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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**CORRELATIVE LIGHT AND ELECTRON MICROSCOPY OF TELOMERIC
REPEAT-BINDING FACTOR 1 IN MAMMALIAN CELLS**

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SCHOOL OF BIOLOGICAL SCIENCES

2019

Correlative Light and Electron Microscopy of Telomeric Repeat-Binding Factor 1 in Mammalian Cells

Eric von Otter

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

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List of abbreviations

CLEM	= Correlative Light and Electron Microscopy
TRF1	= Telomeric Repeat-Binding Factor 1
TRF2	= Telomeric Repeat-Binding Factor 2
eGFP/GFP	= (enhanced) Green Fluorescent Protein
APEX2	= Ascorbate Peroxidase 2
MEF	= Mouse Embryonic Fibroblast
NCP	= Nucleosome Core Particle
NMR	= Nuclear Magnetic Resonance
Cryo-EM	= Cryo-Electron Microscopy
TEM	= Transmission Electron Microscopy
CAP	= Chromatin Architectural Proteins
DAB	= 3,3-Diaminobenzidine
DDR	= DNA Damage Response
FIB	= Focused Ion-Beam
SAEC	= Small Airway Epithelial Cell
CVC	= Chromatin Volume Concentration
PML	= Promyelocytic Leukaemia
POT1	= Protection of Telomeres 1
TIN2	= TERF1-interacting nuclear factor 2
TPP1	= TIN2-binding protein 1
Rap1	= Telomeric repeat-binding factor 2-interacting protein 1
ATR	= Ataxia Telangiectasia and Rad3 Related
ATM	= Ataxia Telangiectasia Mutated
DSB	= Double-Stranded Break
SSB	= Single-Stranded Break
TIF	= Telomere Dysfunction Induced Foci

RPA	= Replication Protein A
ssDNA	= Single-Stranded DNA
NHEJ	= Non-Homologous End Joining
HDR	= Homology-Directed Repair
dsDNA	= Double-Stranded DNA
DC	= Dyskeratosis Congenita
TRFH	= Telomeric Repeat-Binding Factor Hybridization
FRAP	= Fluorescence Recovery After Photobleaching
ITS	= Interstitial Telomeric Site
ChIP-Seq	= Chromatin Immunoprecipitation Sequencing
TAR	= Telomere Associated Repeat
ALT	= Alternative Lengthening of Telomeres
PBS	= Phosphate Buffered Saline
DMEM	= Dulbecco's modified Eagle's medium
PEI	= Polyethylenimine
CB	= Cacodylate Buffer
OsO ₄	= Osmium Tetroxide
kV	= Kilovolt
NEB	= New England Biolabs
PFA	= Paraformaldehyde
NE	= Nuclear Envelope
INM	= Inner Nuclear Membrane
NS	= Normal Salt
LS	= Low Salt
ROI	= Region of Interest

Abstract

Telomeres are specialized nucleoprotein structures at the ends of eukaryotic chromosomes. Telomeric chromatin consists of 'TTAGGG' DNA repeats sequences, nucleosomes and specific telomere-binding proteins called "shelterin". Shelterin is a six-protein complex that is essential for genome stability. Due to the end-replication problem, telomeres shorten with each round of cell division. In stem cells and germ cells, telomere length is maintained by activation of the telomerase enzyme. In somatic cells, however, which have little or no telomerase activity, short telomeres are recognized as sites of DNA damage, resulting in an irreversible cell-cycle withdrawal known as "cellular senescence". As a result, telomere length limits the cellular lifespan to a finite number of divisions. Importantly, one of the hallmarks of cancer is replicative immortality, which requires circumventing the proliferative boundary established by telomere shortening and cellular senescence. Further, the accumulation of senescent cells in human tissues with age has been implicated in age-related disease and human aging. Therefore, telomeric chromatin plays central roles in both cancer and age-related disease.

Shelterin contains two telomere repeat-binding factors (TRF1 and TRF2) that binds specifically to the double stranded region of telomeric DNA. It has been proposed that the end protection is the result of a lariat-structure called the "t-loop" which sequesters the chromosome end, thus shielding it from the DNA damage response and DNA repair machinery. Evidence for the t-loop model is based on imaging of isolated telomeres or reconstitutions of telomeric DNA and purified shelterin components. However, the organization of telomeres inside cells remains largely unknown.

To address this, I have used correlative light and electron microscopy (CLEM) to visualize and study telomeres in mammalian cells. In order to selectively visualize telomeric chromatin by CLEM, a TRF1 construct containing both eGFP and APEX2 tags was generated. EM analysis of cell sections shows that telomeres have a median diameter of 156 nm and 196 nm in HT1080 and MEFs, respectively. Following a low salt treatment of the cells, MEF telomeres de-compact and increase in diameter to 218 nm. Analysis of the ultrastructure of telomeres by electron tomography reveals a mesoporous meshwork of interconnected chromatin fibers and globular domains. Chromatin fibers range from 5 nm to 30 nm in diameter with a mean diameter of 18.5 nm which decreases to 13.1 nm in low salt conditions.

In order to establish a comparative data-set of telomeric and non-telomeric chromatin, nucleosomes were stained for CLEM by eGFP_APEX2 labelling of H2B. The comparison shows that telomeric chromatin fibers more closely resemble heterochromatin regions, whereas telomeric chromatin in low salt conditions more closely resembles euchromatin chromatin in MEFs.

In summary, this thesis uses correlative light and electron microscopy in combination with electron tomography to study telomeric chromatin *in situ* and provide new insights into the ultrastructure of telomeres in mammalian cells.

1. Introduction

By the means of Telescopes, there is nothing so far distant but may be represented to our view; and by the help of Microscopes, there is nothing so small, as to escape our inquiry; hence there is a new visible World discovered to the understanding[...] By this the Earth it self, which lyes so neer us, under our feet, shews quite a new thing to us, and in every little particle of its matter; we now behold almost as great a variety of Creatures, as we were able before to reckon up in the whole Universe it self.

- Preface to Hooke's *Micrographia*, 1665

1.1 The Nucleus

A recent estimate[1] places the number of cells in the human body at 30 trillion (3.0×10^{13}). Out of these only 10%, or 3.0×10^{12} , are estimated to be nucleated cells. Each nucleus contains 3 billion (3.0×10^9) base pairs of DNA – the human genome – which, if combined to a single molecule, would measure 2 meters in length. If all the DNA from each nucleated cell in the human body were laid end-to-end, it would be able to reach the moon and back nearly 10 thousand times. Equally striking is the fact that each of these nuclei contains 2 meters of DNA packed into a volume of only 1 picoliter (pL, 10^{-12} liter) – a volume 50 million times smaller than a drop of water. This feat is achieved through tightly wrapping the DNA around proteins to create the basic unit of genomic DNA organization, *chromatin*.

The name *chromatin* was in 1879 given to “that substance in the cell nucleus which is readily stained” by German cytologist Walter Flemming[2]. At the time of his discovery, Flemming was not able to completely distinguish his “chromatin” from the newly discovered and similar substance “nuclein”, isolated from pus leukocytes in 1869 by Friedrich Miescher. Flemming acknowledged the possibility that they were the same; however, the fact that he suggested a new name indicates that he had some confidence they were not. Flemming was later proven right, Miescher's nuclein consisted of deoxyribonucleic acids, DNA, whereas Flemming's chromatin also included its associated protein component.

Any study of the nucleus necessarily stems from a study of the cell. As is also the case with the development of microscopy; the study of larger individual cells and unicellular organisms naturally came before the study of smaller compartments therein, such as the nucleus. The first mention of a “cell”, named after its similarity to the uniform rooms inhabited by monks in monasteries by the same name, appeared in Robert Hooke's *Micrographia*[3], published in 1665 as one of the first major publications by the Royal Society (Philosophical Transactions of the Royal Society, the oldest scientific journal in the world, was established in the same year). Using a compound microscope and illumination system by his own design - in all likelihood one of the best microscopic set-ups of his time - he studied insects, peacock feathers, scales of fish, leaves, sponges, seaweed and bryozoans to name a few. The mention of a cell is found in Hooke's observations “of the pores of Cork, and other Bodies”:

“...I could exceedingly plainly perceive it to be all perforated and porous, much like a honey-comb, [...] these pores, or *cells*, were not very deep, but consisted of a great many little Boxes, [...] which were indeed the first microscopical pores I ever saw, for I had not met with any Writer or Person, that had made any mention of them before this...” – Robert Hooke, 1665, *Micrographia*, page 113.

Importantly, Hooke was not describing cells by today’s definition, but was rather describing the appearance of the cellulose cell walls of cork, devoid of any living components within. Nor did Hooke intent to suggest anything more than that, describing them as “altogether fill’d with air, perfectly enclosed in little boxes or cells distinct from another”, “empty cavities” and simply “walls”. While Hooke can be credited with coining the term cell, the founder of cell biology is reasonably the Dutch microscopist Antonie van Leeuwenhoek. Van Leeuwenhoek was in all likelihood aware of and inspired by the work of Hooke[4] and even replicated some of Hooke’s findings, specifically his microscopic studies of the mold *mucor* and of the louse. He went on to discover and describe the first bacteria in 1676 – “animacules”, as he called them, which would have measured no larger than 2 μm – and was also the first to observe and describe protozoa. His findings of bacteria and protozoa were later confirmed by Hooke[5]. In 1677, Hooke wrote in “A Letter of the Ingenious and Inquisitive Mr. Leeuwenhoeck of Delft” after initially failing to find any bacteria or protozoa in his water sample:

“I omitted therefore farther to look after them, for about five or six days, when finding it a warm day, I examined again the said water; and then much to wonder I discovered vast multitudes of those exceeding small creatures...”

As with the discovery of the cell, so the discovery of the nucleus was born out of the application of microscopy. Flemming, a microscopist, was also a pioneer in the application of dyes to biological specimen and, notably, the nucleus. Earlier microscopic attempts to visualize the nucleus and its contents were impeded by the nucleus seemingly appearing and disappearing throughout the life of a cell, a result of the refractive index of the nucleus changing between interphase and mitosis. However, with the development of new dyes and advances in cytological staining techniques it became clear that the nucleus and its contents simply adopted different refractive indices at different cell states[6].

From the original discovery of the cell to the discovery of the nucleus and later also chromatin and chromosomes, microscopy has been an indispensable tool. The field has long since progressed passed homemade compound microscopes and through the use of super-resolution microscopy and transmission electron microscopy is now capable of visualizing subcellular compartments at nanometer resolution.

1.2 Chromatin, beads on strings and fibers

1.2.1 The Nucleosome

In order to fit the large amount of DNA into the nucleus, as well as keep it organized and accessible for vital processes such as transcription and mitosis, chromosomes are associated with proteins. The primary protein component of chromosomes is the histone proteins, which together with DNA assemble into chromatin. The fundamental repeating unit of chromatin is the nucleosome, a protein-DNA complex which takes the form of a disc with the dimensions 10.1×5.7 nm[7]–[9]. It consists of ~200 bp's of DNA wrapped around a histone octamer core, which contains two copies each of histones H2A, H2B, H3 and H4[10]–[12]. In addition to the core histones, the nucleosome also includes a histone that binds the DNA linking consecutive nucleosomes together, called linker DNA. This histone is called the linker histone H1[13]. Excluding the linker histone yields a smaller unit, a sub-complex called the Nucleosome Core Particle (NCP) which consists of 147 bp's, or 1.67 turns, of DNA wrapped in a left-handed toroid around a histone core[14]. Linker histone is present throughout chromatin but varies depending on cell type, from 1 H1 molecule per nucleosome in lymphocytes to 0.5 H1 molecule per nucleosome in embryonic stem cells[15]. Histone H1 is further related to the nucleosomal repeat length (NRL) – that is, the average number of bp's between the centers of adjacent nucleosomes – where a higher prevalence of histone H1 corresponds to longer NRL. For example, the NRL in embryonic stem cells is ~184, whereas embryonic stem cells which have been depleted of linker histone have a NRL of ~175[15], [16]. A longer NRL correlated with expression levels of H1, which regulates heterochromatin compaction[17]. Further, NRLs vary between species, cell types and even different areas within a single nucleus[18], [19]. NRL range from 154 bp's in *Schizosaccharomyces pombe*[20] to ~240 bp's in sea urchin (*Strongylocentrotus purpuratus*[21]) sperm. Higher eukaryotes have on average a NRL of 180-200 bp's of DNA[22]. However, it has been clearly demonstrated that NRLs occur in multiples of 10 bp's, indicating that nucleosome orientation relative to adjacent nucleosomes is sensitive to the rotation of DNA which has a helical repeat of ~10 bp[23], [24]. Therefore, the periodicity of NRLs suggests that the most frequent NRL in human cells is 197 bp.

In addition to the canonical histones – H2A, H2B, H3 and H4 – there are also histone variants that differ in amino acid sequence. As a result of their distinct amino acid sequences, inclusion of these histone variants into nucleosomes will modify the nucleosome properties and dynamics. The chromatin content of histone variants will vary between cell types as well as different chromatin regions.

In addition, histone modifications regulate chromatin compaction. Acetylation and phosphorylation, notably on histone H3 and histone H4, decondenses chromatin leading to easier access for the transcription machinery and related proteins. Conversely, other histone marks which do not affect the charge of the nucleosome, such as histone methylations, do not directly impact chromatin compaction but rather recruit protein regulators that do[25]. The structure and organization of chromosomes are thus dependent on many factors, which together generate the complexity necessary to regulate gene expression, differentiation, and respond to DNA damage.

1.2.2 Chromatin

The concept of higher-order structures – primary, secondary, tertiary, and quaternary – commonly applied to proteins, is also applicable here and can be helpful to describe levels of chromatin compaction. According to this analogy, the primary and most basic structure of chromatin is made up by the “beads-on-a-string” conformation, consisting of a string of nucleosomes connected by linker DNA. The secondary structure in the chromatin hierarchy, analogous to the formation of beta sheets and alpha helices from amino acids, would here consist of strings of nucleosomes folding and compacting into a fiber of 30 nm in diameter. Here, variation in repeat length, histone content and epigenetic marks and the resulting variation in propensity to condense and form higher-order structures can be considered analogous of the propensity of different amino acids to form alpha helices or beta sheets. Tertiary chromatin structure would then be composed of 30 nm fibers that have folded further, likely into a supercoil structure of 100-130 nm diameter known as a “chromonema”[26]. After this point, the chromatin is thought to further assemble into 300 and 700 nm “chromatids”, before finally condensing into mitotic chromosomes of approximately 1 μ m in diameter. At this stage, the mitotic chromosome represents an up to 15,000-fold compaction of DNA[27]. For comparison, interphase chromatin represents a 200-1000-fold compaction of DNA. A schematic representation of the various stages of chromatin compaction is shown in Figure 1.

Chromatin structure is dynamic and regulates essential processes in eukaryotes such as DNA transcription, replication, recombination and repair. Indeed, chromatin is itself the substrate for these processes. It follows logically that tightly packed regions of chromatin are harder to access and will obstruct polymerases and thus transcription as well as other processes that require direct access to DNA. Chromatin that is decompacted and readily accessible is called “euchromatin” and, conversely, compact and inaccessible chromatin is called “heterochromatin”. This classification is originally based on observations of differential staining where certain regions of the chromosomes were more readily stained than others and appeared more dense[28]. Heterochromatin is the description given to transcriptionally repressed chromatin that is more condensed in structure. Conversely, euchromatin is structurally “open”, accessible and transcriptionally active. Heterochromatin is further defined into two subcategories, facultative and constitutive. Facultative heterochromatin is the name given to repressed and condensed forms of chromatin that form at various points of the chromosomes, and which varies depending on cell type. This form of heterochromatin is typically used to repress and silence genes during development. As such, facultative heterochromatin is not permanent. By contrast, constitutive heterochromatin occurs largely at the same genomic regions in all cell types and tends to occur in regions that may not carry genes and that contain repetitive DNA elements, such as pericentromeric regions[29]. In addition, remodeling of chromatin between different structural states and the balance between heterochromatin and euchromatin is believed to be central to stem cell pluripotency, differentiation and embryonal development[17]–[20], ageing[34]–[36] and its dysregulation is believed to be involved in certain diseases[37], [38].

In addition to regulation of chromatin compaction by histone proteins, chromatin architectural proteins (“CAPs”) as well as salt concentration in the nucleus also impact chromatin structure. While the atomic structure of the NCP has been well-established by X-ray crystallography[9], [14], nuclear magnetic resonance (NMR) spectroscopy[39] and more recently Cryo-EM[7], higher-order structure of chromatin domains inside cells is largely unknown and debated.

Figure 1: Schematic representation of the levels of chromatin compaction. Starting at the level of individual histones and DNA, the hierarchical model proceeds from an initial beads-on-a-string 11 nm fiber conformation to the higher-order conformation of the 30 nm fibre. Following the 30 nm fibre conformation, a number of intermediate supercoiling steps take place before reaching the compaction of the metaphase chromosome.

1.2.3 Beads on a string

The existence of the 10 nm beads-on-a-string conformation is well-established and is based on abundant and congruent data from many sources, going back almost half a century to the early 1970s. While early X-ray diffraction studies of “nucleohistones”, as chromatin was then called, produced contradictory results – identifying diffraction patterns which suggested repeating features anywhere from 0.8 to 6 nm[40] – they were nonetheless among the first to suggest a repeating feature consisting of both DNA and histones as the basis for chromatin. These early X-ray studies also established a chromatin purification procedure that would be used for

subsequent transmission electron microscopy (TEM) studies. Using this sample preparation method, Ris and Bram in 1971[41] used EM to image lysed nuclei from Calf thymus and were able to observe “100 Å fibrils” with “knobby protuberances”. They further suggested that chromatin – or, rather, “nucleohistone” – consisted of “a network of fibrils about 100 Å in diameter” with occasional features that “is locally thickened to 200 or 300 Å”.

Not much later, Ada and Donald Olins came very close to coining the now ubiquitous description of the 10-nm fiber when they in January of 1974 likened its appearance to “particles on a string”[42]. The first recorded mention of “beads on a string” appeared in a paper by Roger Kornberg, published in the same journal in May of the same year:

“... a chromatin fiber consists of tightly packed DNA and associated protein alternating with more extended DNA and associated protein, rather like beads on a string.”

The Olins’ built upon these findings in a series of seminal papers ranging from 1974 to 1976, where they isolated, fixed and ruptured nuclei from several different sources – chicken erythrocytes, rat thymus, rat liver etc. – and centrifuged these onto carbon-coated grids. These were then air-dried, positively stained and imaged by EM. In their 1974 Science paper, the Olins’ write:

“...chromatin fibers could be readily visualized [...] and revealed irregularly distributed thick and thin regions.”

In retrospect, these images represent the earliest high resolution images of chromatin. Intriguingly, much of the sample preparation procedure used by the Olins’ remains in use still today. For instance, I myself, in my work, utilized a cacodylate buffer, glutaraldehyde fixation as well as osmium tetroxide post-fixation, like the Olins’ in their seminal 74-76 papers.

In an abstract from the ASCB annual meeting in 1973 from C.L.F. Woodcock[43] with the title “Ultrastructure of inactive chromatin”, Woodcock also describes similar features of chromatin. In the abstract Woodcock outlines chromatin preparations from lysed amphibian and avian erythrocytes, sedimented through 0.1 M onto EM grids and goes on to describe:

“relatively untangled chromatin fibers were observed which were composed of arrays of discrete spherical particles about 100 Å in diameter. [...] Mean fiber diameters ranged from less than 100 Å to more than 300Å; [...] Examination of the arrangement of particles [...] suggests that increase in fiber diameter are achieved through irregular coiling and folding...”

This represents one of the earliest structural description of the 30 nm fiber, however, no micrographs or digital records other than the abstract remain.

1.2.4 The 30 nm fiber

In 1976, building upon the understanding that positively charged factors were required for chromatin folding, Finch and Klug imaged nucleosome fibers in the presence of Mg^{2+} as well as

the positively charged linker histone H1 by EM[44]. This confirmed the existence of a “thread” of approximately 300 Å observed by other researchers and further described it as a “solenoidal structure” consisting of 10-nm nucleofilaments, which required linker histone for stabilization. This was the first time the 30 nm chromatin fiber was described in detail. The “solenoidal” model suggested that consecutive nucleosomes were adjacent to their neighbors in sequence and together formed a coiled helix, or “solenoid”. The addition of linker histone here is important as linker histone alters the trajectory of the linker DNA as it enters and exits the nucleosome, which may contribute to the formation of higher-order structures[13]. Further, the addition of cations, in this case magnesium, helps counteract the strong repulsive charges of DNA.

After nearly a decade, a second model for the 30 nm fiber was proposed by Woodcock et al.[45] that suggested that the nucleosomes were organized in a zig-zag manner, where each consecutive nucleosome was instead adjacent to its *second* neighbor, so that the first nucleosome would be adjacent to the third, and the second nucleosome to the fourth, and so on. This conformation was called the “zig-zag” model. To further distinguish these two models, the solenoid model was also defined as a “one-start” helix, which meant to capture the fact that the first nucleosome in the sequence, when repeated, gave rise to the structure. By contrast, the “zig-zag” model was termed the “two-start” helix, because here the repeating motif was a rung consisting of two nucleosomes. Schematic representations of both the solenoid and zig-zag models are shown in Figure 2, C and D.

Up until very recently, high resolution data on the structure and configuration of the 30 nm fiber have been based on *in vitro* data, most frequently in the form X-ray crystallography or, more recently, cryo-EM analysis of reconstituted nucleosome arrays. In 2004[46], a crosslinking study of a nucleosomal array consisting of 12 nucleosome repeats was published by the Richmond laboratory (the same lab had previously solved the nucleosome to 1.9 Å resolution[9], see Figure 2 A). Through crosslinking studies with and without linker histone H1, complemented by negative stain EM of longer arrays, their findings indicated that the reconstituted chromatin fiber was a two-start helix. In the following year, Schalch et al.[47] presented a 9 Å crystal structure of a tetranucleosome. A crystal structure of two of these nucleosomes from the tetranucleosome is shown in Figure 2 B. While the structure itself had insufficient resolution to determine the orientation of linker DNA and histones, they were able to use molecular replacement using the 1.9 Å nucleosome structure to this end. Again, in this structure, the fiber adopted a zig-zag conformation with the DNA alternating back and forth between two stacks of nucleosomes in a manner that is incompatible with the solenoidal, one-start helix. In 2006[48], a paper from the Rhodes laboratory imaged *in vitro* reconstituted nucleosome arrays containing linker histone H5 from chicken erythrocytes and found two distinct classes of fibers which were dependent on the length of the linker DNA. Interestingly, shorter (10 to 40 bp) linker DNA resulted in a 33 nm structure, whereas longer (50 to 70 bp) resulted in a 44 nm structure. Based on this data and the deduced physical constraints of these structures, they suggested a helical model featuring interdigitation of nucleosomes - similar to the solenoidal model.

A 1982 study investigated the effect of transcriptional regulation on chromatin structure by studying the ultrastructure of Balbiani ring genes in the salivary glands *Chironomus tentans* using EM[49]. They used an RNA synthesis inhibitor (5,6-dichloro-l-fi-D-ribofuranosyl-benzimidazole) to image the transcribed gene during various transcriptional states. This inhibitor, importantly, does not interfere with the elongation or termination of transcription but instead blocks its initiation, thus allowing RNA polymerases that have already initiated transcription to complete it. When actively transcribed and coated with polymerases, genes were seen to adopt a 5 nm chromatin fiber conformation. However, after reducing the transcriptional activity with the inhibitor, yielding a state where RNA polymerases were only “sparsely distributed” along the genes, the chromatin fibers instead adopted 10 nm fiber conformations which, following the passage of the final RNA polymerases, condensed gradually into a 25 nm fiber conformation. Based on this, they suggest that genes not directly involved in transcription adopt 25 nm fiber conformations. The EM analysis was performed in plastic-embedded sections of salivary glands, and thus represents an *in situ* view of higher-order chromatin structure in *C. tentans*.

Additional models for the 30 nm fiber have continued to be put forth and the debate continues to this day as to which model is most likely to represent the structure of chromatin *in vivo*[45], [46], [50]–[53]. However, reconciling the available data is complicated by the many factors which influence chromatin folding, which include nucleosome repeat length[16], DNA sequence[54], salt concentration[55], epigenetic state[56], histone H1 content[16], and the presence of CAPs[57] as well as histone variants[58], [59]. For instance, while the original solenoid model included Mg^{2+} and linker histone, a recent Cryo-EM study of tetranucleosomal units which condensed to form 30 nm fibers was imaged entirely without salt[53]. Similarly, the two-start helix model is derived from nucleosome arrays with short NRL of 167 bp’s[47], whereas the one-start helix model contains long NRL ranging from 177-207 bp’s[48]. Further, the exact physiological conditions in the cell and nucleus are not known, as many ions are bound and sequestered by proteins and other factors. As such, it is impossible to perfectly recreate native salt conditions *in vitro*. These disparate findings from decades of *in vitro* chromatin research also highlights the need for examining chromatin *in vivo* or in near-native conditions *in situ*. As a clear consensus for the 30 nm fiber structure continues to evade researchers, an increasing body of work suggests a third possibility: that the 30 nm fiber does not exist *in vivo*.

Figure 2: Schematic representations of higher-order chromatin structure. **A)** Structure of nucleosome solved by X-ray crystallography to 1.9 Å resolution (PDB ID: 1KX5). **B)** One repeating unit of the zig-zag model consisting of two nucleosomes, solved by X-ray crystallography to 9 Å (PDB ID: 1ZBB). **C)** A schematic representation of the two-start helix model, as suggested by Hancock et al [45]. **D)** A schematic representation of the solenoid model, as suggested by Klug et al [44].

1.3 Chromatin as a disordered fiber

As developments and breakthroughs in microscopy and sample preparations brought researchers closer to characterizing chromatin structure inside cells, it was noted that the existence of a 30 nm fiber was absent from nuclear sections in a large number of eukaryotes[60]–[62], or only appears following aldehyde fixation[63] while being absent in cryo-sections. In fact,

when imaged by electron microscopy in thin cellular sections, chromatin appeared to be largely amorphous. The absence of a clear nuclear pattern was in stark contrast to other fibrous or fibrillar structures in the cell, such as actin filaments or microtubules, which were clearly resolvable using the same approach, even when tightly packed or bundled together. One possible explanation for the absence of a clearly defined structure is that chromatin fibers interdigitate between nucleosomes of adjacent chains. Inter-fiber interactions are believed to become increasingly dominant as salt conditions approach physiological, at which point nucleosome fibers are strongly compelled to interdigitate[64]. A more recent advance is focused ion-beam (FIB) milling of vitrified samples coupled with cryo-EM, which has succeeded at imaging ribosomes and nuclear pores inside the cell at high resolution[65], [66]; however, imaging of the nucleus using this technique did not reveal 30-nm fibers[66], [67].

Research findings from different experimental approaches were, concordantly, finding evidence of interphase chromatin as an irregularly folded or disordered fiber, sometimes referred to as a “polymer melt”[41]–[45]. Although certain nuclei appear to contain 30 nm fibers, such as starfish sperm[73] and chicken erythrocytes[50], these cells represent terminally differentiated and transcriptionally silent cell types. As such, the extent to which they represent a universal chromatin structure is unclear. König et al., used both LM and EM to look at *Xenopus laevis* chromosomes at different time-points throughout a reconstitution reaction, and were able to identify fibrous features in the ranges of 60-80 nm and 150-200 nm, but no fibers smaller than this. They do, however, refer to a “complex and closely associated network of small nucleosomal clusters” ranging from 20-40 nm in size, but these do not appear linear or fibrous[74]. Similarly, Belmont et al. in 1989[75], through varying the polyamine as well as fixative protocols, are able to observe 20-30 nm diameter fibers which appear to ‘come undone’ at the periphery of larger and condensed mitotic chromosomes.

A recent EM analysis of nuclei described in Ou et al.’s 2017 paper, *ChromEMT: Visualizing 3D chromatin structure and compaction in interphase and mitotic cells*[76] used fixed cells and a DNA intercalator to generate singlet oxygen through photo-oxidation in the presence of DAB (diaminobenzidine) and subsequently stained the DAB polymer with osmium tetroxide (OsO₄). They applied electron tomography of plastic-embedded sections consisting of interphase and mitotic chromatin in human small airway epithelial cells (SAECs) and human osteosarcoma cells (U2SO). With the resulting 3D data, they look at slices corresponding to a depth of 1.28 nm along the Z-axis of the 3D volume and estimate the “chromatin volume concentration” (“CVC”), a measurement of chromatin density expressed as the percentage of the tomographic volume which is taken up by chromatin. By classifying the data according to chromatin density, or CVC, they found that chromatin fibers occur in a continuous range of fiber diameters which shifts towards higher thicknesses at higher CVCs. Their findings are summarized in Figure 3.

Interphase Chromatin, SAEC

Mitotic Chromatin, U2OS

Figure 3: Summary of fiber dimension from Ou et al. Left columns correspond to tomographic slices of varying (1.28-1.6 nm thickness) to which a filtered threshold has been added. Measurements were performed as indicated with yellow lines on the thresholded areas. The resulting distribution plots are shown to the right in blue and are representative of measurements from the corresponding image stack. CVC: Chromatin Volume Concentration. Interphase Chromatin, SAEC (Fig. 4[76]) features measurements from SAECs in 1.28 nm thick tomographic slices. Mitotic Chromatin, U2OS (Fig. 7[76]) features measurements from mitotic chromosomes in U2OS cells in 1.28 nm thick tomographic slices. (Adapted from ChromEMT: Visualizing 3D chromatin structure and compaction in interphase and mitotic cells, Ou et al., Science 2017, doi: 10.1126/science.aag0025, reprinted with permission from AAAS).

They found that fiber dimensions range from 5-48 nm in diameter, with the vast majority of fibers measuring 5-24 nm. In the tomogram which corresponds to the highest CVC, 47%, the proportion of fibers which range from 24-48 nm constitute ~20% of all fibers. In a sample with a particularly low CVC of 17%, ~5% of fibers are above 24 nm. Most tomograms feature equal or similar proportions of 5-12 nm and 12-24 nm fibers. On the topic of the 30-nm fiber, the group concludes:

“we do not observe higher-order fibers in human interphase and mitotic cells *in situ*. [...] It is possible that 30-nm fibers and 120-nm chromonema are exceedingly rare and/or peculiar to specialized cell types and states. Alternatively, 30- and 120-nm fibers may be induced in *in vitro* conditions and extracted nuclei.”

Considering the absence of evidence for the 30 nm fiber from many *in vivo* studies, it is noteworthy that modern molecular biology textbooks, and indeed many scientific articles, reiterate as fact a hierarchical chromosome organization model wherein a 10 nm fiber condenses to form a uniform and regular 30 nm fiber. This is in contrast with the solenoidal structure originally described by Finch and Klug as “varying in diameter in the range of 300-500 Å”[35, p.

1898]. Indeed, as previously mentioned, Woodcock similarly describes “irregular coiling and folding” giving rise to fibers which exceed 300 Å in diameter.

1.4 Conditions of the nucleus: salt conditions and crowding effects

Capturing chromatin in a native or “physiological” state is difficult *in vitro*, primarily due to the unknown salt concentration inside the nucleus that effects intra- and intermolecular interactions, packing and folding of chromatin, but also due to additional factors such as polyamines (spermidine and spermine) that also have an effect on chromatin compaction. To add to this complexity, the concentrations of salts undergo large variations throughout the cell cycle, with some salts increasing three- to four-fold throughout the cell cycle (Ca^{2+} and Mg^{2+} , from interphase to mitosis). What’s more, isolated chromosomes have been shown to have significantly different salt contents[77] and the exact composition of salt and factors that constitutes physiological settings, is not known. Taken together, this suggests that some findings from *in vitro* studies of chromatin may not be applicable to higher-order chromatin structure *in vivo*.

Further, the histone octamer is a strongly positively charged macromolecule[78]. In humans, it contains ~220 positively charged residues and only ~ 60 negatively charged residues (depending on the isoforms and histone variants included). In other words, the number of lysines and arginines exceed the number of glutamic or aspartic acids. If the linker histone H1 is included, this would in the case of Histone H1.4 mean an additional 66 positively charged residues. By contrast, the phosphate backbone of the approximately 200 bp of DNA which interacts with the nucleosome would carry a negative charge – for each strand in the double helix – and would thus amount to 400 negative charges[79], [80]. In other words, the positively charged histones are not enough to neutralize the negative charge of the DNA. For this reason, it is clear that additional positive factors, such as cations, are required to neutralize this charge and the associated repulsive forces and thus allow for folding to occur. These cations, such as Na^+ and Mg^{2+} , serve to neutralize the negative charges on DNA thus counteracting the repulsive forces that would otherwise interfere with compaction and folding. In other words, at sufficient concentrations of cations, the interactions between nucleosomes and DNA shift from repulsive to attractive, which facilitates chromatin compaction and higher-order folding. At lower salt concentrations, repulsive forces dominate, leading to a decompaction of chromatin and a preference for uncompact chromatin in the form of the beads-on-a-string conformation.

The nucleus is separated from the cytoplasmic compartment by the nuclear membrane. Exchange of material between these two compartments is achieved through transport by the nuclear pore complex. Large macromolecules require transportation across the nuclear membrane, whereas small molecules and ions can freely diffuse through the pore complex through passive diffusion. This passive diffusion leads to the equilibration of small macromolecules as well as ions between nucleoplasm and cytoplasm[81]. Previous work has shown that incubation of cells in hypotonic conditions has a strong effect on chromatin compaction, specifically leading to chromatin decondensation and, depending on conditions,

homogenization of the nucleus and complete disassembly of nucleoli and nuclear bodies[70], [82]–[85]. This hypotonic treatment and resulting decompaction of chromatin also lead to swelling of nuclei and, if the treatment sufficiently harsh or long, may lead to lysis. Such treatment makes possible a fast and reliable method of decompacting and “opening up” chromatin in the cell.

While much effort and attention has been given replicating the polar conditions of the nucleus, comparatively little emphasis has been placed on understanding and recreating the nonpolar nuclear milieu. It is well established that, if present in sufficient amounts, the miscellaneous macromolecules in the nucleus will place a constraint on any interactions and folding of chromatin. The nuclear macromolecular nonpolar milieu has been estimated at 100mg/ml[86], [87], and is likely to impact chromatin dynamics as well as, potentially, chromatin structure and compaction. In addition to interactions resulting from net charge or dipole, other interactions may also depend on shape, size and concentration. At sufficient concentration, even inert molecules will act through steric repulsion, “crowding” or “confinement” effects. In other words, due to their being essentially impenetrable, it is possible for background molecules to exclude chromatin, both from the surrounding solutes as well as denying chromatin the space required for higher-order structure.

Experiments using crowding agents such as BSA, polyethylene glycol or dextran, show that they clearly impact protein folding, protein assembly and enzymatic activity[88], [89]. One example relevant to the nucleus is the 2004 paper[87] by Hancock, demonstrating that the disassembly of PML (promyelocytic leukaemia) bodies, nucleoli and the reduction of nucleolar transcription in low salt conditions are rescued and reassembled upon addition of polyethylene glycol or dextran, indicating that the loss of organization and enzymatic activity was not a result of low cation concentration, but loss of macromolecular crowding[86]. Subsequent work by other groups has also demonstrated that crowding effects condense chromatin *in vivo*[90], [91].

1.5 Telomeres

Telomeres are specialized nucleoprotein structures found at the ends of eukaryotic chromosomes[92]. Originally named by H.J. Muller in 1938, who used X-rays to induce mutations as well as chromosomal breakages and fusions, found that the ends of chromosomes were particularly resistant to such damage[93]. He hypothesized that chromosome termini must have a special function which involves “sealing” the chromosome end. Muller gave this hypothetical structure the name “telomere” (telos (τέλος) for “end” and meros (μέρος) for “part”, in Greek - literally “end part”)[94]. In humans, telomeres consist of “TTAGGG” repeats which vary in length between 5,000-20,000 bp’s, before terminating in single-stranded 3’ G-rich overhang ranging from 50-500 nucleotides in length[95]–[97]. In somatic cells, telomere length varies depending on age, tissue and proliferative history of the cell[98]. Telomere length also varies between species, with a lower limit of 5,000 bp’s found in humans and aves and an upper limit of 150,000 bp’s in laboratory mice[99]. This unique DNA sequence is bound by six proteins that together constitute the “shelterin” complex, thus named for its role in protecting – or sheltering –

telomere ends. Nucleosomes are also present at telomeres. Telomeric chromatin has been shown to have a short nucleosome repeat (NRL of 160-165 bp's), low nucleosome stability and higher nucleosomal mobility[100][101] and features linker DNA up to 40 bp shorter than non-telomeric chromatin[102]. Further, telomeres contain low levels of linker histone H1[102], [103].

The presence of histone modifications such as H3K9me3 (trimethylation of Lys9 on histone H3), K4K20me3 and the presence heterochromatin protein 1 at telomeric chromatin is consistent with constitutive heterochromatin[104]. Further, there is evidence that the removal of certain acetylations, such as H4Lys16Ac, which are present after histone synthesis is required for regulation of accessory proteins at telomeres. This is interesting because this histone mark has been shown to be required for decompaction[56] of certain higher-order structures. As such, its presence at telomeres is consistent with a compact chromatin state. The epigenetic state of telomeres is debated, however, and it is possible that studies of the epigenetic state of telomeric chromatin also include, and are confounded by, the epigenetic state of the subtelomeric regions[105]. Telomeres are also enriched in histone variant H3.3, which is deposited at repressive chromatin regions and is believed to promote heterochromatin compaction following trimethylation at H3.3K9[106].

Telomere length is variable and dynamic, in addition to varying between species and different cell types within an organism, telomere length also varies between the telomeres of a single cell. That is, individual telomeres within a given nucleus are not identical, but will comprise a spectrum of telomere lengths[107]–[109]. Due to an inability of the DNA replication machinery to completely replicate the ends of DNA, telomeres are successively shortened as a result of cellular proliferation[110], [111]. This shortening of telomeres, or “telomere attrition”, stems from the semi-discontinuous approach by which DNA is replicated, and the requirement of a RNA primer to initiate DNA synthesis in the 3' to 5' direction, ultimately resulting in a gap in the lagging strand upon enzymatic degradation of the terminal primer. Intriguingly, while this primer is only 8-12 base pairs in length[112], a mammalian telomere will lose 50-200 base pairs of telomeric repeats in each cell division. As such, it can be deduced that downstream processing following DNA replication, such as 5' resection to generate a 3' overhang, also plays a part in telomere attrition[113]–[115]. This shortcoming inherent to the replication machinery is called the “end replication problem”[111]. Telomere attrition continues until a critical length is reached which yields a persistent DNA damage response (DDR) foci at the telomere. At this point, the cell enters into a state of irreversible loss of proliferative potential known as “replicative senescence” and is also said to have entered “cellular senescence”. Further, it is possible a single critically short telomere is sufficient to induce replicative senescence[108], [117], [118]. The term “replicative senescence” was coined by Hayflick and Moorhead in 1961, upon observing the withdrawal from continued proliferation of cultured human fibroblasts following prolonged cultivation[119]. The discovery established the “Hayflick limit” which describes the approximately 50-60 passages a primary human fibroblast is capable of completing before reaching senescence. The replicative limit for other somatic cells are also in this order of magnitude. Importantly, Hayflick and Moorhead disproved the reigning paradigm that primary cells were immortal. This replicative

limit dependent on telomere attrition is best understood as a proliferative boundary and tumor suppressor mechanism in that it removes aged cells from continued cell cycle proliferation; in other words, it removes cells that are more likely to have accumulated oncogenic or otherwise deleterious mutations[120].

The solution to the end replication problem, and the reason offspring do not inherit short telomeres from their parents, was encountered in the form of a telomere terminal transferase which, through the use of an integral RNA template for reverse transcription of telomeres, is capable of completing the replication of chromosome ends. The enzyme was discovered by Carol Greider and Elizabeth Blackburn in 1985, and was subsequently named “telomerase”[121], [122]. The two later shared the Nobel Prize in Physiology or Medicine with Jack Szostak with the motivation “for the discovery of how chromosomes are protected by telomeres and the enzyme telomerase”. Telomerase activity is transcriptionally repressed in the vast majority of human tissues and is expressed only in embryonic stem cells, male germ line cells, activated lymphocytes and certain types of stem cell populations[123]–[125]. In germ line cells as well as embryonic stem cells, telomerase is expressed at sufficient levels to maintain telomeres at a more or less constant length. Non-embryonic stem cells (multipotent stem cells) experience telomere erosion albeit at a significantly reduced rate[126].

As mentioned above, cellular senescence can be viewed as a tumor suppressor mechanism in that it removes from continued proliferation aged cells, which naturally have a higher risk of carrying oncogenic mutations. In doing so, it not only lowers the probability that pre-cancerous cells will gain the mutations required for oncogenesis, it also directly interferes with the infinite replicative potential necessary for immortalization – one of the so-called “hallmarks” of cancer[127]. In other words, immortalization and oncogenesis necessitates overcoming the proliferative boundary established by telomere shortening and senescence. In an estimated 90-95% of cancers, this boundary is circumvented through an aberrant upregulation and reactivation of the enzyme telomerase, which then maintains telomeres at sufficient length to evade senescence and attain immortalization[128]. In the remaining 5-10% of cancers, immortalization is attained through an alternative mechanism that utilizes homologous recombination to maintain telomere length. The pathway through which this is achieved is known as the Alternative Lengthening of Telomeres (ALT) pathway[129]. While maintained above the critical length at which replicative senescence occurs, cancer cells nonetheless have relatively short telomeres[117].

In addition to its role in tumor suppression, telomeres protect chromosome ends from being recognized as DNA breaks. In the absence of the protective telomeric cap established by the shelterin complex, chromosome ends are indistinguishable from ends generated by chromosome breakage or fragmentation. As a result, unprotected or uncapped chromosome ends are recognized as DNA breaks which may result in deleterious repair and recombination events, leading to telomere-telomere fusions and genome instability[130]. This eukaryotic predicament is referred to as the “end protection problem”. Further, in mammals, the presence of the

specialized shelterin proteins at telomeres serves to repress all aspects of the DDR, as well as regulate telomere maintenance by recruitment the enzyme telomerase[96].

1.6 Telomere structure

1.6.1 The Shelterin complex

In humans, shelterin is a six-protein complex which associates with telomeric DNA sequences (Figure 4). Out of the six, two paralogous proteins, Telomeric Repeat Factor 1 (TRF1) and 2 (TRF2), form the main interface between shelterin and telomeric DNA by binding to double-stranded telomeric repeats. A third protein, Protection of Telomeres 1 (POT1), interacts with single-stranded telomeric repeats of the 3' overhang. Mouse shelterin is similar to human shelterin, except that mice have two types of POT1 (POT1a and POT1b). These DNA-binding proteins are connected through two 'bridging' proteins; TRF1-Interacting Nuclear Factor 2 (TIN2) and TIN2-interacting protein (TPP1). TIN2 is essential for the structural integrity of shelterin and serves as the 'lynchpin' of the complex, binding to both telomeric repeat factors[131] as well as TPP1[132]. TPP1, in turn, connects POT1 and TIN2 and is essential for recruitment of telomerase[133]. Finally, The Repressor Activator Protein 1 (Rap1) binds to TRF2. All six shelterin proteins are ubiquitously expressed *in vivo*[92].

In addition to existing as a six-protein complex, shelterin has also been shown to exist as subcomplexes. Several studies have shown that smaller complexes, for instance lacking either TRF2 or TRF1, still localize to telomeres[131], [134]. Cellular extracts have also identified subcomplexes where TRF1 and TRF2 are completely absent[134]. In contrast to the other shelterin components, the TRF2-interaction partner Rap1 has been shown to have additional functions and binds to non-telomeric regions where it is known to function as a transcriptional regulator[97]. The existence of subcomplexes is further evidenced by *in vivo* pull-down studies looking at stoichiometry and relative component amounts in HeLa1.3 and HTC75 cells[136], where the shelterin proteins do not exist in amounts proportional to the assumed structure and stoichiometry of the shelterin complex. HeLa1.3 is a HeLa-derived cell line with long telomeres (average telomere length of ~23 kb), conversely, HTC75 is derived HT1080 fibrosarcoma fibroblasts and have significantly shorter telomeres (average telomere length of ~4kb). In these studies, TRF2 is more abundant than TRF1 both in cell extracts as well as in chromatin-bound fractions and is present in ~1.5 times excess in HeLa1.3 cells and nearly 5 times excess in HTC75. Further, TRF2, Rap1 and TIN2 are most abundant and are present in roughly equimolar amounts (~1:1.1 for TRF2 and Rap1, ~1:1.7 for TRF2 and TIN2 for the chromatin-bound fractions). Due to the difference in telomere length, it is likely that the relative amounts in HeLa1.3 cells rather than HTC75 cells more closely correspond to the Mouse Embryonic Fibroblasts (~19kb) used in this thesis.

Figure 4: The shelterin complex. Graphical representation of the shelterin complex bound to telomeric repeat sequences and the telomerase holoenzyme (Telomerase catalytic subunit, TERT and Telomerase RNA Component, TERC).

1.6.2 Telomeres and DNA damage

As previously mentioned, telomeres protect chromosome ends from being recognized as DNA breaks or otherwise damaged DNA. In the absence of telomere protection, telomeres are recognized as damaged DNA sites, which leads to the activation of the DDR. The cellular DNA damage response is coordinated and regulated by the DDR signaling pathway which, in eukaryotes, is orchestrated by two main kinases: ATR (Ataxia Telangiectasia and Rad3-related protein) and ATM (Ataxia Telangiectasia Mutated protein); these kinases are at the very top of the signal cascade. In the presence and recognition of DNA lesions, these kinases will initiate the cascade by phosphorylating and recruiting downstream response proteins, activators and effectors. These include p53, 53BP1, PTIP, H2AX, Brca1, Chk1 and Chk2 to name a few, and initiation of the cascade may, depending on the type of lesion and source of genotoxic stress, result in DNA repair, transcriptional regulation, chromatin remodeling, checkpoint activation, cell cycle exit (senescence) or programmed cell death (apoptosis)[137]. Whereas ATM is primarily involved in responding to double-stranded breaks (DSBs), ATR is activated in response to a broader variety of DNA damage and lesions, including DSBs, single-stranded breaks (SSBs) and replication stresses. Following DDR activation, both ATM and ATR will phosphorylate histone H2AX in nucleosomes near the site of damage, which promotes local chromatin remodeling as well as the accumulation of other DDR factors. This remodeling and factor accumulation produces cytologically detectable foci, so-called telomere dysfunction-induced foci, or TIFs, that may extend for megabases around the site of damage, and are believed to facilitate repair[138]. ATR activation and recruitment appears to be dependent on the presence of RPA (Replication Protein A) coated ssDNA however it is possible that the activation and recruitment of ATR to DSBs, which is known to occur, are the result nucleolytic processing and subsequent production of single-stranded intermediates formed during resection and repair of DSBs. As such, it is possible that ATR is in fact reacting to DSBs as well. Similarly, if ATR signaling is impaired, ATM will respond to collapsed replication forks[139]. As such, the functions of ATM and ATR are, at least partially, redundant.

Deletion studies of individual shelterin components have clearly demonstrated that shelterin represses ATM and ATR signaling, as well as inhibits repair attempts of the nonhomologous DNA end joining (NHEJ) and homology directed repair (HDR) pathways. Specifically, POT1 is the key player in the suppression of the ATR signaling pathway, and TRF2 for the suppression of the ATM pathway[140]. The mechanism by which TRF2 inhibits ATM signaling has yet to be fully understood, but it is hypothesized to act through two complementary approaches. The first approach is that TRF2 is vital for the formation of a hypothesized telomeric lariat structure *in vivo* called the 't-loop', which serves to sequester the 3' end into telomeric dsDNA upstream through strand invasion and in doing so hides the chromosome end from detection by the DDR (See Figure 5). For the second, the current view is that TRF2 both inhibits the ATM kinase directly[141] and also interferes with 53BP1 ubiquitylation and accumulation, which are required for ATM activation[142]. I will return to the subject of the t-loop in the next section. In addition to inhibiting ATM, TRF2 is also believed to inhibit NHEJ, and telomeres depleted of TRF2 quickly become substrates for NHEJ and are fused together.

Figure 5: Telomeres and the t-loop. Graphical representation of a telomere, showing the subtelomeric region transitioning through degenerate telomeric repeat sequences into the telomeric DNA of a telomere, which terminates into the 3' overhang. Below, a representation of lariat structure resulting from looping of the telomeric DNA and subsequent strand invasion of the 3' overhang into telomeric dsDNA, forming the t-loop.

NHEJ and HDR are the two major pathways that respond to and repair dsDNA breaks. Homology-directed repair, as the name suggests, requires homologous sequences to aid in repair and thus requires a diploid organism or haploid organisms in a transient diploid state, such as during S phase when sister chromatids are spatially close. By contrast, NHEJ does not require a homologous template for repair and will instead ligate broken DNA ends together. In mammalian cells, NHEJ is the most frequently implemented pathway for DSB repair, while HDR is preferred only for DSBs at replication forks. Out of the two, NHEJ is more efficient but also more error prone. In the absence of inhibition from shelterin components, the NHEJ and HDR repair pathways will result in telomere end-to-end fusions or rapid telomere resection leading to telomere loss, respectively[143]. Ultimately, cells lacking telomere protection end up in ATM/ATR-mediated cell cycle arrest, followed by cellular senescence or apoptosis.

Due to the close interactions and structural interdependence between the shelterin proteins, it is hard to delineate the outcome of individual protein depletions in isolation. Interfering with

TRF2 will by extension interfere with Rap1. Similarly, interfering with TRF1 or TRF2 will perturb TIN2. As an example of their interdependence of the shelterin components, depletion of both TRF1 and TRF2 results in complete loss of shelterin as well as activation of both ATM and ATR[144]. In addition, not all shelterin components can be knocked down *in vivo* due to the resulting lethality or significantly reduced viability of cells in culture.

The close association between shelterin and the DDR also extends also into dysfunction. For instance, a persistent and irresolvable or otherwise overactive DDR is believed to be a hallmark of telomere dysfunction. An illustrating example is replicative senescence, wherein telomere attrition has progressed until the telomere length is insufficient to bind the required number of shelterin complexes in order to uphold end protection. The resulting telomere uncapping gives rise to a persistent, irresolvable DDR – uncapped telomeres require either more telomeric repeats from telomerase or additional shelterin complexes, neither of which are achievable through DNA repair – at critically short telomeres. This persistent DDR signaling leads, through the ATM pathway, to the sustained activity of the p53 tumor suppressor which, in turn, results in sustained transcription of factors that halt cellular proliferation. What follows is irreversible cell cycle withdrawal – a hallmark of cellular senescence[145]–[148]. Correspondingly, inactivation of the p53 pathway allows for cells to circumvent senescence and for telomere attrition to continue, typically resulting in the formation of dicentric chromosomes through telomere-telomere fusions[149].

Further, certain genetic telomere disorders are associated with, and may be exacerbated by, the DDR. Such disorders tend to result from mutations that interfere with the normal function of shelterin and associated proteins. One common example of such a disorder is dyskeratosis congenita which, in addition to being associated with bone marrow failure as well as high risk of leukemia and solid tumors, also carries some characteristics of premature ageing[150]. Patients suffering from dyskeratosis congenita, or DC, have significantly shortened germline telomeres and the disease frequently features pathogenic genetic variants of TRF1, TRF2, TPP1, TIN2, TERC, TERT and other factors required for proper telomere and telomerase function[151]. The bone marrow failure associated with DC arises from stem cell exhaustion, caused by dysfunctional and short telomeres which, while likely affecting all telomeres, particularly impacts cell types with high turnover rates such as hematopoietic stem cells. Stem cell exhaustion occurs when these populations of cells prematurely enter cellular senescence or undergo apoptosis as a result of dysfunctional telomeres and DDR activation, and further increases the risk for cellular defects and tumors in these populations as well as cells of this lineage [152], [153]. Therefore, in addition to protection offered by the putative t-loop model, it is well established that the shelterin complex also contributes to end protection through the repression and inhibition of the DDR.

1.6.3 The T-loop

It is still unclear by what mechanism telomere deprotection takes place, and what happens when a telomere transitions from being sufficiently long, to becoming critically short which results in activation of the DDR. One model for telomere-end protection is the telomere-loop model (or t-

loop), which suggests that mammalian telomeres are folded into a lariat like structure that is stabilized by TRF2. The “t-loop” model further suggests that these loops serve to sequester and hide chromosome ends from repair enzymes, making them inaccessible to the DDR machinery[154]. Originally described in 1999[155], the t-loop is believed to have come about as a primitive solution for chromosome end stabilization following the eukaryotic switch to linear genomes in evolutionary history, which would have occurred prior to the existence of telomeric DNA-binding proteins or telomerase[156]. A second model suggests that shelterin components act on telomeric chromatin to significantly compact telomeres and that this compaction alone is sufficient to render them inaccessible, without the need for a higher-order t-loop structure [157]. The results presented in the paper proposing the compaction model, however, have come under scrutiny and have been questioned by two subsequent papers[158], [159]. A third possibility is that no shelterin-dependent compaction or lariat structure is required, and that shelterin’s repression of the various DDR components alone is sufficient, along with the already compact telomeric chromatin structure. In all three cases, replicative senescence is attributed to an insufficient number of shelterin binding sites leading to insufficient shelterin to form the t-loop, compact telomeres or repress the DDR, respectively.

Several imaging studies have attempted to visualize and study the structure of the putative t-loop. Griffith et al., generated artificial telomeric DNA incubated with purified TRF2 *in vitro* which was subsequently subjected to rotary shadowing with tungsten. When subsequently imaged in an electron microscope, they found that some of the complexes had formed “large duplex loops” and, further, that TRF2 was present at the junction of the loop and the tail in all loops. Exchanging TRF2 with TRF1 also yielded loops, but at a reduced frequency, and TRF1 was not observed at the loop-tail junction. They subsequently repeated the procedure with psoralen cross-linking of the DNA followed by deproteinization, and spread the samples using the classical Kleinschmidt spread[160] with similar results. An additional study by Nikitina and Woodcock[161] provided evidence for the t-loop by successfully isolating telomeres with partly intact telomeric chromatin from mouse lymphocytes and chicken erythrocytes, which feature a reduced complement of chromosomal nonhistone proteins and reduced nuclear matrix. The isolated telomeres, or more accurately telomere-enriched chromatin fractions, were further negatively stained with uranyl acetate and imaged under various salt conditions, reaching a maximum NaCl concentration of 75 mM – approximately half of what is believed to be physiological. The resulting micrographs clearly showed t-loop structures, which at high salt condensed into 30 nm fibers, apparently in lariat conformation[161] (See Figure 6, below). Common to all the t-loop papers discussed above, however, is the fact that only a subset of telomeres adopts the t-loop formation. In the case of the 2014 Woodcock and Nikitina paper, 25% of loops (less, still, if fibers deemed too short to form loops are included). For the 1999 Griffith paper, t-loop formation occurred in 17% and 20% of model DNA to which TRF2 had been added. They further estimated that 35-40% of the model DNA carried sufficiently long overhangs to form lariats and thus suggest that the frequency of t-loop formation was, in fact, high. Importantly, removal of the 3’ hang – which according to the t-

loop model should make strand invasion and thus lariat formation possible – still resulted in 7% loop formation.

Figure 6: Micrographs of loop structures in telomere-enriched chromatin from Nikitina and Woodcock. Telomere-enriched chromatin fixed with glutaraldehyde under various NaCl conditions. **A)** 5 mM NaCl. **B, C)** 25 mM NaCl. **D-G)** 75 mM NaCl. The beads-on-a-string conformation is observed at lower salt concentrations, whereas increased ionic strength yields 30 nm fibers. Arrows indicate putative loop-tail junctions. **A-E)** Telomere-enriched chromatin from chicken erythrocyte. **F, G)** Telomere-enriched chromatin from mouse spleen lymphocytes. (Reprinted with permission from Rockefeller University Press, ©2004 Nikitina and Woodcock. Originally published in *Journal of Cell Biology*[161]. <https://doi.org/10.1083/jcb.200403118>).

Evidence for the role of TRF2 in t-loop formation has also been proposed by super-resolution imaging studies showing that formation of lariat-like structures in chromatin spreads from isolated nuclei is dependent upon the presence of TRF2[162], [163]. However, again the t-loops occur in a small subset of telomeres following cytocentrifugation (see Figure 7, below). AFM

studies have also suggested that TRF2 is able to bend telomeric DNA around its TRFH domain *in vitro* and thereby mediate the formation of looped structures [164].

Figure 7: STORM Imaging of Telomeres in Intact MEFs and Mouse Splenocytes. **Fig 1, B)** 3D-STORM (STORM) image of MEF telomeres visualized by fluorescence in situ hybridization. The Z coordinates in the 3D STORM images shown here are color coded according to the colored scale bar above Fig 2, D. **Fig 1, D)** T-loop-like structures observed in a small number of telomeres from mouse splenocytes following cyto centrifugation. Left, large field view showing several telomeres. Right, examples of individual telomeres. **Fig 2, D):** STORM imaging revealing T-loops after chromatin spreading. (Adapted and reprinted with permission from Elsevier, ©2013 Doksani et al., originally published in Cell[162]. <https://doi.org/10.1016/j.cell.2013.09.048>).

Taken together, evidence for the t-loop has been primarily based on various *in vitro* imaging experiments of decompacted and isolated telomeres using TEM and super-resolution microscopy. Common to all studies is the presence of distinct loop structures, however these only occur in a subset of telomeres imaged. While the model offers a congruent mechanism for telomere protection and a structure that is dependent on sufficient telomere length to be upheld, the t-loop has not been observed inside cells the structure of telomeric chromatin inside cells remains largely unknown.

1.6.4 Telomeric Repeat Factor 1, TRF1

As previously mentioned, TRF1 along with TRF2 are essential for telomere-end protection and are integral for anchoring the shelterin complex to double-stranded telomeric repeats as well as provide specificity for telomeric DNA sequences[165]. The two paralogs share a domain organization, with both containing a TRF homology (TRFH) domain and C-terminal SANT/Myb domains, which are connected through a flexible hinge region. [166]. They differ however in their

N-terminal domains, where TRF1 has an acidic domain and TRF2 has a basic domain[167]. *In vivo*, TRF1 and TRF2 exist primarily as homodimers through an interaction between these dimerization domains. While the TRFH domains in TRF1 and TRF2 share a 52% sequence identity, the telomeric repeat factor's respective TRFH domains are nonetheless able to differentiate between the two, and do not allow for formations of heterodimers[168]. Similarly, although evolutionarily related and overall structurally similar, their respective TRFH domains recognize distinct interaction partners. Both repeat factors interact with TIN2, however this interaction is based in the TRFH domain of TRF1 and the hinge domain for TRF2[169]. Myb-like homeodomains belonging to both TRF1 and TRF2 bind to two half sites of the 5'-YTAGGGTTR-3' sequence motif with nanomolar affinity[170].

Since telomeric chromatin also contains nucleosomes which share the same substrate of telomeric DNA, it is possible competition of binding between TRFs and nucleosomes impact chromatin structure. *In vitro* evidence suggests that TRF2 in particular is impeded by the presence of telomeric nucleosomes whereas TRF1 is not. In addition, TRF1 may affect nucleosome spacing and has been shown to induce nucleosome sliding in telomeric nucleosomal arrays *in vitro*[171], [172]. A recent study (preprint) has reported that TIN2, in the presence of TPP1, is capable of accommodating both TRF1 and TRF2 without competitive binding[173].

A 2004 study[174] looked at the impact of tagging telomeric proteins with GFP in HeLa cells, and further used immunofluorescence to investigate whether GFP-tagged TRF1 and TRF2 are displaced from telomeres by their endogenous counterparts in cells expressing comparable amounts of each. They found that GFP-TRF1 readily colocalized with endogenous TRF2 at telomeres, and immunofluorescence suggested a similar telomere-bound to nucleoplasmic ratio for both tagged and untagged TRF1 and TRF2, indicating that GFP-tagged proteins are not displaced by their endogenous proteins. The study thus concluded that telomere-binding characteristics were not significantly impacted by the use of GFP-TRF fusion proteins. Fluorescence recovery after photobleaching (FRAP) studies further showed that the association of TRF1 and TRF2 with telomeres in living cells is highly dynamic in, with fluorescence recovery in bleached telomeres taking place within seconds of bleaching. By contrast, the same experiment with histone variant H2B revealed no recovery within 3 minutes after bleaching. TRF1 and TRF2 also display similar binding kinetics ($t_{1/2} = 9.4 \pm 1.3s$ and $8.1 \pm 1.5s$, respectively) and residency times of less than a minute.

While TRF1 deletion is lethal in mice and results in growth arrest and senescence in culture, conditional allele knockouts experiments have nonetheless proven to be informative. The deletion studies have shown that replication of the telomere in the absence of TRF1 results in activation of the ATR kinase during replication (S phase) and yields a fragile telomere phenotype in metaphase. This phenotype is reminiscent to the one resulting from replication inhibition by aphidicolin, which similarly gives rise to breaks, lesions and gaps that are visible in metaphase chromosomes[175]. In addition, the appearance of fragile sites is consistent with improper replication, i.e. replication fork stalling or replication fork collapse, at common non-telomeric

fragile loci which experience a higher rate of recombination and are associated with hotspots for chromosomal rearrangements and deletions in cancer. Deficiencies or inhibition of either the ATR pathway or HDR, both of which are involved in responding to replication fork collapse and fork stalling, also exacerbate the frequency of breaks or damage at fragile sites[176]. Taken together, these data indicate that TRF1 is required for the replication of telomeres. Whereas TRF1 is indirectly related to ATR signaling, TRF2 is directly involved in the repression of the ATM signaling pathway[177].

While telomeres have undergone significant change over evolutionary time, mouse and human telomeres remain structurally and functionally similar. One exception is, as previously noted, Pot1[178]. The *Mus musculus* and *Homo sapiens* interspecies sequence identity for TRF1 is 65% and sequence similarity is 76.5%[168], based on BLOSUM62 Blast results between TERF1_HUMAN sequence P54274-1 and TERF1_MOUSE sequence P70371-1, yielding an expected value (E-value) of 0.0 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, blastp suite-2sequences). Sequence identity between the TRFH domains is 83% and Myb-domains is 84%. Importantly, their Myb-related DNA-binding domains are nearly identical and bind to telomeric DNA with the same sequence preference. Broccoli et al., upon conducting a comparison of the two genes and proteins, concluded that biochemically the two are “nearly indistinguishable” and, further, that “TRF1 is functionally conserved”[167].

1.6.5 Subtelomeric regions

Whereas TRF1 and TRF2, as previously established, bind with high affinity to telomeric repeats, it is nonetheless the case that telomeric sequences, or at least telomere-like sequences, occur elsewhere in the genome outside of telomeres. The two primary examples of such extratelomeric sites are subtelomeric regions as well as interstitial telomeric sites (ITSs). Subtelomeric regions are variable and dynamic regions directly proximal to telomeres that act as a transition from telomeric repeats and chromosome-specific sequences. They are composed of mosaics of segmental block duplications, low copy subtelomeric repeat sequences and degenerate telomeric repeats. In humans, subtelomeres tend to be heterochromatic, with substantial H3K9me3 and CpG methylations[105]. It is estimated that in humans, subtelomeres make up 25% of the terminal 500 kb, a number that increases to 80% for the most distal 100 kb[179]. As such, the combined length of subtelomeric sequences ($0.25 \times 500 = 125$ kb) outnumber telomere length many times over, even in organisms with long telomeres. Importantly, due to the high degree of variability exhibited in subtelomeres, the size, sequence content, duplication frequency and organization are all different for each subtelomere[180]–[182].

It has been suggested that studies intending to image telomeres by GFP-tagging or FISH may inadvertently target and image these subtelomeric sequences. An extensive 2011 ChIP-Seq study identifying the binding profiles of TRF1 and TRF2 in a BJ-HELTRasmc tumor cell line found many potential TRF-binding sites[183]. For instance, an analysis of ITSs containing TRF-binding sites came up with 48 different loci. However, and importantly, the mean length of the ITS sequences was only 159 bp's, which is unlikely to interfere with telomere fluorescence or labeling. Further,

the vast majority of these sites are degenerate telomeric repeats. In the same study, out of the 714 human ITSs listed in their databased used (RepeatMasker, UCSC) only 8% showed up in TRF-bound ChIP-Seq results. The longest continuous TRF-bound sequence identified in all TRF ChIP-seq results spanned 400 bp and was one of only two reads above 200 bp in length. Another subtelomeric sequence analysis, limited to one human chromosome arm (4p), established that the sequence feature most similar to telomeric sequences consisted of 22 almost continuous copies of a 6mer, which contained three perfect telomeric repeats as well as an overall sequence similarity of 72% [184]. While such a stretch may be similar enough for TRF-binding, it is nonetheless a short and largely degenerate sequence and is unlikely to generate sufficient fluorescent signal to interfere with imaging of telomeres.

There exist also a G-rich canonical telomere-associated repeat (TAR) sequence, parts of which are almost always found within the most distal 2 kb of a subtelomere, although which parts and the degree of similarity vary. TAR-similar sequences are also found adjacent to (TTAGGG) $_n$ -like islands, and more distantly related versions of the TAR sequence are frequently found in pericentomeric regions[179]. However, the canonical TAR sequence itself – originally described in 1990[181] by Brown et al., and spanning a total of 2111 bp – contains a single perfect “TTAGGG”, and the same sequence also houses the only complete TRF binding motif. As such, while subtelomeres are large and interspersed with varyingly degenerate TTAGGG-like sequences these are all short, relatively scarce and unlikely to contain perfect TRF binding motifs. A 2001 paper places the number ITSs composed of four or more telomere repeats to number over one hundred, but with varying degrees of degeneracy[185].

1.7 Correlative microscopy, APEX and APEX2

Electron microscopy is increasingly becoming an important tool in cell biology as is it capable of offering unique structural insights into cellular structures and architecture. However, the relative vastness of a cell when imaged at high magnification makes it difficult and time-consuming to localize and image small or rare cellular structures. Further, when imaging small structures at high resolution it is important to locate the features of interest with high precision. For these reasons, methods which speed up and facilitate identification and localization is often required in order for any quantitative analysis to be performed. What’s more, structures with unknown cellular localizations, unknown appearance and morphology or that do not stand out from the cellular background are similarly difficult to study by EM. While telomeres are present in large numbers in each cell, they are nonetheless small (roughly 200 nm in diameter) and are, importantly, indistinguishable from non-telomeric chromatin without specific labelling or staining. By utilizing a correlative approach where the structure of interest, in this case telomeres, is labelled for both light and electron microscopy, it is possible to overcome these limitations.

In the work presented here, telomeres were imaged using Correlative Light and Electron Microscopy (CLEM). To this end, a combination of two tags were used in tandem to label telomeres – an APEX tag for EM and fluorescent eGFP tag for LM. APEX is an engineered genetically encoded 28kDa EM tag derived from a plant ascorbate peroxidase by Martell et

al.[186]. In the context of correlative imaging, APEX is used to generate an osmiophilic polymer from 3,3'-diaminobenzidine (DAB) monomers, upon the addition of a hydrogen peroxide catalyst. This osmiophilic DAB polymer, which is generated locally at the structure of interest, can then be stained with Osmium Tetroxide to generate contrast in EM. This contrast distinguishes telomeres from the nuclear background as well as non-telomeric chromatin, while also providing increased contrast for EM. By also correlating the APEX-stain with eGFP fluorescence, it is further possible to distinguish telomeres from other sources of contrast in EM, such as dirt or particularly dense nuclear bodies and structures. Imaging of GFP fluorescence also makes it possible to image the live cells in order to ensure good cellular health and sufficient transfection efficiency, prior to DAB polymerization by APEX. APEX2 is a derivative of APEX, obtained through directed evolution by the same group, and offers an improved catalytic efficiency and activity in cells[187]. The APEX and APEX2 reaction is further unaffected by chemical fixation of the enzyme, allowing for fixation to take place before any imaging is performed. This greatly simplifies the subsequent correlation due to inhibiting movement of the cells and cellular structures during imaging and sample preparation. As such, APEX2 can suitably be used in combination with eGFP to visualize, identify and study cellular structures at high magnification in EM.

The application of DAB polymerization for use in electron microscopy was first established in 1982 where it was combined with microinjected fluorescent dyes and polymerized by photooxidation[188]. Subsequent developments have allowed for specific labelling by using immunostaining[189], [190] and, more recently, endogenous enzymes such as APEX. To date, CLEM has been successfully employed to study a plethora of cellular events and structures and is frequently coupled with electron tomography[191].

APEX is an engineered genetically encoded 28kDa EM tag derived from a plant ascorbate peroxidase by Martell et al.[186]. In the context of correlative imaging, APEX is used to generate an osmiophilic polymer from 3,3'-diaminobenzidine (DAB) monomers, upon the addition of a hydrogen peroxide catalyst. This osmiophilic DAB polymer, which is generated locally at the structure of interest, can then be stained with Osmium Tetroxide to generate contrast in EM. By subsequently correlating eGFP fluorescence from light microscopy with the APEX-labelling in EM, it is possible to identify and distinguish the labelled structure from other sources of contrast in EM. APEX2 is a derivative of APEX, obtained through directed evolution by the same group, and offers an improved catalytic efficiency and activity in cells[187]. The APEX and APEX2 reaction is not effected by chemical fixation. This allows for fixation to take place before any imaging is performed, which makes possible correlation the fluorescence images obtained early in the sample preparation with EM micrographs obtained at the end. As such, APEX2 can suitably be used in combination with eGFP to visualize, identify and study cellular structures at high magnification in EM.

2. Materials and Methods

2.1 Cloning

APEX2 (Lam 2015, AddGene, kindly supplied by Alice Ting) was cloned into Clontech pEGFP-C1 vector (Clontech, Cat#6084-1, USA) C-terminally of GFP using PCR based cloning utilizing the restriction sites BglII and SacI (NEB, Cat#R0144S/R0156S, Singapore). Full length long isoform TRF1 (Homo sapiens telomeric repeat binding factor 1 (TERF1), transcript variant 1, mRNA, NCBI NM_017489.2, Laboratory of Daniela Rhodes) was subsequently inserted C-terminally of APEX2 using EcoRI and BamHI (Thermo Scientific, Cat#ER0271/Cat#ER0051, USA) restriction sites. Yielding a linker of 15 bp of the sequence “TCCGGACTCAGATCT” between EGFP and APEX2, and a linker of 21 bp linker of the sequence “GGAGCTCAAGCTTCGAATTCT” between TRF1 and APEX2. This construct was used for all TRF1-experiments.

For the histone construct, histone H2B (HIST1H2BJ, NM_021058.3) was amplified from a pEGFP_N1 vector[192] kindly supplied by Dr. Gabriela Davey (Davey Lab, NTU), and inserted into two separate pC1 Clontech vectors, firstly into the pEGFP-C1 vector in the same orientation as the TRF1 construct and, secondly, into the same vector missing the EGFP-gene, yielding an N-terminal APEX2 tag alone. The cloning procedure of the second construct, APEX2_H2B, required the reintroduction of Kozak fragments upstream of the APEX2 gene, and necessitated an additional PCR and purification step. In the tandem EGFP-APEX2 vector, the linker between the two tags is 15 bp and of the sequence “TCCGGACTCAGATCT” (same as in the TRF1 construct). Further, the linker between APEX2 and H2B in both vectors is 29 bp and of the sequence “AAGCTTCGAATTCTGCAGTCGACGGTACC”.

Constructs were amplified through transformation into competent NEB10 β cells grown under kanamycin selection using standard protocols. All amplification products were purified by Gel Extraction Purification using the Nucleospin® Gel and PCR Clean-up kits (Macherey Nagel, USA), according to manufacturer instructions, following agarose gel electrophoresis in a 1% agarose gel (Agarose (Certified Megabase Agarose), Bio-Rad, Cat#1613108, USA). Sequences for the constructs can be found in Appendix A1.

Figure 8: Graphical representation of the cloned constructs. Numbers correspond to number of amino acids. The length of the protein blocks corresponds to their sequence length, whereas linkers do not. The TRF2 construct was supplied by Dr. Alexander Ludwig.

2.2 Cell culture

Cell lines used in this study include iMEFs (Immortalized mouse embryonic fibroblasts) kindly provided by Ben Nichols' group at the MRC LMB and HT1080 cells (human fibrosarcoma cells) kindly provided by Zbynek Bozdech group at SBS. Cells were grown on fibronectin-coated plates (Sigma Aldrich, F1141-1MG, Singapore), seeded using a 1:400 dilution in PBS, for 2 hours at 37°C, and were subsequently washed with PBS. All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM 1X + GlutaMAX, 4.5g/l D-Glucose, 110 mg/L Sodium Pyruvate, Life Technologies, Singapore) supplemented with 10% FBS (Fetal Bovine Serum, FBS Gold, PAA Laboratories, Canada) and penicillin/streptomycin (PS, Thermo Scientific, USA) and incubated at 37°C, 5% CO₂ and 20% O₂.

2.3 Transfections

24 hours prior to transfection, cells were seeded onto 35 mm glass-bottom MatTek dishes (MatTek, Cat#P35G-1.5-15-C, USA). 1-2 hours prior to transfection cells were given fresh DMEM + 10% FBS media without penicillin/streptomycin. Overexpression of fusion proteins took place in media lacking penstrep. MEF cells were transfected using polyethylenimine (PEI, stock: 1 mg/ml; Polysciences, Cat#23966, USA) at a cell density of 20-40% using a 3:1 ratio of PEI:DNA (w/w). DNA and PEI were added separately to two aliquots of pure DMEM and, following a brief incubation in RT, both solutions are combined and incubated for an additional 30 minutes at room temperature. Upon completion of the incubation, the combined mixture is added dropwise onto the cells.

For transfection of MEFs in a 35 mm MatTek dishes, 1.25 µg or 2 µg of DNA were used for expression of TRF1 and H2B constructs, respectively. In all PEI transfection, overexpression took place for 16 hours before cell fixation. Calcium phosphate was used for transfections of HT1080. To achieve this, a 1 M solution of CaCl₂ was diluted to 125 mM in a final volume of 550 µl to which a volume corresponding to 2 µg of construct DNA was added dropwise. The eppendorf tube was mixed by flicking following each added drop. The resulting mixture was then added dropwise to 550 µl of 2xHBS buffer (50 mM HEPES, 10 mM KCl, 280 mM NaCl, 1.5 mM Na₂PO₄, pH 7.1) and

the mixture was incubated at 37°C for 30 minutes. Following incubation, the mixture was added to the cells and incubated for 16 hours before fixation.

2.4 CLEM procedure

2.4.1 Fixation

Initial transfection optimization experiments utilized a 10 minute 4% PFA fixation, following which the samples were mounted onto glass slips using 50% glycerol as a mounting medium and sealed with nail polish. The procedure employed for CLEM (correlative light- and electron microscopy) is based largely on the methods outlined in Shu et al.[193] and Martell et al.[186]. For normal salt conditions, following the cell culture, transfection and expression steps outlined above, cells were fixed with a 2% glutaraldehyde (EMS, Cat#16220, USA) solution prepared in 0.1 M Sodium Cacodylate Buffer (CB, EMS, Cat#12310, USA) at pH 7.4 containing 2 mM CaCl_2 . The cells were incubated in fixation solution for 5 minutes in RT, following which the solution was exchanged for fresh solution and subsequently incubated for 1 hour on ice.

For the hypoosmotic treatment of cells to induce chromatin decompaction, the term “low salt” treatment will be used henceforth. For low salt treatment, the fixation step was preceded by an incubation in 0.3X PBS for 1 minute after which cells were fixed with 2% glutaraldehyde in 0.3X PBS for 5 minutes at RT, after which the fixative was changed and the cells incubated for 1 hour on ice, still in 0.3X PBS. The procedure for all conditions was identical for all subsequent steps, which were all carried out on ice. The samples were washed with cold CB for 3x3 minutes, blocked and quenched for 6x5 minutes in cold CB containing 50 mM Glycine and then rinsed for 5x2 minutes in cold CB.

2.4.2 Light microscopy image acquisition

After cells were imaged on a CorrSight spinning disc confocal microscope (FEI/Thermo Fischer Scientific) using a cooling stage (FEI/Julabo) at 4°C. The CorrSight equipped with an Orca R2 CCD camera (Hamamatsu), a 40x and 63x oil immersion objective (EC Plan-Neofluar 40x/1.30 Oil M27, Zeiss, Cat#420460-9900-000, Singapore) and a 488 nm laser line with standard filter sets. Confocal fluorescence images at consecutive focus depths were collected with a step size of 300 nm, yielding an image stack of optical slices of the fluorescence in transfected cells. These were acquired from marked regions of interest using FEI's Live Acquisition software (v. 2.6.0.24) and were collected at 40x nominal magnification. The same regions of interest were subsequently also imaged in transmitted light, and their positions saved in the software.

2.4.3 APEX2/DAB reaction

After confocal and transmitted light microscopy, 0.03% (v/v) of H_2O_2 (ICM pharma, Singapore) was added to a freshly prepared solution 2.5 mM 3,3-diaminobenzidine (DAB)(Sigma-Aldrich, Cat#D8001, USA), prepared through diluting a 25 mM DAB solution 10x in 0.1M HCl. Following addition of H_2O_2 to the diluted DAB solution, 2 ml of the mixture was then aspirated into a syringe and filtered through a 0.2 μm filter (Acrodisc® Syringe Filters with Supor® Membrane, Pall,

Singapore) before adding the solution to the cells. The reaction was allowed to proceed for 1.5-2 minutes before the reaction was terminated with 5x2 minutes washes with cold CB. After DAB polymerization, transmitted light images were again recorded of transfected cells.

2.4.4 Post-fixation and uranyl acetate staining

Following completion of the post-reaction image acquisition, samples were post-fixed with a 1% solution of osmium tetroxide (OsO_4) (EMS, Cat#19152, USA) in cold CB containing 2 mM CaCl_2 for 60 minutes and were subsequently washed 5x2 minutes in cold MilliQ water. The samples were then stained with a filtered 1% uranyl acetate prepared in CB for 1 hour. After uranyl acetate staining, the cells were washed five times two minutes with cold MilliQ filtered water.

2.4.5 Dehydration

Samples were then dehydrated using a cold graded ethanol series consisting of 2-3 minute incubations with 20%, 50%, 70%, 90%, 100% and 100% ethanol. An additional two incubations with 100% anhydrous ethanol for 3 minutes each was performed at RT, at which point any remaining condensation in or around the dish was manually removed with Kimwipes (Kimberly-Clark™ Professional Kimtech Science™ Kimwipes™ Delicate Task Wipers, Singapore).

2.4.6 Resin infiltration

Durcupan ACM resin ((11.4 g component A, 10 g component B, 0.3 g component C, 0.1 g component D) (Sigma-Aldrich, Cat#44611/44612/44613/44614, USA)) was prepared was mixed with anhydrous 100% ethanol and added to the cells at a ratio of 1:3, 1:1 and 3:1. Each infiltration step proceeded for 30-45 minutes at RT on a rocking stage, covered by a plastic cover to prevent dust contamination. 100% Durcupan resin was then added to the cells and incubated overnight. The following day, pure resin was exchanged 3-6 times for 1 hour each after which the samples were placed in a 60°C oven for 48 h for resin polymerization.

2.4.7 Sample excision and mounting

After resin polymerization, transfected cells were localized using a light microscope and areas of interest were sawed out using a jeweler's saw. The sawed out resin blocks were then cleaned from debris with MilliQ and Kimwipes and mounted on dummy resin blocks using super glue (Yamayo, Superglue RED, Japan). The samples were then left overnight covered by a protective plastic cover in a low-light environment.

2.4.8 Ultramicrotome sectioning

Samples were first trimmed by hand using a razor (Single-Edge Razor Blade Carbon Steel, Electron Microscopy Sciences) into a trapezoid shape of 1-2mm in size, and any remaining debris or glass particles were manually removed. The sample was then mounted and aligned on a Leica EM UC7 Ultramicrotome fitted with a diamond knife (Ultra 35° Diamond Knife DiATOME). Ultrathin sections (70-140 nm thick) were sectioned for EM analysis. The resulting sections were collected

from the aqueous ultramicrotome reservoir on floated 100 mesh copper grids (VECO, EMS, Cat#011-Cu, USA) and left to dry under cover in a low-light environment.

2.4.9 Carbon coating

The grids were next mounted in a High Vacuum Carbon Sputter Coater (EM ACE600, Leica) and coated with a 1.5-3 nm layer of carbon (Coating settings were 3×10^{-6} mbar, 120 V, 20.6° sample tilt, coating angle 88° and with a rotational speed at setting 5). Thicker sections intended for tomography were coated a second time on the opposite surface of the grid.

2.4.10 Gold fiducial coating

Prior to fiducial coating, the grids were placed on glass slides (Microscope Slides, BMH880103 TWIN END FROSTED, Biomedica, USA) covered with a stretched layer of Parafilm (Parafilm M 4", All-Purpose Laboratory Film, Bemis, USA) and glow discharged (208c, Cressington Scientific) 5 mA, 10×10^{-4} mbar for 60 on the section side of the grid and 45 seconds on the opposite side. The grids were then submerged into a filtered 10 nm gold fiducial solution (900 µl BB International 10 nm Gold Solution Cat#EMGC10, USA) in 1400 µl 0.1 % BSA, filtered through a 0.2 µm Syringe Filter for 1-1.5 minutes before being left to air dry under plastic cover in a low-light environment.

2.5 Fluorescent foci quantification

Using the 3D object counter in Fiji[194], an intensity threshold including fluorescent punctae but excluding fluorescent background was applied to optical slices of individual cells in a fluorescent image stack.

2.6 TEM imaging and correlation procedure

TEM micrographs were acquired on a Tecnai T12 (FEI) operated at 120 kV fitted with a 4k x 4kx Eagle CCD camera (FEI). Prior to acquiring images, sections were irradiated ("baked") at low magnification for 15 minutes. Cells of interest were located using tiled transmitted light image maps of post-DAB and fluorescence images of areas of interest, collected on the CorrSight. Electron micrographs of transfected cells were acquired at low magnification. Optical slices from the fluorescent stacks with focus depth corresponding to the section were identified, based on a comparison on nuclear features and morphology, and were subsequently overlaid with the micrograph and correlated in Adobe Photoshop (CS6, Version 13.0). Typically, minor changes in the nuclear morphology which occur as a result of infiltration, dehydration and sectioning were corrected for to correlate fluorescence and EM images. Following identification of telomeres by correlation, higher magnification EM images were recorded using a nominal magnification of 23-30,000x and an applied defocus of -2µm to -5µm.

2.7 Telomere size measurements

Size estimations of telomeres were performed on high magnification micrographs of ultrathin 70 nm sections in both HT1080 and MEFs. Telomere dimensions for non-spherical morphologies

were estimated by averaging their width and height using the line measurement tool in Fiji[194]. For round telomeres, a representative diameter for the structure was used.

2.8 Electron tomography and 3D reconstruction

140 nm thick sections used for tomography were first screened on a Tecnai T12 (FEI) and baked as described above. The grids were then transferred to a 200kV Tecnai Arctica (FEI) equipped with a Falcon III detector. Dual axis tilt series were recorded orthogonally respective one another at -65° to $+65^\circ$ with micrographs acquired at every degree. This yielded a complete data set of 232 images, 131 for each tilt series. Following completion of the first tilt series, the sample holder and grid were taken out of the microscope manually rotated by 90° before collecting the second tilt series. Tilt series were collected at a nominal magnification of 39,000x, yielding a pixel size of 0.27 nm, and at an applied defocus of $-2\ \mu\text{m}$ to $-3\ \mu\text{m}$ and dosage of $10\text{-}15\text{e}/\text{\AA}^2$ per micrograph. The dual tilt series were then reconstructed by filtered backprojection using the the IMOD software package[195], [196] (version 4.9.0), during which all tomograms were binned by a factor 2. The resulting reconstruction was then loaded into 3dmod and used to create an image stack of consecutive tomographic slices representing the internal structure at depth intervals of 0.54 nm in x/y/z direction (corresponding to the original pixel size post binning). For line scan analysis, the tomograms were binned in the z-direction by a factor of 10, for extraction of 5.4 nm thick tomographic slices. These were subsequently lowpass filtered to 20 Å using the “3D Gaussian” function in Fiji[194]. 2D fiber measurements were carried out on resolvable fibrous features from these binned and filtered tomographic slices with using the measurement tool in ImageJ/Fiji[194]. Mid-point fiber measurements were carried out on unfiltered and unbinned tomographic slices. Line scans were performed using the plot profile tool in ImageJ/Fiji and were subsequently exported to excel where they were visualized as graphs.

Tomographic reconstructions (x/y/z pixel size 0.54 nm) were visualized using surface rendering in Amira (Thermo Scientific, version 6.5.0). This was achieved through assigning a numerical threshold corresponding to the intensity of the DAB/OsO₄ stained telomeric chromatin, using the “interactive threshold” function. Contiguous domains of the tomogram were then identified and assigned a numerical number and color using the “labeling” function. The labelled contiguous domain corresponding to the central telomere was subsequently excised from non-contiguous and peripheral chromatin, as well as background intensity, using the “arithmetic” operation. This yielded a 3D surface rendering which excludes densities outside of telomeric chromatin. The extracted volume was visualized by surface rendering using the “generate surface” operation, with a minimal smoothing of “1.5”. Finally, images from the resulting surface render were generated using Amira. Images of cross-sections of the surface rendered volume were similarly generated in Amira, using the “region of interest” or “ROI” crop operation.

Figure 9: Overview of the tomography dual tilt procedure. A) One tilt acquisition with micrographs obtained every angle, tilted from +65 to -65. B) Following the initial tilt series, the sample (section, pink) is rotated 90 degrees and the same tomography procedure is used to acquire a second tilt series.

2.9 Center point telomere fiber measurements

Tomographic slices of telomeres from low salt-treated cells were manually screened for apparent resolvable fibrous features. Identified fibers were then traced in Z in order to identify the start and end points of the fiber and the center point was determined to be located at the tomographic slice half-way between these two extremes. 2D fiber dimension measurements were then carried out in triplets at the center point using the line measurement tool in Fiji[194].

2.10 3D Segmentation of chromatin features

Segmentations of isolated fibers and other chromatin features were generated from unbinned filtered tomographic slices (corresponding to 0.54 nm thickness in Z). Again, the threshold value

corresponding to the darkly stained intensity was applied and extracted by applying a threshold value (as described above). Features were manually segmented by manual selection of thresholded areas corresponding to the feature of interest. Manual segmentation was terminated in tomographic sections where the feature in question could no longer be unambiguously resolved. Extracted and segmented areas were then visualized in 3D by surface render as previously described, using the “generate surface” operation. The dimensions of these segmented structures were then measured in Amira and images generated.

3. Results

The goal of the work presented in this thesis was to investigate the structure of telomeres in mammalian cells. To achieve this, the work was divided into four parts:

1. Firstly, generate eGFP and APEX2 constructs of TRF1 and H2B for correlative light and electron microscopic analysis.
2. Secondly, characterize the shape and diameter of telomeres in HT1080 (human fibrosarcoma cell line) and Mouse Embryonic Fibroblasts (MEFs) by transmission electron microscopy.
3. Thirdly, apply electron tomography and image analysis to study the 3D structure of telomeres in MEFs.
4. Finally, compare the ultrastructure of telomeres to non-telomeric chromatin.

3.1 Cloning of TRF1 and H2B constructs

3.1.1 Cloning of correlative TRF1 constructs

Figure 10: Gel electrophoresis of PCR products and digestion fragments of TRF1 constructs. **Left)** PCR products (lane 1 and 2) from amplification of the TRF1 gene (1320bps) from a pET-31b(+) vector using primers with 5' extension restriction sites for EcoRI and BamHI. Molecular weight reference (M) is a 1 kb DNA ladder (NEB). **Right)** Electrophoretic analysis of the C1_EGFP_APEX2_TRF1 construct digested with EcoRI and BamHI. High molecular weight bands correspond to the empty vector (5481 bps) from which TRF1 has been excised and the low molecular weight bands to TRF1 alone (lane 1 and 2). Molecular weight reference (M) is a Generuler 1kb ladder (Thermo Scientific).

In order to visualize telomeric chromatin by correlative microscopy, a construct was generated containing EGFP, APEX2 and human Telomeric Repeat-binding Factor 1 (TRF1). First, the TRF1 gene was amplified by Polymerase Chain Reaction from a pET-31b(+) vector (EMB Biosciences),

resulting in a product of the expected size (1320 bps) as shown by electrophoretic analysis using a 1% agarose gel (Figure 10, left). This product was then cloned into a pEGFP_APEX2_C1 vector (Clontech) using EcoRI/BamHI restriction sites. The final construct thus contained EGFP_APEX2_TRF1. Following transformation, amplification in NEB® 10β Competent E. coli cells and purification of the product through gel extraction, a restriction enzyme digestion using EcoRI/BamHI was carried out. The digestion products were analyzed by gel electrophoresis showing bands corresponding to the pEGFP_C1 + APEX2 backbone (appeared close to the 5000 bp reference ladder, expected size 4731 bps) and corresponding to the TRF1 insert alone (Figure 10, right).

3.1.2 Cloning of correlative H2B constructs

Figure 11: Gel electrophoresis of PCR products and digestion fragments of H2B constructs. **Left)** PCR product from amplification of H2B gene insert from a pEGFP_N1 vector using primers with 5' extension restriction sites for KpnI and BamHI, theoretical product size 381 bps. 100 bp DNA ladder (M) from (NEB). **Middle)** Analysis of the C1_EGFP_APEX2_H2B construct with BglII/BamHI, theoretical product sizes are 1173 and 4690 bps of DNA. **Right)** Analysis of the final C1_APEX2_H2B construct digested with KpnI/XhoI, theoretical product sizes 807 and 4329 bps.

As a control for labeling nucleosomes, a histone H2B construct was made utilizing 5' extension during PCR to add EcoRI and BamHI restriction sites to the insert. H2B was subsequently cloned into a pEGFP_APEX2_C1 vector. Similarly, this construct was analyzed by gel electrophoresis (Figure 11) following PCR amplification of the insert, which yielded a band of the expected size (381 bps of DNA). Following transformation and amplification in NEB 10β Competent E. coli cells and purification of the amplified product through gel extraction, an analytical restriction enzyme digestion using enzymes XhoI/BamHI was carried out on six colonies. Out of these, 2 colonies expressed plasmids with the expected increase in product length (Figure 11, middle, lanes 3 and 6).

In addition, a third construct was made containing APEX2_H2B without eGFP (Figure 11, right). It was digested with NheI and BglII, resulting in an excision of the EGFP gene as well as the Kozak-sequence upstream. In order to re-introduce this Kozak consensus sequence, two synthetic oligos were purchased. When duplexed, these contained a sense 5' "CTAGC" sequence corresponding to a digested NheI and an antisense 5' "TCTAG" sequence corresponding to a digested BglII end, as well as an internal XhoI site. The digested vector was then ligated with the duplex. Further, the APEX2 insert was amplified with 5' extensions which included a Kozak fragment along with BglII and SacI sites. This APEX2 insert was then cloned into the vector using BglII and SacI. Following transformation and amplification in NEB 10 β Competent E. coli cells, the final product from four colonies was digested with KpnI and XhoI, showing that the XhoI site was present. The analytical digestion yielded bands corresponding to 807 bps of the insert and introduced oligo and 4329 bps corresponding to the backbone (Figure 11, right, lanes 1-4).

After cloning, the plasmids were amplified in NEB 10 β cells, purified using a PureLink Quick Plasmid Miniprep Kit (Life Technologies, Cat#K210011, Singapore) and sequenced (1st Base Asia, Singapore, single pass DNA sequencing service).

3.2 Microscopic analysis of transfected constructs in HT1080 and MEF

3.2.1 Expression of correlative TRF1 construct in HT1080

Next, human fibrosarcoma cells (HT1080) grown on 35 mm glass-bottomed MatTek dishes were transiently transfected with the eGFP_APEX2_TRF1 plasmid using calcium phosphate. After 16 hours of transfection, cells were washed with PBS, fixed in 2% glutaraldehyde and processed for correlative microscopy analysis as described in Materials and Methods (section 2.4) and Results (section 3.3). Representative fluorescence images of transfected HT1080 cells are shown in Figure 12 (C-G), as well as transmitted light images recorded before and after DAB polymerization reaction (A-B). Clear fluorescent foci can be seen in transfected cells, corresponding to individual telomeres labelled with the eGFP_APEX2_TRF1 fusion protein.

Figure 12: Transmitted light and confocal fluorescence microscopy of HT1080 cells expressing eGFP_APEX2_TRF1. **A)** Transmission light image of HT1080 cells before DAB polymerization. Arrows indicate a transfected cell. Arrows with asterisk indicate an untransfected cell. **B)** Transmission light image of the same area after the DAB polymerization reaction. The transfected cell appears darker. **C)** Fluorescence image of the same area as shown in A and B. Bright foci inside the nucleus correspond to individual telomeres. **D-G)** Gallery of representative fluorescence images. Scale bar 10 μm .

3.2.2 Expression of correlative TRF1 and H2B constructs in MEF

Mouse embryonic fibroblasts (MEFs) were transfected and processed for correlative microscopy as described above, except that polyethylenimine was used as a transfection reagent instead of calcium phosphate. Representative fluorescence images for transfected MEF cells are shown in Figure 13 (C-F), as well as transmitted light images before and after the DAB polymerization (A, B). Clear fluorescent foci characteristic of labelled telomeres are visible throughout the nuclei in the fluorescence images. Further, stained punctae following the APEX2 reaction and DAB polymerization are visible in confocal images (Figure 13 B).

Figure 13: Transmitted light and confocal fluorescence microscopy images of fixed MEF cells transfected with TRF1 construct. **A)** Transmission light image of fixed MEF cells transfected with a correlative TRF1 construct. **B)** Transmission light image of the same area following the DAB polymerization reaction. **C)** Confocal fluorescence image of the same area. **D-G)** Confocal fluorescence images of representative cells. Arrows without asterisks indicate transfected cells, arrows with asterisk indicate untransfected cells. Scale bar 10 μm .

In order to de-compact chromatin, MEFs were incubated in 0.3X PBS for 1 minute and then fixed with 2% glutaraldehyde in 0.3X PBS. Cells incubated and fixed in 0.3X PBS will here be referred to as “low salt conditions”. Representative fluorescence and transmitted light images of transfected MEF cells after low salt treatment are shown in Figure 14. Here again fluorescent foci characteristic of telomeres are clearly visible in transfected cells.

Figure 14: Transmitted light and confocal fluorescence microscopy of MEF cells after low salt treatment. A) Transmission light image of fixed a MEF cell expressing eGFP_APEX2_TRF1. **B)** Transmission light image of the same the same cell after DAB polymerization reaction. **C)** Confocal fluorescence image of same area as shown in A and B. **D-G)** Confocal fluorescence images of representative cells. Scale bar 10 μ m.

In order to optimize transfection efficiency and labeling of telomeric chromatin, different concentrations of DNA and transfection reagents were screened for both HT1080 and MEF cell lines. HT1080 cells consistently displayed higher levels of background fluorescence in the nucleus as compared to MEFs (Figure 12 D, G, Figure 13 C-F). Further, as seen in Figure 12 G, fluorescent signal was also detected in nucleoli in HT1080 cells. The unspecific nuclear signal, likely resulting from the presence of unbound or excess TRF1-construct, yielded significantly higher levels of DAB polymer and OsO_4 stain throughout the nucleus in HT1080 cells as compared to MEFs (see Figure 12 B and Figure 13 B). The majority of transfected MEFs displayed specific telomere labelling with low amounts of background fluorescence (see Figures 13, 14) and further displayed no nucleolar staining. No apparent difference in terms of labeling of telomeric chromatin and fluorescent signal was observed after low salt treatment of MEFs. The APEX2 reaction in MEFs yields readily distinguishable DAB punctae at telomeres, whereas this is not apparent in HT1080 cells. This is likely the result of HT1080 cells having shorter telomeres compared to MEFs.

The same transfection and imaging approach was applied to label all chromatin in the nucleus expressing the eGFP_APEX2_H2B fusion protein. As expected, the fluorescence signal was apparent throughout the nucleus with the exception of nucleoli (Figure 15 C-F). This is expected as nucleoli contain factors that are primarily engaged in transcription of ribosomal genes and assembly of ribosomal subunits.

Figure 15: Transmitted light- and confocal fluorescence microscopy images of fixed MEF cells transfected with H2B construct. A) Transmission light image of fixed MEF cells transfected with a correlative H2B construct. B) Transmission light image of the same area following the APEX2 polymerization reaction. C) Confocal fluorescence image of the same area. D-G) Confocal fluorescence images of representative cells. Arrows without asterisk indicate transfected cells, arrows with asterisk indicate untransfected cells. Scale bar 10 μ m.

A control experiment was also performed where MEFs were co-transfected with eGFP_TRF2 and APEX2_H2B and processed for correlative TEM as described previously. The aim of the experiment was to determine if dense features in the nucleus in APEX2-labelled H2B which had been observed in previous correlative H2B experiments colocalized with telomeres. The experiment showed no co-localization of fluorescently labelled TRF2 with any dense structures or nuclear bodies seen in the nucleus.

3.2.3 Quantification of fluorescently labelled telomeres

To quantify the number of fluorescent labelled telomeres per nucleus, I collected Z-stacks of eGFP_APEX2_TRF1 transfected cells and analyzed the images using a plugin called 3D Object Counter in Fiji/Image J[194]. A chromosome-spread analysis carried out by Barbara Hübner in our research group indicate that the MEF cell line used in this study is polyploid and contain 74 chromosomes instead of 40 (data not shown). Analysis of 166 cells, including both normal and low salt conditions, showed that the MEFs had on average 85 foci (Figure 16). Interestingly, the number of foci increased from 74 to 95 in low salt treated cells. This is likely the result of an increase in cells in S and G2 phase for the low salt condition, when compared to the cells used for normal salt condition.

Figure 16: Quantification of fluorescently labelled telomeres in MEFs. Violin plots of the number of foci (dots) corresponding to the number of telomeres per nuclei. The shape of the violin plot corresponds to the relative distribution of number of fluorescent foci. Wider areas indicate a higher frequency of cells. The black vertical line corresponds to the Q1-Q3 interquartile range. Black central spheres indicates the average. The number of cells (n) analyzed is shown below the X-axis.

3.3 CLEM procedure

Figure 17: Overview of an APEX2 correlative light and electron microscopy (CLEM) experiment. Illustration of sample preparation steps (1-9) from transfection of cells to resin embedding and polymerization (**left**). Cells are imaged on a confocal microscope before (Pre DAB) and after (Post DAB) DAB polymerization (**right**). Magnified area below shows individual telomeres (indicated by arrows) that appear dark after DAB treatment.

Figure 18: Correlation of APEX2-derived DAB staining and eGFP fluorescence. **A)** Transmission light image of a MEF cell transfected with the eGFP_APEX2_TRF1 construct. **B)** The same cell following the hydrogen peroxide catalyzed APEX2 reaction that produces a local deposit of DAB (dark punctae inside the nucleus). **C)** Fluorescent optical slice corresponding to the same focus depth as the transmission light image in A and B. Arrows indicate individual telomeres.

3.4 Correlation of fluorescence images with electron micrographs of telomeric chromatin

Having established transfection and labeling of telomeric chromatin, I next prepared cells for electron microscopy analysis as summarized in Figure 17. The steps (fixation, dehydration, staining and embedding in resin) are commonly used in conventional EM analysis of cells. However, in an APEX2 experiment there is one additional step to generate electron dense contrast (Figure 17, step 4). Hydrogen peroxide and DAB are at this stage added to the cells, and the APEX2 reaction is allowed to take place for 1.5-2 minutes. Figure 18 shows the appearance of dark DAB punctae inside the nucleus and the corresponding fluorescence image of labelled TRF1. The DAB polymer is then post-stained with OsO_4 .

The sample preparation procedure used in an APEX2 experiment may distort or shrink the sample. Further, small errors during ultramicrotome sectioning will give a potential offset in angle between the EM section and the optical slice. After cutting ultrathin (70 nm) sections, I next correlated fluorescence images (optical slices) to images recorded on a transmission electron microscope. The correlation is necessary in order to identify telomeric chromatin from other high contrast regions in the nucleus, such as nuclear bodies and contaminants. As seen in Figure 19, correlation between EM contrast and fluorescence is possible with a high degree of accuracy.

Dark regions outside the nucleus are peroxisomes (Figure 19 A, indicated by asterisks), which contain endogenous peroxidases and thus give rise to DAB polymerization in the absence of the APEX2 enzyme. The superimposition (Figure 19 B) shows an increased number of fluorescently labelled telomeres as compared to stained telomeres in EM. This is due to the fact that the resolution of fluorescence microscopy in Z has an axial resolution of approximately 800 nm, whereas the physical cellular section used for imaging in EM has a thickness of 70 nm. As such, a superimposition of the two necessarily includes a larger portion of the depth of the cell in fluorescence, and thus more fluorescent foci in the light microscope when compared to the number of stained telomere foci in the physical section imaged by EM.

Figure 19: Correlation of light and electron micrographs of telomeric chromatin. A) Electron micrograph of a MEF cell transfected with eGFP_APEX2_TRF1. Telomeres are indicated with white arrows. Other high contrast regions (peroxisomes and carbon deposits on top of the section) are indicated with black asterisks. B-C) Superimposition of the EM image (70 nm) shown in A and corresponding optical slice (Captured at focus depth intervals of 300 nm, axial resolution ~800 nm). C) Higher magnification EM images of three telomeres shown in C (labelled 1,2,3).

3.5 TEM of telomere structure in micrographs of ultrathin sections

3.5.1 TEM analysis of HT1080 telomeres

Having established correlation of the fluorescent punctae with the APEX2-derived DAB polymer post-stained with OsO₄, I proceeded to collect electron micrographs of telomeres imaged at higher magnification (23-30,000k) on a 120kV transmission electron microscope. Representative telomeres from both HT1080 and MEFs are shown in Figure 20 and 21, respectively. In micrographs of HT1080 and MEF cells, telomeres appear spherical, elongated or have an irregular shape. For telomeres with irregular or elongated shapes, the length and width were measured and averaged to give a representative diameter. The mean diameter of telomeres in MEFs was 196 nm and 156 nm in HT1080 cells (Figure 23). Assuming a spherical structure, this corresponds to a nearly two-fold increase in volume of MEF telomeres as compared to HT1080 telomeres ($V = (4/3)\pi r^3$ for radii of 78 and 98 nm which, respectively, gives spheres of $\sim 1.99 \times 10^6$ and $\sim 3.94 \times 10^6$ nm³ in volume, corresponding to a ~ 1.98 times increase in volume). This is expected because human telomeres are shorter than mouse telomeres. Telomeric length analysis by southern blot analysis (carried out by Bilal Ahsan in Sara Sandin's group, data not shown) shows that the telomere length in HT1080 and MEF cells were 5,300 bp's and 19,200 bp's, respectively.

Figure 20: Gallery of electron micrographs of HT1080 telomeres. A-L) Micrographs of telomeres in 70 nm thin cell sections. Less compacted regions are indicated by white arrows. Black arrows indicate protruding features.

3.5.2 TEM analysis of MEF telomeres

Figure 21: Gallery of electron micrographs of MEF telomeres. A-X) Micrographs of 70 nm thin cell sections. Less compacted regions are indicated by white arrows. Black arrows indicate protruding features.

Figure 22: Electron micrographs of representative telomeres from low salt-treated MEFs. A-X) Micrographs of 70 nm thin cell sections. Less compacted regions are indicated by white arrows. Black arrows indicate protruding features.

In order to decondense telomeric chromatin to facilitate EM analysis, MEF cells were treated with low salt buffer (0.3X PBS) for 1 minute prior to, and during fixation. This low salt, or hypoosmotic, treatment of the cells is a well-established technique in chromatin biology where it is used to decompact or decondense chromatin in the cell. Here, the low salt treatment was used in an attempt to open up the telomeric chromatin and allow for easier visualization of telomeric ultrastructure and internal organization of telomeres in the cell. Henceforth, the term “low salt telomere” is used to refer to telomeres in cells which have undergone low salt-treatment. Similarly, the term “normal salt telomere” is used to refer to telomeres in cells which have not undergone low salt-treatment.

The sample preparation procedure was otherwise identical to previous samples investigated. Figure 22 shows a gallery of representative telomeres obtained from low salt treated MEFs, imaged at 30,000x magnification. Comparisons of normal salt (Figure 21) and low salt (Figure 22) conditions reveal striking differences in EM contrast and ultrastructure. Under normal salt conditions, telomeres display a homogenous EM density and largely compact structure (Figure 21 C, F and G). In contrast, fibrous networks are seen in the decompacted telomeres under low salt conditions. Protrusions of chromatin fibers with a diameter of 20-50 nm were present in both normal and low salt conditions (Black arrows in Figure 21 and Figure 22). Whereas these fibers were only occasionally observed under normal salt conditions (Figure 21 D, H, M and P) the protrusions are present in nearly all telomeres from low salt-treated cells analyzed.

Figure 23: Telomere size measurements. A-C) Telomeres with a round shape were measured as shown in A and B. The diameter of irregularly shaped or elongated telomeres were estimated by calculating the average of length and width as shown in C. D) Box plots of telomere size for the different cell types and salt conditions. The number of telomeres measured for each condition is indicated below each plot. (*) indicates a probability value of <0.05 , (**) of $p<0.001$, values determined using Welch's t-test. NS=Normal Salt (0.1M CB Buffer), LS=Low Salt (0.3X PBS).

The size span of normal salt MEFs significantly shifted both for smallest and largest telomere measured (120-300 nm) as compared to HT1080 telomeres (80-250 nm). The same trend was apparent comparing telomeres from normal salt cells with telomeres from low salt treated MEFs (150-380 nm). Similarly, the median telomere size increases from normal salt HT1080 to normal salt MEF (difference of 40 nm, $p<0.05$) as well as from normal salt MEF to low salt MEF (difference of 22 nm, $p<0.001$). A comparison of telomeres from normal salt and low salt conditions shows an 11% increase in median diameter, which would correspond to an approximately 38% increase in volume.

3.5.3 TEM analysis of MEF telomeres located at the nuclear envelope

Telomeres have in the literature been reported to be enriched close to the nuclear envelope (NE) during post mitotic assembly[197] and during interphase[198]. Telomeric tethering to the nuclear envelope is further believed to be of importance during recombination of homologous chromosomes in meiosis[199]. I therefore analyzed the ultrastructure of telomeres localized close to the NE.

As indicated in Figure 24 B, E, F and G, protrusions of chromatin fibers towards the NE suggest that the telomeres are in close contact with the NE. In A, C and in particular D in Figure 24, the telomeres appear to be directly in contact with the nuclear envelope. In Figure 24 B and G, the telomere protrusions feature stronger contrasts (derived from DAB polymerization resulting from presence of the TRF1 correlative fusion protein and OsO₄ staining) as compared to surrounding heterochromatin regions. This indicates that the protrusion contains TRF1 and is thus telomeric chromatin. This is not the case in Figure 24 C and E, where no increase in stain is observed between the telomere region and NE. This suggests that these telomeres are embedded in lamina associated heterochromatin domains and not in direct contact with the NE. However, as telomeres in close proximity to the nuclear envelope are limited to only a handful of examples in the data presented here, these are too few to quantify or otherwise characterize this potential interaction between telomeres and the nuclear envelope.

Figure 24: Electron micrographs of MEF telomeres localized close to the nuclear envelope. A-D) Micrographs of telomeres from low salt treated (0.3X PBS) MEFs. **E-H)** Micrographs of normal salt telomeres. White squares indicate the area displayed at higher magnification below. Black arrows indicate protrusions of chromatin fibers. Scale bar 200 nm.

3.6 Electron tomographic analysis of MEF telomeres

The EM analysis of telomeres *in situ* and features of telomere structures from individual micrographs are limited to 2D, due to the fact that a single micrograph is a projection of the object imaged. Whereas the analysis of high magnification micrographs offers interesting insights into the overall structure, size and appearance of telomeres *in situ*, individual micrographs alone cannot give information about the 3D structure of an object. One method of obtaining structural

3D information is electron tomography. For electron tomographic analysis, I prepared 140 nm thick sections of normal and low salt treated MEFs. The tomographic sections were coated with carbon and 10 nm colloidal gold particles were added for alignment. Dual axis tomographic tilt series were recorded on a 200kV Tecnai Arctica using a nominal magnification of 39,000X. One micrograph was recorded per degree from -65° to +65° yielding a data set containing 131 images per tilt series. These tomograms were then reconstructed using the IMOD software[196], binned by a factor of 2 and lowpass filtered to 20Å. The final pixel size was 0.54 nm. A total of 10 normal salt tomograms and 8 low salt tomograms were collected and reconstructed as shown in Table 1. From the 3D reconstruction, tomographic slices were binned in groups of 10 in the Z direction resulting in tomographic slices corresponding to 5.4 nm in depth. In addition, telomeres were visualized using surface rendering in Amira (Thermo Scientific). The image processing steps are illustrated in Figure 25. Tomograms and sequential tomographic slices series of all tomograms are included in Appendix A3 and A4, respectively.

Tomogram	Dose [e ⁻ /Å ² s]	Exposure [s]	Defocus [μm]	Threshold	Residual error, SD [†]
tomo_ns_1	7.5	2	-2	140	1.037, 0.573
tomo_ns_3	7.5	2	-4	141	0.752, 0.433
tomo_ns_4	7.5	2	-4	141	0.617, 0.332
tomo_ns_5	7.5	2	-2	139	0.974, 0.537
tomo_ns_6	7.5	2	-2	140	0.975, 0.554
tomo_ns_7	7.5	2	-3	141	0.977, 0.543
tomo_ns_10	7.5	2	-3	140	0.687, 0.404
tomo_ns_12	7.5	2	-2	140	0.631, 0.379
tomo_ns_13	7.5	2	-4	141	0.750, 0.422
tomo_ns_15	7.5	2	-2	141	1.306, 0.705
tomo_ns_17	7.5	2	-3	141	0.630, 0.366
tomo_ls_1	7.5	2	-2	142	0.595, 0.383
tomo_ls_5	7.5	2	-2	141	0.511, 0.296
tomo_ls_6	7.5	2	-2	141	0.582, 0.367
tomo_ls_7	7.5	2	-2	142	0.545, 0.349
tomo_ls_8	7.5	2	-2	141	0.647, 0.375
tomo_ls_9	7.5	2	-2	147	0.585, 0.342
tomo_ls_10	7.5	2	-2	141	0.664, 0.446
tomo_ls_11	7.5	2	-2	140	0.524, 0.329

Table 1: Summary of tomographic data and eTomo reconstruction collection. Dual axis tomograms were collected on a 200kV Tecnai Arctica equipped with a falcon III detector. ns=normal salt. ls=low salt. Dose per

image was $15\text{e}/\text{\AA}^2$. Threshold values for 3D surface rendering in Amira are listed. *Residual errors are shown for the second tilt, following fiducial transfer from the first.

Figure 25: Image processing and analysis of an electron tomogram. **A)** Electron micrograph recorded at 0° as part of a tomographic tilt series. The image has not been contrast adjusted or filtered. **B)** Average Z-projection of the full tomogram. **C)** 5.4 nm thick tomographic slice filtered to $20\text{ \AA}$. **D)** 0.54 nm thick tomographic slice. **E-H)** Same images as presented in A-D, adjusted for contrast for clarity. **I-J)** Individual processing steps for tomographic rendering in Amira (6.5.0). **I)** 0.54 nm thick tomographic slice, same as shown in H). **J)** A threshold has been added in blue using the “interactive threshold” function in Amira, which corresponds to the pixel values of the stained telomere. **K)** The selected threshold is assigned different colors depending on connectivity in 3D using the “labelling” function (default settings: “interpretation:3D”, “Neighborhood:26”). All contiguous regions in 3D are assigned a color. Here, the central telomere along with all contiguous regions is colored green. **L)** Contiguous density corresponding to the central telomere and connected fibers, shown in blue, is extracted

signal using Amira's "arithmetic" function. **M-P)** Different views of the 3D surface rendered telomere. Top view (**M**), +45 rotations around the X-axis (**N**, **O**) and side view (**P**). The scale bar is 100 nm.

Figure 26: Two-page gallery of 18 telomeres reconstructed by electron tomography. 5.4 nm thick tomographic slice, extracted from the centre of the tomogram, is shown next to the surface rendered 3D volume (black background). The tomograms are given identifiers in the bottom left. LS = Low Salt; NS = Normal Salt.

Figure 27: Sequential tomographic slices of a telomere from a normal salt-treated MEF. Each image corresponds to a 5.4 nm thick tomographic slice extracted from tomogram tomo_ns_1 (NS1)(Table 1). The scale bar 200 nm.

Figure 28: Sequential tomographic slices of a telomere from a low salt-treated MEF. Each image corresponds to a 5.4 nm thick tomographic slice extracted from tomo_ls_1 (LS1)(Table 1). The scale bar 200 nm.

Figure 29: Comparison of telomeres from normal and low salt-treated MEFs. Three 5.4 nm thick tomographic slices from tomograms shown in Figure 27 and Figure 28. **A-C)** Normal salt telomere (slices number 3, 9 and 12). White arrows indicate low-density regions and gaps inside telomeres. **D-F)** Low salt telomere (slices number 1, 5 and 7). White arrows indicate low-density regions and gaps inside telomeres. **F)** A large low-density pocket is highlighted with a dotted line that measures 81 nm in length. The scale bar is 200 nm.

3.7 Line scan analysis of internal telomere structure in MEF tomograms

To analyze density variations and the internal structure of telomeric chromatin, line scans of tomographic slices were carried out on normal and low salt MEFs. In Figure 30, black lines indicate the placement of the line scan and the accompanying graph is shown in blue below each image. The distances between repetitive features are given in nm, below the corresponding graph. A low numerical value (intensity) corresponds to a dark pixel value in the image. Repetitive features, measuring 25-50 nm in length, were found in telomeres from both normal and low salt conditions. However, a fibrous ultrastructure was less apparent in the normal salt telomere. For example, Figure 30 J shows a highly compacted region in the centre of the telomere that measures 166 nm in length. In contrast, the telomere from the low salt condition is de-compacted and individual chromatin fibers can be clearly distinguished in the tomogram. Low salt fibers have a diameter of 23-46 nm. Line scans of the normal salt telomere have, on average, larger diameters. The propensity for highly compacted regions and larger diameters in normal salt telomeres also consists for comparisons with other tomograms.

Figure 30: Line scan analysis of telomeres from normal and low salt-treated MEFs. All images are 5.4 nm thick tomographic slices. **A-D)** Four identical tomographic slices of a low salt telomere with line scan measurements in different orientations as indicated (black lines terminating in arrows). **E-H)** Four tomographic slices of a normal salt telomere, at different Z heights of the tomographic stack. **I-L)** Same images as shown in E-H with line scans indicated (black lines terminating in arrows). The intensity plots are shown below each image. Low intensity corresponds to dark pixels in the image. Tomograms used for the line scan analysis was tomo_ns_17 (NS17) and tomo_ls_5 (LS5).

3.8 Measurements of telomeric chromatin fiber dimensions in binned tomographic slices

Displaying the tomographic data as binned tomographic slices corresponding to different depths of the structure allows for measurements of compacted regions and fibers to be performed. In order to determine the start and end point of these regions with higher precision, the measurements were performed on unfiltered tomographic slices. Distinguishable fibers were the focus for these measurements. In order to obtain representative fiber dimensions, measurements were carried out in triplets along the fiber and subsequently averaged. Figure 31 demonstrates how the measurements were carried out, with the average diameter given in nanometers with the symbol " $\emptyset$ ". Figure 31 A shows a telomere from normal salt conditions and Figure 31 B shows a telomere from low salt conditions. In addition to fiber measurements (box regions 1-3), examples of highly compacted regions inside the telomeres are also highlighted (box region 4). A summary and comparison of all fiber width measurements carried out as described in Figure 31, from both normal and low salt telomeres, is shown as a distribution plot in Figure 32. A total of 702 measurements were carried out (234 fibers, in triplets), out of which 329 were from low salt telomeres and 373 were from normal salt telomeres. The mean diameter of fibers were thinner in low salt telomeres, possibly indicating that the structure has opened up and allowed for smaller features to be measured. The low salt distribution shows a clear peak at 12.0 nm (" $\ast 1$ ") and a mean fiber width of 13.1 nm (median 12.65 nm). By comparison, the normal salt has a main peak at 17.7 nm (" $\ast 3$ ") and a mean fiber diameter of 18.5 nm (median 18.1 nm). Taken together, telomeric chromatin fibers have a combined peak at 14.4 nm (" $\ast 2$ ") and a mean fiber diameter of 15.9 nm. In the low data set, a second peak is visible at 23 nm (" $\ast 4$ "). This peak is also seen in the normal salt data set. Further, a fifth and smaller peak is visible in all three plots around 30-35 nm (" $\ast 5$ "). The interquartile (Q1-Q3) fiber diameter range is 10.1 – 15.6 nm for low salt fibers and 14.7 – 21.8 nm for normal salt fibers. The smallest diameter measured in either data set was 4.7 nm, the vast majority occur within the span of 8-30 nm (94.1% for normal salt, 85.9% for low salt). For normal salt fibers, 84.5% occur within the span 10-25 nm, whereas only 67.2% for low salt telomeres. Due to the shift to higher fiber dimensions seen in normal salt telomeres, more than a third (35.5%) are above 20 nm in diameter. The corresponding number for low salt telomeres is 6.7%.

Figure 31: Telomere fiber width measurements. Images of 5.4 nm thick tomographic slices. Four areas (left) highlighted with a white box are shown at higher magnification (right) where measurements on resolvable fibers are shown. **A)** Normal salt telomere. **B)** Low salt telomere. Fiber measurements were carried out in triplets as indicated with white lines (box A1-3 and B1-3) and averaged. The average fiber width (in nm) are indicated with the symbol $\varnothing$. Examples of areas excluded from fiber width measurements are shown in box A4 and B4. These include areas too dense to resolve individual chromatin fibers. The scale bar is 50 nm (left) and 25 nm (right).

Figure 32: Distribution plot of telomere fiber diameters. Three superimposed distribution plots corresponding to fiber measurements carried out on normal salt telomeres (pink), low salt telomeres (green) and the two data sets combined (blue). Peaks are indicated with numbered asterisks in black (*1, *2, *3) whereas shoulders or areas of interest are numbered asterisks in gray (*4, *5). The frequency has been normalized relative to the most frequent fiber diameter in each data set.

3.8.1 Measurements of telomeric chromatin fiber dimensions at their mid-point in binned tomographic slices

When performing fiber measurements in binned tomographic slices it is possible that measurements are performed on fibers that are only partially included in the tomographic slice. As a result, such measurements would measure peripheral and thinner parts of the fiber, thus underestimating its dimension. It is further possible that features which appear as continuous fibers in the top-down binned view along the Z-axis are, in reality, separate and disconnected. For this reason, I performed a second round of measurements on fiber width, this time including only clearly distinguishable fibers perpendicular to the Z-axis and which feature a clear start and end point. Since normal salt telomeres were too compacted to trace individual fibers, I included only tomograms of the low salt telomeres in this analysis. The majority of low salt chromatin fibers had a diameter of 10-20 nm in diameter. As such, identifying the start and end point of fibers of this scale in steps of 5.4 nm from the binned tomographic slices would be difficult. Therefore, unfiltered and unbinned tomographic slices, corresponding to 0.54 nm thick tomographic slices, were used. The contrast and brightness were adjusted for clarity. As shown in Figure 33, the starting point is tomographic slice 1 (first square in the top left in A, B, C and D)

corresponding to the starting point of the fiber as indicated with a white arrow. The fiber is then traced through the tomographic slices in the Z direction until the density is no longer apparent. The depth and this number of this last tomographic slice is taken as the end point of the fiber, and the fiber measurements were carried out in the middle. In A) the fiber is present through slices 1-51. The central measurements were thus done in slice 26. These measurements confirm the presence of separate, distinguishable fibers of the same approximate dimensions as previously described. The fibers shown here have a diameter of 16.1 to 31.2 nm.

Figure 33: Central slice measurements of chromatin fiber widths of telomeres from low salt-treated MEFs. Gallery of tomographic slices corresponding to a thickness of 0.54 nm. The number in the top right corner of the image indicates the tomographic slice depth in Z, where the beginning of the fiber is defined as slice 1. Arrows indicate the start and the end of fibers in Z. **A)** Measurement in tomo_ls_1 (LS1). **B)** Measurement in tomo_ls_9 (LS9). **C)** Measurement in tomo_ls_7 (LS7). **D)** Measurement in tomo_ls_10 (LS10).

The previous sections have focused on analyzing and measuring the structure of telomeres and chromatin fibers in data derived from 3D reconstructions of tomograms, which were processed and presented as 2D tomographic slices. While presenting 3D data as 2D may reduce its complexity and make it more accessible to analyses, this is done at the expense of the larger structural context and scope. As such, in addition to studying individual features, analyses on full 3D tomograms were also performed. For this analysis, the tomograms were processed in Amira 6.5.0 as shown in Figure 25. In Amira, pixel values corresponding to darkly stained regions is manually specified and extracted from the data set (Figure 25, J). The extracted values are then processed using Amira's "labelling" function which identifies contiguous regions of stain in 3D and assigns each a numerical value (Figure 25, K). The contiguous region corresponding to the telomere is then selected and extracted as a separate volume (Figure 25, L). Finally, the extracted 3D volume of the telomeres is displayed by surface rendering (Figure 25, M-P).

3.9 Fiber measurements and fiber tracing in tomographic 3D slices of telomeres

Following measurements in 2D of tomographic slices, a second analysis was performed with the intention of corroborating the fiber dimension data from tomographic slices with the corresponding data from surface rendered 3D slices. To this end, a tomographic slice from a normal salt telomere was selected and the 3D surface render was cropped to the corresponding thickness of 5.4 nm (Figure 34). The same was also done for a telomere from a low salt treated MEF (Figure 35).

Figure 34: 2D and 3D analysis of a telomere from a normal salt-treated MEF. A) Electron micrograph showing the 0 degrees image of the tilt series. The scale bar is 200 nm. B) Close-up view of the same image shown in A), the scale bar is 100 nm. C) Unfiltered and unbinned 0.54 nm thick tomographic slice. D) 5.4 nm thick tomographic slice (10x binning in Z). E) Surface rendered 3D representation of the entire tomogram. F) Surface rendered representation of a 5.4 nm thick section of the tomogram (same region as shown in D). Contiguous densities as determined by appearance in D) and F) are highlighted with a blue line.

Figure 35: 2D and 3D analysis of a telomere from a low salt-treated MEF. **A)** Electron micrograph of the 0 degrees image in the tilt series. The scale bar is 200 nm. **B)** Close-up view of the same image shown in A). The scale bar is 100 nm. **C)** Unfiltered and unbinned 0.54 nm thick tomographic slice. **D)** 5.4 nm thick tomographic slice (10X binning in Z). **E)** Surface rendered 3D representation of the entire tomogram. **F)** Surface rendered representation of a 5.4 nm thick section of the tomogram (same region as shown in D). Contiguous densities as determined by appearance in D and F are highlighted with a blue line.

Figure 36: Surface rendered tomographic slices of MEF telomeres. Surface rendered 3D representations of 12

MEF telomeres. A 5.4 nm thick central region of the density is shown. **A-F)** Low salt telomeres. **G-L)** Normal salt telomeres. Blue line measurement and corresponding fiber width (asterisk) are indicated. Parallel fibers are indicated with arrows. The scale bar is 100 nm.

The combination of gaussian filtering and extracting volumes of contiguous density yields a clear cross-section view of the internal structure of telomeres. The fiber dimensions found here are in accordance with fiber measurements in the 5.4 nm tomographic slices. In both conditions, fibers range from 10 to 30 nm whereas the low salt telomeres contain a larger proportion of diameters in the size range of 8-20 nm. Here, the internal structure is comparable to a mesoporous network of fibers ranging from 8-30 nm in diameter with internal pores and holes ranging from a few nanometers up to 50 nm in normal salt conditions, and up to 80 nm in low salt conditions. In addition to fibrous features and a mesoporous structure, telomeres also feature highly compacted and dense regions. These areas appear featureless when viewed as surface rendered 3D volumes. Dense regions constitute a substantial portion of telomeres and are more frequently seen in normal salt telomeres. Further, many fibers are organized in parallel lines (see arrows in Figure 36 B-E and G). This phenomenon is clearly seen in Figure 36 B and G where the arrow, in both cases, indicates the first fiber which is followed by subsequent fibers in the same orientation as the first. In these two cases, the initial fiber is found in the bottom left of the tomographic slice, and the aligned fibers propagate diagonally towards the top right. In Figure 36 B, C and G, the arrow is orthogonal to the orientation of the aligned fibers. In Figure 36 E, in order to not obscure the tomographic volume, the arrow follows the orientation of the aligned fibers.

3.10 Measurements and structural analysis of segmented telomeric fibers in 3D

Analysis of telomere structure in binned tomographic slices or surface rendered 3D slices make it easier to visualize and interpret the internal structure of telomeric chromatin. However, by ignoring the larger 3D context there is some ambiguity as to whether features that appear fibrous in thin slices are fibers or part of larger domains. To address this, manual segmentation was performed on low salt telomeres to identify and visualize entire fibers in 3D. Manual segmentation involved identifying darkly stained regions that could be clearly distinguished from other parts of the telomeres. In addition, it is possible to identify and view subareas of tomographic data using the ROI (Region of Interest) function in Amira. Examples of fibers generated by both methods are shown in Figure 37.

Figure 37: Gallery of telomeric chromatin fibers from low salt-treated MEFs. A1) A 0.54 nm thick tomographic slice filtered to 20 Å. The area indicated with a white square is shown at higher magnification in A3. **A2)** Same tomographic slice with a threshold added corresponding to telomeric chromatin in blue. The area indicated with a white square is shown in A4. **A3)** Zoomed view of A1, a white arrow indicates a fiber pointing down in Z. **A4)** Zoomed view of A2. Not indicated threshold has been made transparent for clarity. **B)** Four views of a segmented chromatin fiber. Fiber dimensions are indicated in nanometers. Asterisks indicate areas of connectivity with the surrounding telomere density, which have been excluded for clarity. **C-G)** Gallery segmented chromatin fibers showing different morphology, including branched and looped regions.

Through manual segmentation and applying the ROI tool to tomographic data sets in Amira, it is possible to isolate and visualize individual fibers and structural features in 3D. Based on representative tomograms shown in Figure 37, fiber dimension varies from 5.1 nm (Figure 37 E, unrotated) to 33 nm (Figure 37 F, first rotation). Fibers below 8 nm or above 25 nm were rare. In addition to distinguishable fibers (Figure 37 B, D, G) there are globular regions (Figure 37 C). Similarly, it is possible that larger dense features are several fibers packed too tightly to be resolved, or it may represent non-fibrous structures. In addition to this approach, it is also possible to view and analyze telomeric fibers and features at the periphery of the telomeres, where the structure tends to be less condensed and not obscured by adjacent chromatin. An example of two such structures can be seen in Figure 38.

Figure 38: Surface rendered 3D representation of fibrous features in low salt telomeric tomograms. A) Images of tomo_ls_11 (LS11). **B)** Images of tomo_ls_6. Areas indicated with white rectangles are shown rotated and at higher magnification below. Scale bars as indicated.

3.11 Establishing a comparative data set of bulk using H2B-APEX2 labelling

Having characterized the ultrastructure of telomeres in MEFs, I next established a correlative imaging approach to visualize all chromatin in the nucleus. To this end, a eGFP_APEX2 construct with H2B was generated. H2B is one of the core histones and is expected to be present in all nucleosomes in chromatin. In addition to a comparison of structural features of telomeric chromatin and non-telomeric chromatin (bulk chromatin), the H2B experiments were also intended to investigate whether higher expression levels of the correlative construct had an impact on chromatin fiber thickness due to an increased level of DAB/OsO₄ stain. Transfected cells were generated and screened for expression level based on post-DAB images, fluorescence images as well as relative amount of EM staining. Using this approach, five transfected MEF cells with varying expression levels were selected for tomography. In Figure 39, cell 6 (C6) and cell 2 (C2) were defined as “high” expressing cells. Cell 4, cell 5 and cell 1 (not shown, comparable expression to cell 5) were defined as “low” expressing cells. From these cells, 10 dual axis tilt series were collected at various nuclear locations and reconstructed in 3D as described previously. The areas selected for 7 of the dual tilt series collected are shown below, in white squares. Sequential 5.4 nm thick tomographic slices of bulk chromatin tomograms can be found in Appendix A5.

Figure 39: Overview of cells and expression levels used for data collection of H2B tomograms. Cells 4 and 5 are low expressing cells, whereas cells 2 and 6 are high expressing cells. The relative intensity levels of all of the images have been maintained. A post DAB transmission image of Cell 2 was not available as it had been obscured by debris in the buffer. *Untransfected cell. **transfected cells not analyzed by tomography.

Following 3D reconstruction of the tilt series, the H2B tomograms were processed in the same way as the TRF1 tomograms. A lowpass filter of 20 Å was applied and 5.5 nm thick tomographic slices were generated. A selection of tomographic slices from cells with various expression levels are shown in Figure 40. A summary of tilt series acquisition and reconstruction parameters is shown in Tables 2 and 3. In total, 10 tomograms from MEFs of various expression levels were collected.

Tomogram	Cell type	Construct	Magnification (x10 ³)	Pixel Size	Expression
TomoC1A1	MEF	EGFP_APEX2_H2B	39	0.2787	Low
TomoC1A2	MEF	EGFP_APEX2_H2B	39	0.2787	Low
TomoC2A1	MEF	EGFP_APEX2_H2B	39	0.2787	High
TomoC2A2	MEF	EGFP_APEX2_H2B	39	0.2787	High
TomoC2A3	MEF	EGFP_APEX2_H2B	39	0.2787	High
TomoC2A4	MEF	EGFP_APEX2_H2B	39	0.2787	High
TomoC2A5	MEF	EGFP_APEX2_H2B	39	0.2787	High
TomoC4A1	MEF	EGFP_APEX2_H2B	39	0.2787	Low

TomoC5A1	MEF	EGFP_APEX2_H2B	39	0.2787	Low
TomoC6A2	MEF	EGFP_APEX2_H2B	39	0.2787	High

Table 2: Summary of H2B tomographic data collection. The pixel size of the original raw micrographs in each tilt series. Following 3D reconstruction, the tomograms were binned by a factor of 2. Expression level was based primarily on stain in EM, fluorescence intensity and post DAB stain in transmitted light images.

Tomogram	Dose [$e^-/\text{\AA}^2s$]	Exposure [s]	Defocus [μm]	Threshold	Residual error, SD [†]
TomoC1A1	15	1	-2	139	0.593, 0.326
TomoC1A2	15	1	-2	139	0.734, 0.421
TomoC2A1	15	1	-2	141	0.748, 0.443
TomoC2A2	15	1	-2	141	0.729, 0.416
TomoC2A3	15	1	-2	155	0.666, 0.373
TomoC2A4	15	1	-2	139	0.739, 0.429
TomoC2A5	15	1	-2	142	0.709, 0.397
TomoC4A1	15	1	-2	145	0.540, 0.302
TomoC5A1	15	1	-2	150	0.651, 0.374
TomoC6A2	15	1	-2	136	0.579, 0.325

Table 3: Defocus, threshold and alignment errors for dual axis tilt series of H2B. Dose per image was $15e^-/\text{\AA}^2$. Threshold value used in Amira for surface rendering are listed. [†]Residual errors are shown for the second tilt, following fiducial transfer from the first.

Figure 40: Tomographic analysis of H2B-APEX2 labelled chromatin in MEFs. Images of 5.5 nm thick tomographic slices. The relative chromatin concentration has been divided into low (left column, A-J), medium (middle column, B-J) and high (right column, C-K), Areas shown in C and K are in close proximity to the nuclear envelope, visible in the top portion of each tomographic slice. The scale bar is 100 nm.

3.12 Line scan analysis of bulk chromatin structure in MEF tomograms

The H2B tomographic data was analyzed using a line scan analysis as shown in Figure 41.

Figure 41: Line scan analysis of H2B-labelled chromatin in MEFs. Tomographic data acquired from 140 nm sections of MEFs transfected with a correlative eGFP_APEX2_H2B construct. All images shown are 5.5 nm thick tomographic slices with the corresponding intensity plot shown below. The line scanned area is shown in cyan. Repetitive low-intensity features in the intensity graph area indicated, connected by black lines and the distance between features are given in nanometers. **A-H)** Line scans of low expressing cells. **I-P)** Line scans of high expressing cells. **E, F)** Regions featuring particularly dense chromatin regions. **I, J, P)** Regions in close proximity to the nuclear envelope. The scale bar is 100 nm.

3.13 Measurements of bulk chromatin fiber dimensions in binned tomographic slices

In contrast to telomeric chromatin, bulk chromatin is distributed throughout the nucleus, with more condense regions close to the nuclear envelope and nucleoli, as expected. Overall, H2B labelled chromatin appears less compacted as compared to telomeric chromatin. The line scans show dispersed densities and larger areas devoid of chromatin fibers. This can be seen in Figure 41 A, D, N, K L and O which measure what, viewed like this in 2D, appears to be separate and parallel chromatin fibers. Here, the distances separating individual fibers range from 20-30 nm (Figure 41 A, D, K and O) which is comparable to some features of low salt telomeric chromatin, to 35-70 nm (Figure 41 N, O) which is rarely encountered in telomeric chromatin data. Similar

distances are also frequently observed in essentially all chromatin regions that are either not in close proximity to the nuclear envelope or within a local area of higher packing. It is therefore clear that the majority of bulk chromatin features a lower chromatin concentration, or less dense chromatin packing, than telomeric chromatin. However, there are areas of higher chromatin concentration spanning hundreds of nanometers, as well as individual local densities of less than 100 nm that occur within otherwise dispersed chromatin (see Figure 41 H, M). Line scans carried out in areas with more densely packed chromatin are more comparable to normal salt telomeric chromatin. This suggests that heterochromatin regions in the nucleus may be similar in compaction to that of telomeric chromatin. More dispersed regions of bulk chromatin are more comparable to low salt telomeric chromatin.

Figure 42: Distribution plot of bulk chromatin fiber diameters. Two superimposed distribution plots of fiber measurements carried out on bulk chromatin in low expressing MEFs (yellow) and high expressing MEFs (red). Peaks are indicated with numbered asterisks in black (*1, *2, *3) whereas less distinct shoulders or areas of interest are numbered asterisks in gray (*4, *5). The frequency has been normalized relative to the most frequent fiber diameter in each data set.

In addition to line scan analysis, 2D fiber measurements were also carried out. Here, the data set was split into “low”, for data acquired from cells expressing lower levels of H2B construct, and “high” for high expressing cells. Importantly, these measurements were carried out on distinguishable fibers and dense areas of chromatin were excluded. The main peak bulk chromatin fiber dimensions in both data sets (*1 and *2, in Figure 42) are 11.9 and 12.4 nm,

respectively. Indicating that in both data sets, the most frequently occurring fiber has a diameter of 12 nm. In the low expressing data set, a second peak of similar frequency occurs at 15.5 nm. From this second peak there is a steep drop-off in fiber frequencies until a final shoulder occurs at 23.5 nm in fiber thickness (5*). In contrast to this drop-off, the high expressing data set features higher relative frequencies of fiber diameters above 17 nm and extends to above 30 nm. For the entire data set, only 5 fibers were measured as ~30 nm or above. The mean fiber diameter was 13.9 nm (median 13.6 nm) for low expressing bulk chromatin (n=171) and 15.73 nm (median 15.0 nm) for high expressing bulk chromatin (n=331). The mean fiber diameter for the combined data set is 15.11 nm (median 14.42 nm). Importantly, this shows that the difference in fiber dimensions between high and low expressing cells is only 1.8 nm for mean fiber dimensions, 1.27 with regard to median diameter, and 0.5 nm with regards to the most frequently occurring fiber dimension. The interquartile range (Q1-Q3) for fiber diameters are 11.81 – 19.31 nm and 10.88 – 16.33 nm for the high and low expressing H2B data sets, respectively.

3.14 Comparison of chromatin dimensions and features from telomeric and bulk chromatin

Comparing the H2B data to those obtained from telomeric chromatin, it is evident that disperse bulk chromatin diameters are comparable to telomeric chromatin fibers encountered in low salt-treated MEFs (Table 4). The main peaks in both H2B and low salt TRF1 data sets all occur within a close range of 11.9 to 12.4 nm (H2B low expr. 11.9, TRF1 low salt 12.0, H2B high expr. 12.4 nm). Importantly, all H2B data was collected under normal salt conditions. For this reason, in order to accurately contrast bulk chromatin with telomeric chromatin as they occur under physiological conditions, a comparison between the H2B and normal salt TRF1 tomograms is more informative. In this case, normal salt telomeric fibers are significantly thicker, with respect to median diameter (18.08 vs. 14.42 nm), mean diameter (18.47 vs. 15.11 nm) as well as most frequently occurring diameter 17.7 vs. 12.2 nm). Further, thick chromatin fibers (30 nm and above) were more frequent in telomeric chromatin.

TRF1	Peak [nm]	Median [nm]	Average [nm]	Sample size [n]	IQR
Normal Salt	17.7	18.08	18.47	373	14.66-21.77
Low Salt	12.0	12.64	13.11	329	10.11-15.57
Total	14.4	15.29	15.12	702	11.68-19.24
H2B					
High expres.	12.4	15.00	15.73	331	11.81-19.31
Low expres.	11.9	13.63	13.93	171	10.88-16.33

Table 4: Table summarizing fiber measurements in telomeres (TRF1 labelled) and bulk chromatin (H2B labelled). Peak refers to main peak or most frequently occurring fiber dimension. IQR = interquartile range, Quartile 1-Quartile 3.

In order to compare telomeric chromatin to bulk chromatin, a gallery of tomographic slices of H2B, normal salt TRF1 and low salt TRF1 is shown in Figures 43 and 44. Similar features of

corresponding scale and dimensions are indicated with white arrows. Bulk chromatin in close proximity to the nuclear envelope (Figure 44, C) and particularly dense areas of chromatin in the nucleus (Figure 43, A) appear similar to normal salt telomeres. Areas of intermediate densities in bulk chromatin contains features which resemble low salt telomeres (Figure 43 C and D; Figure 44 A and B).

Figure 43: Comparison of telomeric and bulk chromatin regions. **A)** Dispersed area of bulk chromatin from a low expressing cell (cell 4 area 1, C4A1). **B)** Normal salt telomere, NS10 **C)** Disperse area of bulk chromatin from a high expressing cell (cell 2 area 5, C2A5). **D)** Low salt telomere, LS6. **E-F)** Higher magnification images of the areas indicated in C and D. White arrows indicate features of similar dimensions and appearance.

Figure 44: Comparison of telomeric and bulk chromatin. **A)** Dispersed area of bulk chromatin from a high expressing cell (cell 2 area 5, C2A5). **B)** Low salt telomere, LS9 **C)** Dense area of bulk chromatin from a high expressing cell (cell 2 area 1, C2A1). **D)** Normal salt telomere, NS5. **E-F)** Higher magnification views of areas indicated in C and D. White arrows indicate features of similar dimensions and appearance.

4. Discussion

To date, no study has succeeded at imaging the structure and organization of telomeres in cells at high enough resolution to visualize its ultrastructure. Additionally, while it is known that telomeric chromatin consists of nucleosomes and shelterin complexes, as well as specific histone variants, epigenetic marks and NRLs, it nonetheless remains unclear if telomeric chromatin differs from non-telomeric chromatin in structure and appearance.

In order to address these questions, I have applied correlative light and electron microscopy (CLEM) and electron tomography with the aim of imaging and analyzing telomeres and telomeric chromatin *in situ* at high resolution. Using this technique, I was able to identify and selectively visualize telomeric chromatin by EM as well as obtain and study 3D data of telomeres in the cell. The estimated resolution in our electron tomography of APEX2 and resin embedded cells is 5 nm, which is approximately four times higher than STORM microscopy. A graphical summary of some of the findings are shown in Figure 45.

In cells, telomeres appear as mesoporous bundles of chromatin which ranges in size from 100-300 nm in diameter, depending on cell type and salt conditions. As is expected, the longer telomeres present in MEFs resulted in significantly larger telomere sizes when compared with HT1080, with a difference of about 40 nm in median diameter (Figure 23). Further, lowering the ionic strength decompacted telomeres and increased the size in MEFs from 196 nm to 218 nm. Telomeres in both HT1080 and MEFs display chromatin protrusions and looped fiber regions (indicated with black arrows, Figures 20, 21 and 22). What's more, these features increase in frequency following low salt treatment in MEFs where telomeres take on the appearance of decondensed network of fibers. In low salt conditions, individual fibrous features and protrusions range from 20-50 nm (in particular, see Figure 22, D, E, L, Q, R, T). Low salt telomeres similarly display increased internal gaps and areas of apparent decompaction as compared to normal salt MEFs and HT1080 telomeres. In Figure 22 D, E and T (indicated with white arrows) several examples of such internal gaps with dimensions exceeding 50 nm are visible. Figure 22 O appears to show a decompacted cleft that nearly runs the length of the telomere. Further, several low salt telomeres give the appearance of having decondensed to such an extent as to consist entirely of a fibrous network (Figure 22 E, Q) and have lost the dense and uniform telomere structure seen in most normal salt telomeres. Within these particularly decondensed telomeres, fibers in the range of 15-30 nm can be observed. Particularly striking fiber protrusions are seen in Figure 22 G, H, V.

4.1 Telomeres at the nuclear envelope

Telomeres are frequently observed in close proximity to the nuclear envelope in the literature, raising the question if they anchor to the nuclear envelope or alternatively embed within the lamina associated heterochromatin domains without interacting with the nuclear envelope directly [197], [198]. Several telomeres in close proximity to the NE were identified in both normal salt and low salt MEF samples (Figure 24). Out of these telomeres, one in particular appears to

very clearly be anchored to the NE with a distinct thick and stained (indicating TRF1 content) protrusion (Figure 24 B), providing evidence that anchoring can occur. Other NE-proximal telomeres appear further away from the NE and are embedded within the lamina associated heterochromatin region (Figure 24 E, G and H). Others appear to be directly touching the NE (Figure 24 A, D). While the presence of telomeres in close proximity to the NE are interesting, they are too few for any further analysis to be performed.

4.2 Internal organization of telomeres

When viewed as binned tomographic slices (Figures 27, 28) the internal structure of telomeres is reminiscent of the electron micrographs of decondensed telomeres. While minimal internal gaps and areas of apparent decompaction were visible in the normal salt MEFs electron micrographs, here the appearance is that of a mesoporous lattice consisting of intertwining fibers (see also Figure 29).

The apparent parallel alignment of fibers within the telomere (Figure 36 B-E, G) may represent an efficient way of organizing chromatin in tightly packed chromatin structures, such as telomeres. It is possible such parallel lattice-like organization is suited specifically for packing of telomeres, as the varying thickness of telomeric chromatin fibers and the additional presence of the shelterin complex likely makes for a less uniform fiber as compared to non-telomeric chromatin. Together with the apparent compact nature of telomeres *in situ*, the overall compact lattice-like organization may be an example of an efficient packing of disordered, and inactive, regions of chromatin.

The internal gaps in binned normal salt tomographic slices are nonetheless small in size, and rarely exceed 30 nm in diameter. Larger gaps are found towards the periphery of telomeres, as well as in tomograms of telomeres that do not span the entire depth of the section, but terminate within it (see Appendix A4, NS6 and NS7). This is similar to the decompaction observed in the periphery of telomeres in electron micrographs (Figures 21, 22). The telomeric decompaction observed in electron micrographs following low salt treatment of MEFs is also seen in tomographic slices. Here, binned tomographic slices of telomeres from low salt-treated MEFs show significantly larger gaps (Figure 29) as compared to normal salt telomeres. What's more, these gaps also make up a larger proportion of the structure, and individual gaps are larger and continuous for longer distances. Striking examples revealing the extent of the decompaction possible can be seen in Appendix 4 (LS5 and LS6) where a large portion of the structure is made up of internal gaps. A surface rendered tomographic slice of LS6 is shown in Figure 36 A, where a continuous open cleft runs through the structure (indicated with a teal arrow, Figure 36 A) highlighting the openness of the structure. Similarly decondensed internal structures are shown in Figure 35 D and F. In addition to fibers, these structures also feature dense regions which are reminiscent of a node of chromatin fibers, formed from chromatin connecting and intertwining. It is unclear to what extent these are distinct structural features of telomeric chromatin and to what extent these are simply tightly packed yet separate fibers that cannot be resolved. One such dense region is highlighted and measured in Figure 36 I, with similar areas present in Figure 36 K.

4.3 Structural features of telomeric chromatin

4.3.1 Chromatin globules

An example of a non-fibrous feature which has been manually segmented is shown in Figure 37 C. This globular structure is completely separate and resolvable from the rest of the telomere and is connected only through the 10 nm thick “neck” (measured as 9.0-9.7 nm in Figure 37 C, the asterisk in the fourth frame indicates the connection to the rest of the structure). This structure indicates that telomeres do not only consist of fibers, but of globular domains as well. Alternatively, it is possible that this globule represents the termination of a fiber and perhaps the terminus of the telomere itself. However, based on a comparison previous work, in particular the telomere-enriched chromatin loop–tail structures in mouse spleen lymphocytes shown in Nikitina and Woodcock’s 2004 paper[123] (Figure 6), the scale suggests that the segmented globule is too small to contain a loop terminus.

4.3.2 Chromatin fibers

The chromatin fibers visualized by electron tomography and measured in binned tomographic slices vary in diameter from 5 nm 38 nm, with the vast majority, across all data sets, varying between 5 and 30 nm (~97% across all fiber measurements). In the case of telomeric chromatin, there is however a clear shift towards higher fiber dimensions in the normal salt data set, which also features a wider range of fiber dimensions as compared to the low salt data. The normal salt telomeric data represent the highest proportion of fibers in the 12-24 nm range (74%) as well as the highest median diameter (18.08 nm), average diameter (18.47 nm) and most frequently occurring diameter (17.7 nm), by a large margin. The closest comparison is found in the high expressing H2B data set. Here, the smallest difference – between average fiber dimension – is 2.74 nm smaller in the high expressing H2B data than in normal salt telomeric fibers, a difference of nearly 20% in thickness. A comparison of their main peaks, or most frequently occurring fiber dimension, yields a difference of 5.3 nm, or 43% in thickness. As such, there exists a significant difference between even the strongly DAB-coated and OsO₄ stained H2B chromatin fibers and telomeric chromatin. There are several reasons to expect thicker chromatin fibers in telomeric chromatin as compared to bulk chromatin. Firstly, telomeric chromatin features the added presence of shelterin complexes in addition to nucleosomes. Secondly, the nucleosome repeat length has been measured as being shorter in telomeres, thus resulting in a higher number of nucleosomes per unit of DNA length. Thirdly, while some doubt has recently been cast on the epigenetic state of telomeres, the possibility that the epigenetic state of telomeric chromatin further contributes to thicker fiber dimensions remains. In addition to this, one must also consider the added difficulty of resolving distinguishable fibers in the significantly more tightly packed telomeric structure. As a result, it is possible that the shift towards thicker fibers observed in the normal salt telomere data is the result of misidentification of two or several separate fibers as one thick fiber. Intriguingly, the low salt data is of similar distribution of fiber dimensions as well as diameter range as the H2B data. As such, a significant decrease in fiber thickness has resulted from the low-salt treatment of the MEFs. The most likely explanation is a disruption of

the higher-order packing present in normal salt conditions, due to the lower ionic strength of the low salt condition.

The presence of the six-protein complex shelterin is likely the largest contributor to difference in fiber dimensions. Based on a comparison of fiber dimensions between normal salt telomeric chromatin and non-telomeric chromatin, the shelterin complex in combination with additional differences in chromatin structure together result in several additional nanometers. In a comparison between most frequently occurring fiber dimensions in these data sets, which likely corresponds to the most stable and dominant chromatin structure, this gives an increase of 5 nm in telomeric fiber thickness as compared to bulk chromatin (5.3 nm when compared to high expressing H2B cells, 5.8 nm when compared to low expressing H2B cells). Interestingly, following the low-salt treatment of MEFs, this difference in thickness between most frequent fiber dimension disappears entirely. This drastic reduction is likely a result of a decompacted chromatin fiber following the reduction in ionic strength in the nuclear milieu, which results in a thinner chromatin fiber. Based on DNA damage response studies performed by Barbara Hübner in Sara Sandin's laboratory in MEFs transfected with correlative TRF1 and TRF2-construct, no increase in DDR activation, as measured by colocalization with 53BP1 as well as overall 53BP1 staining, was visible following low-salt treatment (data not shown). Further, these data show no clear difference between transfected cells and untransfected controls, suggesting that the transiently expressed TRF-constructs do not interfere with telomere structure enough to yield a DDR. As such, it is unlikely that the reduction in fiber thickness following the low salt treatment is related to loss of shelterin. This would suggest that, under low salt conditions, the most common fiber dimension contains both nucleosomes and shelterin together, while being of the same thickness as the most common fiber dimension non-telomeric chromatin.

In addition to showing large variety in fiber dimensions between fibers, fibers also display large variations within the individual fibers (Figure 37). In the segmented fiber shown in Figure 37 B, which represents a comparatively homogeneously thick fiber, the thickness varies from 17.2 nm to 32.8 nm within ~50 nm along the fiber. Figure 37 E, by contrast, shows a more extreme example. Here, a thin "neck" of 5.1 nm connects significantly thicker chromatin regions, which approaches 20 nm in thickness at other points along the same structure. This variability is less extreme in non-telomeric chromatin, which also features less compact chromatin, which may contribute to more readily resolvable fibers which appear less variable. Similarly, whereas 2D measurements of fiber dimensions in both H2B data sets yield very similar thicknesses for the most frequently occurring fiber dimensions (~12 nm in both cases). This is likely to represent a genuine and prevalent fiber species. By contrast, measurements in telomeric chromatin is limited to resolvable fibrous features which means that the measurements are not an accurate representation of all fibers present in telomeric chromatin, but rather a representation of resolvable fibers.

4.4 Hierarchical chromatin models and the 30 nm fiber

Regardless, the large and frequent variation in fiber dimensions, in both H2B and TRF1 samples, is difficult to reconcile with a hierarchical chromatin folding model with distinct and dominant fibers corresponding to discrete dimensions. In the case of telomeric chromatin, the presence of the shelterin complex in addition to characteristic epigenetic marks and histone variants of course renders the discussion on hierarchical folding largely meaningless; the broader debate on the presence or absence of a 30-nm fiber deals with non-telomeric chromatin. However, while still keeping the limitation of only measuring and segmenting resolvable fibers in mind, it is still possible to establish that the available data do not suggest the presence of discrete stable and more prevalent fiber populations. Rather, the normal salt fiber dimension distribution (Figure 32, pink distribution) is reminiscent of a normal distribution; the distribution peaks at 17.7 nm with a hint of a shoulder (Figure 32, marked “*4”) and plateau from ~28-33 nm. This is also the case for the broad range of fiber distributions observed in non-telomeric fiber dimension distributions. It is possible such a distribution may result from intermediately folded fibers which populate the distribution between discrete and more dominant higher-order conformational states. However, the hierarchical model suggest that higher-order fiber conformations are more stable and thus more dominant, which would suggest a higher abundance of these discrete conformations within the distribution. In the data presented here, fiber dimensions corresponding to discrete 30 nm states are present in the high expressing cells, but this dimension does not stand out as significantly more abundant within the distribution. What’s more, no fibers above 28 nm are observed in the low-expressing H2B cells. More prominent shoulders are present in the H2B distribution, notably *3, *4 and *5 (Figure 42), however these do not correspond to any proposed higher-order structures as all occur below 25 nm in diameter.

Further, a transcriptionally regulated fiber which cycles from 5 to 25 nm depending on transcriptional state, as suggested by Daneholt et al.[49], may similarly be compatible with a broad distribution of fiber dimensions representing genes at various transcriptional states. In the data presented here, fiber dimension corresponding to this 25 nm fiber are present, and at higher frequency than are 30 nm dimensions. Further, both the telomeric chromatin in low salt treated MEFs and low expressing H2B data show an increase in fiber dimensions just below 25 nm. However, a model where genes not undergoing active transcription adopt a 25 nm conformation would likely yield much higher frequencies than are observed here.

Taken together, the sum of the H2B data is more consistent with a disordered 10 nm chromatin fiber, than with discrete hierarchies of chromatin packing. The presence of very similar main peaks in both low and high expressing data sets (11.9 and 12.4 nm, respectively) could correspond to the proposed 10 nm beads-on-a-string conformation, which can fold and condense further but, by contrast to *in vitro* folding studies, does not adopt significantly more dominant conformations corresponding to a 25 or 30 nm fiber. Rather, the fiber folds in an irregular fashion, yielding intermediate fiber dimensions and conformations resulting in a broad distribution of fiber dimensions. Fiber dimensions measured at below 10 nm in diameter, are likely DAB-coated and OsO₄-stained DNA alone, or chromatin structures that are not entirely captured within a single binned tomographic slice and as a result have been measured peripherally.

It is at this point interesting to note the early work of the Olins', Woodcock as well as Finch and Klug[42]–[44]. As previously mentioned, Woodcock in his 1973 abstract refers to “fiber diameters” which “range” from less than 100 Å to more than 300 Å. He goes on to describe that the increase in fiber diameter are achieved through “irregular” coiling and folding. Similarly, the Olins in their 1974 Science paper refer to “irregularly distributed thick and thin regions”. (Here it is admittedly somewhat unclear to what extent they mean to refer to the irregularity of which thin regions coalesce to form thicker regions, or to the irregular thickness of the fibers themselves). Further, Finch and Klug in their 1976 solenoid paper similarly refer to “thick fibers” “of a diameter that is somewhat variable, even along the same fiber, but is usually in the range of 300-500Å”.

In large part, the non-telomeric chromatin findings presented in this thesis are also consistent with those reported by the Ellisman and O'Shea groups in 2017[76], which also proposes a disordered chromatin fiber model, and further shares many methodological similarities with my work. In their paper, they also utilize correlative fluorescence and electron microscopy, DAB-polymerization and osmium staining, as well as electron tomography to study bulk chromatin. Notably, they use a fluorescent DNA dye to polymerize DAB through photo-activation. Further, human osteosarcoma (U2SO) cells and human small airway epithelial cells (SAECs) were used, and 8-tilt series were collected on thicker (250 nm) sections. The tomograms were reconstructed into subvolumes of 138 nm thickness and the data was primarily analyzed in tomographic slices corresponding to 1.28 – 1.60 nm in depth. Strikingly, they find similar chromatin fiber diameters in all conditions (Figure 3) with the bulk occurring between 5-36 nm, and a small subset (<9%) with dimensions above that. In higher density chromatin areas and metaphase chromatin, the data consistently shows approximately equal parts of 5-12 nm fiber diameters as 12-24 nm diameters, which together constitute ~80% of fibers in all conditions.

These distributions are similar to those reported here in this thesis, with some exceptions. In the data presented here the majority of fibers occur within the span of 5-24 nm in diameter (~96% for H2B fibers from low expressing cells, ~93% for high expressing cells). However, whereas the data presented in their paper frequently shows similar or equal distributions between 5-12 and 12-24 nm fiber dimensions, there is a consistent and significant shift in my data towards the latter. Further, the highest proportion of fibers observed in the interval 12-24 nm interval is ~40% in their paper; the lowest proportion in my data is 62%. Further, their fiber dimension distributions for higher density areas (25-47% “chromatin volume concentration” or “CVC”) includes a separate bin of 36-48 nm in diameter. In my work, fibers above 36 nm occurred only in telomeric chromatin. However, it is clear from the representative 2D measurements shown in their paper that their measurements include more irregular and bulky features, whereas my measurements have focused on distinguishable fibrous features. This may account for some of the differences in measurements. Further, were I to measure bulky and irregular features as well as particularly in denser chromatin areas (see Figure 33 H, I, J, P) these would likely correspond to dimensions above 36 nm. Ou et al. also report thinner chromatin chains in interphase subvolumes with lower chromatin densities (<20% CVC) where (~60%) of the fibers measured are in the 5- to 12 nm

range. The corresponding number for my data is ~35% for low expressing cells and ~27% for high expressing cells. However, looking specifically at the tomogram featuring lowest apparent chromosome concentration, C5A1, I find that 44% the fibers measured therein fall into the 5- to 12 nm range. As such, the most dispersed regions in my data set are closer to the values reported in their work. As these 2D measurements are somewhat subjective, some disagreement is expected.

They further estimate, based on 3D modelling and iterative fitting of nucleosome-linker histone units into their tomographic 3D volumes, that the addition of DAB polymers and OsO₄ staining adds 1-2 nm to increased thickness. A comparison of the main peaks in the two H2B data sets suggest a difference in thickness of 0.5 nm between high expressing cells and low expressing cells with regards to their most frequent chromatin fiber species and, further, the difference in median fiber thickness between the two is 1.37 nm (Table 4). If accurate, subtracting the additional 1-2 nm of added thickness from DAB and OsO₄ staining would place the most frequent chromatin species in both of my H2B data sets at a thickness corresponding to 10 nm. It is worthwhile to note whereas the work by Ou et al. includes both mitotic and interphase chromosomes, my own work has focused on interphase chromosomes. As such, based on my work alone it not possible to rule out that the 30 nm fiber exists as a higher-order structure in mitotic chromosomes. However, a clear and dominant 30 nm is not present in the mitotic chromatin shown in the work by Ou et al.

A visual comparison of the appearance of the telomeric chromatin visualized in this work and in previous attempts at visualizing telomere structure reveals some important differences. Firstly, while previous studies have continued to show decondensed and relaxed chromatin fibers in lariat configuration (the t-loop), the telomeric chromatin shown in this thesis is a densely packed network of chromatin features. This clear difference in appearance emphasizes the importance of imaging chromatin structures as close to their native environment as possible. The complexity of the structure as it appears *in situ* combined with the relatively dense packing of chromatin fibers as compared to chromatin presented in *in vitro* work precludes unambiguously identifying lariat-like structures. As such, my work presented here either lacks sufficient resolution, or the processing capability required to analyze the complex connections of fibers in the tomographic data to locate and identify a t-loop, or both. The work that, based on appearance of the chromatin fibers, does come closest to the fibrous structure of the *in situ* telomere is likely the higher salt conditions displayed in Woodcock's 2004 paper (shown in Figure 4, G). As previously mentioned, the 2004 Woodcock paper is the only EM study which succeeds at retaining part of the chromatin component during telomere isolation. Here, a 75 mM NaCl concentration has condensed a telomere-enriched chromatin fraction from a mouse spleen lymphocyte into a contiguous 30 nm fiber which clearly forms a lariat structure surrounding an internal gap. This most condensed structure makes for an interesting comparison with fibers visualized in the cell in the present study. The dimension of the condensed isolated chromatin is approximately 30 nm, which corresponds to a minority of fibers encountered in the present study. Conversely, the

internal gap is large and constitutes a considerable proportion of the entire structure, which is rarely encountered *in situ*.

4.5 Salt conditions of the nucleus

As previously discussed, the native or physiological salt conditions of the nucleus are difficult to estimate accurately. While the DMEM medium and FBS used for culturing of cells contains a complex mix of salts, downstream processing of the cellular samples EM and electron tomography requires the use of certain buffers with other salt conditions. For all sample preparations other than low salt TRF1, cells were washed with 0.1 M Sodium Cacodylate buffer (CB buffer) and subsequently also incubated in CB buffer during fixation. As such, while the cells may retain some salt from the culturing medium, they are primarily subject to the salt conditions of the CB buffer. The sodium concentration of the CB buffer already exceeds the highest salt concentration used by Woodcock et al. and, further, is within the range of estimates for nuclear sodium concentrations[200], [201]. Importantly, however, by imaging chromatin while in the nucleus the rest of the nuclear environment is maintained, including nonpolar molecules and nuclear proteins.

4.6 Estimated resolution of tomographic data

Cryo-EM tomography of cells has the potential to reach lateral resolutions of just a few nanometers, typically ranging from 2-5 nm [202], [203]. Importantly, the tomographic data collected here is not of cryogenically frozen samples but plastic embedded samples where the presence of plastic, as well as heavy stains such as uranyl acetate and OsO₄, lower the resolution. It is possible to estimate the resolution of a tomographic data set by comparing it to known structures, or by fitting known structures into the tomographic volume. This is not possible here as telomeric chromatin is not well characterized and, further, no clear examples of nucleosomes or NCP are distinguishable in the tomographic data. It is, however, possible to estimate a probable resolution based on the methodology and technique used. Such an approach would suggest a lateral resolution of approximately 5-10 nm. The resolution in the axial dimension is lower, due to the loss of information from the “missing wedge”[204]. The missing wedge, in short, represents the missing information resulting from the fact that, beyond a certain tilt angle, radiation is no longer transmitted through the section due to its thickness along the beam path. In this case, tomographic sections were tilted $\pm 65^\circ$ in two orthogonal tilts. As such, due to the dual tilt series employed in this study, the missing wedge is actually missing square pyramid. As such, the resolution of the data sets employed here is anisotropic and features a lower resolution in the Z-direction.

4.7 Fluorescent dot quantification

In the telomere fluorescent foci quantification comparison between normal and low salt data sets (Figure 16), there is a significant increase in the average number of dots observed in the lower salt data. The analysis has two main limitations. Firstly, it is more likely to neglect short telomeres which due to their reduced fluorescence cannot be clearly distinguished from the

minimal background fluorescence. Secondly, due to the limits to axial and lateral resolution (estimated to be ~800 nm and ~250nm, respectively) two sufficiently close, adjacent, telomeres run the risk of being misrecognized as a single dot. Variations in expression level between the conditions arising from their separate transfections and sample preparations may give rise to some differences in background fluorescence as well as telomeric fluorescence. Further, it is possible that cells from the low salt conditions contain a larger proportion of cells in S or G2 phase, which would naturally yield a larger number of telomeres. In comparing these results with the fluorescently labelled metaphase spreads carried out by Barbara Hübner, the number of TRF1-punctae was estimated to be just above 100 per cell (data not shown). This number more closely corresponds to the low salt telomere quantification.

4.8 Validity of telomeric and non-telomeric chromatin comparison

The validity of the comparison of non-telomeric chromatin to telomeric chromatin presented in this work depends, in part, on how well the fusion proteins utilized label and display chromatin. For instance, if TRF1 occurs at a significantly different frequency at telomeric chromatin than the frequency at which H2B is found in non-telomeric chromatin, this would also give rise to different frequencies of APEX2 and thus different levels of staining. In a 2010 paper investigating the *in vivo* stoichiometry of telomere-bound shelterin components, Takai et al. found[136] that telomeres in the HeLa1.3 cell line, with telomere lengths that are similar to the MEFs used here (~23kb in HeLa1.3, ~19kb in MEFs) contained 270 chromatin bound TRF1 molecules per telomere. This corresponds to one TRF1 homodimer per 170 bp of telomeric DNA. The corresponding figure for H2B, assuming a 100% occupancy of the canonical H2B and a nucleosomal repeat length of 201 bp, is two copies of H2B per 201 bp. Histone H2B has limited histone variants, most of which are associated with oocytes and zygotes which decrease with differentiation[205]. As such, an estimate close to 100% is not unreasonable. As such, based on these calculations, TRF1 and H2B occur in comparable frequency per bp. Other factors may contribute to differences in the incorporation of fusion proteins into chromatin, but this calculation nonetheless serves as an indication. If a TRF1 fusion protein were to be exchanged for TRF2, the difference would be larger still (420 chromatin-bound TRF2 molecules in HeLa1.3[136]). A TIN2 construct would yield a similar overrepresentation of telomeric APEX2-molecules as would TRF2 but, due to the structural importance of TIN, is likely to give rise to structural issues and potentially uncapping and DDR activation.

4.9 Transient expression and expression levels of telomeric repeat factors

The correlative constructs utilized in this work were transiently expressed under a CMV promoter, which has been shown to give variable expression levels in different cell lines[206]. Regulating expression level of constructs from transient expression is difficult. In this work, some regulation is possible by excluding from analyses cells which feature unusually high or low expression levels, as determined by fluorescence intensity and background fluorescence. Further, it has previously been suggested that TRF2 acts to compact telomeres[207], [208]. Considering the homology between TRF1 and TRF2, and their shared substrate of telomeric dsDNA, it is possible that they

compete for binding. X-ray crystal structures determined for both proteins suggests that they bind to the same sequences[170]. If so, it is conceivable that an increase in TRF1 through transient expression may result in displacement of TRF2 dimers, and vice versa. If true, it follows that a perturbation of TRF2 levels through overexpression of either TRF1 or TRF2 may have an effect on telomeric compaction and, potentially, ultrastructure. One recent paper reported an 8-fold decompaction of telomeric volume as measured by super-resolution microscopy of photoactivatable monomeric GFP-tagged telomeres upon RNAi depletion of TRF1. However, it was quickly contradicted by two rigorous replications published in the same issue of *Genes and Development* by the Lingner and de Lange groups[118],[119]. No other research has linked TRF1 levels to telomere compaction. As part of their work, the Lingner group utilized a large field-of-view STORM set-up to study telomere dimensions using a large sample size. A sample size of 1208 telomeres was used for analysis of HeLa L (“L” for “long”) telomeres, and 2629 telomeres were used for an analysis of HeLa S (“S” for “short”) telomeres. HeLa L cells have an average telomere length of 33 kb, and HeLa S cells an average length of 11 kb. Both groups report telomere dimensions by radius of gyration, R_g , which is described as the root mean square of the distance between the centre of the cluster and individual localization. As such, it is roughly analogous to the radius of an irregular 3D shape. The Lingner group found no difference in compaction upon TRF1 or TRF2 depletion ($n > 900$ telomeres for each condition[159]). By contrast, the de Lange group ($n > 10$ cells for each condition) found various compactations and decompactations in R_g , ranging from -6% (a decrease in R_g ; a compaction) to 31% (an increase in R_g ; a decompaction) with an average of +8% over 6 independent experiments following cre-mediated deletion of TRF1. Corresponding experiments with deletion of TRF2 yield some decompaction (R_g change of $\leq 20\%$) but are also able to trace the majority of the contribution towards higher R_g values to a subset of abnormally large telomeres, and like the Lingner group offer the caveat that this decompaction may be explained by clustering of dysfunctional telomeres. They conclude that only minimal decompaction can be observed following cre-mediated deletion of TRF1 or TRF2, which may be, in part or wholly, explained by clustering of dysfunctional telomeres. As such, the decompaction reported by the initial report are likely exaggerated, and deletion or strong reduction of TRF1 or TRF2, or both together, at most result in minimal changes in telomeric volume. Further, based on the R_g 's reported, they calculate that the compaction in length observed in HeLa S gives to a 27-fold compaction (R_g of 68 nm for 11kb). This same calculation for the normal salt MEF telomere dimensions measured in this work gives a 33-fold compaction in length (diameter 196 nm, 19 kb). Further, the measured dimensions of telomeres roughly correspond to those reported in my work, as well as other published research (180 nm mean telomere diameter for MEFs[162]). As such, the telomere sizes and level of compaction described in this work corresponds well with previous published work. Based on these findings, it seems unlikely that a transient overexpression of TRF1 has any significant impact on telomere decompaction. A recent preprint further suggests that competitive binding of TRF1 and TRF2 only occurs in the absence of TPP1[173] and does not occur *in vivo* upon expression of with fluorescently labelled fusion proteins of TRF1 or TRF2[174]. Further, competition resulting in displacement of either TRF1 and

TRF2 is likely to trigger an activation of the DDR, which is not significantly higher in cells transfected with either TRF1 or TRF2 correlative constructs compared with an untransfected control. Further, previous studies using transient expression of TRF1 constructs have been carried out without any initiation of checkpoint activation, as determined by the lack of phosphorylation of H2AX[197], indicating that transient expression of TRF1 does not disrupt shelterin or alter telomere structure sufficiently to induce DNA damage.

However, in addition to competition between TRF-proteins, several publications suggest that overexpression of TRF2 in mice[209] as well as humans[210] impacts nucleosome spacing in telomeric chromatin. Similarly, TRF1 has been implicated in interfering with the organization of telomeric nucleosomal arrays *in vitro*[172]. As such, it is possible that overexpression of TRF1 displaces nucleosomes from telomeric chromatin. However, such competitive binding is expected to occur already as a result of telomere shortening, which naturally reduces the number of available bindings sites on the telomeric chromatin. As such, if TRF1 competes with nucleosomes, this competition already occurs naturally within cells. The extent to which excess TRF1 or TRF2 may impact telomere structure is however unclear and requires further investigation. To limit the contribution of excess TRF1 protein and better regulate expression levels, future studies should aim to establish expression of constructs under control of the endogenous promoter using the CRISPR/Cas9 genome editing approach[211].

4.10 Differences in appearance between telomeres from normal and low salt conditions

Some difference in appearance is obvious upon comparison of certain normal salt tomographic slices with low salt tomographic slices. Specifically, the edges of chromatin features in low salt telomeres are more distinct and well-defined than those observed in normal salt telomeres (See Figure 29 and Figures 27, 28). It is unclear exactly what the cause of this difference is. One possibility is that this is caused by a difference in expression level of the TRF1 construct arising from the separate transfection and sample preparations of the two salt conditions, even when much care has been taken to ensure that the cells imaged from each condition are of similar expression levels. These, in turn, may lead to small differences in DAB/OsO₄ coating and thus, potentially, appearance. However, it is also possible that the two conditions have equivalent expression levels, i.e. a similar number of fusion proteins per telomere, but that the increase in telomeric volume in the low salt condition nonetheless results in a lower level of fusion protein per unit volume. This further means that a 70 nm section of normal salt telomere contains a larger proportion of a full telomere than does a 70 nm section of a low salt telomere. The same goes for a 140 nm section. In other words, a 70 nm section is able to “capture” more of a ~200 nm telomere than it does a ~220 nm telomere (35% of the full depth vs. 31.8% with respect to depth in Z). In the same manner, due to the fact that the decompaction and telomeric volume increase takes place prior to the DAB polymerization reaction in the low salt samples, a lower number of APEX2 molecules per unit volume is expected, which may give rise to differences in stain and appearance, even given identical expression levels.

4.11 Telomere size and physical section thickness

Using 140 nm thick tomographic sections to capture and study structures in the range of 200 nm inevitably leads to incomplete structures, in this case telomeres, captured in each section. This is a necessity stemming from the need to collect images at high tilt angles where thicker sections are opaque and would thus limit the data collection to lower angles. As such, it is possible that some aspect of the telomeric structure is lost as a result of this section thickness. However, due to the large number of telomeres captured and studied in this work, the tomograms collected include both top and bottom of telomere structures (See LS5, LS6, LS11 and NS7 in Appendix A4). As such, while none of these sections contain complete telomeres, they together constitute several full telomeres.

4.12 Dotty patterns in tomographic slices

The beads-on-a-string like features visible in tomographic slices of both telomeric and bulk chromatin are intriguing (Appendix A2). However, based on the fact that the dots are equally conspicuous and dark in both TRF1-derived and H2B-derived stain, they are unlikely to be nucleosomes, as these would then be expected to be darker in the H2B-expressing cells. Further, the dimensions of the dots are too small to correspond to a fiber consisting of 11 nm nucleosomes, and similarly show too small distances between particles (Appendix A2, “Comparison of dotty patterns in telomeric and bulk chromatin”, Page 119).

Figure 45: Graphical summary and schematic representation of research findings. Summary of research findings using idealized schematic representations. **A)** Very brief overview of the correlative procedure, allowing for identification and correlation of telomeres. **B)** Schematic representation of a stained telomere viewed in 2D as imaged by TEM. **C)** Schematic representation of a full depth 3D surface render of a telomere. **D)** Schematic representation of a 5.4 nm tomographic slice from a surface rendered telomere. The lines in light blue suggest an idealized and speculative model for the lattice-like internal organization observed in some telomeres. **E)** Schematic representations of segmented chromatin features, showing both fibrous, globular as well as more complicated higher-order chromatin structures. Line measurements are indicated with distances in nm.

5. Conclusions

This thesis presents a detailed characterization of the ultrastructure of telomeres, telomeric chromatin and the internal organization of telomeres in the nucleus. The combination of CLEM and electron tomography presented herein allows for the imaging and analysis of chromatin and telomeric chromatin at 5-10 nm resolution. In the cell, telomeres are compact spheroid structures of approximately 200 nm in diameter in MEFs, and 150 nm in HT1080. Short incubations of cells in a hypotonic, “low salt”, solution decompact chromatin *in situ* and thus facilitate imaging and analysis of individual distinguishable fibers and features as well as the internal organization of telomeres. When analyzed in 3D, telomeres are revealed to consist of a complex organization of fibrous and globular features. A recurrent feature of telomeres when viewed as in cross-section are fibrous features arranged in parallel lines, in an organization reminiscent of a lattice (See Figure 45 D for an idealized representation). Together, these features constitute a mesoporous network, with distinguishable telomeric chromatin fibers ranging primarily from 5-30 nm in diameter and internal gaps ranging from 5-50 nm. In addition to distinguishable fibrous and globular features, large portions appear as dense clusters often exceeding 50 nm in diameter. These may consist of individual chromatin features too densely packed to be resolved by the technique employed here or, alternatively, as native higher-order chromatin structures. Taken together, the various analyses of chromatin structure and ultrastructure, from both telomeric and non-telomeric chromatin, are more consistent with a chromatin fiber of variable diameter rather than with models proposing discrete hierarchies of higher-order chromatin folding.

Due to the electron microscope used for this study having an accelerating voltage of 200 kV, the sample thickness for tomography was limited to approximately 140 nm. As cellular sections of this thickness are insufficient to contain a full telomere, this work necessarily suffers from an inability to image and analyse entire telomeres. It is likely that cutting-edge electron microscopes with accelerating voltages of 300 kV would be able to accommodate thicker sections (250-300 nm) which would allow for entire telomeres to be imaged and analyzed by tomography. It is further possible that the higher resolution offered by these cutting-edge microscopes would facilitate tracing of the entire telomere, which could yield additional insights into its internal organization and may further enable identification and visualization of the t-loop, if present. A second limitation of the work presented here is the use of transient expression, which may impose changes in expression levels between experiments and conditions and thus interfere with comparisons between these. Further, it is possible that overexpression of TRF1 may interfere with or otherwise alter telomere structure and function. As such, a better alternative would be to ensure endogenous expression levels of the correlative construct(s) throughout all experiments using CRISPR/Cas9 genome editing. Finally, the use of immortalized cells for investigations into cellular biology may yield a distorted view of the phenomenon which is being studied. For this reason, the use of primary cells is preferred.

6. Future Work

Having established that it is possible to identify and image telomeric chromatin at high magnification *in situ*, future work should seek to image thicker cellular sections from primary cells expressing endogenous levels of correlative construct, using cutting-edge 300 kV electron microscopes. Such an experiment would likely yield additional important insights into telomere structure and organization *in situ* and, if performed under low salt conditions, may offer sufficient resolution to allow for tracing of the entire telomere. This would make possible the identification of the t-loop *in situ*.

Further, it is possible that recent advances in super-resolution CLEM and cryo-CLEM[212], [213] may offer such correlative accuracy between super-resolution microscopy and EM that telomeres can be identified without the need for APEX2-labelling. If so, combining super-resolution microscopy or low-dose super-resolution microscopy with the native cellular conditions offered by cryo-EM would make for a compelling project.

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Appendix

A1. Proteins and Constructs

Telomeric Repeat-Binding Factor 1 (TRF1), Isoform 1, Homo Sapiens (NP_059523.2)

439 a.a., 50.2 kDa

MAEDVSSAAPSPRGCADGRDADPTEEQMAETERNDEEQFECQELLECCQVQVGAPEEEEEEEEDAGLVAEA
EAVAAGWMLDFLCLSLCRAFRDGRSEDFRRTRNSAEAIHGLSSLTACQLRTIYICQFLTRIAAGKTLDAQFEN
DERITPLESALMIWGSIEKEHDKLHEEIQNLIKQAIIVCMENGNFKEAEVFERIFGDPNSHMPFKSKLLMIIS
QKDTFHSFFQHFSYNHMMMEIKISYVNYVLSEKSSTFLMKAAAKVVESKRTRTITSQDKPSGNDVEMETEANL
DTRKSVSDKQSAVTESESGTVSLLRSHKNLFLSKLQHGTQQQDLNKKERRVGTGPQSTKKKKESRRATESRIPVS
KSQPVTPPEKHRARKRQAWLWEEDKNLRSGVRKYGEGNWSKILLHYKFNNRTSVMLKDRWRTMKKLKLISS
DSED

eGFP_APEX2_TRF1

934 a.a., 105.6 kDa

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTCLKFICTTGKLPVPWPTLVTTLTYGVCFS
RYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYN
YNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRD
HMLVLEFVTAAGITLGMDELYKSLRSMGKSYPTVSADYQDAVEKAKKKLRGFIAEKRCAPLMRLAFHSAG
TFDKGKTGGPFGTIKHPAELAHSANGLDIAVRLLEPLKAEPILSYADFYQLAGVVAVEVTGGPKVPFHPGR
EDKPEPPPEGRLPDPTKGSDDLVDVFGKAMGLTDQDIVALSGGHTIGAAHKERSGFEGPWTSNPLIFDNSYF
TELLSGEKEGELLQLPSDKALLSDPVFRPLVDKYAADEDAFFADYAEAHQKLSELGFADAGAAQASNSAVDMAE
DVSSAAPSPRGCADGRDADPTEEQMAETERNDEEQFECQELLECCQVQVGAPEEEEEEEEDAGLVAEAEAVA
AGWMLDFLCLSLCRAFRDGRSEDFRRTRNSAEAIHGLSSLTACQLRTIYICQFLTRIAAGKTLDAQFENDERIT
PLESALMIWGSIEKEHDKLHEEIQNLIKQAIIVCMENGNFKEAEVFERIFGDPNSHMPFKSKLLMIISQKDTF
HSFFQHFSYNHMMMEIKISYVNYVLSEKSSTFLMKAAAKVVESKRTRTITSQDKPSGNDVEMETEANLDTRKS
VSDKQSAVTESESGTVSLLRSHKNLFLSKLQHGTQQQDLNKKERRVGTGPQSTKKKKESRRATESRIPVSKSQP
VTPEKHRARKRQAWLWEEDKNLRSGVRKYGEGNWSKILLHYKFNNRTSVMLKDRWRTMKKLKLISSDSED

Histone H2B type I-J, Homo Sapiens (NP_066402.2)

126 a.a., 13.9 kDa

MPEPAKSAPAPKKGSKKAVTKAQKKDGKKRKRSRKESYSIYVYKVLKQVHPDTGISSKAMGIMNSFVNDIFER
IAGEASRLAHYNKRSTITSREIQTAVRLLLPGELAKHAVSEGTKAVTKYTSK

eGFP_APEX2_H2B

632 a.a., 69.4 kDa

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTCLKFICTTGKLPVPWPTLVTTLTYGVCFS
RYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYN

YNSHNVYIMADKQKNGIKVNFKIRHNIEDGQVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRD
HMLLEFVTAAGITLGMDELYKSGLRSMGKSYPTVSADYQDAVEKAKKKLRGFIAEKRCAPLMRLAFHSAG
TFDKGKTGGPFGTIKHPAELAHSANGLDIAVRLLEPLKAEFPILSYADFYQLAGVVAVEVTGGPKVPFHPGR
EDKPEPPPEGRLPDATKGSDHLRDVFGKAMGLTDQDIVALSGGHTIGAANKERSGFEGPWTSNPLIFDNSYF
TELLSKEKEGLLQLPSDKALLSDPVFRPLVDKYAADEDAFFADYAEAHQKLSLGFADAGAQASNSAVDGTM
PEPAKSAPAPKKGSKKAVTKAQKKDGKKRKRKRKESYSIYVYKVLKQVHPDTGISSKAMGIMNSFVNDIFERIA
GEASRLAHYNKRSTITSREIQTAVRLLLPGLAKHAVSEGTKAVTKYTSK

APEX2_H2B

388 a.a., 41.9 kDa

MGKSYPTVSADYQDAVEKAKKKLRGFIAEKRCAPLMRLAFHSAGTFDKGKTGGPFGTIKHPAELAHSANN
GLDIAVRLLEPLKAEFPILSYADFYQLAGVVAVEVTGGPKVPFHPGREDKPEPPPEGRLPDATKGSDHLRDV
FGKAMGLTDQDIVALSGGHTIGAANKERSGFEGPWTSNPLIFDNSYFTELLSKEKEGLLQLPSDKALLSDPVFR
PLVDKYAADEDAFFADYAEAHQKLSLGFADAGAQASNSAVDGTMPPEPAKSAPAPKKGSKKAVTKAQKKD
GKKRKRKRKESYSIYVYKVLKQVHPDTGISSKAMGIMNSFVNDIFERIAGEASRLAHYNKRSTITSREIQTAVR
LLPGLAKHAVSEGTKAVTKYTSK

A2. Recurring Chromatin Features, H2B

Patterns of dots in a line found in H2B-APEX2 labelled chromatin.

Gallery of dotted chromatin features present in all tomograms. Features are reminiscent of beads-on-a-string fiber, but individual dots are too small (typically less than 7nm). Distances between dots similarly do not correspond to what is expected from beads-on-a-string conformation.

Patterns of zig-zags and stacked discs found in H2B-APEX2 labelled chromatin.

Gallery of dotted chromatin features present in all tomograms. Features resembling of zig-zags and stacked discs. Feature thickness ranges from 10-30+ nm.

Separate large and darkly stained spheroids present in H2B-APEX2 labelled chromatin.

Gallery of darkly stained spheroids seen in bulk chromatin. Some spheroids reach 50 nm in diameter. Frequently occurs separate from peripheral chromatin.

Clusters/chains of smaller spheroids.

Gallery of clustered small spheroids. Similar in appearance to the previously shown dark spheroids that occur in clusters. Approximately 20 nm in diameter.

Comparison of dotty patterns in telomeric and bulk chromatin

Comparison of dotted chromatin feature present in all tomograms. Top row) Features from filtered and binned tomographic slices of H2B chromatin. **A-D)** Features from filtered and binned tomographic slices of low salt telomeric chromatin. **E-H)** Features from filtered and binned tomographic slices of low salt telomeric chromatin.

A3. 3D Representations of TRF1 Tomograms

LS1 Full volume 3D surface render of a low salt telomere, produced in Amira as described previously.

LS5 Full volume 3D surface render of a low salt telomere, produced in Amira as described previously.

LS6 Full volume 3D surface render of a low salt telomere, produced in Amira as described previously.

LS7 Full volume 3D surface render of a low salt telomere, produced in Amira as described previously.

LS8 Full volume 3D surface render of a low salt telomere, produced in Amira as described previously.

LS9 Full volume 3D surface render of a low salt telomere, produced in Amira as described previously.

LS10 Full volume 3D surface render of a low salt telomere, produced in Amira as described previously.

LS11 Full volume 3D surface render of a low salt telomere, produced in Amira as described previously.

NS1 Full volume 3D surface render of a normal salt telomere, produced in Amira as described previously.

NS3 Full volume 3D surface render of a normal salt telomere, produced in Amira as described previously.

NS4 Full volume 3D surface render of a normal salt telomere, produced in Amira as described previously.

NS5 Full volume 3D surface render of a normal salt telomere, produced in Amira as described previously.

NS6 Full volume 3D surface render of a normal salt telomere, produced in Amira as described previously.

NS7 Full volume 3D surface render of a normal salt telomere, produced in Amira as described previously.

NS10 Full volume 3D surface render of a normal salt telomere, produced in Amira as described previously.

NS12 Full volume 3D surface render of a normal salt telomere, produced in Amira as described previously.

NS15 Full volume 3D surface render of a normal salt telomere, produced in Amira as described previously.

NS17 Full volume 3D surface render of a normal salt telomere, produced in Amira as described previously.

A4 Filtered and Binned Consecutive Tomographic Slices, TRF1

LS1 Sequential tomographic slices of a low salt telomere. Scale bar 200 nm.

LS5 Sequential tomographic slices of a low salt telomere. Scale bar 200 nm.

LS6 Sequential tomographic slices of a low salt telomere. Scale bar 200 nm.

LS7 Sequential tomographic slices of a low salt telomere. Scale bar 200 nm.

LS8 Sequential tomographic slices of a low salt telomere. Scale bar 200 nm.

LS9 Sequential tomographic slices of a low salt telomere. Scale bar 200 nm.

LS10 Sequential tomographic slices of a low salt telomere. Scale bar 200 nm.

LS11 Sequential tomographic slices of a low salt telomere. Scale bar 200 nm.

NS1 Sequential tomographic slices of a normal salt telomere. Scale bar 200 nm.

NS3 Sequential tomographic slices of a normal salt telomere. Scale bar 200 nm.

NS4 Sequential tomographic slices of a normal salt telomere. Scale bar 200 nm.

NS5 Sequential tomographic slices of a normal salt telomere. Scale bar 200 nm.

NS6 Sequential tomographic slices of a normal salt telomere. Scale bar 200 nm.

NS7 Sequential tomographic slices of a normal salt telomere. Scale bar 200 nm. Heterochromatin is seen towards the bottom in some tomographic slices.

NS10 Sequential tomographic slices of a normal salt telomere. Scale bar 200 nm.

NS12 Sequential tomographic slices of a normal salt telomere. Scale bar 200 nm.

NS15 Sequential tomographic slices of a normal salt telomere. Scale bar 200 nm.

NS17 Sequential tomographic slices of a normal salt telomere. Scale bar 200 nm.

A5 Filtered and Binned Consecutive Tomographic Slices, H2B

Cell 1, Area 1 (C1A1) Sequential tomographic slices of bulk chromatin. Scale bar 200 nm.

C1A2 Sequential tomographic slices of bulk chromatin. Scale bar 200 nm.

C2A1 Close to nuclear envelope (top). Sequential tomographic slices of bulk chromatin. Scale bar 200 nm. The nuclear envelope is visible towards the top in most tomographic slices.

C2A2 Sequential tomographic slices of bulk chromatin. Scale bar 200 nm.

C2A3 Sequential tomographic slices of bulk chromatin. Scale bar 200 nm.

C2A4 Sequential tomographic slices of bulk chromatin. Scale bar 200 nm. The nuclear envelope is visible towards the top in all tomographic slices.

C2A5 Sequential tomographic slices of bulk chromatin. Scale bar 200 nm.

C4A1 Sequential tomographic slices of bulk chromatin. Scale bar 200 nm.

C5A1 Sequential tomographic slices of bulk chromatin. Scale bar 200 nm.

C6A2 Sequential tomographic slices of bulk chromatin. Scale bar 200 nm. The nuclear envelope is visible towards the bottom left in all tomographic slices.