figure 19 shows that with an increase in the concentration of NaCl, the amount of chromatin in the supernatant increased. Although more than 50 % of the chromatin was released at 350 mM NaCl, telomeric DNA remained undetectable by Southern blot analysis in any of the soluble fractions (Figure 19, bottom panel).

Figure 19: Telomere isolation using increasing amounts of NaCl at pH 8.0. **Lane A:** 100 mM NaCl. **Lane B:** 150 mM NaCl. **Lane C:** 200 mM NaCl. **Lane D:** 250 mM NaCl. **Lane E:** 300 mM NaCl. **Lane F:** 350 mM NaCl. **Top panel:** Relative amount of insoluble (blue) and soluble (pink) DNA. **Middle panel:** Agarose gel and stained with ethidium bromide. **Bottom panel:** Telomeric southern blot analysis. Chromatin isolated from HT1080 cell line was used as positive control.

3.1.1.3 Telomere isolation under a variable concentration of NaCl in alkaline pH

Next, I tried to improve the solubility of telomeric chromatin using different buffer conditions. This included increasing the pH to 9.0, 10 mM EDTA, 2 mM EGTA, 1 % NP-40, 5 mM DTT and a broader range of NaCl concentration (200 - 2000 mM NaCl). Consistent with the above-mentioned results, increasing the NaCl concentration increased the amount of chromatin released into the supernatant. The top panel in Figure 20 (lane C) shows that over 90 % of bulk chromatin is soluble at 600 mM NaCl. However, telomeric DNA was only released into the supernatant using extremely high salt concentration (2 M NaCl) at which point all DNA-binding proteins, such as histones, are known to dissociate (Figure 20, bottom panel, lanes A-C).

Figure 20: Telomere isolation using increasing amounts of NaCl at pH 9.0. **Lane A:** 200 mM NaCl. **Lane B:** 400 mM NaCl. **Lane C:** 600 mM NaCl. **Lane D:** 2000 mM NaCl. **Top panel:** Relative amount of insoluble (blue) and soluble (pink) DNA. **Middle panel:** Agarose gel and stained with ethidium bromide. **Bottom panel:** Telomeric southern blot analysis.

Telomere association with the nuclear matrix has previously been characterized in interphase HeLa cells. Titia de Lange group has shown that telomeric DNA and TRF1 associate with the nuclear matrix (96), and lamin A/C, lamin B, p160, hnRNAP D1/D2 which are known nuclear matrix proteins (163). Whether telomeric chromatin is insoluble due to interactions with the nuclear matrix is debatable. Telomeres remain insoluble in interphase cells (95) and metaphase cells (presented in this study). Since there is no 'nuclear matrix' during metaphase suggesting that there may be other factors that contribute to the insolubility of

telomeric chromatin. Given that a number of RNA-binding proteins are known to associate with chromatin (164), I treated the samples with RNase A (ref. section 2.5) to see if the telomeric chromatin solubility improves. The obtained results show that RNA did not contribute significantly towards the insolubility of metaphase telomeres. Fluorescent *in-situ* hybridization data published by Joaquin Lingner's group show that TERRA remains bound to a subset of chromosome ends during metaphase (4). TERRA has the potential to form G-quadruplex with telomeric DNA (165) or RNA/proteins hybrids (166). RNase H, which specifically cleaves RNA from RNA-DNA hybrid, was not included in the experiments presented herein. Therefore, it remains unknown whether TERRA may play a role in telomere insolubility.

Iconic research by Laemmli's group shows that DNAase treated metaphase chromosomes contain a Pronase-resistant proteins scaffold, which is precipitated even after washing with 2 M NaCl (167). Telomeric regions of Pronase treated chromosomes also contain RNase-resistant RNA, which is stable at 600 mM KCl (168). It is possible that telomeres in metaphase chromosomes associate with such nuclease-resistant RNA factors. Further studies are required to characterize which factors that influence telomere solubility in mammalian cells.

3.1.1.4 Isolation of telomeres from hESCs cells

In differentiated cells, telomeres interact with a fibrous network of Lamin A/C, which is mediated by TRF2 (160). Notably, Lamin A/C is not expressed in hESCs (169). Additionally, embryonic stem cells express a high level of telomerase to maintain their telomere length (170). The available literature does not provide information on whether telomeres are attached to the nuclear matrix or soluble in hESCs. However, the absence of Lamin A/C gives hope that telomeres could

potentially be more soluble in hESCs as compared to differentiated cells. To test this hypothesis, chromatin from isolated hESCs nuclei was digested with a cocktail of restriction enzymes. To identify conditions required to obtain telomeres in a soluble state, the sample was equally divided into different tubes and a chromatin-release step was carried out using a range of NaCl concentration (200 – 2000 mM) and a buffer that contained 10 mM Tris HCl pH 9, 10 mM EDTA, 2 mM EGTA, 1 % NP-40 and 5 mM DTT.

As observed in the previous experiments, the high concentration of NaCl increased chromatin solubility. The top panel in Figure 21 shows that 50 % of the bulk chromatin is soluble at 200 mM NaCl. The amount of soluble chromatin is increased to 99 % in the presence of 400 mM NaCl. However, except for the condition where 2 M NaCl is used, telomeric DNA is enriched in the insoluble fractions (Figure 21, bottom panel). Notably, faint signals of telomeric DNA are detectable in the soluble fractions at 400-600 mM NaCl but a strong telomere signal is only observed at 2 M NaCl (Figure 21, bottom panel).

Figure 21: Telomere isolation from hESCs under variable salt concentration and pH 9.0. **Lane A:** 200 mM NaCl. **Lane B:** 400 mM NaCl. **Lane C:** 600 mM NaCl. **Lane D:** 2000 mM NaCl. **Top panel:** Relative amount of insoluble (blue) and soluble (pink) DNA. **Middle panel:** Agarose gel and stained with ethidium bromide. **Bottom panel:** Telomeric southern blot analysis. Chromatin isolated from HT1080 cell line was used as positive control.

3.1.1.5 Isolation of intact telomeres from mouse heart and kidney cells

Since telomeric chromatin could not be isolated at sufficient quantities for EM analysis from cells in culture, I next investigated if it could be isolated from tissue.

In these experiments, nuclei from kidney and heart tissue (C57BL/6 mice) were purified followed by chromatin digestion and sucrose gradient fractionation as described above. Next, I carried out the chromatin-release step using 10 mM Tris HCl pH 7.5, 10 mM EDTA, 2 mM EGTA, 1 % NP-40, 5 mM DTT and a range of

NaCl concentrations (100 – 2000 mM). Although a small amount of telomeric DNA was detectable at 600-1000 mM NaCl, 2M NaCl was again required to release the majority of telomeric DNA into the supernatant (Figure 22).

Figure 22: Telomere isolation from mouse heart and kidney cells. Southern blot detection of telomeric DNA. Agarose gels stained with ethidium bromide are shown in the top panel and southern blot of the same samples below. Bulk chromatin isolated from hESCs was used as positive control.

Previous studies have suggested that telomeres are attached to the nuclear matrix in adherent and non-adherent HeLa cells, Raji (Burkitt's lymphoma cell

line), human peripheral blood lymphocytes, histiocytic lymphoma, differentiated HL-60 (95), human Rhabdomyosarcoma, non-transformed lymphoblastoid cells and mouse renal adenocarcinoma (96). These studies were conducted on lithium diiodosalicylate (LIS) treated nuclei. The LIS procedure (171) removes all histones and other nuclear factors resulting in 'halo' nuclei that contain naked chromosomal DNA and insoluble nuclear remnant. The naked DNA in 'halo' nuclei was digested by restriction enzymes and telomeric DNA was found to be in the insoluble fractions (95, 96). In agreement with the above-mentioned studies, I also found that telomeric chromatin isolated from heart and kidney tissue is insoluble. However, the previously described metaphase analysis (section 3.1.1.1) suggests that the insolubility of telomeric chromatin does not depend on nuclear matrix interactions but an inherent property of telomeric DNA/chromatin.

Results from previous and current section show that telomeric chromatin either could not be isolated at all or the quantity of the isolated telomeres was insufficient for EM analysis. Neither previous studies (95),(96) nor my investigative attempts have explored the possibility of telomere isolation from Hutchinson-Gilford progeria syndrome (HGPS) cells. HGPS is an extremely rare premature aging disease caused by a heterozygous mutation in *LMNA*, the gene that encodes for A-type lamins; A and C (172) . The mutated lamin A protein is termed as progerin, which harbors a deletion of 50 amino acids within its C terminus. Progerin induces various cellular defects such as alterations in gene expression of proteins of various signaling pathways, change in nuclear shape and structure, compromised DNA repair and chromatin organization. These cellular defects ultimately leads to premature senescence and aging (173). The

most relevant fact to discuss here is that Progeria patients have extremely short telomeres. It is also noteworthy that telomeres in other cells types have been reported to be anchored to lamin A/C (95, 96), which is mutated in Progeria cells. Due to their small size and compromised anchorage site, it is intriguing to speculate that telomeres in Progeria cells might be soluble and could serve as a potential candidate for telomere isolation for structural studies.

3.1.1.6 Isolation of intact telomeres from mouse spleen-lymphocytes

Isolation of native telomeres was also attempted using mouse spleen lymphocytes, based on a previously reported study where telomeres had been successfully isolated at low salt concentrations using this particular cell type (94). The chromatin release step was carried out using a buffer that contained 75 mM NaCl, 10 mM Tris HCl pH 7.5, 1 mM EDTA, 0.2 mM EGTA, 0.1 % NP-40, and 1 mM DTT. Restriction enzyme digested chromatin was fractionated using 5-40% sucrose density gradient followed by Southern blot analysis. The obtained results show that telomeric chromatin from mouse spleen lymphocytes is soluble at 75mM NaCl and present in different fractions of the sucrose gradient (Figure 23). Fraction 7 of the sucrose gradient fractions has the maximum enrichment of telomeric chromatin. Telomere enrichment was determined by comparing the intensity of the telomeric DNA signal in southern blot against the total amount of DNA present in each fraction. Representative negatively stained electron micrographs of fraction 7 show lariat-like telomeric chromatin up to 1 micrometer in size (Figure 23 B). However, western blot analysis could not detect the presence of either TRF1 or TRF2 in the isolated telomeric chromatin. To ensure that this was not due to protein degradation during chromatin isolation I took the total cell lysates of mouse spleen-lymphocytes, liver, heart and kidney for western

blot analysis. Except for mouse spleen-lymphocytes (red asterisks in Figure 23C) the antibodies gave a strong signal for both TRF1 and TRF2 in all the other tissue. These results suggest that TRF1 and TRF2 are not present, or at low abundance, in mouse spleen-lymphocytes. This is also discussed, but not shown, in the previously published EM analysis of isolated telomeres from mouse spleen lymphocytes (94). Interestingly, Northern blot analysis has shown that a comparable expression of TRF1 mRNA in mouse kidney and spleen tissue (174). The discrepancy between the amount of TRF1 mRNA and TRF1 protein levels in mouse spleen remains unclear.

Figure 23: Telomere isolation from mouse spleen-lymphocytes. **A:** Density gradient ultracentrifugation and telomeric DNA detection by Southern blot analysis. Fractions 1-9 are labelled from left to right at the bottom of the blot where P (pellet) represents an insoluble fraction. Genomic DNA of the human HT1080 cell line was used as positive control. Fraction 7, enriched with telomeric DNA is highlighted by white circles in agarose gel and Southern blot. **B:** Representative transmission electron microscopic images showing the morphology of negatively stained telomeric chromatin in fraction 7. **C:** Western blot analysis of shelterin protein using cell lysates of the mentioned samples. Cell lysate obtained from HT1080 cell line was used as positive control.

Although TRF1 and TRF2 were not present in telomeric chromatin isolated from mouse spleen lymphocytes, I prepared cryo-EM grids to try to analyze the structure of the chromatin. I used holey grids coated with an extra layer of carbon, or a monolayer of Graphene oxide, to try to capture telomeric chromatin in vitrified ice. However, despite numerous attempts (Table 2), the purified chromatin could not be detected on cryo-EM grids. This could be due to the low amounts of material and that chromatin might stick to the filter paper during cryo-EM grid preparation. It is possible that the large size of the chromatin samples analyzed makes it challenging to capture in ice.

Titia de Lange *et al* (95, 96) reported that telomeres in HeLa cells are strongly anchored to the salt-insoluble fraction of the nucleus – generally referred to as the nuclear matrix (97, 98). Given that TRF1 was one of the components identified in insoluble nuclear matrix and a small fraction of TRF1-depleted telomeric chromatin could be released from HeLa nuclei, Titia de Lange proposed that TRF1 has an anchoring function (95, 96). This hypothesis is supported by my observation that TRF1 was not detectable in mouse quiescent lymphocytes by Western blot analysis. In contrast, the TRF1 was present in other mouse cell types. Interestingly, homologue of TRF1 has been reported to be undetectable in chicken erythrocytes – the second known cell to have soluble telomeres (94). Contrary to undetectable/low abundance of TRF1, Northern blot analysis has shown a comparable expression of TRF1 mRNA in mouse kidney and spleen tissue (174). At present, the discrepancy between the amount of TRF1 mRNA and TRF1 protein levels in mouse spleen and the implications of an undetectable/low abundance of TRF1 in these cells are unclear. It is also unclear

how these cells avoid activation of DNA repair mechanism in absence of TRF1 and TRF2.

TRF1 in conjunction with tankyrase, TIN1 and POT1 represses telomerase activity in dividing cells, thereby regulates telomere length. Since unstimulated lymphocytes have little or undetectable telomerase activity (175), it will be interesting to understand how telomerase is repressed in these cells. It is possible that TRF1-mediated regulation is not needed and these cells repress telomerase through an alternative. Quiescent mouse spleen lymphocytes are non-dividing cells with small nuclei packed with compact heterochromatin and very little cytoplasm (176). These characteristics point towards a minimum nuclear activity; hence, it is also possible that these cells require very less TRF1, which is undetectable by Western blot analysis. A complete understanding of telomere biology in quiescent mouse spleen lymphocytes necessitates a detailed characterization of telomeric chromatin and shelterin proteins in the context of DNA repair mechanism and regulation of telomerase in these cells.

3.2 Chapter II: Reconstitution of telomeric DNA and TRF1

3.2.1.1 Purification of telomeric DNA

The DNA used in these experiments was purified from a pUC 57 plasmid. The plasmid contained 24 telomeric DNA repeats in tandem, corresponding to 12 TRF1 dimer-binding sites. The telomeric DNA insert was flanked by SmaI and PstI restriction enzyme sites (Figure 24A) and PEG precipitation was used for purification (Figure 24B).

Figure 24: Purification of telomeric DNA containing 24 'TTAGGG' repeats. **A:** Schematic representation of the telomeric DNA construct. **B:** Telomeric DNA purification by PEG precipitation.

Cryo-EM analysis of the purified telomeric DNA was carried out using a Volta phase plate (Figure 25). It is a device made of continuous thin carbon film that is positioned in the back focal plane of the objective lens and generates phase contrast without the need for defocusing (177, 178). This results in a strong boost of image contrast, that makes it easier to directly visualize naked DNA, determine particle orientation in micrographs and solve structures of small complexes by single particle analysis (179) (180) (181).

Figure 25: Cryo-EM analysis of telomeric DNA. Representative cryo-EM images of DNA recorded without a phase plate (-PP, left) and with a phase plate (+PP, middle). Power spectra showing a 90° phase shift (right).

3.2.1.2 *In-vitro* reconstitution of telomeric DNA with purified hTRF1

First, it was important to identify molar ratios of hTRF1 to DNA for EM analysis. In this context, a DNA-hTRF1 binding electrophoretic mobility shift assay was performed where telomeric DNA was titrated against increasing amounts of hTRF1. The electrophoretic mobility shift assay was carried out using buffers that contained 10-150 mM NaCl, all of which showed a similar DNA-hTRF1 binding pattern. As expected, at 1:1 molar ratio hTRF1 dimers completely saturated telomeric DNA (Figure 26A). The sample where 4 out of 12 binding sites (0.3:1 molar ratio) were occupied by hTRF1 was selected for EM analysis. Clear

densities representing hTRF1 dimers bound to DNA could not be observed in cryo-EM images. Instead, the images show free DNA and heterogeneous densities of hTRF1 ranging 7-8 nm in dimensions as separate entities (Figure 26 B, C). Given that the telomeric DNA in the sample is sub-saturated with hTRF1, it is expected to observe free DNA molecules in the images. However, densities that unambiguously represent telomeric DNA bound hTRF1 could not be observed. The most likely explanation for perturbed DNA-binding could be that the nucleoprotein complex dissociates during cryo-EM grid preparation. This notion is further supported by the presence of large protein aggregates with free pools of DNA observed in cryo-EM images of the sample where all DNA binding sites (1:1 molar ratio) were completely saturated by hTRF1 (Figure 26D).

Figure 26: DNA-hTRF1 binding assay and cryo-EM analysis. **A:** Telomeric DNA-hTRF1 binding electrophoretic mobility shift assay using a buffer that contained 50 mM NaCl (left), 150 mM NaCl (middle) and 10-20 mM NaCl (right). Numbers at the top of the gels correspond to molar ratios (number of hTRF1 dimers bound to telomeric DNA). Free telomeric DNA was used as a control. **B:** Cryo-EM images of the sample highlighted with a black asterisk in A. A gallery of potential TRF1 particles is shown below. **C:** Size estimation of hTRF1 particles. **D:** Cryo-EM image of the sample highlighted with a blue asterisk in A, where all 12 binding sites (1:1 molar ratio) are occupied by hTRF1.

During cryo-EM grid preparation, complexes are exposed to the air-water interface. A recent cryo-EM study shows that as many as 90% of the protein complexes analyzed were adsorbed to the air-water interface and partly denatured. Protein denaturation during sample preparation can be reduced by using an electron microscopy support grids with a continuous layer of carbon or hydrophilized Graphene (182). Therefore, I also tried using 2 nm thick carbon coated grids for cryo-EM analysis of reconstituted DNA-hTRF1. However, this did not improve the cryo-EM grid preparation. Similar observations, where nucleoprotein complexes fall apart during cryo-EM grid preparation, have been observed in other research projects in Sara Sandin's laboratory. Although proposed methods for cryo-EM grid preparations have proven to preserve the structural integrity of proteins in general, systematic studies aiming at improving the sample preparation of nucleoprotein complexes for cryo-EM are required.

3.3 Chapter III: *In-situ* analysis of telomeric chromatin in ALT cancer cells

The technique of correlative light and electron microscopy (CLEM) was employed for *in-situ* analysis of telomeres in ALT cancer cells. To selectively stain telomeres, I transfected cells using a vector containing hTRF1 fused to eGFP and APEX2. Telomeres in transfected cells were localized in a fluorescence microscope with the help of the eGFP signal. In a subsequent step, APEX2 mediated osmium tetroxide staining was used to generate an electron dense signal of telomeric chromatin (Figure 14). Finally, cells were embedded in resin, sectioned and analyzed by electron tomography.

3.3.1.1 Biochemical characterization of U2OS cells

A U2OS cancer cell line was used for CLEM and electron tomographic analysis of telomeric chromatin. As expected, Southern blot analysis of telomeric DNA shows a smear of hybridization signal for telomeres smaller than 3 kb with strongest hybridization signal at 25 kb (Figure 27 A). In contrast to telomerase-positive cell lines, such as HT1080 cells, U2OS cells maintain telomere length by telomerase-independent ALT mechanism (183). Western blot analysis confirmed that these cells are telomerase negative and not devoided of shelterin proteins (Figure 27).

Figure 27: Biochemical characterization of U2OS cells. **A:** Southern blot analysis of telomeric DNA. U2OS cells have telomeric DNA ranging from as small as >3 kb with strongest hybridization signal at 25 kb. The higher molecular weight band, most likely, is undigested genomic DNA. **B:** Western blot analysis of telomerase and shelterin proteins in U2OS cells. Cell lysate of HT1080 cell line was used as positive control. Red asterisk highlights the absence of telomerase in U2OS cells.

U2OS cells are known to have heterogeneous telomeres, ranging in size from undetectable to > 50 kb in length (24, 42, 184-187). The results presented in Figure 27 agree with the published data. The extremely high molecular weight band might reflect homologous recombination of telomeric DNA with subtelomeric DNA, (188). In addition, sequence polymorphism of enzyme-restriction enzyme cleavage sites in the subtelomeric regions (189) could also explain the pattern of the telomeric signal seen in the Southern blot.

3.3.1.2 Transient transfection and telomere count in U2OS cells

Cells were transiently transfected by a vector that contained eGFP-APEX2 tagged hTRF1 (Figure 28 A). I next screened for optimal transfection conditions. The obtained results (Table 3 Pg. 48, Material and Methods section) indicated that at best, a maximum of 10% transfection efficiency could be achieved (Figure

28 B). Moreover, non-specific eGFP/background fluorescence signal remained undetectable in the cytoplasm or nucleus of the transfected cells (Figure 28 B). The numbers of telomeres in 56 transfected cells were counted using the fluorescence signal of eGFP (ref. section 2.20). The obtained results show that the number of telomeres in transfected cells varies from a minimum of 54 to a maximum of 103 with a mean value of 71 (Figure 28 C, D).

Figure 28: **A:** Schematic illustration of the eGFP-APEX2-hTRF1 construct. **B:** Confocal light microscopic image of U2OS cells transiently transfected by eGFP-APEX2-hTRF1 (white foci). Arrowheads indicate transfected cells. DAPI was used to stain all nuclei (red). **C:** Nucleus of a transfected cell to illustrate how the fluorescence signal was extracted from the images. **D:** Violin plot of the number of telomeres measured from 56 transfected cells. The thickness of the plot boundary represents data frequency.

Like most of the other cancerous cell lines, U2OS cells are aneuploid. The average chromosome numbers in these cells are reported to be 65-77 (27, 190,

191). Therefore, the minimum expected number of telomeres in these cells is 130. However, different approaches to characterize telomere number in fixed and live ALT cells identified ~70 telomeres (27, 190). In agreement with the published data, Figure 17 shows that the mean value of the number of telomeres in eGFP-APEX2 tagged hTRF1 transfected cells is 71. There are three important factors that most likely attribute to these observations. First, presumably as a recombination by-product, approximately 6-7% of the chromosomes in U2OS and other ALT cells are telomere-free (24, 26). This would reduce the minimum expected number of telomeres in these cells to 120. Second, like other ALT cells, a subpopulation of telomeres in U2OS cells are clustered (27), which would lead to the counting of clustered telomeres as one. Figure 17, 20, 21 and 23 show that indeed, fluorescent signal of some telomeres in the eGFP-APEX2 tagged hTRF1 transfected cells is high, indicating that some telomeres in the transfected cells are most likely clustered. Interestingly, time-lapse live-cell imaging using fluorescently labelled PNA probe against telomeric DNA show that telomeres in U2OS dynamically cluster and separate, indicating a reversible and dynamic behavior of cluster formation (190). Third, U2OS cells also have extremely short telomeres (43) that embrace fluorescent signal too weak to be detected or discriminated from the non-specific background (26, 184).

The biological relevance of telomere clustering in ALT cells has been recently addressed (28). The published data suggest that telomere clustering is induced by telomere-associated orphan nuclear receptors (COUP-TF2 and TR4) that otherwise are long-known to regulate gene expression. These proteins have been identified to bind with telomeric repeat-variant (5'-GGGTCA-3' instead of 5'-GGGTTA-3') prevalent in ~2 kb DNA preceding human telomeres (192) and

mediate telomere-telomere recombination and drive targeted telomere insertions in the chromosome (28).

In addition, ALT cells have extra-chromosomal single and double-stranded DNA called 'c-circles' and 't-circles', respectively that range from 0.7- 56.8 kb in length (24, 29, 30). These extrachromosomal DNA molecules are proposed to act as a substrate for homologous recombination to confer t-circle mediated telomere elongation in ALT cells. Although the extra-chromosomal telomeric DNA has also been reported in yeast, plants, vertebrates and even in telomerase positive cell lines (31-33), its existence in ALT cells is generally considered as one of the hallmarks of ALT cells. With the exception of U2OS cells, quantitative FISH, differential centrifugation-based separation of DNA followed by Southern blot analysis, and a newly developed technique of Halo-FISH based studies report that the amount of extra-chromosomal DNA in different ALT cells is 10-15% of the total telomeric content (24, 25). In contrast, the extra-chromosomal DNA in U2OS cells merely constitutes < 4% of the total telomeric content. Whether the chromatin architecture of extrachromosomal double-stranded DNA species is comparable to that of telomeres remains unknown.

3.3.1.3 Spatial distribution of telomeres within the nuclear volume

The spatial distribution of 263 telomeres in relation to the nuclear envelope was analyzed using a similar approach as described in section 2.20 and Figure 28 C. Telomeres and the boundary of nuclear membrane was identified using the eGFP signal and DAPI stain. The obtained results show that telomeres are distributed throughout the nuclear volume with a mean distance of 1.1 μm from their center to the nuclear envelope. Notably, ~15% of the telomeres within a distance of 500 nm and ~50 % of the telomeres were found to be in the range of 0.9-1.5 μm from

the nuclear envelope (Figure 29). Taking into consideration the measured z-height of the nuclei (4.4 - 5.3 μm), this shows that a large proportion of telomeres in U2OS cells localize close to the nuclear envelope.

Figure 29: Spatial distribution of telomeres in U2OS cells. **A:** Nucleus of an eGFP-APEX2 tagged hTRF1 transfected cell. Five telomeres are boxed, which represent telomeres in the vicinity, proximal and distal to the nuclear envelope. **B:** Sequential xz, xy and yz slices of the nucleus shown in A. The bottom insets display xz, xy and yz slices to trace telomeres boxed in A. **C:** Graphical representation of the spatial distribution of 263 telomeres from different cells in relation to their nuclear envelope.

Several studies report that telomere clustering to nuclear envelope is required for proper meiotic pairing and the recombination of homologous chromosomes in yeast and mammalian cells (193-195). Conversely, the majority of telomeres of primary cells and human cancer lines including U2OS cells are reported to be either in the nucleoplasm or randomly distributed within nucleus during interphase (96, 190, 196, 197). The above-mentioned studies discuss the spatial distribution of telomeres in general but not include in measurements of the distance of telomeres to the nuclear envelope.

The most comparable results to those presented here are from the live-cell imaging of U2OS and IMR90 cells by time-lapse confocal microscopy (197). The authors report that telomere localization in three nuclear zones, I, II and III that are similar to the terms 'in the vicinity', 'proximal' or 'distal' to the nuclear envelope, used here. The published results show that ~24% of the telomeres localize in zone I, ~43% in zone II (cumulative ~67%), which agrees to ~15% telomeres 'in the vicinity' and ~50% 'proximal' to nuclear envelop (cumulative ~65%), observed here. Interestingly, telomeres percentage in the two zones reversed at the end of mitosis and lasted during the whole process of the nuclear assembly formation (197). Since the percentage of telomeres in the zone I and II remained unchanged, telomeres most likely re-distributed in these zones after mitosis. Such dynamic behavior of telomeres in U2OS cells is also observed using live-cell imaging where the majority of telomeres followed a constrained diffusive motion, and a sub-population engaged in association and dissociation to form dynamic clusters (43).

3.3.1.4 Correlative light and electron microscopy of telomeres

Next, CLEM experiments were performed for electron tomographic analysis of telomeric chromatin inside cells. Figure 30 illustrates the workflow for the CLEM experiments.

Figure 30: Schematic illustration showing the workflow for cellular analysis of telomeres in U2OS cells.

In a typical CLEM experiment, telomeres of eGFP-APEX2 tagged hTRF1 transfected cells were identified in a confocal fluorescence light microscope by the eGFP signal (Figure 30, step 3) whereas APEX2 osmium stain was used to identify the telomeres by EM (Figure 30, step 14). Figure 31 shows that telomeres imaged in confocal light microscope could be unambiguously correlated with electron microscopic images of plastic embedded cell sections by EM. Initial CLEM experiments conducted in this study were performed without bloc Uranyl Acetate staining (step 7, Figure 30). However, subsequent experiments were performed using weak Uranyl acetate staining to enhance other the contrast of other chromatin regions and nuclear components.

Figure 31: Correlative light and electron microscopy of telomeric chromatin in ALT cancer cells. **A/B left panel:** Confocal light microscope images of z-stack slices recorded at step 3 of the workflow illustrated in Figure 30. The green colored fluorescence signals of eGFP represent telomeres. **A/B middle panel:** Electron micrographs of plastic embedded sections of cells that were not stained (A) and stained with Uranyl Acetate. Telomeres enclosed in the sections can be appreciated by an electron-dense osmium stain generated by the APEX2 tag. **A/B right panel:** Composite image of a confocal Z-slice and electron micrograph. Telomeres are highlighted with white circles. **C/D:** Electron micrographs of telomeres labelled in B.

For two-dimensional characterization of telomeres, low (11,000X) and high (30,000X) magnification EM images of telomeres were recorded using a 120kV transmission electron microscope (T12 FEI) in three independent experiments. A gallery of representative EM images is shown in Figure 32 A. Occasionally some electron dense particles (Figure 32B) of variable size and structure were observed on the cell sections, which did not correlate with the eGFP signal in the corresponding confocal light microscopic image. Such non-correlated densities, referred here as contaminations, are likely to be dust particles or small carbon particles since the grids were carbon coated to prevent charging before EM analysis (section 2.19).

Interestingly, a non-telomeric nuclear structure of near-perfect circle shape was also observed in a few cell sections. These nuclear bodies were easily distinguishable from telomeres due to their unique homogenous structural morphology. Figure 32 C shows a side-by-side comparison what appears to be a nuclear body and a telomere of similar size, shape and contrast. Even having a close resemblance, the given example shows that telomere is distinguishable from the nuclear body due to its characteristic perforated morphology. Nonetheless, the correlation between EM contrast and the eGFP signal was used here to differentiate between telomeric chromatin other electron-dense nuclear objects.

Figure 32: EM analysis of 70 nm thick cell section. **A:** Representative high magnification (30000X) images of telomeres (encircled in white). Composite CLEM image is shown in the left panel. Except for the cell # 1, all cells were stained with Uranyl acetate stain. **B:** Examples of contaminants and other electron-dense nuclear objects highlighted in red **C:** Magnified images of a nuclear body (left) and telomere (right). White arrowheads indicate a filamentous ultrastructure of telomeric chromatin.

3.3.1.5 Shape and size of telomeres

Visual inspection of the EM images suggests that the telomeres have a variable shape (Figure 33 A). Some appear spherical or elongated, whereas other telomeres have an irregular structure. In order to measure the size of the telomeres, thresholding was applied to include only the darkest contrast regions in the EM images (Figure 33 B). The box plot and tabulated statistical values in Figure 33 (C, E) shows that 68% of the telomeres have an irregular structure, 15% are spherical and 7% are elongated. These results contrast with observations of a recent stochastic optical reconstruction microscopy-based study, which report an ovoid appearance of telomeres in HeLa cells (198). The discrepancy in the results could be due to the difference in cell types and the resolution of the techniques used both studies.

Telomere size was determined by measuring the average of telomere length along x and y-axis. Majority of telomeres are heterogeneous in shape, which posed a challenge for accurate estimation of telomere size in many cases. In this context, telomere size was calculated after taking the average of multiple measurements at different axial points. The obtained results show that telomeres have a size range of 184-600 nm and a mean diameter of 342 nm. Interpretation of data in the context of telomere shape shows that heterogeneous, elongated and spherical shaped telomeres have sizes of 345 nm, 361 nm and 324 nm, respectively.

Figure 33: The size and shape of telomeric chromatin in U2OS cells. **A:** representative raw micrographs of ‘heterogeneous’ (irregular), ‘elongated’ and ‘spherical’ telomeres. **B:** extracted densities after thresholding. **C:** Box plot representation of telomere shape and size (**D**) of 69 telomeres **E:** Tabulated statistical values of data presented in C and D.

3.3.1.6 Electron tomographic analysis of telomeric chromatin

For 3D EM analysis of telomeric chromatin, tomographic tilt series were collected using 150 nm thick cell sections coated with colloidal gold particles. A total number of 15 cells in 6 cell sections were screened on a 120kV transmission electron microscope (T12 FEI). Final selection of telomeres for tomographic analysis was made after considering the following factors. First, I screened cells that expressed different amounts of eGFP-APEX2-hTRF1. Second, telomeres from selected cells denote the spatial distribution of telomeres categorized in Figure 29, i.e. including the telomeres in the vicinity, proximal and distal to the nuclear envelope. Third, telomeres representative of heterogeneous, elongated and spherical shapes categorized in Figure 33. Finally, I excluded cells that were too close to the grid bar or telomeres that did not have sufficient amounts of gold particles on the section. 10-20 gold particles are generally required for alignments of tomographic tilt series. Based on the above-mentioned criteria, a total number of 11 telomeres in four cells were selected for tomographic analysis (Figure 34) using a 200kV transmission electron microscope (Tecnai Arctica FEI) equipped with a direct electron detector (Falcon III FEI). The grids were tilted $\pm 65^\circ$ and an image was recorded at every degree using the TOMO software (FEI). Except for T7 (telomere # 7) and T11 (telomere # 11), dual-axis tilt series were recorded by manually rotating the grid 90 degrees and restart data collection. The single-axis and dual-axis tilt series were then aligned and reconstructed in 3D (Figure 34B) using the IMOD software package. Finally, the tomograms were low-pass filtered to 20Å to remove noise and 10 nm tomographic slices were extracted from the 3D reconstructions for image analysis. Inspection of the 10nm thick tomographic slices reveals a complex network of interconnected chromatin fibers (Figure 35).

Figure 34: Tomography analysis of telomeric chromatin **A:** left panel EM images of four cells (C1-C4) in 150 nm sections. **A:** middle panel, corresponding confocal images. The green colored fluorescence signal of eGFP represents telomeres. **A:** right panel, composite CLEM image. Telomeres are highlighted with white circles. Telomeres selected for tomographic analysis are labelled as T1 through T11. **B:** Tomographic slices. Arrowheads indicate the nuclear envelope (NE). The border between telomeric chromatin and other chromatin regions is highlighted in cyan.

Figure 35: Montage of 10 nm thick tomographic slices of telomere # 1 (left). Boxed areas are shown at higher magnification (middle and right). Discernible chromatin fibers are highlighted in blue and compact densities in red.

3.3.1.7 3D image analysis of tomograms

For 3D analysis of the reconstructed tomograms, I applied a 40Å low-pass filter to reduce noise, make it easier to identify high-contrast telomeric regions and to trace fibers within the 3D volume. Densities with low and high contrast were identified using interactive thresholding in software called Amira (Thermo Fisher). This process was carried out iteratively while inspecting and adjusting the

threshold to ensure precision at the pixel level. Finally, 3D densities corresponding to telomeres (high contrast regions in the tomograms) were extracted based on the contrast difference between telomeres and the surrounding non-telomeric chromatin. Figure 36 shows the steps involved in 3D volume extraction from a reconstructed tomogram.

Figure 36: Three-dimensional volume extraction of a telomere. **A:** One z-slice of tomogram # 5. **B:** Superimposition of the image shown in A and map of high contrast densities. Densities with similar contrast intensity are coloured with the same colour. Densities interpreted as telomeric chromatin is shown in dark orange. **C:** Surface rendering telomere in grey. **D:** same as C but shown without the EM density. **E:** Two-dimensional projection of tomogram. **F:** Surface rendered tomogram. A telomere is shown in yellow and the rest of the EM density in grey. **G:** Non-telomeric volume. **H:** Final extracted 3D volume of the telomere.

Three-dimensional volume extraction of telomeres from all tomograms was carried out as explained above. Figure 37 shows tomographic slices next to the corresponding 3D surface rendered telomere.

A

Tomogram # 11

Figure 37: Tomographic analysis of telomeric chromatin **A:** 6nm thick tomographic slices of the extracted telomeres **B:** Two-dimensional projections of entire tomogram (top). 3D surface rendered tomogram (bottom).

Inspection of the surface rendered tomograms shows that the telomeres have 'less-dense' and 'more-dense' regions (Figure 38). In agreement with the analysis of tomographic slices, 3D surface rendered volumes also reveal telomeric chromatin as a network of interconnected fibers of variable thickness. Independent of the assigned zones, heterogeneity and interconnection seems a universal characteristic of telomeric chromatin (Figure 38). Chromatin fiber contours were depicted as skeletonized projections of the volume to trace the fibers in 3D (Figure 39). In areas where the chromatin is highly condensed, it is more challenging to interpret the structure (Figure 39).

Figure 38: Tomographic analysis of telomeric chromatin. 3D rendered volumes of telomeres are shown in the left panel. Red and blue boxes highlight zones of 'less-dense' and 'more-dense' chromatin, respectively. Boxed areas are magnified in the middle (less-dense) and right panel (more-dense).

Figure 39: Examples of 'less-dense' and more-dense telomere regions. The full telomere is shown in yellow, extracted sub-regions of the tomograms in cyan and the trace of chromatin fibers as a skeleton (dark grey).

Chromatin fiber thickness was measured in the ‘less-dense’ regions where they are clearly distinguishable in the tomograms (Figure 40 A). The obtained results show that telomeric chromatin fibers vary in thickness and range from 10 nm to 34 nm in diameter.

Figure 40: Thickness measurement of telomeric chromatin fibers. **A:** Two representative chromatin fibers. The thickness of each fiber was measured by taking an average of measurements along the fiber axis. **B:** Violin plot representing telomeric chromatin fiber thickness measured from entire dataset (left). The thickness of the plot boundary represents data frequency. Statistical values of the data are tabulated in the right panel.

Besides variability in fiber thickness, a highly interconnected structural morphology is the most noticeable characteristic of telomeric chromatin. To characterize this, the number of connections in chromatin fibers that are traceable

at least for 40 nm along their path were counted (Figure 41). Minimum fiber length per connection was calculated after dividing the length of the chromatin fiber by the number of connections. Chromatin fibers representing all three groups of thicknesses (≤ 15 nm, ≤ 25 nm or ≤ 35 nm) and from less and more-dense areas were included in this analysis. In total, 17 fibers from different tomograms were analyzed. The obtained results show telomeric chromatin fibers are connected within a minimum length of 10 nm to a maximum of 33 nm. The mean fiber length per connection was 21.46 nm.

Figure 41: Fiber connections **A:** 3D rendered volume of telomere #2 is shown in yellow. The extracted density is shown in cyan. **B:** Gallery of telomeric chromatin fibers. Red arrowheads indicated fiber interactions. **C:** Violin plot representing minimum fiber length per connection measured from 17 fibers. Statistical values of the data are mentioned adjacent to the plot.

Since the early work of Aaron Klug (81) Cristopher Woodcock (82), John Finch and Kornberg (92) to the recent publication of Mark Ellisman and Clodagh O'Shea groups (147), bulk chromatin – in general – has been reported to be comprised of fibers of variable thickness. This reflects chromatin compaction, which is dependent on ionic strength, transcriptional and epigenetic status and cell-cycle stage. Telomeric chromatin, as observed in the tomograms presented in this thesis, is also comprised of fibers of variable thickness, however, having shelterin as its integral part, factors attributable to their variability must be different than merely an outcome of chromatin compaction. Telomeric chromatin contains 10nm nucleosomes and it is therefore not surprising that chromatin fibers with a thickness of 10-15 nm were observed in the tomograms. In addition to nucleosomes, telomeres are also decorated with shelterin proteins and TERRA at high abundance. The exact diameter of the entire shelterin complex is not known and it is also not known if shelterin complexes decorate telomeric chromatin as discrete particles. In addition to binding to the naked DNA, hTRF1 can also recognize and bind to telomeric nucleosomes (91, 199). It is possible that fibers with the mean thickness value of 20 nm represent telomeric chromatin fibers containing nucleosome-bound hTRF1. The epigenetic state of telomeric chromatin, which remains to be established could also render telomeric chromatin thicker than the bulk chromatin. The crystal structures of DNA binding domains of hTRF1 (PDB:1W0T) and hTRF2 (PDB:1W0U) show that these proteins bind to the same telomeric sequence (60). Biochemical evidence from *in-vitro* studies shows that telomeric nucleosomes favors hTRF1 binding on adjacent linker DNA but disfavor hTRF2. Given that TERRA transcription is upregulated in U2OS cells and they are more prone to accumulate TERRA-

telomeric-DNA heteroduplexes (42), it is also possible that the fibers in the thickness range of 15-25 represent such heteroduplexes. Telomeric chromatin fibers of 25-35 nm thickness may represent a high order state of a subpopulation of telomeric chromatin. Given the complex structure of telomeric chromatin observed in the tomograms, it is also possible that two separate fibers – interwind or spatially interacting with each other – appear as one thick fiber of 25-35 nm thickness.

Available literature lacks the information of whether and how telomeric chromatin fibers interact. A worth emphasizing point is that each homodimer of hTRF1 and hTRF2 can potentially bind with two TIN2 proteins. It is possible that distal telomeric chromatin fibers are extensively crosslinked through TIN2 mediated bridging of hTRF1 and hTRF2. On the other hand, biochemical and fluorescence microscopic data favor the role of TERRA in bridging the fibers through RNA-DNA heteroduplex formation. A mutual existence of both cannot be excluded.

The data published by Woodcock *et al* 2004 is the only study that provides electron microscopic glimpse of partially intact telomeric-chromatin that was isolated from mouse quiescent lymphocytes and avian erythrocytes. The authors of this study used variable ionic strength (5-75 mM NaCl) during electron microscopic imaging. Notably, at 5 mM NaCl, the chromatin displayed an 'open'/'beads-on-a-string' morphology whereas at 75 mM NaCl telomeric chromatin condensed into a contiguous '30 nm fiber'. Telomeric chromatin is shown to be adopt/retained the 't-loop' conformation at both ionic strengths. These ionic-strength dependent open/condensed states of telomeric chromatin makes for an interesting comparison with telomeric fibers observed in the

presented tomographic data. The highest ionic-strength used in those experiments was 75 mM NaCl, which is significantly lower than the physiological concentration. Conversely, the intra-nuclear sodium concentration in my samples is within the physiological range. Due to a fundamental difference in ionic strength, unsurprisingly, I observe a minority of fibers with a thickness of ~30 nm. I infer that the observed variability in the fiber thickness is an inherent characteristic of telomeric chromatin instead of an ionic-strength induced artefact.

Telomeric chromatin in the images published by Woodcock are shown to adopt t-loop – although the percentage reported in the text is 25%. According to the t-loop model, the single-stranded G-overhang folds back into the double-stranded region forming a large lasso-like structure. I could not observe any t-loop structures in the tomograms since the telomeric chromatin appeared highly compacted inside cells.

In this study, I used APEX2 labelling to enhance the contrast of TRF1 to differentiate telomeric chromatin from non-telomeric chromatin. A limitation of this technique is that it requires cells to be fixed, dehydrated and resin embedded for EM analysis. Furthermore, there is a risk of compression (shrinkage) during the dehydration step. In tomography, there is also a limited tilt range that results in anisotropic resolution due to the missing wedge. Taken together, I, therefore, estimate that the resolution obtained in the tomographic analysis is limited to 3-5nm.

4 Conclusion

A telomere is an exceptionally large heterochromatin domain that contains many thousands of shelterin proteins, TERRA and nucleosomes. Telomere maintenance is implicated to play a pivotal role in cancer and ageing. A wealth of information exists on the functional role of individual shelterin proteins, but little is known about the structure of native telomeric chromatin. In this study, I tried to isolate telomeres for EM analysis from native sources using metaphase chromosomes, hES cells, mouse heart, kidney and spleen lymphocytes. With the exception of spleen lymphocytes, telomeric chromatin was found to be insoluble. Similar to a previously reported study, telomeres could be isolated from spleen lymphocytes. However, there was no detectable level of TRF1 or TRF2 in these cells and the amount of material obtained was not sufficient for cryo-EM analysis. A low abundance of TRF1 and TRF2 in mouse spleen lymphocytes has been reported but it is not clear how these cells protect the chromosome ends from being recognized as DNA-breaks. Since isolation of intact telomeres from native sources was not successful, I next tried to reconstitute long telomeric DNA with hTRF1. Although the reconstitution worked well, hTRF1 was shown to dissociate from the DNA during cryo-EM grid preparation. The third approach used in this study was correlative light and electron microscopy (CLEM) of telomeres in ALT cancer cells. The presented data show that telomeres of eGFP-APEX2 tagged hTRF1 transfected cells can be imaged in the confocal light microscope and traced back in EM images of plastic embedded cell sections for tomographic analysis. Analysis of the nuclear distribution of telomeres revealed that a majority of telomeres were close to the nuclear envelope. EM analysis showed that the telomeres were heterogeneous in shape and size and that the mean diameter

was 345nm. Next, I used electron tomography to analyze the ultrastructure of telomeres inside cells. This revealed that telomeric chromatin in ALT cells is highly compacted and is composed of a complex network of interconnected fibers. Approximately 75% of chromatin fibers have a thickness in the range of 10-25 nm.

5 Future Directions

The present study provides the first insight into how telomeric chromatin is organized in ALT cancer cells. However, there are a number of questions that need to be answered. For instance, how does the structure of telomeric chromatin differ from other heterochromatin regions in the nucleus? How many nucleosomes are present at telomeres in ALT cells and how can we differentiate between nucleosomes and shelterin proteins in electron tomograms? Which factors influence the compaction of telomeric chromatin? What is also not known is if the subtelomeric region is embedded in the telomeric region in the tomograms analyzed here. It is difficult to envision that these questions can be addressed without having structural information at a resolution which is sufficient to resolve individual telomeric components in their native state. Cryo-electron tomography has been shown to achieve such a high resolution for ultracellular structures of pleomorphic nature. In this regard, cryo-correlative light and electron microscopy could be used in future studies of telomeres. However, one major challenge is that telomeres are relatively small objects that cannot be differentiated from other heterochromatin regions just by shape or appearance. Therefore, probes similar to APEX2 need to be developed for cryo-EM.

6 References

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