Relationships between nuclear lamins and telomere biology in progeroid laminopathies and cancer

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Doctor of Philosophy

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Abstract

Telomeres are essential for maintaining the integrity of the genome. Shortening and dysfunction of telomeres initiates cellular senescence, halting further cell division, and instigating alterations in biological processes which contribute to ageing. Progeroid syndromes are disorders of ageing. Patients exhibit not only an early external appearance of old age, but several age-related diseases including osteoporosis, muscle wasting and cancer.

Laminopathies are diseases caused by mutations in LMNA, the gene encoding the key nuclear matrix component lamin A. Some mutations result in progeroid phenotypes that are associated with elevated levels of senescent cells. The rate of telomere shortening in progeria cells was found to be accelerated relative to healthy controls, confirming a role for lamin A function in telomere biology.

In order for a cell to bypass the protective mechanism of senescence, it must initiate a telomere maintenance mechanism (TMM), resulting in cancer. Several biological changes accompanying the alternative lengthening of telomeres (ALT) TMM overlap with mechanisms affected by changes in lamin biology. As such, lamins and related nuclear proteins were investigated in ALT cells and found to differ from tumour-derived cells which activate the telomerase-based TMM. It was found that although nuclear levels of lamins differed between cells of the two TMMs, the interaction of lamin A with chromatin was not altered. Localisation studies of lamin A revealed different distribution of lamins in the nucleus. An excess of intranuclear lamin observed in ALT cells is proposed to be permissive for recombination-based processes required for telomere elongation through ALT, whereas laminopathy lines exhibit a reduced intranuclear lamin, and an accompanying DNA damage repair deficiency. These findings reinforce the importance of lamin organisation and intranuclear network stability in chromatin maintenance, and highlight a new role for lamins in ALT cancers.
Acknowledgements

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Thanks go to my family for their understanding and support, and for still being there at the other end of the phone regardless of how long it’s been. A special thanks to my mum and my gran for their long-standing belief in me, and for getting some of that to rub off.

Finally, to Jake, who has provided unwavering encouragement. Thank you for your love and company in these last four years. Never surrender.
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## Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>ALT</td>
<td>Alternative lengthening of telomeres</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CSR</td>
<td>Class switch recombination</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSB</td>
<td>Double-strand break</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double-stranded DNA</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FC</td>
<td>Final concentration</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent in situ hybridisation</td>
</tr>
<tr>
<td>FTI</td>
<td>Farnesyl transferase inhibitor</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>HGPS</td>
<td>Hutchinson-Gilford progeria syndrome</td>
</tr>
<tr>
<td>HMW</td>
<td>High molecular weight</td>
</tr>
<tr>
<td>hTERT</td>
<td>Human telomerase reverse transcriptase</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IF</td>
<td>Intermediate filament</td>
</tr>
<tr>
<td>IMS</td>
<td>Industrial methylated spirit</td>
</tr>
<tr>
<td>INM</td>
<td>Inner nuclear membrane</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IQR</td>
<td>Inter-quartile range</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LA</td>
<td>Lamin A</td>
</tr>
<tr>
<td>Lamin A/C</td>
<td>Lamins A and C</td>
</tr>
<tr>
<td>LMW</td>
<td>Low molecular weight</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MBq</td>
<td>Mega Becquerel</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
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<td>Millilitre</td>
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</tr>
<tr>
<td>NPC</td>
<td>Nuclear pore complex</td>
</tr>
<tr>
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</tr>
<tr>
<td>ONM</td>
<td>Outer nuclear membrane</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Population doubling</td>
</tr>
<tr>
<td>pg</td>
<td>Picogram</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PNA</td>
<td>Peptide nucleic acid</td>
</tr>
<tr>
<td>PNS</td>
<td>Perinuclear space</td>
</tr>
<tr>
<td>Q-FISH</td>
<td>Quantitative-fluorescent in situ hybridisation</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>Quantitative-polymerase chain reaction</td>
</tr>
<tr>
<td>rcf</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>RD</td>
<td>Restrictive dermopathy</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline-sodium citrate</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>STELA</td>
<td>Single telomere length analysis</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
</tr>
<tr>
<td>Tel+</td>
<td>Telomerase positive</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>TERC</td>
<td>Telomerase RNA component</td>
</tr>
<tr>
<td>TRF</td>
<td>Terminal restriction fragment</td>
</tr>
<tr>
<td>WS</td>
<td>Werner syndrome</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μl</td>
<td>Microlitre</td>
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<tr>
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Chapter 1   Introduction

1.1 The nuclear lamina

1.1.1 Overview

In eukaryotic organisms, the nucleus serves to compartmentalise essential genetic processes away from the other activities of the cell. This segregation is made possible by the nuclear envelope, a structure comprising of a double membrane, nuclear pore complexes (NPCs) and the lamina. The outer nuclear membrane (ONM) is contiguous with the endoplasmic reticulum and is resident to ribosomes. NPCs span the nuclear membranes, providing a channel which allows the passage of RNA and proteins in and out of the nucleus. The inner nuclear membrane (INM) is home to a large variety of integrated proteins, the composition of which varies between tissue- and cell-type (Schirmer and Gerace, 2005; Schirmer et al., 2003). The nuclear lamina is a fibrous meshwork composed of several proteins, primarily the lamins from which it derives its name, as well as numerous INM proteins which interact with lamins and anchor the lamina to the INM and perform several other nuclear functions. Two types of lamins, A- and B-types, form the core of the lamina, which provides the nucleus with its structural stability and shape, while interactions with binding-partners allow the anchorage of chromatin to the lamina, and of the nucleus within the cell. At least one B-type lamin is present constitutively in metazoan cells, while A-type lamins are regulated developmentally, with particular importance in skeletal and cardiac development (Ho et al., 2013; Solovei et al., 2013; Swift et al., 2013).

Mutations in the lamin A gene may have dire consequences for the cell and the individual, as is observed in the numerous and diverse disorders that arise from
lamin A dysfunction, termed laminopathies. The structure of the lamina serves to protect the integrity of the nucleus and its contents, and contributes to the co-ordination of nuclear membrane break-down and re-assembly during cell division (Patel et al., 2014). The lamina is essential for the organisational distribution of chromatin throughout the nucleus, and chromatin attachment to the INM (Dechat et al., 2008). Lamins are also thought to form an intranuclear network, providing a scaffolding for the anchorage of replication, transcription and splicing factors (Broers and Ramaekers, 2004).

The discovery of mutations in LMNA leading to progeroid disorders has led to considerable research efforts into characterising the molecular basis of rapid ageing in these individuals. As patients are not exposed to a life-time’s worth of environmental factors, progeria is considered as an ideal model for the molecular processes behind the normal ageing process (Olive et al., 2010).

1.1.2 Properties of lamins

Nuclear lamins comprise the family of type V intermediate filament (IF) proteins present in all metazoans. Vertebrates have three lamin genes encoding seven distinct isoforms (Cohen et al., 2001), which may be classified into two major types of lamin, A- and B-type, according to sequence and biochemistry (Dechat et al., 2008). In humans, the B-type lamins are encoded at two separate loci, LMNB1 on chromosome 5q which encodes lamin B1, and LMNB2 located on chromosome 19p which encodes lamins B2 and B3. Lamins B1 and B2 are expressed throughout the cell lifespan (Stewart and Burke, 1987). A-type lamins are encoded by the LMNA gene located at chromosome 1q and are differentially expressed through alternative splicing (Figure 1.1:1a). Lamin A and lamin C are the most abundant and are expressed in nearly all differentiated cells (Dechat et al., 2008). Lamin C2, an additional splice variant, is germ-cell specific (Furukawa et al., 1994; Nakajima and Abe, 1995), as is lamin B3 (Furukawa and Hotta, 1993), and it is likely that both contribute to
spermatogenesis. A minor isoform, lamin AΔ10, is also produced by LMNA in small amounts in several somatic cell types (Machiels et al., 1996). Evolutionarily, it would seem that lamins hold the ancestral origin of IF proteins (Weber et al., 1989), and that lamin B originated first, as invertebrates, which express only a single lamin, express only B-type lamins (Erber et al., 1999).

Caenorhabditis elegans have one lamin gene, lmn-1, that resembles a B-type lamin (Riemer et al., 1993). Reduction of lmn-1 results in chromosomal abnormalities, nuclear deformities, and embryonic lethality (Liu et al., 2000).

**Figure 1.1.1** Schematic representations of A-type lamins a LMNA gene transcript including exon numbers and alternative splice sites used for the three major A-type lamin isoforms. Lamin AΔ10 is initiated within intron 1 b Lamin A translated protein indicating the relative positions of the head, rod and tail domains, the nuclear localisation signal (NLS), immunoglobulin-like fold (Ig-LF), and known regions of interaction with the proteins indicated. Adapted from Scharner et al. (2010).
While several invertebrates express only one lamin, *Drosophila melanogaster* express two forms of lamin: lamin Dm₀, a B-type lamin that is processed at the nuclear periphery into two forms, Dm₁ and Dm₂ (Gruenbaum et al., 1988), and the developmentally regulated A-type DmC (Bossie and Sanders, 1993; Riemer et al., 1995). Fungi and plants do not express lamins.

Despite significant sequence homology and functional overlap, A- and B-type lamins are distinguishable by several differing biological factors. Lamin A and C function is associated with developed cells (Stewart and Burke, 1987), while lamin B is required for development. Targetted mutations in functional regions of *Lmnb1* results in neonatal death and severe cellular abnormalities including dysmorphic nuclei, differentiation problems and accelerated senescence in mice (Vergnes et al., 2004). Unlike lamin A/C, B-type lamins are essential for viability in mammalian cells (Harborth et al., 2001). Lamin A/C expression correlates with the later stages of development (Constantinescu et al., 2006; Riemer et al., 1995; Rober et al., 1989), although this may not be the case in all organisms (Foster et al., 2007; Hall et al., 2005). Lamin B expression decreases during the lifespan of cultured cells (Shimi et al., 2011) and, upon the induction of lamin A/C expression, lamin B1 expression is reduced in chicken embryo development (Lehner et al., 1987). In mitosis, B-type lamins remain associated with the nuclear membrane while A-type lamins solubilise and disperse during mitosis prior to re-assembly (Dechat et al., 2007; Gerace and Blobel, 1980; Lee et al., 2000). Post-translational processing also differs between different lamins (see section 1.1.2.1).

All lamins consist of a small N-terminal globular head excluding lamin C₂ (Furukawa et al., 1994), a large C-terminal tail, and a central rod domain (Figure 1.1:1), through which lamins may homodimerise to form the basic lamin unit (Taniura et al., 1995). The rod domain consists of four α-helical coils (1A, 1B, 2A and 2B), joined by three linkers (L1, L12 and L2). Sequences at the rod ends
mediate head-to-tail polymer formation, which may then associate to form the characteristic 10nm filaments of IFs (Stuurman et al., 1998, 1996), although the globular head domain may also have a regulatory role in A-type lamin polymer formation (Izumi et al., 2000). IF proteins form a wide array of insoluble and complex higher-order structures which have been observed in vitro for several IF proteins, including keratin and vimentin filaments and aggregates (Coulombe et al., 1990; Hatzfeld and Weber, 1991). This organisation creates high tensile strength, allowing IF proteins to absorb mechanical stress, thus protecting cells from damage. IF proteins may also transduce mechanical pressure to signalling and gene regulation (Herrmann et al., 2009).

IF structures, including the lamina, are highly insoluble (Goldman et al., 1986), and investigation of higher-order lamin organisation has been made difficult by the propensity of lamins to form large paracrystal structures in vitro, which do not occur naturally in vivo. Although the meshwork of A- and B-type lamins form the core framework of the lamina, the inability in vitro to form higher-order structures suggest a dependence on the association of lamina-associated proteins for organisation (Gant et al., 1999). Although visualisation of higher-order lamin structures has not been possible in mammals, head-to-tail dimers have been observed in vitro assembling into long protofilaments in a staggered, anti-parallel manner, which may then associate laterally and form 10nm filaments in Caenorhabditis elegans (Ben-Harush et al., 2009) and an extremely organised lattice structure has been observed in Xenopus laevis oocytes (Stuurman et al., 1998).

The structure and stability of the lamina network conserves the integrity of the nucleus. The lamina is also crucial for sensing and responding to mechanical stress. The ability of the cell to sense mechanical strain is crucial to many biological processes. Mechanical stress initiates a response cascade, whereby physical forces induce conformation change in mechano-sensitive proteins of
the cytoskeleton, exposing or altering molecular recognition sites, thereby affecting biochemical signal transduction (Vogel and Sheetz, 2006). Mouse embryonic fibroblasts (MEFs) lacking lamin A (Lmna⁻/⁻) exhibit altered mechanotransduction, a loss of nuclear integrity and stiffness, and lowered viability in response to stress (Broers et al., 2004; Lammerding et al., 2004).

Recent evidence suggests that the natural variation in amounts of lamin A in the nuclear volume could be attributable to developmental regulation in relation to mechanical forces. The expression level of lamin A responds to mechanical stress during differentiation of tissue-types, and is proportionally increased in cells that are exposed to high levels of mechanical strain, for example bone and muscle tissue (Swift et al., 2013). High levels of lamin A/C result in a stiff nuclear envelope that is less responsive to conformational change, while lamin B does not contribute (Lammerding et al., 2006; Swift et al., 2013).

1.1.2.1 Lamin processing and localisation

Lamins are extensively decorated with post-translational modifications, including SUMOylation, acetylation and phosphorylation (Simon and Wilson, 2013), however, most research interest is focused at the C-terminus, where a series of processing events must be carried out in order to produce mature lamin A, as illustrated in Figure 1.1:2a. Translocation to the nucleus after translation is mediated via a C-terminal nuclear localisation signal (NLS) consensus sequence resembling that of the SV40 large T-antigen (Loewinger and McKeon, 1988; Wu et al., 2014). However, localisation is also highly dependent on a C-terminal CaaX box and the processing stages that modify the C-terminus. With the exception of lamin C, all lamins are translated as precursor molecules, terminating with CaaX (one cysteine, followed by two aliphatic residues and any amino acid; CSIM in lamin A and CAIM in B-type lamins (Holtz et al., 1989)). This sequence serves to mark several proteins for farnesylation via farnesyltransferase (FTase) activity (Beck et al., 1990; Casey and Seabra, 1996;
Farnsworth et al., 1989). After farnesylation, proteolytic cleavage between the cysteine and first aliphatic residue is carried out by RCE or ZMPSTE24 (Maske et al., 2003; Young et al., 2005) and the exposed cysteine residue is carboxymethylated by isoprenylcysteine carboxyl methyltransferase (ICM) (Clarke et al., 1988; Dai et al., 1998).

This form of lamin A, termed prelamin A, is then processed further, in an apparently unique additional cleavage at the C-terminus (Figure 1.1:2a). The second cleavage step, carried out by ZMPSTE24, releases the terminal fifteen amino acids along with the farnesyl and methyl modifications (Corrigan et al., 2005). As lamin C does not contain the CaaX motif, and is therefore never farnesylated, this final maturation step of lamin A represents the major difference between A- and B-type lamins, as B-type lamins remain farnesylated. Lamin A processing is also unique among farnesylated proteins in that processing steps may occur in the nucleus, rather than at the ER (Clarke et al.,

![Figure 1.1:2](image_url)

**Figure 1.1:2** Lamin A post-translational processing of the C-terminal tail domain to produce mature lamin a from a WT lamin A nascent protein, or b mutant nascent lamin A resulting from aberrant activation of a cryptic splice site in exon 11. The ZMPSTE24 recognition site is absent in the mutant protein.
The CaaX box may also play a role in the formation of higher-order lamin structures (Izumi et al., 2000).

The role of this cleavage-modification-cleavage is thought to aid localisation of lamin A to the INM. It would seem that farnesylation is required for localisation of prelamin A to the nuclear periphery, perhaps due to the hydrophobic nature of the farnesyl and carboxymethyl groups (Hennekes and Nigg, 1994; Lutz et al., 1992). Prelamin A may then be cleaved \textit{in situ} (Lutz et al., 1992; Sasseville and Raymond, 1995; Sinensky et al., 1994). Indeed, peripheral localisation of human lamin C, which lacks its own temporal farnesylation signal, is dependent on the re-location of lamin A (Vaughan et al., 2001). However, farnesylation is not required in the mouse system. Mouse lamin A is able to locate to the periphery in the absence of a farnesyl tag (Davies et al., 2010), and lamin C is also able to integrate into the lamina without lamin A (Fong et al. 2006).

Not all lamin locates to the periphery, and this may be crucial for cell biology. A sub-fraction of A-type lamins that remains in the nucleoplasm is purported to form an intranuclear skeleton-like structure, which may aid recruitment of transcription factories and DNA replication factors, tethering them to specific nuclear regions (Dechat et al., 2008, 2007; Shumaker, 2003). The organisation of intranuclear lamins may be mediated by LAP2α (Gant et al., 1999), and this organisation results in a more motile and soluble structure than the lamin A found at the periphery (Dahl et al., 2006). A fraction of B-type lamin is also retained in the intranuclear region, and may too aid DNA replication (Moir et al., 1994).
1.1.3 Additional lamina components and lamin-interacting partners

Numerous non-lamin proteins contribute to the functions of the lamina and, in the cases where function has been elucidated, these interactions are known to be important for organisation and positioning of the lamina, as well as chromatin interactions with the lamina. Most interactions have been confirmed through *in vitro* assays. Interactions are described below and summarised in Figure 1.1:3.

![Figure 1.1:3 Schematic of the nuclear membranes, including some of the known associated factors (not to scale). Represented is the peripheral lamina, composed of a meshwork of A- and B-type lamins and their associated factors as indicated in the legend. ER=endoplasmic reticulum, PNS=perinuclear space, NPC=nuclear pore complex, INM=inner nuclear membrane, ONM=outer nuclear membrane.](image)

**Figure 1.1:3** Schematic of the nuclear membranes, including some of the known associated factors (not to scale). Represented is the peripheral lamina, composed of a meshwork of A- and B-type lamins and their associated factors as indicated in the legend. ER=endoplasmic reticulum, PNS=perinuclear space, NPC=nuclear pore complex, INM=inner nuclear membrane, ONM=outer nuclear membrane.

1.1.3.1 The LINC complex

The nucleus is held in position in the cytoplasm via a complex of proteins known as the linker of nucleoskeleton and cytoskeleton (LINC). The LINC complex spans the INM and ONM, providing a physical bridge between the nucleoplasm and the cytoskeleton. This complex interacts with and connects elements of the lamina and the cytoskeleton, allowing mechanotransduction
signalling between the two compartments, and absorbing mechanical strain (Brosig et al., 2010; Chambliss et al., 2013; Lombardi et al., 2011). The complex consists of a lamina-interacting, INM-associated protein, and an ONM-associated factor capable of tethering to the cytoskeleton and binding within the perinuclear space (PNS). In humans, the Sad1 and UNC84 domain-containing (SUN) proteins SUN1 and SUN2 (encoded by the SUN1 and SUN2 genes respectively) form the basal INM unit for the LINC complex. Most SUN proteins consist of a large N-terminal nucleoplasmic region, a central transmembrane domain and a coiled-coil domain, and a C-terminal SUN domain which spans the PNS. The luminal C-terminus SUN domain is highly conserved evolutionarily, from plants to yeast to mammals (Méjat and Misteli, 2010). Homotrimerisation of SUN proteins is mediated by the SUN domain (Zhou et al., 2012) and both the SUN and coiled-coil domains recruit and bind the ONM LINC element by means of a Klarsicht/ANC-1/Syne-1 homology (KASH) domain (Sosa et al., 2012). In mammals, the KASH-domain containing protein family is composed of nesprins (nuclear envelope with spectrin repeats). The nesprin family is large and extremely diverse, including isoforms ranging wildly in size (from ~45kDa to ~1MDa), encoded by four genes (SYNE1-4). Although most nesprins translocate to the ONM to form the LINC complex with SUN proteins, specificity to the ONM or INM is dependent on the size of the nesprin and its interaction partners. Small nesprins, including nesprin-1α, may be transported through NPCs to the INM and interact directly with lamin A/C (Mislow et al., 2002). Nesprin-1α targets muscle A-kinase anchoring protein (mAKAP) to the nucleus of muscle cells (Pare et al., 2005), which may have importance in disease development in the disorder Emery-Dreifuss muscular dystrophy (EDMD, see section 1.1.4.1). Nesprin-1 and -2 mainly bind actin, while nesprin-4 associates with microtubules and nesprin-3 is able to bind IFs and nuclear microtubules indirectly through its own interacting partners (Ketema and Sonnenberg, 2011).
1.1.3.2 LEM domain-containing proteins and BAF

The LEM domain is so-named due to its initial discovery in three proteins: LAP2, Emerin and MAN1, all of which are able to bind lamins. Located towards the N-terminus, the LEM domain consists of a motif that has evolved for specific binding of barrier to autointegration factor (BAF) (Brachner and Foisner, 2011). BAF is able to bind double stranded DNA in a non-specific manner (Zheng et al., 2000), and is a key factor in mediating chromatin organisation through coordination with the lamina.

Six LAP2 isoforms are encoded by alternative splicing of the LAP2 locus, and most bind the lamina through lamin B1 (Foisner and Gerace, 1993). As well as a LEM-domain, LAP2s contain a second, LEM-like, direct DNA binding motif (Cai et al., 2001). LAP2α is the exception of the LAP2 group, as it does not contain a transmembrane domain and so does not associate at the nuclear periphery, but with intranuclear A-type lamins (Dechat et al., 2000). This interaction is required for formation of the intranuclear lamin structure, and may coordinate chromatin organisation within the nucleus (Dorner et al., 2007; Gant et al., 1999). As well as interacting directly with A-type lamins, LAP2α also interacts with retinoblastoma protein Rb, providing anchorage within the nucleoplasm that is essential for Rb function, suggesting a role for lamins in cancer (Markiewicz et al., 2002), as well as a potential route for delayed cell division observed in patients with lamin A mutations (Dechat et al., 2007).

Emerin and MAN1 (encoded by genes EMD and MAN1, respectively) share much homology and there is a large redundancy between the two proteins in terms of their function in chromosome segregation at mitosis in C. elegans, (Liu et al., 2003). Emerin and MAN1 are able to bind one another, as well as BAF (Mansharamani and Wilson, 2005; Shimi et al., 2004). Emerin is also able to bind lamin A (Lee et al., 2001), which is required for emerin localisation to the nucleus (Vaughan et al., 2001). Emerin also stabilises the LINC complex through
interactions with nesprin-1α and -2β (Mislow et al., 2002; Wheeler et al., 2007) and SUN1/2 (Haque et al., 2010). Mutations in the EMD gene lead to the X-linked form of EDMD (Bione et al., 1994).

BAF is a small protein (only 10kDa) that forms dimers that are able to multimerise in vitro upon binding DNA (Zheng et al., 2000). As well as binding DNA, BAF may be able to influence heterochromatin density (Segura-Totten et al., 2002). BAF is able to bind LAPs, emerin and nesprins through the LEM domain of each and this interaction is essential for proper nuclear re-assembly of the lamina after mitosis, and for cell viability (Lee et al., 2001; Mansharamani and Wilson, 2005; Shumaker et al., 2001).

1.1.3.3 Other binding partners
Large numbers of novel nuclear envelope transmembrane (NETs) and linker proteins have been identified (Schirmer and Gerace, 2005), of which, most of the best characterised are described above. However several other factors may be pertinent to lamin biology. An actin-binding site has been identified and confirmed in lamins, and lamin A contains an additional site that is able to mediate F-actin bundling (Simon et al., 2010). NPC components NUP153 and NUP88 have been shown to associate directly with lamin A through purified pull-down methods (Al-Haboubi et al., 2011; Lussi et al., 2011), confirming an early report of NPC association with the lamina (Aaronson and Blobel, 1975).

Overexpression of several NETs results in redistribution of chromatin in the nucleus, as well as relocalisation of specific chromosomes (Zuleger et al., 2013). This induces tissue-specific expression patterns, in accordance with findings that the nuclear envelope proteome differs greatly between cell-types (Korfali et al., 2012). Lamin A/C has been shown to bind the transcription factors MAPK/MAK/ MRK overlapping kinase (MOK2) (Dreuillet et al., 2002; Harper et al., 2009) and sterol regulatory element binding protein 1 (SREBP1) (Lloyd et al.,
Mutations in lamin A resulting in familial partial lipodystrophy (FPLD, see section 1.1.4.1) affect the affinity of lamin A to SREBP1, potentially affecting adipocyte differentiation which SREBP1 is required for (Lloyd et al., 2002). Reduction of lamin A or emerin compromises the differentiation potential of muscle cells, associated with reduced levels of retinoblastoma protein (pRb), Myosin D, desmin, M-cadherin and other factors involved in muscle differentiation (Frock et al., 2006). In this study, transcript levels of myosin D and desmin were found to be lowered in Lmna\(^{-/-}\) myoblasts (Frock et al., 2006), suggesting that lamin A could be mediating transcriptional activation, while lamin A/C regulates pRb levels through inhibition of its proteasomal degradation (Johnson et al., 2004). Although no direct binding has been identified between lamins and the transcription factor megakaryoblastic leukaemia 1 (MKL1), a mechanosensitive transcription factor important in cardiac development, it’s normal nuclear distribution is dependent on the presence of lamin A/C (Ho et al., 2013), reinforcing the importance of A-type lamins in cardiac development.

Intranuclear A- and B-type lamins are able to mediate organisation of discreet compartments containing RNA polymerase II and its associated splicing factors in human and mammalian cells (Jagatheesan et al., 1999; Kumaran et al., 2002; Spann et al., 2002). This association between lamins and splicing factors may be cell-type dependent and required only during differentiation (Muralikrishna et al., 2001). Lamins are also involved in DNA replication, and disruption of the intranuclear lamin network affects the distribution of replication factors and DNA synthesis efficiency (Spann et al., 1997).

The Lamin B receptor (LBR), aside from binding B-type lamins (Ye and Worman, 1994), is able to directly bind chromatin, anchoring it to the lamina (Pyrpasopoulou et al., 1996), as well as bind heterochromatin proteins HP1-\(\alpha\) and HP1-\(\gamma\) (Ye and Worman, 1996; Ye et al., 1997). Interestingly, lamin A is able
to bind the replication factor proliferating cell nuclear antigen PCNA, which may have implications in DNA damage and cell cycle progression, as PCNA can be relocalized in the presence of mislocalized, N-terminally truncated lamin A (Spann et al., 1997). Lamins may interact with DNA in a more direct manner through histones (Taniura et al., 1995), or even direct binding to duplex DNA (Stierlé et al., 2003).

1.1.4 Lamin A dysfunction and laminopathies

The identification of mutations in LMNA has revealed a host of functions that are reliant upon normal lamin A function. Such roles include chromatin organisation and anchorage (Dechat et al., 2008), transcription (Shimi et al., 2008), RNA splicing (Kumaran et al., 2002), replication (Spann et al., 1997), cellular and nuclear stability (Lombardi et al., 2011) and cell cycle progression (Dechat et al., 2007). These diverse roles are mirrored by a wide variety of disorders associated with mutations throughout the various domains of lamin A, with symptoms that include muscular dystrophy and myopathy, cardiomyopathy, lipodystrophy and progeria. Diseases caused by mutations in genes encoding lamins and lamina-associated proteins are collectively termed laminopathies. Mutations in lamin B are rarely reported, most likely due to the requirement of a base-level of functionality for viability (Harborth et al., 2001). Therefore the most commonly recorded lamin B mutations involve duplications (Padiath and Fu, 2010), however mutations in LMNB2 have been implicated as modifiers in acquired partial lipodystrophy (Hegele et al., 2006).

1.1.4.1 Non-progeroid laminopathies

Mutations occurring throughout lamin A result in several non-progeroid disorders that tend to manifest as muscular dystrophies, but include dilated cardiomyopathy (DCM), familial partial lipodystrophy (FPLD) and the neuropathy Charcot-Marie-Tooth disorder (CMT).
Muscle wasting disorders include Emery-Dreifuss muscular dystrophy (EDMD), limb girdle muscular dystrophy subtype B1 (LGMDB1) and congenital muscular dystrophy (CMD). EDMD is mainly caused by mutations in LMNA and EMD. Symptoms include tendon contracture, muscular dystrophy and heart defects (Emery, 1989). Mutations in EMD, which encodes emerin, cause the X-linked form of the disease, and single mutations may result in broad phenotypic variation (Hoeltzenbein et al., 1999). Rarer cases of EDMD result from mutations in genes encoding nesprin-1 and -2 (Zhang et al., 2007) and putatively SUN1 and -2 (Meinke et al., 2014). LMNA mutations may be dominant or recessive and two LMNA EDMD mutations result in the condition CMD, as well as different amino acid changes at codon 249 (Figure 1.1:4).

CMD is caused by LMNA mutations affecting the rod domain and C-terminal head. Muscle wasting may be accompanied by delayed development and joint contractures, although there is a broad range of phenotypic variation (Quijano-Roy et al., 2008). Symptoms include ventricular dilation and hypertrophy, potentially leading to heart failure. Lamin A is mutated in >10% of DCM individuals, and mutation may be inherited or sporadic. Mutations are located along the length of lamin A (Figure 1.1:4). A genotype-phenotype analysis revealed that individuals with lamin A mutations are prone to an earlier age of onset and more severe symptoms (Taylor et al., 2003). Limb girdle muscular dystrophy is also caused by mutations in several genes, however those identified in LMNA cluster in the C-terminal tail domain of lamin A (Figure 1.1:4), and may also result in EDMD (Muchir et al., 2000).

Familial partial lipodystrophy (FPLD), results from missense mutations in regions (Figure 1.1:4). FPLD patients exhibit a normal appearance until after puberty, when subcutaneous fat is then lost from the limbs and trunk, while simultaneously accumulating in the face and neck. Other symptoms include prominent musculature, altered skin pigmentation and diabetes (Köbberling
and Dunnigan, 1986). Expression of residue R482 FPLD mutants holds no effect on myocyte differentiation (Favreau et al., 2004), while adipocyte differentiation is affected (Boguslavsky et al., 2006).

Charcot-Marie-Tooth disease is a genetically and symptomatically heterogeneous collection of common neuropathic disorders with many subtypes, a small proportion of which stem from a single homozygous mutation at residue R298 in lamin A (De Sandre-Giovannoli et al., 2002), resulting in subtype 2. Common symptoms include muscle weakness and wasting.

Generally, muscular dystrophy is associated with mutations which impact the function and stability of the LINC complex, as highlighted by the gene mutations in LINC components that cause EDMD. Mutations in lamin A that result in muscular dystrophy and cardiomyopathy also result in impaired nuclear response to mechanical stress (Zwerger et al., 2013). Emerin localisation is affected by the presence of muscular dystrophy-associated LMNA mutants.

**Figure 1.1.4** Lamin A amino acid changes resulting in laminopathies. DCM=Dilated cardiomyopathy, EDMD=Emery-Dreifuss muscular dystrophy, CMD=congenital muscular dystrophy, FPLD=familial partial lipodystrophy, CMT2=Charcot-Marie-Tooth disorder type 2, LGMDB1=limb girdle muscular dystrophy type B1, NT=N-terminus, CT=C-Terminus. V440M and R584H from Hegele et al. (2000), R482W/Q/L from Shackleton et al. (2000). Adapted from Scharner et al. (2010).
(Manju et al., 2006), and mislocalisation of emerin is associated with impaired function of MKL1 (Ho et al., 2013). Several of the mutation locations in \textit{LMNA} that associate with FPLD overlap the locations of atypical Hutchinson-Gilford Progeria Syndrome (HGPS) and atypical Werner syndrome (WS). Lipodystrophy is a common symptom of HGPS, indicating that these regions of lamin A as specific to adipocyte function. This is most likely through disruption of the interaction with SREBP1 which binds between lamin A residues 227 and 487, where the majority of mutations in FPLD cases reside (Lloyd et al., 2002). However, over-expression of WT lamin A affects adipocyte differentiation and inhibits lipid synthesis and accumulation, again suggesting that aberrant accumulation of even WT lamin A may be sufficient for disease (Boguslavsky et al., 2006), and almost all laminopathies present with abnormal distribution of adipose tissue.

1.1.5 Hutchinson-Gilford progeria syndrome

HGPS is an extremely rare condition affecting around one child in every eight million (Pollex and Hegele, 2004). The vast majority of children with HGPS are said to have classical HGPS and carry a single base \textit{de novo} mutation (1824C>T) in \textit{LMNA} (Eriksson et al., 2003; Hennekam, 2006). Children suffering from this classical form of the disease are born with a completely normal appearance but within 1 to 2 years develop notable features including alopecia, loss of subcutaneous fat and very slow growth. Further symptoms include hypo- and hyper-pigmentation of the skin, sclerodermatous skin, arthritis and osteoporosis. General frailty and short stature as well as distinctive, small facial features, prominent veins due to subcutaneous fat loss and aged-looking skin produce an overall appearance of old age. Due to the overlap with symptoms of normal ageing, HGPS is categorised as a segmental premature ageing syndrome. The average age of death is approximately 13 years and is almost
invariably due to cardiac failure due to atherosclerosis or stroke (Al-Shali and Hegele, 2004).

1.1.5.1 Lamin A processing and progerin expression in classical HGPS

In classical HGPS, the 1824C>T mutation activates a cryptic splice site in exon 11, consequently excluding the remaining 150bp of the exon (Eriksson et al., 2003). The resultant 50 amino acid deletion includes the final ZMPSTE24 cleavage site required for release of the modified C-terminus (Figure 1.1:2b). Activation of this site is "leaky", in that some of the transcripts produced by the mutant allele are WT. The WT allele produces WT transcripts at a normal rate, leading to an overall ratio of 2:3 mutant to WT transcripts (Reddel and Weiss, 2004).

The omission of the ZMPSTE24 processing step resulting from the loss of its recognition site produces a form of lamin A that lacks 50 amino acids but retains farnesylation (Figure 1.1:2). This protein is termed progerin or LAΔ50 (Eriksson et al., 2003; Hennekam, 2006). Lamin C transcription is terminated in exon 10 and as such is unaffected by the mutation (Figure 1.1:1). Cells that express progerin exhibit several nuclear deformities, including blebbing of the nuclear membrane, altered mechanosensitivity and dissociation from the nuclear periphery and change in composition of heterochromatin (Goldman et al., 2004).

The nuclei of HGPS cells display striking morphology, exhibiting lobulations, blebs, folds and wrinkles (Figure 1.1:5) at high levels in cell populations, particularly at later passages ex vivo (Scaffidi and Misteli, 2005). HGPS cells with abnormal morphology within a sample population display higher levels of DNA damage than wild-type or HGPS cells with a normal nuclear appearance, indicating that alterations to the physical organisation of the lamina may have a role in disease (Constantinescu et al., 2010). Interestingly, progerin is expressed
at low levels in the cells of healthy individuals and may accumulate with age, which is caused by sporadic activation of the cryptic splice site mutated in HGPS (Scaffidi and Misteli, 2006). The presence of progerin in the cells of normally aged individuals is associated with an increase in nuclear dysmorphia, as well as other cellular symptoms common to HGPS (Scaffidi and Misteli, 2006), including shortened telomeres and increased senescence (Cao et al., 2011). Evidence also suggests that with increasing numbers of cellular divisions, progerin transcripts are upregulated (Rodriguez et al., 2009). This indicates that progerin progressively accumulates in the nucleus, even in normally aged individuals, causing nuclear deformities, and that this process is accelerated in HGPS cells. Lamin B organisation is also altered in HGPS cells (Goldman et al., 2004; Taimen et al., 2009), and global levels are depleted (Scaffidi and Misteli, 2005) indicating that lamin B and its interactors could also contribute to the HGPS phenotype.

![Figure 1.1:5](image)

**Figure 1.1:5** Examples of nuclei in a normal human foreskin fibroblast (control), classical HGPS, late onset HGPS (T623S) and restrictive dermopathy (RD) fibroblasts immunostained for lamin A/C. Images provided by the Shackleton laboratory and reproduced with permission.

### 1.1.5.2 Impact of progerin on nuclear lamin organisation

The presence of progerin results in an over-accumulation of A-type lamins at the nuclear periphery. The lamina thickens in HGPS cells, and this effect worsens with the age of cells in culture (Goldman et al., 2004). Progerin as well as WT lamin is integrated into the lamina matrix more readily in HGPS cells, (Scaffidi and Misteli, 2005). It is possible that accumulation of progerin at the lamina is
due to aberrant localisation signalling from the farnesyl group, which instigates morphological changes at the nuclear membrane (Capell et al., 2005). However, though retention of the farnesyl group is sufficient to cause abnormal nuclear morphology, several non-classical mutants which do not aberrantly retain C-terminal farnesylation are also able to trigger nuclear dysmorphia (Huang et al., 2008; Taimen et al., 2009).

The proclivity of progerin for integration into the lamina holds other implications for the cell, as it results in reduced levels of progerin and lamin A levels in the nucleoplasm (Scaffidi and Misteli, 2005). Intranuclear lamin A may aggregate and coordinate the association of DNA damage response (DDR) proteins and DNA in subnuclear compartments (Dechat et al., 2008; Shumaker, 2003). This depletion of lamin A from the nuclear space in HGPS cells (visible in Figure 1.1:5) could therefore alter expression levels if transcription factor recruitment is impaired. Alternatively, disrupted interactions at the periphery or within the nuclear space that affect chromatin-associated factors may be responsible for the alterations seen in heterochromatin observed in HGPS (see below).

The stability of the lamina network is drastically altered in HGPS patients. Fluorescence Recovery after Photobleaching (FRAP) assays have demonstrated a progressive and severe decrease in A-type lamin mobility in HGPS nuclei with cellular age (Dahl et al., 2006; Scaffidi and Misteli, 2005). This has consequences for the mechanistic response of HGPS nuclei to external pressures. The HGPS lamina loses elasticity in a progressive manner and has a reduced ability to respond to mechanical stress (Verstraeten et al., 2008), as does the lamina of cells ectopically expressing progerin (Dahl et al., 2006). This mechanistic effect could itself be responsible for dissociation of chromatin and chromatin associated factors from the lamina through steric hindrance (Scaffidi and Misteli, 2005). It has also been suggested that increased cellular sensitivity to
mechanical strain contributes to loss of vascular smooth muscle cells, and therefore early death in HGPS patients caused by atherosclerosis (Verstraeten et al., 2008).

1.1.5.3 Impact of progerin on chromatin

The locality of chromatin in the nucleus is important for, and indicative of expression levels (Croft et al., 1999). Heterochromatin is thought to interact with lamin A either directly or via LEM or other lamina-associated proteins (see sections 1.1.3.2 and 1.1.3.3) and all lamins bind DNA in vitro (Shoeman and Traub, 1990). Lamin A- and lamin B-associated chromatin is generally heterochromatic and transcriptionally silent (Guelen et al., 2008; Kubben et al., 2012). Dissociation of heterochromatin from the nuclear periphery is a key marker of HGPS cells (Dechat et al., 2008; Goldman et al., 2004), and a generalised increase in global decondensation has been observed (Csoka, 2004; Shumaker et al., 2006). Trimethylation of histone 3, lysine residue 9 (H3K9me3), a marker of constitutive chromatin, is reduced in HGPS cells and subsequently chromatin interaction with HP1-α, a binding partner of LBR (Ye et al., 1997) which binds through H3K9me3, is also reduced (Shumaker et al., 2006). In contrast, trimethylation of histone 4, lysine 20 (H4K20me3), a facultative chromatin marker, is upregulated in HGPS and progerin-expressing HeLa cells (Shumaker et al., 2006). Progerin associates with almost 100 additional genes when compared with WT lamin A, while losing interaction with only 8 upon expression in MEFs (Kubben et al., 2012). As a consequence, the global expression profile of HGPS cells is altered, with the greatest effects on transcription factor and extracellular matrix protein expression (Csoka et al., 2004). Heterochromatin alterations are observed before abnormal nuclear morphology is observed (Shumaker et al., 2006), implying that lobulation of the nuclear membrane cannot be directly linked to chromatin alterations. DNA methylation is also affected in HGPS, altering expression of several genes.
including the inflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells NF-κB (Heyn et al., 2013).

A constitutively active DDR (Csoka, 2004), high incidence of double strand breaks (DSBs) and large numbers of persistent DNA damage foci in HGPS and other laminopathy and laminopathy-model cells are widely reported (Gonzalez-Suarez et al., 2009; Li and Zou, 2005; Liu et al., 2008, 2006). Liu et al. demonstrated a delayed response of p53 binding protein 1 53BP1, a key mediator of DDR factors, to recruit to γ-H2AX foci (a marker for DSBs) in Zmpste24−/− MEFs and HGPS cells following γ-irradiation, as well as activated checkpoint protein CHK1 (Liu et al., 2005). This and other studies have recorded the involvement of global DNA repair factors ataxia-telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR), in the DDR of HGPS cells (see section 1.3.1), working through CHK1 and CHK2 to halt cells at the G1/S phase and G2/M checkpoint (d’Adda di Fagagna et al., 2003; Liu et al., 2006), presumably causing the slow cell cycle progression of HGPS cells. In 2008, Liu et al. suggested this could explain their finding that XPA (normally exclusively a factor in nucleotide excision repair and unrelated to DSB repair) is bound aberrantly to DSB ends (Liu et al., 2008). This is a potential mechanism for DSB accumulation as XPA could be blocking binding of required factors and contributing to the formation of permanent lesions. Lamins may also regulate cycle through control of retinoblastoma protein (pRB) nuclear localisation and proteasomal degradation (Johnson et al., 2004).

### 1.1.5.4 Franesyl transferase treatment

Farnesyl transferase inhibitors (FTIs) block the action of farnesyl transferases and have been employed experimentally to lower farnesylated lamin A levels. FTIs have proven to dramatically reduce lamin A accumulation at the nuclear periphery and alleviate nuclear dysmorphia in HGPS cells ex vivo (Capell et al., 2005; Constantinescu et al., 2010; Toth et al., 2005), in cells ectopically
expressing lamin A mutants (Capell et al., 2005; Mallampalli et al., 2005) and in
mouse models (Loren G Fong et al., 2006; Yang et al., 2008, 2006, 2005). Several
other symptoms of HGPS nuclear dysfunction are also improved with FTIs,
including a return of chromatin localisation and the global gene expression
profile (Columbaro et al., 2005; Scaffidi and Misteli, 2005) and DSB repair
capacity (Constantinescu et al., 2010). Mice expressing a progerin allele
(LmnaHG/+ ) exhibit similar symptoms to humans with HGPS, including a normal
appearance at birth followed by growth deficiency, loss of subcutaneous fat,
bone weakening and reduced survival (Yang et al., 2006). Administering FTIs to
these mice and genetically identical MEFs not only improves nuclear
dysmorphia (Yang et al., 2005), but also improves the developmental
deficiencies described above (Yang et al., 2006), as well as improving survival
(Yang et al., 2008).

However, certain symptoms of HGPS are not addressed by FTIs, such as DNA
damage that has already accumulated (Constantinescu et al., 2010; Liu et al.,
2006), or mechanosensitivity, despite a correction of increased nuclear stiffness
(Houben et al., 2007). As increased senescence and/or apoptosis due to
mechanosensitivity in arterial cells could be the root of atherosclerosis in HGPS
patients (Verstraeten et al., 2008), the major cause of death in HGPS, FTIs may
not protect against early fatality.

1.1.6 Atypical HGPS and other progeroid laminopathies

Atypical HGPS conditions are so termed as they derive from mutations other
than the typical G608G, and have a tendency to deviate from the classical
phenotype, frequently displaying symptoms from other laminopathy syndromes
(Smallwood and Shackleton, 2010). ~80% of HGPS cases are a result of the
classical G608G mutation, while the remainder are caused by other, less well
classified mutations (Hennekam, 2006). Atypical mutations span the length of
LMNA and range from apparently simple missense mutations to alternative
mechanisms of cryptic splice site activation (Figure 1.1:6). These atypical mutations may result in a range of phenotypes present in classical HGPS, but generally to a less severe extent, and in some cases symptoms resemble or overlap with other laminopathy syndromes. A subset of individuals share many symptoms of Mandibuloacral Dysplasia (MAD), which in many ways resembles a milder form of HGPS (Hennekam, 2006).

MAD is a very rare disorder caused by mutations in various locations in the LMNA gene (Figure 1.1:6), and results in mandibular and clavicular hypoplasia, loss of subcutaneous fat, dappled skin pigmentation and progeroid features (Simha et al., 2003). In a similar manner to atypical HGPS, manifestation of symptoms varies between individuals (Ahmad et al., 2010; Bai et al., 2014; Garg et al., 2018).

**Figure 1.1:6** Lamin A amino acid changes resulting in progeroid disorders. Mutations are limited to the first half of the rod domain and the C-terminal head domain. V169fs= frameshift from codon 169 to stop codon (Scharner et al., 2010), G567del= intronic donor splice site deletion IVS11+1G>A, resulting in deletion of exon 11 (Doh et al., 2009). G608G, although a silent mutation, sporadically initiates splicing within exon 11, as does the T623S mutation. One instance of G608G resulting in RD phenotype has been recorded (Navarro et al., 2004). Adapted from Kudlow et al. 2007. HGPS mutants V607V and G567del from Moulson et al. 2007. T528M and M540T do not cause disease except when inherited together (Navarro et al., 2004). HGPS=Hutchinson-Gilford Progeria Syndrome, A-WS=atypical Werner Syndrome, MAD=Mandibuloacral dysplasia, RD=restrictive dermopathy, NT=N-terminus, CT=C-Terminus.
et al., 2005; Novelli et al., 2002; Simha et al., 2003). At the severe end of the spectrum is another rare disorder, Restrictive Dermopathy (RD), which exhibits several symptoms of HGPS and MAD, alongside several other severe phenotypes.

RD is usually caused by homozygous or compound heterozygous ZMPSTE24 mutations that eliminate ZMPSTE24 function (Moulson et al., 2005; Navarro et al., 2013, 2005). Due to the role of ZMPSTE24 in lamin A processing (see section 1.1.2.1), loss of function results in an absence of native lamin A expression, which is replaced by prelamin A. Prelamin A is a partially processed form of lamin A which retains the farnesyl modification. This results in devastating consequences for the individual, evident by intrauterine growth retardation and neonatal death. Symptoms include tight, sclerotic and easily broken skin, particularly evident at joints, which are stiff and retracted. Shared symptoms

**Figure 1.1:7** Reverse transcriptase PCR results from T623S, HGPS and control cells (U2OS) for WT and mutant transcripts. Data was generated in the Shackleton lab and is reproduced with permission. au=arbitrary units.

<table>
<thead>
<tr>
<th>Position</th>
<th>1863–1869</th>
<th>Position</th>
<th>1818–1824</th>
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<tbody>
<tr>
<td>Consensus</td>
<td>GGT__AGT</td>
<td>Consensus</td>
<td>GGT__AGT</td>
</tr>
<tr>
<td>LMNA WT</td>
<td>GGT__ACT</td>
<td>LMNA WT</td>
<td>GGT_GGGC</td>
</tr>
<tr>
<td>T623S</td>
<td>GGT_CAGT</td>
<td>G608G</td>
<td>GGT_GGGT</td>
</tr>
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Table 1.1-1 Position and sequence of cryptic splice sites mutated in T623S and G608G LMNA, compared to the consensus major-class splice activation sequence. Consensus bases are given in bold. Mutant transcript sequences are given for bases 1863–1869 in T623S and bases 1818–1824 in HGPS (G608G).
with MAD and HGPS include prominent blood vessels, small facial bones, clavicular hypoplasia and hair loss (Navarro et al., 2004). At the cellular level, RD nuclei resemble those of HGPS patients, exhibiting blebbing and altered chromatin organisation (Columbaro et al., 2010). ZMPSTE24 mutations may also cause HGPS and MAD-like phenotypes (Agarwal et al., 2006, 2003; Denecke et al., 2006; Shackleton et al., 2005), the milder symptoms of which are due to, and whose severity negatively correlate with, residual ZMPSTE24 function (Barrowman et al., 2012).

Two cases of RD resulting from mutations in LMNA have been reported. One individual expressed a lamin A form lacking the 90 amino acids (termed lamin A Δ90) encoded by exon 11 due to an intronic splice site mutation (Figure 1.1:6). The other was genotyped as having the classical G608G mutation, suggesting that genetic modifiers may affect the disease outcome (Navarro et al., 2004). Although their conditions were extremely severe and resulted in very early death, their symptoms were milder than classical RD, perhaps due to residual expression of WT lamin A (Navarro et al., 2004).

An additional mutation, T623S, results from a single base change 1868C>G resulting in deletion of exon 11 codons. This is a result of activation of a cryptic splice site, different to that affected by the classical 1824C>T mutation, but results in a similar transcript that lacks the ZMPSTE24 binding site, as well as 35 amino acids, and as such is termed lamin A Δ35 (Figure 1.1:6 and Table 1.1-1). The T623S phenotype exhibits the symptoms of classical HGPS but at a slower rate, and with a later age of onset (Fukuchi et al., 2004; Shalev et al., 2007). As progressive loss of exon 11 in mutant transcripts correlates with an increase in severity, this could demonstrate a functional importance of this region of lamin A. However, it has been shown that over-expression of even wild-type lamin A in human fibroblasts induces HGPS-like characteristics (Huang et al., 2008), and that farnesylated lamin A accumulates at the nuclear periphery over time in
HGPS fibroblasts, correlating with an increased nuclear membrane aberrations and chromatin localization disruption (Goldman et al. 2004; McClintock et al. 2006).

The classical HGPS 1824C>T mutation adheres more strongly to the consensus than the T623S 1868C>G mutation (Patel & Steitz 2003 and Table 3.1-1). As such, the 1868 (T623S) mutation confers a splice site that is activated less frequently than the classical 1824 mutation. Reverse transcriptase-PCR carried out in the Shackleton laboratory demonstrates a 2.5-fold increase in progerin transcripts compared to Δ35. This is in agreement with Western blot results which demonstrate a higher proportion of progerin relative to WT lamin A when compared to Δ35 and WT lamin A (Figure 3.1:2, p86). Also, two additional HGPS mutations which cause elevated activation of the splice site used in classical HGPS, V607V (1821G>A) and an intronic mutation (IVS11+1G>A), result in higher levels of progerin expression and more severe phenotypes (Moulson et al., 2007). It is therefore extremely likely that the delayed phenotype manifestations in the atypical T623S form are a result of slower accumulation of farnesylated lamin A at the nuclear periphery. It is possible that increased expression of lamin A Δ90 could be a causative factor in the severity of its accompanying RD-like syndrome, although this would require confirmation.

Werner Syndrome (WS) is a progeroid syndrome usually caused by mutations in the WRN gene which encodes the helicase WRN (see section 1.2.3.6). However, ~20% of patients are referred to as atypical and have mutations in LMNA (Chen et al., 2003; Doh et al., 2009). Mutations cluster in the first half of the rod domain of lamin A (Figure 1.1:6). WS symptoms are variable between individuals and do not become apparent until later in life. Symptoms include short stature, premature hair greying and loss, cataracts, osteoporosis, atherosclerosis and similar facial characteristics to HGPS patients (Huang et al., 2006; Kudlow et al., 2007). Unlike HGPS, WS patients are susceptible to a variety of cancers (Goto et
al., 1996). Cancer and cataracts are more representative of symptoms of natural ageing, however individuals with \textit{LMNA} mutations may not present with these symptoms and onset is earlier (Chen et al., 2003), indicative of inherent differences in disease mechanisms between disease caused by \textit{LMNA} and \textit{WRN} mutations.

A number of genotype-phenotype correlations have been established. All mutations that result in retention of the C-terminal cysteine residue (and therefore, retain the farnesyl modification) result in the characteristic presence of dysmorphic nuclei in patient cells (Bergo et al., 2002; Eriksson et al., 2003; Mallampalli et al., 2005; Navarro et al., 2004). N-terminal point mutations do not cause nuclear blebbing, and generally result in atypical symptoms (Smallwood and Shackleton, 2010). Interestingly, farnesylation retention is not required for the change in nuclear morphology as one N-terminal missense mutation (E578V – see section 3.1.3) with abnormal nuclei shows (Fukuchi et al., 2004). Mutations affecting residues that become buried within the immunoglobulin-like fold located in the C-terminal globular region associate with skeletal muscle phenotypes, while exposed residues associate with lipodystrophy (Scharner et al., 2014).

### 1.2 Telomeres

#### 1.2.1 Overview

Telomeres are highly conserved repetitive sequences located at the termini of linear chromosomes. In humans, telomeres consist of a tandem array of the hexamer TTAGGG and form a nucleoprotein complex with a set of proteins known as shelterin. Shelterin is able to bind to telomeres and mediate the formation of a t-loop, a structure in which the end of a telomere is sterically hidden from DNA damage recognition factors. This prevents the cell from
recognising the telomere terminus as a double strand break (DSB), which could result in telomere-telomere fusion, exit from cell cycle and/or genomic instability.

Due to the end replication problem, telomeres shorten with each cell division in the absence of a telomere maintenance mechanism (TMM). When telomeres reach a critical length senescence is initiated. The accumulation of senescent cells is thought to be a key factor in the ageing phenotype. If senescence is bypassed telomeres may continue to shorten until the cell reaches crisis. At this stage, telomeres are so short that association of shelterin is compromised, exposing the telomere to DNA damage recognition factors. Efforts by the cell to erroneously repair this “break” lead to genomic instability. Mutations or epigenetic changes that permit the cell to adopt a TMM allow the cell to continue proliferating and this is a key step in carcinogenesis.

1.2.2 Telomere structure

Human telomeres consist of a long tract of canonical TTAGGG repeats, normally extending between 5 and 15kb at the chromosome terminus (Samassekou et al., 2010). Telomere length is highly variable between different cell types (de Lange et al., 1990; Engelhardt et al., 1997), different telomere ends in a single cell (Graakjaer et al., 2004; Lansdorp et al., 1996), and between alleles of sister chromatids (Baird et al., 2003). Telomere length is partially inherited (Graakjaer et al., 2006; Slagboom et al., 1994) with a bias towards paternal inheritance (Nordfjäll et al., 2005), and may be determined by genetic factors (Baird et al., 2006). Loci that may contribute to heritable telomere length have been identified on chromosome 12 and the X chromosome (Nawrot et al., 2004; Vasa-Nicotera et al., 2005). Opposing findings in mice (Garcia-Cao et al., 2004), one study in twins has established that the contribution of environmental and epigenetic factors are minor in human telomere length determination (Graakjaer et al., 2004).
With advances in techniques that are capable of measuring single telomere lengths, it has become apparent that individual telomeres show tendencies towards individual characteristics. Short arm telomeres tend to be longer than their long arm counterparts (Samassekou et al., 2009). The 17p telomere has a strong tendency to be the shortest or one of the shortest telomeres (Britt-Compton et al., 2006; Martens et al., 1998), while 3p, 4p and 5p are among the longest (Samassekou et al., 2009). Unusual length dynamics are also apparent at the telomeres of the sex chromosomes (Mayer et al., 2006; Perner et al., 2003).

Adjacent to the 5-15kb long TTAGGG tract is a region of the telomere (Figure 1.2:1) which includes canonical TTAGGG repeats but mainly consists of degenerate repeats, including TCAGGG, TGAGGG and TTGGG which may extend for ~2kb (Allshire et al., 1989; Baird et al., 1995). Degenerate telomere sequences may also be present within the main body of the telomere, which may have consequences for telomeric stability (Mendez-Bermudez et al., 2009). Directly adjacent to the telomere is a region of subtelomeric DNA. While some

![Figure 1.2:1](image)

**Figure 1.2:1** Sequence composition of the telomere and subterminal regions of human chromosomes. The telomere consists of ~2-20kb repeat tracts of the conserved TTAGGG repeat and the 3’ overhang at the most distal (terminal) end of the chromosome. Immediately adjacent to the telomere is the subtelomeric region, consisting of a patchwork of degenerate and canonical telomere repeats.
subtelomeres show little homology to others, such as 7q (Brown et al., 1990; Riethman et al., 1993), historical duplications, reshuffling and exchange events have resulted in a patchwork-like composition (Baird et al., 2000, 1995; Brown et al., 1990; Cross et al., 1990; Martin-Gallardo et al., 1995; Monfouilloux et al., 1998), and large amounts of homology exist across different chromosome ends (>99.5% between duplicons) (Riethman et al., 2005). Because of this, and the repetitive nature of these sequences, accurately defining subtelomeric regions is a difficult process (Bailey et al., 2001). A schematic representation of subterminal chromosome structure is provided in Figure 1.2.1.

The telomere terminus is marked by a 3’ G-rich overhang which is thought to invade double stranded telomeric DNA to form a lariat termed the t-loop. A short D-loop also results from the invasion of the 3’ end into the normal duplex DNA (Griffith et al., 1999). This process is thought to be essential for telomere function and is orchestrated by the components of the multi-protein complex termed shelterin.

1.2.2.1 Shelterin components

The telomere-specific complex shelterin recognises and binds telomere TTAGGG repeats to form a nucleoprotein complex, protecting telomere ends from recognition by the DNA damage response (DDR), which could otherwise recognise telomere termini as double strand breaks (DSBs). Shelterin is composed of six proteins: TRF1, TRF2, RAP1, TIN2, TPP1, and POT1.

Telomere repeat-binding factors TRF1 and TRF2 are detectable along entire stretches of telomeric repeats and are responsible for recruiting all other shelterin components to telomeres (Palm and de Lange, 2008). Each contains a C-terminal SANT/Myb domain that binds to the 7nt sequence TAGGGTT with extremely high specificity (Court et al., 2005; Hanaoka et al., 2005). Both also contain a TRF homology domain (TRFH) which mediates homodimerisation.
Despite a large degree of homology between the two proteins they are unable to directly interact (Broccoli et al., 1997). The TRFH also serves as a docking site for several telomere binding factors (Chen et al., 2008). RAP1 binds TRF2, increasing its affinity for telomeric sequence, and is able to bind DNA directly (Arat and Griffith, 2012). TRF1-interacting nuclear factor 2, TIN2 acts as a linchpin in shelterin, stabilising the complex through its simultaneous binding of both TRF1 and TRF2 (Ye et al., 2004). TIN2 also binds Tripeptidyl Peptidase 1 TPP1 (Frescas and de Lange, 2014), through which protection of telomeres 1 (POT1) is recruited (Hockemeyer et al., 2007). POT1 recognises G-rich single stranded telomeric DNA and is able to bind the overhang (Baumann and Cech, 2001), and the D-loop of the t-loop conformation (de Lange, 2005).

1.2.2.2 The 3’ overhang

The single-stranded G-rich overhang is conserved in most organisms (Henderson and Blackburn, 1989). Electron microscope images of a lariat at the terminus of telomeric DNA suggested that the overhang, through mediation by TRF2, is able to invade double stranded telomeric sequence to form the t-loop (Griffith et al., 1999). Recent innovations in microscopy have confirmed these findings, and have excluded a role in t-loop formation for all shelterin components except TRF2 (Doksani et al., 2013).

The overhang may range in size from ~12-300nt (Dai et al., 2010; Makarov et al., 1997; McElligott and Wellingr, 1997; Stewart et al., 2003; Zhao et al., 2008) and may be dependent on cell type and divisional age of the cell (Stewart et al., 2003). The overhang is partly derived through the end-replication problem (Levy et al., 1992), so called because DNA polymerase requires a primer in order to initiate replication, and upon detachment of this primer there remains an unreplicated section of DNA relative to at least the size of the primer (Olovnikov, 1973). Therefore, the nascent strand created by lagging-stand synthesis will be missing genomic sequence at the 5’ end. Semiconservative
replication results in one daughter copy with an overhang in place (lagging strand synthesis), and one blunt end (leading strand synthesis), which requires processing to obtain an overhang. This processing is carried out on nascent duplex telomeres derived from leading strand synthesis by the nuclease Apollo. Apollo is recruited to telomeres through TRF2 and its nuclease activity is regulated by POT1 (Wu et al., 2012). Exonuclease I (ExoI) may generate long overhangs at both telomere ends, which are subsequently filled in by the CTC1, STN1 and TEN1 (CST) complex (Wang et al., 2012; Wu et al., 2012). Leading strand daughter duplex DNA shortens by ~30nt, while the lagging strand shortens by ~100nt (Zhao et al., 2008). The final RNA primer is positioned ~70-100nt from the ends during replication (Chow et al., 2012), which could account for increased lagging-strand shortening, however, nuclease digestion would seem to be carried out on both lagging- and leading-strand-derived telomeres as the C-rich strand sequence shows a strong tendency (~80%) to end after the first cysteine of the CCCAAT-5’ repeat (Sfeir et al., 2005). To add further complexity, the overhang may in fact be extended through resection, then shortened through fill-in in a cell cycle-dependent manner (Dai et al., 2010). This could be regulated by differential association of telomere-associated proteins at S/G2 (Verdun and Karlseder, 2006; Verdun et al., 2005). Ultimately this process leads to the shortening of telomeres with every cell division.

1.2.3 Telomeres, DNA damage and ageing

Telomere shortening has been reproducibly observed in cultured primary cells (Harley et al., 1990; Lansdorp et al., 1996), as well as in vivo (Allsopp et al., 1995; Kajstura et al., 2000; Notaro et al., 1997). As continued shortening of chromosomes would result in erosion of essential genetic code, telomeres ensure the protection of the genome. Thus, upon shortening to a critical length, a signalling pathway is set in motion that halts further cell division, as well as telomere shortening. The result is senescence, a state in which the cell may no
longer divide, and several biological and morphological changes occur in the cell (Campisi, 2013). This process is a natural defence against catastrophic DNA damage events that could result from fusion-breakage cycles (see section 1.2.3.5), and could ultimately lead to cancer. Tissues accumulate senescent cells with age (Herbig et al., 2006; Lawless et al., 2010; Wang et al., 2009), and it is likely that this is the cause of several functional changes which lead to tissue dysfunction and disease associated with ageing (Artandi and DePinho, 2010; Cawthon et al., 2003; Krtolica et al., 2001).

1.2.3.1 The mitotic clock and telomere-associated senescence

Limited replicative capacity of cultured cells was first observed by Hayflick in 1961 (Hayflick and Moorhead, 1961). Serving as a mitotic clock, the number of times a cell divides ex vivo has a predictable limit, and this is often termed the Hayflick limit after its discoverer. Telomeres shorten progressively with continual cell division (Harley et al., 1990), and it is telomere length that dictates the potential replicative capacity of a cell (Allsopp and Harley, 1995; Allsopp et al., 1992). Telomeres shorten by ~30-160bp per cell division (Allsopp et al., 1992; Baird et al., 2003; Counter et al., 1992; Harley et al., 1990; Levy et al., 1992; Vaziri et al., 1993), until reaching a critical length. This process initiates cell cycle exit, entering the cell into a non-dividing state where much of its expression profile is altered, termed replicative senescence (Bodnar et al., 1998). Upon critical shortening, elements of shelterin may dissociate from telomere ends, disrupting the secondary structure of telomeres and activating a DDR-like response involving many global DDR proteins (see section 1.2.3.5 and d’Adda di Fagagna et al. 2003). Current understanding suggests that five telomeres or more must shorten critically and exhibit a DDR (see below) before senescence is initiated (Kaul et al., 2011). Genomic DNA damage may also induce an alternative senescent state that has distinct downstream effectors from the senescence pathway activated by shortened telomeres (Cesare et al., 2013).
1.2.3.2 DNA damage response

Much of the DDR relies heavily on blanket signals provided by modification of factors readily available in the nucleus. The first responder to double stranded breaks is the MRN complex, consisting of Mre11, Rad50 and Nbs1. MRN binds and processes damaged DNA ends (Carson et al., 2003; Moreno-Herrero et al., 2005; Uziel et al., 2003). The key activator ataxia-telangiectasia mutated (ATM), a serine/threonine kinase, is then recruited to double strand breaks, or in the case of single strand breaks ataxia telangiectasia and Rad3 related (ATR), and activates several DDR factors through phosphorylation. This includes phosphorylation of serine residue 139 of histone variant H2AX (Burma et al., 2001). This phosphorylated form is termed γ-H2AX, and spreads rapidly on H2AX over hundreds of kilobases from the initial site of damage detection (Rogakou et al., 1999). γ-H2AX is a marker of DNA damage and is key in the recruitment of DNA repair effectors (Paull et al., 2000; Sharma et al., 2012).

As well as recruiting DNA repair proteins, ATM will also initiate a rapid halt to cell cycle progression by phosphorylation of CHK1 and CHK2 (at residues serine 345, and threonine 68 respectively), which, now activated, inhibit with their downstream effectors Cdc25A and Cdc25C phosphatases to bring about arrest at G1/S or G2/M (Li and Zou, 2005). Upon completion of repair, cell cycle may continue and the loss of phosphorylation of H2AX may play a role in resuming the cell cycle (Downey and Durocher, 2006). p53, a powerful tumour suppressor, is activated by ATM. It induces transcription of p21 which in turn may upregulate p16INK4A. Should damage be irreparable, senescence is initiated through this increased transcription of p21 and p16INK4A (Campisi and d’Adda di Fagagna, 2007). Double-strand breaks may then be repaired by homologous recombination (HR) or non-homologous end joining (NHEJ).
1.2.3.3 Repair via homologous recombination

Double-strand breaks form as a result of exposure to reactive oxygen species and ionizing radiation, and may be formed during replication due to fork collapse. Whether the cell carries out HR or NHEJ is largely dependent on the stage of the cell cycle, as well as the nature of the lesion (Symington and Gautier, 2011). HR carries out fidelitous repair by using a sister-chromatid as a template for recombination that is only available during S-phase (Saleh-Gohari and Helleday, 2004), when replication fork collapse may occur. Current knowledge of the HR repair system derives mostly from what has been learnt.

![Diagram of DNA damage repair by homologous recombination](image)

**Figure 1.2:2** DNA damage repair by homologous recombination. (a) DNA damage is induced and detected by DDR factors. 5' resection is carried out and strand invasion is mediated by RPA. Following DNA synthesis, HR repair may be resolved by one of the three mechanisms shown in figures b-d. (b) Integration of the remaining strand and Holliday junction resolution resulting in crossover or non-crossover, and conservation of genetic data. (c) The extended strand is displaced and re-anneals to the remaining strand. Processing and extension result in conservation of genetic data. (d) A replication fork may form and continue processively to the chromosome terminus. Any mutations will be copied. Adapted from (Deriano and Roth, 2013)
from the yeast homologous system. Breaks are usually not clean in that blunt ends do not generally result and so processing of the ends of a break is required before repair can be carried out. HR repair is dependent upon cyclin dependent kinase 1 (CDK1), which is required for 5’-3’ resection of DNA ends at the site of damage (Ira et al., 2004). This is mediated by the MRN complex, or in yeast the MRX complex, and carried out by the exo...

1.2.3.4 Repair via non-homologous end-joining

Much of what is understood about NHEJ derives from studying the programmed rearrangement of antibody gene loci. Early in their development, T and B lymphocytes carry out V(D)J recombination in the Variable Diverse and Joining regions of the heavy and light antibody chain genes. Rearrangement of these three regions provides a basis for antibody diversity. V(D)J recombination is initiated by recombination activating gene proteins (RAGs), which induce DSBs, and completed by classical-NHEJ. A second process, immunoglobulin class switch recombination (CSR), takes place in mature B lymphocytes, directing class changes of the antibody heavy chain. CSR also employs DSBs, but resolves them with both classical- and atypical-NHEJ in a 53BP1-dependent manner (Bothmer et al., 2010).
In G0, G1 and early S phases of the cell cycle, HR is repressed and NHEJ is the major effector of DSB repair. NHEJ does not require a homologous sister-chromatid and instead will anneal broken ends to one another despite an absence of sequence homology, or ligate sequences based on small sections of homologous sequence (microhomologies), regardless of whether the two sequences correspond to the same locus, which can result in deletions (Roth and Wilson, 1986). NHEJ is also able to compensate for loss of HR in cells deficient in key HR proteins (Takata et al., 1998). A major regulator of NHEJ repair is 53BP1, which is able to direct repair to be carried out through one of two routes (Bothmer et al., 2011, 2010): classical NHEJ (c-NHEJ) or alternative NHEJ (a-NHEJ) (Figure 1.2:3).

In c-NHEJ, Ku, a heterodimer of Ku70 and Ku86, is first to bind DNA and mediates binding of other NHEJ factors (Fattah et al., 2010). Ku binds and maintains the proximity of the two ends of the break to one another, while also recruiting DNA-dependent protein kinase catalytic subunit (DNA-PKcs), as well as DNA ligase IV and its cofactors XRCC4 and XRCC4-like factor (XLF). DNA-PK

**Figure 1.2:3** DNA damage repair by non-homologous end joining. Classical NHEJ (c-NHEJ) results in minimal loss of genomic material, however alternative NHEJ (a-NHEJ) relies upon alignment of microhomologous regions, potentially resulting in large deletions, or translocations. Adapted from (Deriano and Roth, 2013)
aids in complex recruitment and is also able to form a complex with the exonuclease ARTEMIS, which may carry out DNA processing (Ma et al., 2002), before the DNA ligase IV completes ligation on regions of microhomology (Grawunder et al., 1997).

a-NHEJ is the least faithful mode of DSB repair, and frequently results in translocations, deletions and insertions (McVey and Lee, 2008). The discovery of a-NHEJ is relatively recent and as such its mechanisms are less well-defined than other forms of repair. Although first identified as a backup system for c-NHEJ (Kabotyanski et al., 1998; Liang and Jasin, 1996), a-NHEJ is now known to function in the presence of a functional c-NHEJ pathway (Nussenzweig and Nussenzweig, 2007). a-NHEJ relies upon the MRN complex, which may play a direct role in end processing alongside the exonuclease CtIP (Deriano and Roth, 2013), but is carried out independently of Ku, which may be replaced by poly (ADP-ribose) polymerase 1 (PARP1) (Haince et al., 2008). Ligation may be carried out by DNA ligases I and III (Liang et al., 2008).

1.2.3.5 Telomere dysfunction induces senescence through a DNA damage response pathway

Critical shortening or damage at telomeres may result in T-loop deconstruction, loss of the overhang and shelterin dissociation, exposing the telomere (a process termed uncapping) (d’Adda di Fagagna et al., 2003; de Lange, 2005; Karlseder et al., 2002). This results in a DDR that involves the recruitment of factors found at double-strand break sites. These aggregates have been termed Telomere dysfunction induced foci (TIFs) (Takai et al., 2003). TIFs are distinguishable from normal DSB sites by the presence of TRF1 (Takai et al., 2003), although the particulars of downstream effectors are not well enough understood to fully distinguish a telomere-specific response (Palm and de Lange, 2008). Many proteins involved in the ordinary ATM directed response co-localise in TIFs that are associated with senescent cells. These foci, which
include γ-H2AX, 53BP1, the MRN complex and activated ATM, are thought to induce telomere-associated senescence, causing cells to permanently and irreversibly exit the cell cycle (d’Adda di Fagagna et al., 2003). TIFs may also persist in senescent cells that accumulate with age in vivo (Scaffidi and Misteli, 2006; Sedelnikova et al., 2004). It has been shown that telomeric binding proteins are not merely passively shielding telomere ends from detection by the DDR apparatus but that TRF2 actively inhibits ATM activity, while POT1 may inhibit ATR activity (Denchi and de Lange, 2007; Karlseder et al., 2004). p16INK4A is not a contributor to establishing telomere-induced senescence (Herbig et al., 2004), and CHK2 may not be activated despite ATM activation (Cesare et al., 2013).

1.2.3.6 Werner syndrome

Werner Syndrome (WS) is a progeroid syndrome associated with deficiencies in telomere biology. Classical-WS is an autosomal recessive disorder caused by homozygous or compound heterozygous mutations in the RECQL2 gene. RECQL2 encodes WRN, and WS mutations tend to result in truncated forms of WRN which are incapable of relocating to the nucleus (Moser et al., 2000). WRN is a RecQ helicase involved in several cellular processes, including DNA repair, replication and recombination. As well as exhibiting symptoms of premature ageing (see section 1.1.6), patients are susceptible to various forms of cancer (Goto et al., 1996). WS patients also present with genomic instability, DNA repair deficiencies, replication defects and attenuated apoptosis (Croteau et al., 2014). At telomeres, the helicase activity of WRN acts in partnership with exonucleases to unwind and dissociate the telomeric D-loop, mediated by TRF1 and TRF2 (Machwe et al., 2004; Opresko et al., 2004). WRN is required for normal telomere lagging-strand synthesis (Crabbe et al., 2004), telomeric DNA damage processing (Eller et al., 2006), and DNA damage regulation upon telomeric dysfunction (Crabbe et al., 2007). The proliferative capacity of WS cells
is reduced compared to normal cells, but may be restored through telomerase expression (telomerase is able to extend telomeres - see section 1.2.4.1). However, investigation of the rate of telomere shortening in WS has given mixed results, with some cells exhibiting a normal or slightly elevated shortening rate while others exhibited greatly accelerated shortening rates at certain points in proliferative lifespan. Though this was likely as a result of clonal expansions within the population, mean telomere length was extremely short in WS cells at early passages (~6kb) (Baird et al., 2004), perhaps indicating a developmental role rather than a persistent issue. Telomere length is also shorter in vivo in WS than age matched controls in skin fibroblasts (Ishikawa et al., 2011). Together, these results reinforce the importance of telomere length and integrity in telomere-initiated senescence and ageing.

1.2.4 Telomere maintenance mechanisms and cancer

The genomic instability caused by telomere dysfunction may provide an entry point to establishing immortalisation in cancer. Accumulation of mutations leads to further genomic instability and any mutations conferring a growth advantage will result in expansion of neoplastic cell types. A myriad of cellular mechanisms exist to limit proliferation, including senescence induction upon telomere dysfunction. As such, a key feature of cancer cells is their ability to circumvent normal cell signalling responses that halt proliferation. In order to propagate without limit, cancer cells must overcome the imposition of senescence induced by telomere shortening. Through interruption of the p53 and pRb signalling pathways (Hara et al., 1991), cells may bypass senescence and enter crisis – a state in which telomeres continue to erode (Counter et al., 1992). This may result in cycles of telomere fusion, in which uncapped telomeres are joined, followed by uneven breakage at cell division and causing extreme chromosomal instability (Gisselsson et al., 2001; Lo et al., 2002; Maser et al.,
Within this population, cells may contain mutations which lead to the activation of a telomere maintenance mechanism (TMM).

To this effect, cancer cells may take advantage of cellular mechanisms already present in the genetic code, though transcriptionally restricted in most normal cells. The most common TMM that is activated in cancer cells is telomerase, a multi-subunit enzyme capable of extending telomeres through a reverse transcription mechanism. Telomerase may be activated by epigenetic alterations which activate transcription of the telomerase subunit telomerase reverse transcriptase (TERT) (Sui et al., 2013). A second, less well-understood mechanism, termed alternative lengthening of telomeres (ALT) is activated in a minority of cancers, and seems to be dependent upon recombination-based elongation of telomeres. The propensity of a particular cell type to initiate telomerase expression or the ALT system in cancer may depend on inherent differences in expression patterns (Lafferty-Whyte et al., 2009). Cell types of mesenchymal origin are more predisposed to activating ALT, and common ALT cancers include glioblastoma multiforme (Hakin-Smith et al., 2003), liposarcoma (Costa et al., 2006), sarcoma and astrocytoma (Henson et al., 2005). Activation of the ALT and telomerase pathways are not mutually exclusive and both may be employed in human cancer cells (Cerone et al., 2001).

1.2.4.1 Telomerase

Not all cells are prone to telomere shortening. As telomeres are key to maintaining genome integrity, certain human cell types that require telomere length maintenance while continuing to divide, specifically germ cells and stem cells (Kim et al., 1994), are able to lengthen telomeres through expression of telomerase. However, in the remainder of normal cells, telomerase remains inactive and cells whose telomeres reach a critical length will initiate, unless bypassed, the senescence response.
Telomerase is a multi-subunit holoenzyme, the core of which is composed of the catalytic reverse transcriptase subunit hTERT in humans (Harrington et al., 1997) and a telomerase RNA component hTR, which provides a template for DNA synthesis (Feng et al., 1995). Telomerase also binds several auxiliary factors, including the key component Dyskerin, which is required for stable formation of the telomerase complex (Mitchell and Collins, 2000). hTERT is highly conserved and binds telomeres through the shelterin component TPP1, via its interaction with POT1 (Xin et al., 2007). hTR is a 451nt telomeric RNA sequence which binds hTERT and serves as a template for its catalytic activity. hTR may also aid the processivity of telomerase action (Nandakumar and Cech, 2013). In human cells expressing telomerase, telomere length remains heterogeneous, but short telomeres and senescence are avoided, leading to the theory that telomerase specifically targets short telomeres for lengthening (Blackburn, 2001; Zhu et al., 1999). This has been confirmed through ectopic expression of telomerase in various cell types, in which telomere length is regulated to cell type-specific limits (Britt-Compton et al., 2009a). Telomerase may extend the proliferative lifespan of a cell not only through telomere elongation but also through blocking the DDR and apoptosis, and stimulating proliferation (reviewed in Majerská et al. 2011).

Background levels of expression of hTR are present in most tissues, and telomerase expression and subsequent telomere lengthening may be reconstituted in human cells by induced expression of hTERT alone (Vaziri and Benchimol, 1998), with no effect on genomic stability (Ouellette et al., 2000). Due to the biological implications of maintaining telomere length, TERT expression is tightly regulated by several factors related to oncogenesis (Kyo et al., 2008). In contrast to humans, mice express telomerase in most somatic cell types (Kipling, 1997), and is required for cell growth and survival (Lee et al., 1998). Mouse telomere length is highly variable and telomeres can reach up to
150kb (Kipling and Cooke, 1990). The representativeness of the mouse as a model organism for telomere biology must therefore be carefully considered.

**1.2.4.2 The ALT mechanism**

The ALT pathway is activated in ~10-15% of cancers that do not express telomerase (Heaphy et al., 2011). No single event or pathway has been identified as the cause of the ALT pathway, making identification of ALT markers difficult. Robust methods for identifying ALT are of interest due to the poor prognosis associated with ALT in some cancer types (Cesare and Reddel, 2010), although the prognosis of diffuse malignant peritoneal mesothelioma patients exhibiting ALT is variable (Villa et al., 2008), possibly reflecting the heterogeneous nature of ALT pathways. As such, ALT is identified by a collection of characteristics as follows.

ALT telomeres are extremely heterogeneous in length, and can reach lengths greater than 50kb (Bryan et al., 1997, 1995). Losses and gains may occur rapidly (Murnane et al., 1994), most likely due to the high level of recombination at ALT telomeres (see below). It is thought that these extreme lengths are not tolerated by the cell, which initiates a trimming mechanism resulting in an abundance of extrachromosomal linear and circular telomeric DNA in ALT cells, although the mechanisms of this are not fully understood. t-circles are double-stranded circular telomeric sequences, so called as it is thought they are formed by resolution of t-loops (Cesare and Griffith, 2004; Wang et al., 2004). Although abundant in ALT cells, t-circles are also found in telomerase positive cells (Tomaska et al., 2009). More specific to ALT positive cells are c-circles composed of a single strand C-rich circle, with some duplex portions containing the complementary G-rich sequence (Henson et al., 2009). It has been proposed that C-circles derive from G-strand specific degradation of t-circles. Less common, but still present, are G-circles, the inverse manifestation of C-circles,
and extremely large fragments of telomeric DNA containing secondary structures, termed t-complex DNA (Nabetani and Ishikawa, 2009).

Promyelocytic leukaemia (PML) bodies are nuclear structures that contribute to a number of nuclear functions, including tumour suppression (Bernardi and Pandolfi, 2007). The presence of telomeric DNA in PML bodies is associated with ALT and as such these structures are termed ALT-associated PML bodies (APBs). APBs are identifiable by their association with telomeric DNA, as well as telomere-associated proteins, including members of the shelterin complex and DNA repair and recombination factors (Yeager et al., 1999). It would seem that a subset of APBs are able to co-localise with telomeres, whose mobility is increased compared to PML bodies, providing discreet compartments for ALT-related recombination processes to take place (Jegou et al., 2009), although ALT induced telomere lengthening may occur in their absence (Jeyapalan et al., 2008). ALT cells are also more commonly prone to genomic instability than telomerase positive cells (Lovejoy et al., 2012), and exhibit extraordinary instability at the MS32 minisatellite locus (Jeyapalan et al., 2005).

High levels of sister-chromatid exchange (Londoño-Vallejo et al., 2004) and rapid acquirement of telomere length polymorphisms (Murnane et al., 1994) indicate that the ALT mechanism depends on recombination processes to implement telomere elongation. Dunham et al. demonstrated via the insertion of a neomycin resistance marker in telomeres and subtelomeric regions that recombination occurs at telomeres while subtelomeres remain unaffected, and that telomerase positive cells do not employ recombination at telomeres (Dunham et al., 2000). Several models have been proposed as to how ALT cells employ recombination-like processes in order to extend telomeres, including sister-chromatid exchange and homologous recombination, as well as mechanisms that use extrachromosomal linear or circular telomeric DNA as a
template (reviewed in Cesare & Reddel 2010). Due to the heterogeneity of the ALT phenotype, it is possible that several of these mechanisms may apply.

1.3 Links between telomere and lamin biology

Telomere length has been frequently linked to ageing and several reports have shown a link between lamin A malfunction and disruption of normal telomere biology (Allsopp et al., 1992; Decker et al., 2009; Gonzalez-Suarez et al., 2009; Huang et al., 2008). Telomere dysfunction induces the DDR, which may result in entry into senescence. Both the DDR and levels of senescence are elevated in HGPS cells, and damage at telomeres is specifically enhanced (Benson et al., 2010). Two studies so far have shown that classical HGPS cells have shorter telomeres when compared to controls (Allsopp et al., 1992; Decker et al., 2009), suggesting that increased cellular senescence in HGPS is attributable to telomere-induced senescence. Rapid accumulation of TIFs in progerin-expressing cells suggests that telomere damage signalling is occurring in cells prior to critical shortening (Benson et al., 2010), thus senescence may be induced by damaged telomeres that would otherwise continue to shorten before initiating senescence.

1.3.1 DNA damage and lamin deficiencies

Ectopic expression of progerin in normal fibroblasts produces increased damage signalling at telomeres – a fourfold increase in γ-H2AX association with telomeres (Benson et al., 2010). These data mirror the accumulation of γ-H2AX in senescing mesenchymal cells, which is coupled with aggregation of telomeres with intranuclear lamin structures (Raz et al., 2008). The DDR is greatly perturbed in HGPS cells. DNA damage accumulates and is slow to be repaired. Patient cells display a deficiency in 53BP1 recruitment to sites of damage, and lesions induced by ionising radiation (Liu et al., 2005). Similarly, 53BP1
recruitment is slow in both unirradiated (Gonzalez-Suarez et al., 2009) and irradiated $Lmna^{-/-}$ MEFs (Redwood et al., 2011), in which a reduction in 53BP1 levels is observed (Gonzalez-Suarez et al., 2011). $Lmna^{-/-}$ MEFs c- and a-NHEJ deficiencies may be rescued through 53BP1 expression (Gonzalez-Suarez et al., 2011; Redwood et al., 2011).

The importance of 53BP1 in NHEJ has been highlighted by its requirement for V(D)J recombination. During the DDR, ATM phosphorylates, and thereby activates 53BP1 which then protects and promotes DSB end-joining (Bothmer et al., 2011; Rybanska-Spaeder et al., 2013) in a γ-H2AX-dependent manner (Difilippantonio et al., 2008). 53BP1 also seems to play a key role in controlling which mode of repair is carried out on a DNA break. 53BP1 preferentially activates c-NHEJ in CSR (Bothmer et al., 2011) and co-operates with breast cancer 1, early onset (BRCA1/Brca1) to activate NHEJ preferentially over HR in mice (Bunting et al., 2010). 53BP1 plays a specific role in telomere damage repair, being essential for NHEJ of dysfunctional telomeres. This process increases telomere motility, is dependent on ATM, and requires a 53BP1-H4K20me1 interaction for optimal repair (Dimitrova et al., 2008). An interesting study on the cells of ataxia telangiectasia (AT) patients, who have defective ATM (from whom the protein derives its name), revealed that telomeres are more frequently associated with the lamina in AT cells than control cells (Smilenov et al., 1999), indicating a connection between ATM, telomeres and the lamina which could be explored.

It seems that rapid shortening events are not the cause of telomere attrition in HGPS from data obtained in a telomere length study in lamin A stably transfected cells (Huang et al., 2008), which suggests a steady but increased rate of loss as measured by Q-PCR, however more data is required to confirm these conclusions. Chromosome orientation fluorescence in situ hybridisation) has shown no increase in sister-chromatid exchange (Gonzalez-Suarez et al., 2009),
demonstrating no increase in recombination activity at telomeres in HGPS cells. This suggests that the shortening process is replication-dependent or resultant of a compensatory increase in division in cells in a population where a high proportion of senescent cells put a strain on tissue maintenance.

1.3.2 Telomeres, heterochromatin, lamins and nuclear localisation

Telomeres may associate with the nuclear lamina in humans (de Lange, 1992; Ludérus et al., 1996), and a direct interaction between telomeres and lamina has been confirmed via chromatin immunoprecipitation (ChIP) assay in mice (Gonzalez-Suarez et al., 2009). This extensive study by Gonzalez-Suarez et al. into the effects of lamin A deficiency on telomeres in Lmna⁻/⁻ mice provides convincing evidence for a critical interaction between the two with data showing altered telomere localisation toward the nuclear periphery and an increase in TIF formation. This study also identified a NHEJ repair deficiency in Lmna⁻/⁻ cells, which the authors attributed to reduced 53BP1 after establishing no changes in the cellular levels of several DNA repair factors (Gonzalez-Suarez et al., 2009).

Telomeres have a specific heterochromatin profile which is crucial in maintaining telomere function (Michishita et al., 2008) and nuclear localisation (Uhlírová et al., 2010). Both lamin A reduction and histone methylation affect telomere localisation in MEFs (Uhlírová et al., 2010). Telomeric heterochromatin contains high levels of H3K9 methylation and H4K20Me3, both of which are greatly effected in HGPS cells (Columbaro et al., 2005; Dechat et al., 2008; Scaffidi and Misteli, 2005; Shumaker et al., 2006). The lamin A mutant E145K cell line shows an increase in telomere association with the lamina (Taimen et al., 2009), and HGPS telomere mobility in the nucleus is reduced (De Vos et al.,
This suggests that telomere localisation may be altered in HGPS cells due to epigenetic remodelling at telomeres.

Another overlap of telomere and lamin processes is the interaction of both within the nucleoplasm with LAP2α during mitosis and nuclear assembly (Dechat et al., 2004, 2000). LAP2α specifically binds intranuclear lamin A (Dechat et al., 2000) and is thought to bind telomeres via the non-specific double stranded DNA binding protein BAF (Dechat et al., 2004). This mediated and temporal interaction could provide critical information as to the role of intranuclear lamin A in genome organisation during mitosis, which in turn could be investigated in laminopathy disorders. Loss of lamins also result in an decreased association of telomeres with PML bodies in MEFs (Uhlírová et al., 2010).

1.3.3 Telomere length is reduced by lamin A deficiencies

Two studies have recorded telomere length directly in HGPS cells. The first identified a reduction in average telomere length in HGPS patient fibroblasts versus age matched and parental controls (Allsopp et al., 1992). The second observed shorter telomeres in patient cell lines versus controls, and an increased variability of length versus controls (Decker et al., 2009). These studies, have the inherent drawback of measuring telomere length at a single time point. Telomere length varies greatly between individuals, and in various tissues of the same individual. Allowing for the different periods of time that cells were cultured ex vivo in the above experiments, a single measurement of telomere length may not be representative, as telomeres shorten with every cell division. Additionally, due to inter-individual differences, a measurement at one time point that yields a short average telomere length may simply be attributable to natural inter-individual variation. As such, control samples are also difficult to select.
An additional study overcame these barriers by ectopic expression of four mutant forms of lamin A known to induce the HGPS cellular phenotype in fibroblasts containing an excisable hTERT construct (Huang et al., 2008). Ectopic expression of telomerase is known to inhibit telomere shortening as well as lengthen telomeres in normally telomerase-negative, and can reverse the proliferative deficiencies in HGPS cells (Benson et al., 2010; Kudlow et al., 2008; Ouellette et al., 2000). Thus, hTERT expression allowed normalisation of telomere length. The construct was then excised and lamin A mutants were then expressed. Measurement of telomere length across several time points revealed an accelerated telomere shortening rate in lamin A transfected cells versus controls. Interestingly, WT lamin A over-expression also seemed to induce an acceleration in telomere shortening.

Expression of telomerase can reverse proliferative deficiencies in HGPS cells and may inhibit telomere shortening, as can be seen in various normal cell lines (Benson et al., 2010; Kudlow et al., 2008; Ouellette et al., 2000). These studies have shown that some of the effects of telomerase induction may be reversing the p53, p21, p16 and pRB negative effects on cell cycle progression whose expression is altered upon telomerase transduction, potentially via interference with the altered mechanics of the telomere-lamina interaction. This coordinates with findings that p53 suppression allows some recovery of replicative potential (Kudlow et al., 2008) as does ATM/ATR suppression (Liu et al., 2006). Zmpste24, p53 double knockout mice have also been bred and display increased lifespan and weight gain (Varela et al., 2005). In terms of experimental design, it is notable that a significant proportion of separate clonal expansions of HGPS cells fail to immortalise upon transformation by telomerase (Wyllie et al., 2000).
1.4 Objectives

The first issue to be address by this project is the unknown contribution of HGPS molecular mechanisms to telomere shortening. Although short telomeres have been observed in cell lines derived from classical HGPS patients, issues regarding cell line derivation and the varied replicative history of cell lines tested question the conclusiveness of these results. Additionally, these results are not informative of any ongoing process that may affect telomere length. As such, it was decided that telomere length should be recorded in HGPS cells over the course of their proliferative lifespan. By recording the mean number of cell divisions in an HGPS population it is possible to derive a mean telomere shortening rate per cell division, which may be directly compared to control lines. Thus the hypothesis that short telomeres previously observed in HGPS cells are the result of an elevated telomere shortening rate, rather than sudden loss of telomeric sequence, may be tested.

Considerable research has been carried out on classical HGPS cells, however, due to the rarity of the condition, and the even scarcer prevalence of atypical HGPS, considerably less is known about atypical HGPS mutants. It was therefore decided that telomere shortening analysis would also be carried out in a number of atypical HGPS cell lines, as well as an RD cell line for comparison. Farnesylated forms of mutated lamin A are associated with nuclear membrane deformity, however one atypical patient line with a C-terminal head domain mutation that leaves lamin A processing unaffected (E578V) also exhibits some nuclear membrane blebbing (Csoka 2004; Toth et al. 2005). Although the rescue of nuclear membrane morphology may correspond with protection against the acquisition of new DNA damage lesions (Constantinescu et al., 2010), the failure to repair DNA damage upon treatment with FTIs suggests some separation between these two consequences of farnesylated lamin A accumulation (Scaffidi and Misteli, 2005)(Liu et al., 2006). Thus the E578V line was selected to test
whether telomere biology is altered in the presence of aberrant nuclear morphology, but not farnesylated lamin A.

Upon establishing telomere shortening rate, I aimed to extricate any underlying factors that may contribute to any potential changes in telomere biology in patient cells. Potential mechanisms were to be identified based on telomere dynamics recorded by the shortening analysis. This was made possible by the use of single telomere length analysis (STELA) for establishing length, which allows a high resolution of the dynamics of single telomeres.

Telomere integrity and sufficient activation of senescence is crucial for avoiding the development of cancer. HGPS patients do not typically develop cancer, and this suggests that genomic integrity is not compromised in patients, despite high levels of DNA damage. This implies that damage is either repaired more slowly or results in premature passage into senescence. However, previously published data suggest that A-type lamins impact telomere biology, including telomere localisation within the nucleus. The mobility and localisation of telomeres has shown to be altered in ALT cells. As such, it was hypothesised that lamin biology may be altered in ALT cells, and experiments were designed to observe differences between lamin:telomere interactions in telomerase positive and ALT cells. This was first explored by comparison of cellular levels and nuclear localisation of lamins and lamin-associated factors in ALT and telomerase positive cells. As chromatin is altered in both laminopathy and ALT cells, it was hypothesised that the altered levels and localisation of lamins discovered via these methods would result in a disruption of the interaction between chromatin and lamins, and this was explored using chromatin immunoprecipitation (ChIP).
Chapter 2       Materials and Methods

2.1 Cell culture

2.1.1 Subculturing cell lines

All cell lines were grown in appropriately supplemented media (see Table 2.1-1) at 37°C in 5% CO₂. Adherent cells were subcultured as follows: upon reaching confluency of 80-100% (cell line dependent), media was aspirated off, cells were washed in 1X phosphate buffered saline (PBS) before the addition of 0.25% Trypsin unless otherwise stated in Table 2.1-1. Cells were returned to 37°C in order to promote cell detachment, trypsin was inactivated with media, and cells re-plated in fresh flasks in the following total volumes of media: 8ml in 25cm² flask, 12ml in 75cm² flask, 20ml in 175cm² flask. Suspension cells were mixed thoroughly via resuspension with a pipette in media before being split into fresh flasks at dilutions between 1:2 and 1:32, in the same volume of fresh media.

Tissue culture plastics were purchased from Greiner Bio-One, and media and trypsin products from Invitrogen Gibco.

2.1.2 Population growth measurement

Cell counts were performed using a haemocytometer.

Cells were subcultured 1:2 (1:3 or 1:4 when necessary) at between 80 and 100% confluence and mean population doubling (PD) calculated using the equation:

\[ PD = \frac{\ln(\text{count at harvest}) - \ln(\text{initial cell number})}{\ln 2} \]

Counts were adjusted for viability, as assessed by the Trypan Blue assay.
2.1.3 Trypan Blue staining

Trypan Blue is a stain that selectively stains dead cells or tissue, while uptake is excluded from living cells, allowing an estimation of viability levels. 150μl Hyclone Trypan Blue Solution (Thermo Scientific) was added to 150μl resuspened cells in media. The solution was mixed well, left for 3 minutes then loaded onto a haemocytometer. 300-400 cells were counted for each passage and the proportion of dead (blue) cells recorded and excluded from cell counts when calculating PD.

2.1.4 β-galactosidase Senescence assay

β-galactosidase is present at higher concentrations in senescent cells and may be used as a maker to identify senescence by the addition of X-gal in sub-optimal conditions for enzymatic activity (see section 3.2.1). The assay was carried out on each patient and control primary cell line at as many time-points as possible. Cells were seeded onto 13mm HCl acid-treated glass coverslips in 24-well-plates between 24 and 72 hours before assay (~2x10⁴ cells per coverslip). Cells were washed once in PBS, fixed for 3 minutes in 1X fixation solution (2% formldehyde, 0.2% gluteraldehyde in 1X PBS), washed twice with 1X PBS then incubated in a staining solution for 16 hours at 37°C, protected from light and without CO₂ supplementation. Staining solution (90mM citric acid (C₆H₈O₇)/sodium phosphate (NaH₂PO₄) buffer pH5.98, 150mM NaCl, 2mM MgCl₂ 5mM potassium ferricyanide K₃[Fe(CN)₆], 5mM potassium ferrocyanide K₄[Fe(CN)₆], 400μg X-gal) was prepared as a fresh solution each time. Cells were then washed in 1X PBS 3 times and fixed in 100% methanol for 30 seconds before air-drying and mounting with Invitrogen ProLong Gold. β-galactosidase activity was recorded via manual counting on an Olympus BH-2 BHS microscope. A minimum of 400 cells were counted and re-counted on each coverslip, and the average percentage of β-galactosidase positive cells was calculated.
### 2.1.5 Cell lines

<table>
<thead>
<tr>
<th>Name</th>
<th>Immortalisation status</th>
<th>Growth media</th>
<th>Telomere maintenance</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>G608G (AG11498)</td>
<td>Primary line</td>
<td>DMEM + 20% FBS + 1X pen/strep</td>
<td>N/A</td>
<td>Skin fibroblast, 14 year old male. Heterozygous c. 1864C&gt;G in LMNA Purchased from Coriell Institute</td>
</tr>
<tr>
<td>E578V (AG04110)</td>
<td>Primary line</td>
<td>DMEM + 20% FBS + 1X pen/strep</td>
<td>N/A</td>
<td>Skin fibroblast, 13 year old female. Heterozygous c. 1945A&gt;T in LMNA Purchased from Coriell Institute</td>
</tr>
<tr>
<td>RD</td>
<td>Primary line</td>
<td>DMEM + 20% FBS + 1X pen/strep</td>
<td>N/A</td>
<td>Skin fibroblast, 1 day old, restricitive dermopathy German (neonatally fatal). Homozygous null c. 1085_1086insT in ZMPSTE24 Gift from prof. Manfred Wehnert, Ernst Moritz Arndt University</td>
</tr>
<tr>
<td>T623S</td>
<td>Primary line</td>
<td>DMEM + 20% FBS + 1X pen/strep</td>
<td>N/A</td>
<td>Skin fibroblast, 10 year old British Caucasian female. Heterozygous c. 1868C&gt;G in LMNA Received from Great Ormond Street Hospital</td>
</tr>
<tr>
<td>S143F</td>
<td>Primary line</td>
<td>DMEM + 20% FBS + 1X pen/strep</td>
<td>N/A</td>
<td>Skin fibroblast, 6 year old German. Heterozygous c. 428C&gt;T in LMNA Gift from Prof. Manfred Wehnert, Ernst Moritz Arndt University</td>
</tr>
<tr>
<td>NHDF</td>
<td>Primary line</td>
<td>DMEM + 10% FBS + 1X pen/strep</td>
<td>N/A</td>
<td>Normal Human Diploid Fibroblast. Skin fibroblast, healthy adult male. Shackleton lab long-term stocks</td>
</tr>
<tr>
<td>155-BR</td>
<td>Primary line</td>
<td>DMEM + 15% FBS + 1% NEAA</td>
<td>N/A</td>
<td>Skin fibroblast, healthy 55 year old male</td>
</tr>
<tr>
<td>161-BR</td>
<td>Primary line</td>
<td>DMEM + 15% FBS + 1% NEAA</td>
<td>N/A</td>
<td>Skin fibroblast, healthy 49 year old male</td>
</tr>
<tr>
<td>HT1080</td>
<td>Tumour derived</td>
<td>DMEM + 10% FBS</td>
<td>Tel+</td>
<td>Fibrosarcoma, 35 year old male. p53 mutated, exhibits pseudodiploidy.</td>
</tr>
<tr>
<td>U20S</td>
<td>Tumour derived</td>
<td>DMEM + 10% FBS</td>
<td>ALT</td>
<td>Osteosarcoma, female, DSB repair deficent</td>
</tr>
<tr>
<td>WI-38</td>
<td>SV40</td>
<td>DMEM + 10% FBS</td>
<td>ALT</td>
<td>Fetal lung fibroblast, female. Werner’s –ve, DSB repair deficent</td>
</tr>
<tr>
<td>VA13/2RA (WI38ALT)*</td>
<td>SV40</td>
<td>DMEM + 10% FBS</td>
<td>ALT</td>
<td>Pluripotent embryonic male lung, derived from metastasis of testis carcinoma</td>
</tr>
<tr>
<td>NT2D1</td>
<td>Tumour derived</td>
<td>High glucose DMEM +10% FBS</td>
<td>Tel+</td>
<td>Pluripotent embryonic male lung, derived from metastasis of testis carcinoma</td>
</tr>
<tr>
<td>JFCF6T.5K post crisis</td>
<td>SV40</td>
<td>DMEM + 20% FBS Trypsin LE</td>
<td>ALT</td>
<td>Jejunal fibroblast, cystic fibrosis</td>
</tr>
<tr>
<td>Cell Line</td>
<td>Source</td>
<td>Culture Media</td>
<td>Genetic Modification</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>--------</td>
<td>---------------</td>
<td>----------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>JFCF.6T-1J/11E</td>
<td>SV40</td>
<td>DMEM + 10% FBS</td>
<td>ALT</td>
<td>Jejunal fibroblast, cystic fibrosis</td>
</tr>
<tr>
<td>JFCF.6T-1J/11C</td>
<td>SV40</td>
<td>DMEM + 10% FBS</td>
<td>Tel+</td>
<td>Jejunal fibroblast, cystic fibrosis</td>
</tr>
<tr>
<td>llcf/a2 post crisis</td>
<td>Tumour derived</td>
<td>RPMI + 20% FBS</td>
<td>ALT</td>
<td>Breast fibroblast, female with Li-Fraumeni. p53 null</td>
</tr>
<tr>
<td>HeLa</td>
<td>Tumour derived</td>
<td>DMEM + 10% FBS</td>
<td>Tel+</td>
<td>Adenocarcinoma, 31 year old black female. HPV positive</td>
</tr>
<tr>
<td>Saos-2</td>
<td>Tumour derived</td>
<td>DMEM + 15% FBS</td>
<td>ALT</td>
<td>Osteosarcoma, female.</td>
</tr>
<tr>
<td>LoVo</td>
<td>Tumour derived</td>
<td>DMEM + 10% FBS Trypsin LE</td>
<td>Tel+</td>
<td>Colon, male. MSH2 deficient</td>
</tr>
<tr>
<td>SW480</td>
<td>Tumour derived</td>
<td>DMEM + 10% FBS Trypsin LE</td>
<td>Tel+</td>
<td>Adenocarcinoma male. MSH2 deficient</td>
</tr>
<tr>
<td>SUSM-1</td>
<td>Chemical</td>
<td>DMEM + 10% FBS</td>
<td>ALT</td>
<td>Embryonic liver fibroblast, DSB repair deficient</td>
</tr>
<tr>
<td>T24</td>
<td>Tumour derived</td>
<td>RPMI + 10% FBS</td>
<td>Tel+</td>
<td>Bladder carcinoma</td>
</tr>
<tr>
<td>WV</td>
<td>SV40</td>
<td>DMEM + 10% FBS</td>
<td>ALT</td>
<td>Fibroblast, 45 year old male, Werner syndrome</td>
</tr>
</tbody>
</table>

Table 2.1-1 Summary of all information available for all cell lines used in this study. Abreviations: DMEM: Dulbecco’s Modified Eagle Medium (1X) with GlutaMAX-I, 1g/L D-Glucose,sodium pyruvate (Invitrogen GIBCO #21885-025) was used for all cell lines except NT2D1, which was cultured in high glucose (4.5g/L) DMEM without sodium pyruvate (Invitrogen GIBCO #61965-026); FCS: fetal calf serum; NEAA: non-essential amino acid (Invitrogen GIBCO); pen/strep: penicillin/streptomycin, 1X=100U/100µg/ml (GE Healthcare PAA) * WI-38 VA13/2RA is referred to as WI3ALT herein.
2.1.6 DNA/protein pellet preparation

Both DNA and whole cell extracts were derived from cell pellets collected in the following manner. Cells were trysinised and pelleted at 1400 rpm for 5 minutes in a Sorvall Legend RT benchtop centrifuge. Media was removed, cells resuspended in 1X PBS and centrifugated 1400 rpm for 5 minutes. PBS was removed and the pellet snap-frozen in dry ice or liquid nitrogen before storage at -80°C.

2.2 Genomic DNA extractions

2.2.1 DNA extractions

Small cell pellets (1x10^5-5x10^6 cells) were thawed quickly at room temperature and resuspended in 50μl of 1X SSC (see Appendix A). 50μl lysis buffer (100mM Tris-HCl pH7.5, 100mM NaCl, 10mM EDTA, 1% Sarkosyl) was added and lysates were incubated at room temperature for 20 minutes with the addition of RNase (Sigma), FC 10μg/ml. Lysates were then incubated at 55°C for 6 hours with addition of Proteinase K (Sigma), FC 100 μg/ml. 100μl phenol:chloroform:isoamyl alcohol (25:24:1) was added to Qiagen MaxTract High density 1.5ml phase-lock tubes, followed by lysates and mixed thoroughly. Tubes were centrifugated at 4000 rpm for 5 minutes, and the aqueous phase transferred to a clean tube. 20μl (0.1X lysate volume) 3M sodium acetate (Invitrogen) and 1μl Invitrogen Pellet Paint were added and mixed gently, and 100% ethanol poured gradually onto the surface of the samples. Lysates were left at room temperature for 10 minutes to allow DNA precipitation. Samples were gently inverted to spool the DNA pellet and 100% ethanol removed. Pellets were then washed in 80% ethanol before removing ethanol and drying at room temperature for 15 minutes.

For large pellets (>5x10^6 cells), the above volumes were doubled or scaled up appropriately. 2M sodium acetate was used and Pellet Paint was excluded from the precipitation step. DNA pellets were dissolved in a volume of H2O appropriate to
pellet size (20-100μl) and rotated overnight at 4°C to aid resolubilisation. Quantity and quality of genomic DNA was confirmed using NanoDrop 1000 (Thermo Scientific) and for STELA by electrophoresis of an aliquot of the sample on a 1% agarose gel with 200ng λ high molecular weight marker.

**2.2.2 Hirt DNA extractions**

~5x10^6-1x10^7 cells were trypsinised and washed in PBS as in section 2.1.6. Pellets were resuspended immediately after a PBS wash in 1ml Hirt buffer containing 0.6% SDS and 10mM EDTA pH7.5, and incubated at room temperature for 20 minutes. 5M NaCl was added to FC 1M and lysates were rotated overnight at 4°C. The next day, lysates were centrifuged at 18,000rpm at 4°C. The supernatant was collected and 1ml phenol:chloroform:isoamyl alcohol (25:24:1) was added to Qiagen MaxTract High density 1.5ml phase-lock tubes, followed by lysates and mixed thoroughly. Tubes were centrifugated at 4000 rpm for 5 minutes, and the aqueous phase transferred to a second MaxTract phase-lock tube and phenol:chloroform extraction repeated in order to remove excess NaCl. 50μl (0.05X lysate volume) 2M sodium acetate was added and mixed gently, and 100% ethanol poured gradually onto the surface of the samples. Lysates were left at room temperature for 10 minutes to allow DNA precipitation. Samples were gently inverted to spool the DNA pellet and 100% ethanol removed. Pellets were then washed in 80% ethanol before removing ethanol and drying at room temperature for 15 minutes. DNA pellets were dissolved in a 100μl H₂O rotated overnight at 4°C to aid resolubilisation, before restriction digests and dot blotting (see section 2.8.2).

**2.3 Single Telomere Length Analysis (STELA)**

STELA is a PCR-based approach developed for this purpose and allows sizing of individual telomere lengths of specific chromosome ends in a population. Details can be found in section 3.1.1.
EcoRI (NEB) restriction digests were carried out at 37°C for 2 hours to fragment genomic DNA in reactions of 20μl containing 40U enzyme and 1X NEB EcoRI restriction enzyme buffer. Samples were then re-quantified using a NanoDrop 1000 spectrophotometer and diluted to 10ng/μl before use in STELA PCR reactions.

STELA PCRs were carried using a method adapted from Baird et al. 2003. In a specifically allocated PCR hood, digested genomic DNA was incubated with 0.9μM Telorette 2 primer for 20 minutes prior to diluting in 1ng/μl tRNA carrier solution to a DNA concentration between 2.5ng-10ng/μl. PCR reactions were set up as master mixes before aliquoting into a 96-well plate. Water controls were included for each primer set used for amplification. Each 10μl reaction contained 0.01μM teltail primer, 0.3μM subtelomere-specific primer, 0.9μl 11.1X PCR buffer (see Appendix Table 1), 0.1μl Taq (KAPA Biosystems): PWO (Genaxxon) (5:1, FC 0.5U and 0.05U respectively), 10ng tRNA and 1μl DNA/tRNA/Telorette 2 mix (final DNA concentration of 250pg-1ng). See Table 2.14-1 for primer sequences.

Initially, PCR was conducted on digested DNA samples at different concentrations to optimise the number of bands produced by STELA so as not to over-crowd lanes or leave lanes too sparse. Loading dye (0.1X TBE (see Appendix A), 6% glycerol, 0.1mg/ml bromophenol blue) was added to each well. Products were run overnight at 120V on 40cm 0.8% HGT agarose gels in 0.5X TBE with 100ng Fermentas GeneRuler™ 1Kb DNA ladder and 100ng Fermentas GeneRuler™ High Range DNA ladder until the largest band of the marker ran 6cm from wells. Gels were imaged using Gene Genius Bio Imaging System and GeneSnap software from SynGene to confirm marker running and absence of smears, before trimming to 28-30cm in length and Southern blotting (section 2.7).

2.3.1 PCR cycling conditions

STELA PCRs were carried out in Applied Biosystems Veriti 96-well thermal cyclers under the following conditions:
12q: 32 cycles of 20 seconds at 96°C, 40 seconds at 66°C, 10 minutes at 68°C. 1 cycle of 10 minutes at 68°C.

XpYp: 32 cycles of 20 seconds at 96°C, 40 seconds at 64°C, 10 minutes at 68°C. 1 cycle of 10 minutes at 68°C.

17p: 32 cycles of 20 seconds at 96°C, 40 seconds at 65°C, 12 minutes at 68°C. 1 cycle of 12 minutes at 68°C.

2.4 MS32 analysis

PCR was carried out on undigested genomic DNA. Each reaction contained 0.2μM primers MS32B and MS32E, 1X PCR buffer (see Appendix A), 12.5mM Tris, 1ng/μl high molecular weight salmon sperm DNA and 0.06ul of Taq (KAPA Biosystems):cloned Pfu (Stratagene), (20:1 ratio, FC 0.3U and 0.015U respectively).

2.4.1 PCR cycling conditions

PCRs were carried out in Applied Biosystems Veriti 96-well thermal cyclers under the following conditions: 32 cycles of 20 seconds at 96°C, 40 seconds at 62°C, 10 minutes at 70°C. 1 cycle of 1 minute at 56°C, 10 minutes at 70°C

Products were size separated by electrophoresis overnight in a 20cm 1% agarose gel before Southern Blotting (section 2.7).

2.5 DSN overhang assay

2.5.1 Digests

The Duplex Specific Nuclease (DSN) exhibits extremely specific preference for digest of double-stranded DNA while leaving single stranded DNA intact (Shagin et al., 2002). This allows near-complete digestion of double-stranded DNA but retention of single stranded telomeric overhangs, which may then be quantified as described
below. DSN assay was carried out as closely as possible to the method described by Zhao et al. 2008.

One day prior to digests, genomic DNA was diluted to 350ng/μl and incubated at 37°C in a water bath overnight to aid complete solubilisation and accurate pipetting.

Digests were set up in pairs, and prior to DSN digest one sample was digested with Exol. Exol degrades DNA in the 3’-5’ direction, digesting the 3’ overhang, allowing this sample to be used as a control for background signal in the hybridisation analysis. Experiments were set up as follows: DSN digest (Experiment): 5μg DNA, 1.7μl Exol buffer (Fermentas), H₂O to 17μl. DSN Exol double digest (Control): 5μg DNA, 10U Exol enzyme (Fermentas), 1.7μl Exol buffer (Fermentas), H₂O to 17μl.

Digests were incubated for 1 hour at 37°C in a water bath before the addition of 0.2U DSN enzyme and 2μl DSN 10X master buffer and a further incubation at 37°C in a water bath, for 2 hours. Reactions were halted by the addition of 0.5μl 0.5M EDTA pH8 and retained on ice until loaded onto an acrylamide gel.

Activity of DSN was previously confirmed as per manufacturer’s instructions by carrying out restriction digest on sample plasmid DNA supplied with the enzyme. DSN enzyme and all related buffers supplied by Evrogen.

### 2.5.2 Gel Electrophoresis

Polyacrylamide denaturing gel mix was prepared containing 6% 19:1 accugel acrylamide 40% (National Diagnostics), 1 X TBE, 7.7M ultra-pure urea (Serva).

Gel mix was filtered via vacuum through 3MM Whatman paper and stored at 4°C, protected from light.

Gels were cast in 16x16cm plates using the Protean II Xi system (Bio-Rad). Prior to casting the gel, glass plates were cleaned with SDS solution, polished with IMS and set up with 0.75mm spacers. To polymerise, 0.25μg/ml APS and 1ul/ml TEMED were
added to the gel mix, which was then poured into the plate set-up and a 15-well comb (Bio-Rad) inserted.

Once set and immediately prior to loading samples, wells were flushed with water and excess urea and water removed with 3MM Whatman paper strips.

Meanwhile, samples were prepared by addition of half the sample volume of formamide denaturing loading buffer (90% formamide, 0.01M EDTA pH8, 0.1μg/ml w/v Xylene cyanol, with or without 0.1μg/ml w/v bromophenol blue) and heated at 65°C in a heat block for 5 minutes before loading into dry wells. Oligonucleotide sequences for TTAGGG(n) markers are given in Table 2.5-1. Wells and Protean II Xi cell were topped up with 1 X TBE running buffer and electrophoresis conducted for approximately 80 minutes or until bromophenol blue had migrated two thirds down the gel.

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<tr>
<th>Name</th>
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Table 2.5-1 Oligonucleotides used as size markers in DSN assay. All oligonucleotides produced by Sigma-Aldrich

2.5.3 Gel transfer

Two pieces of extra thick blotting paper (Bio-Rad) and a positively-charged nylon membrane (Roche) were cut to size and pre-soaked in chilled 0.5X TBE for at least 20 minutes. The gel was placed onto the nylon membrane, on top of one piece of pre-soaked (0.5X TBE) extra thick blotting paper (Bio-Rad), and covered with a second piece of pre-soaked extra thick blotting paper before being placed into BioRad Trans-Blot SD Semi-Dry transfer cell and air bubbles removed by applying an even, rolling pressure in each direction across the sandwich. Transfer was carried out at constant 400mA for 50 minutes. The membrane was then rinsed briefly in 0.5X TBE, dried at 80°C for 15 minutes and cross linked (700kJ/cm², CL-1000 UV cross-linker, UVP).
2.6 Chromatin Immunoprecipitation (ChIP)

ChIP buffers were prepared in advance and filter sterilised with 0.4μm syringe filter.

2.6.1 Cross-link and cell harvest

Cells to be harvested were cultured in 175cm² flasks to ~90% confluency. In 10ml of media, formaldehyde was added directly to adherent cells in tissue culture flasks at a final concentration of 1% (v/v) and incubated for 10 minutes at room temperature. 125mM glycine was added to halt cross-linking and the flasks were incubated at room temperature for 5 minutes. Flasks were then transferred onto ice. Cells were first washed in 10ml ice cold 1X PBS then scraped in ice-cold 1X PBS and pelleted at 800rcf for 5 minutes at 4°C in a benchtop Sorvall Legend RT centrifuge. PBS was removed and pellets snap-frozen and stored long-term at -80°C.

2.6.2 Bead preparation

One day prior to immunoprecipitation, 40μl IgG Dynabeads (Invitrogen) per ChIP reaction were aliquotted into 1.5ml microcentrifuge screw-cap tubes and pelleted on a magnetic rack (Dynamag-2, Invitrogen) and storage buffer was removed. Beads were resuspended in ChIP-PBST wash buffer (1X PBS, 0.02% Tween 20) and rotated at 18rpm on a Stuart variable-speed rotator at 4°C overnight with the relevant amount of antibody (see Table 2.16-1)

Beads were pelleted on a magnetic rack and washed once in ChIP PBST wash buffer before adding 500μl ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA pH 8.0, 16.7mM Tris-HCl pH 8.1, 150mM NaCl) with 1X protease inhibitor cocktail (PIC) (Sigma Aldrich, P8340) and 75μl cell lysate (see preparation method below).
2.6.3 Cell lysis and chromatin preparation

Initially, ChIP was validated using a two-stage lysis to isolate nucleic DNA. This involved a cell lysis step where ChIP pellets were resuspended to a concentration of \(~5\times10^7\) cells/ml in chilled (4°C) ChIP Lysis Buffer (5mM PIPES, 20mM Tris-HCl pH 8.0, 85mM KCl, 0.5% IGEPAL CA-630 IP-40) with the addition of PIC, and incubated on ice with regular vortexing to lyse cell membranes. Nuclei were then pelleted by centrifugation at 4°C, at 800rcf for 4 minutes. The nuclei were resuspended at the same dilution in chilled (4°C) Nuclear Lysis Buffer (50mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0, 1% SDS) with PIC and retained on ice before sonication (see below). However, it was found that cells did not lyse in ChIP Lysis Buffer, in accordance with a previous report (Schoppee Bortz and Wamhoff, 2011), due to the level of protein cross-linking at the cell membrane. It was also found that cell membranes did lyse in Nuclear Lysis Buffer at room temperature, but that nuclear lysis was absent or minimal (Figure 2.6:1). Intact nuclei were, however, absent from lysates after sonication. As such the lysis method was adapted and carried out as described below.

ChIP cell pellets were resuspended in Nuclear Lysis Buffer to a concentration of \(~5\times10^7\) cells/ml and incubated at room temperature for 10-20 minutes (cell-line dependent), while cell membrane lysis was monitored on an Olympus BH-2 BHS microscope. During incubation, lysates were resuspended with a 0.5mm needle and vortexed regularly to eradicate cell clumps and aid lysis. Nuclei were pelleted at 800rcf at 4°C for 5 minutes (Eppendorf 5415R microcentrifuge), resuspended in same volume Nuclear Lysis Buffer and retained on ice. Lysates were sonicated in a Diagenode standard Bioruptor on high power setting with the addition of 0.5cm of ice as per the manufacturers instructions, for 6 cyles of 30 second on/30 seconds off in 15ml Faclon tubes with a Bioruptor reflecting bar.
In order to verify fragment size, 20μl lysate was reserved for gel confirmation (see section 2.6.3.1). Lysates were then pre-cleared by centrifugation to aid removal of background signal. Lysates were centrifuged at 13K rpm at 4°C for 10 minutes and transferred to a new tube. This step was repeated once. 75μl lysate was then added to the prepared bead:antibody complexes and incubated at room temperature for 1 hour with rotation (Stuart rotator SB3).

![Figure 2.6.1](image)

*Figure 2.6.1* Brightfield images of HT1080 cells in lysis buffers. ChIP lysis buffer failed to lyse cell membranes on ice or at room temperature. Nuclear lysis buffer was able to lyse cell membranes while keeping nuclei intact. a Cells in ChIP lysis buffer after 30 minutes incubation on ice. Cells remain intact and clumped. b Cells in ChIP lysis buffer after 30 minutes incubation at room temperature. Cells remain intact and clumped. c Free nuclei in nuclear lysis buffer after 15 minutes at room temperature. Cell membranes are no longer intact and nuclei are retained.

### 2.6.3.1 Gel confirmation of chromatin shearing

In order to confirm adequate shearing of chromatin to 200bp-1kb fragments, a small aliquot of sonicated material was removed from each sonicated lysate and size separated by gel electrophoresis. Lysates were first incubated overnight at 65°C with rotation with the addition of 20μl IPure purification solution (see section 2.6.5). Lysates were then purified using QIAquick PCR purification kit (Qiagen) as per the manufacturer’s instructions and run on a 1.5% LE agarose gel with Low Molecular Weight (LMW) ladder (NEB). Gels were imaged using Gene Genius Bio Imaging System and GeneSnap software from SynGene.

### 2.6.4 Washes

Beads were washed with rotation (Stuart rotator SB3) at 4°C for 5 minutes in the following chilled (4°C) buffers in the order of: Low Salt Immune Complex Wash Buffer
(0.1% SDS, 1% Triton X-100, 2nM EDTA pH 8.0, 20mM Tris-HCl pH 8.1, 150mM NaCl), High Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2nM EDTA pH 8.0, 20mM Tris-HCl pH 8.1, 500mM NaCl) and Lithium Chloride Immune Complex Buffer (0.25M LiCl, 1% IGEPAL CA-630, 1% deoxycholic acid, 1mM EDTA pH 8.0, 10mM Tris HCl pH 8.1) in order to achieve high-stringency. These washes were followed by two 5 minute washes in TE Wash Buffer (10mM Tris-HCl pH 8.0, 1nM EDTA pH 8.0) to remove excess salts.

2.6.5 Sample purification

DNA purification was carried out using the IPure kit (Diagenode) as per the manufacturer’s instructions. Briefly, samples were incubated at 65°C with rotation overnight in provided solution to elute and reverse cross-linking of DNA:protein complexes. Dynabeads were removed and IPure magnetic beads added and incubated for 1 hour at room temperature before washes in Dynabead wash buffers. DNA was eluted in the elution buffer provided and used directly for dot blot and PCR assays.

2.6.6 Semi-quantitative PCR

PCRs were set up as 20μl reactions as follows: 1X Taq Buffer (KAPA Biosystems), 0.5mM dNTPs (0.125mM of each), 0.3mM of each primer, 1U Taq. Primer sequences may be found in Table 2.14-1. PCRs were carried out in Applied Biosystems Veriti 96-well thermal cyclers under the following conditions: 1 cycle of 1 minute at 95°C. 32 cycles (30 cycles for D4Z4) of 15 seconds at 95°C, 15 seconds at 60°C, 30 seconds at 68°C (two-step PCR was carried out for GAPDH at 95°C and 68°C). 1 cycle of 1 minute at 68°C.

2.6.7 Antibody verification

Antibody suitability for ChIP was confirmed by efficiency and specificity of the pull-down of each protein by its respective antibody. Conjugated antibody:bead
complexes were incubated with ChIP lysates for one hour, then washed briefly in ChIP PBST wash buffer. Beads were then resuspended in mild elution buffer (1% SDS, 100mM NaHCO₃) and rotated at room temperature for 2 minutes. Eluate was retained and Laemmeli buffer (see section 2.13.2) added to both the eluate (mild elution) and the beads (harsh elution). Both mild and harsh elutions were carried out in order to verify antibody binding to both the Dynabeads and the substrate. Most protein:antibody interactions should be released under mild elution conditions, allowing visualisation of the protein, while a harsh elution results in the release of the antibody immunoglobulin chains. The samples were boiled for 10 minutes before loading onto a SDS-PAGE gel. Western blotting was carried out as described in section 2.13 and the antibody used for IP was also used as the primary to verify specificity, except in the case of H3K9me3 (see section 6.2.1).

2.7 Southern Blotting

Gels were submerged and agitated in depurination solution (0.25M HCl) for 7 minutes. Gels were then rinsed in water and submerged in denaturation solution (0.5M NaOH, 1M NaCl) for 20 minutes, changing the solution at 10 minutes, with constant shaking. Gels were again rinsed before washing in neutralisation (0.5M Tris, 3M NaCl pH7.0) solution for 20 minutes (again changing solution once at 10 minutes). DNA was transferred onto 0.45μm Nylon Transfer Membrane (Osmonics), by placing the gel onto 20X SSC wetted 3mm Whatman paper that was in contact with a reservoir of 20X SSC. The membrane was placed on top, followed by two sheets of Whatman previously soaked in 6X SSC. The blotting rig was topped with paper towels and a weight and towels changed regularly for at least 5 hours to allow consistent capillary action. At this point the rig was deconstructed and the membrane was dried for 5 minutes at 80°C. DNA was cross-linked onto the membrane by exposure to UV (short wave length 254nm, 700kJ/cm²) using the CL-1000 UV crosslinker (UVP).
2.8 DNA Dot blot preparation

2.8.1 Denaturing dot blots for ChIP analysis

ChIP samples were prepared in 96-well plates. The DNA was added to each well and diluted in H₂O to standardise samples to a final volume of 10μl. 5 volumes of denaturing mix (0.5M NaOH, 2M NaCl, 25mM EDTA pH 8.0, with bromophenol blue) per sample was added. Samples were resuspended 10 times, centrifugated briefly, and incubated at room temperature for a minimum of 10 minutes. The samples were then loaded onto a pre-soaked (H₂O) 0.45μm Nylon Transfer Membrane (Osmonics), cushioned with two sheets of pre-soaked 3MM Whatman paper using a 96-well Hybri-Dot manifold (Life Technologies), drawn through by vacuum. The DNA was then washed twice with 120ul 2X SSC. Blots were dried for 10 minutes at 80°C and DNA cross-linked onto membrane with UV (700kJ/cm²).

2.8.2 Native dot blots for overhang analysis

DNA samples were partially digested with AluI (NEB), with the addition of 1X NEBuffer 4 at 37°C for three hours. Half the sample was then digested with ExoI for one hour at 37°C. Both ExoI digested and undigested samples were prepared for loading by direct addition of bromophenol blue and loaded onto 0.45μm Nylon Transfer Membrane (Osmonics) as above. Two 150μl washes with 2X SSC were carried out and blots were dried and cross-linked as above.

2.9 DNA radiolabelling

2.9.1 Random-primed labelling of DNA double stranded probes

15ng of PCR amplified probe (see section 2.9.2.1) or commercially bought ladder was initially mixed with water and denatured by boiling for 5 minutes. 6μl 5X OLB (see Appendix Table A2), 12μg bovine serum albumin (BSA), 7.5U DNA polymerase I large Klenow fragment, 1.5μl ³²P α-dCTP (0.555MBq) were added. Reactions were
incubated at room temperature overnight and recovered with the addition 50μl Oligo Stop Solution (20mM NaCl, 20mM Tris-HCl pH 8.0, 2mM EDTA pH 8.0), 30μl high molecular weight salmon sperm DNA (30mg/ml), 25μl 2M sodium acetate and precipitating in 470μl 100% ethanol. Pellets were then washed in 500μl 80% ethanol, resuspended in 500μl H₂O (1.5ml H₂O for ladders) and incubated at 96°C for 5 minutes before adding to hybridisation tubes.

**2.9.1.1 Telomere probe**

PCR reactions for the telomere probe (Teloprobe) were set up in 40μl reactions containing 1X PCR buffer (see Appendix A), 10μM Tel1 primer, 10μM Tel2 primer, 26.6μl H₂O and 4U Taq polymerase (Kapa Biosystems) (see Table 2.14-1 for primer sequences). PCR was carried out in Applied Biosystems Veriti 96-well thermal cyclers. 1ng of existing telomere probe was used as a template. PCR products were run on a 1% LE Agarose gel and products between 300bp and 12kb excised and purified using QIAquick gel extraction kit (Qiagen).

**Cycling conditions:** 15-20 cycles of 40 seconds at 96°C, 40 seconds at 65°C, 5 minutes at 70°C.

**2.9.2 Oligonucleotide probe end-labelling**

Oligonucleotides (see Table 2.1-1 for sequences) were prepared as a master mix containing 1X Kinase mix (70mM Tris-HCl pH7.0, 10mM MgCl₂, 5mM spermidine trichloride, 2mM dithiothreitol), 350U T4 polynucleotide kinase (NEB) and 0.15μl ³²P γ-dATP (55.5 kBq) per probe reaction. 9μl master mix was added to 0.14μM oligonucleotide per reaction. Reactions were incubated for 1 hour at 37°C before overnight incubation at room temperature. Reactions were stopped by addition of kinase stop solution (25mM EDTA pH 8.0, 0.1% SDS, 10μM ATP) at room temperature.
2.9.2.1 Oligonucleotides

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</table>

Table 2.9-1 Sequences of oligonucleotides used directly in end-labelling probe reactions for DSN overhang assay. All oligonucleotides were generated by Sigma-Aldrich.

2.10 Hybridisation conditions

Radiolabelled probes were hybridised to DNA-bound membranes under the following conditions:

2.10.1 STELA and MS32

Dried membranes were rinsed in water and placed between meshes in Hybaid hybridisation tubes (Thermo). Membranes were pre-hybridised for 15 minutes prior to hybridisation overnight with Teloprobe, LMW (NEB) and HMW (Thermo Scientific) marker probes for STELA, and an MS32-specific probe (derived in the Royle laboratory) and 1kb marker (Thermo Scientific) for MS32 blots. Hybridisation and pre-hybridisation were carried out in Modified Church Buffer (0.5M sodium phosphate buffer, 1mM EDTA pH 8.0, 14% SDS) at 65°C. Membranes were then washed in low stringency buffer (1XSSC, 0.1% SDS) in 15 minute intervals. Membranes were then rinsed in water, blotted dry and mounted before exposure to Amersham Phosphor Screens.

2.10.2 Native and denaturing dot blots

Pre-hybridisation was performed in TMAC hybridisation solution (3M TMAC, 0.6% SDS, 1mM EDTA pH 8.0, 10mM Na₃PO₄, 5X Denhardt’s (see Appendix A), 4ug/ul S. cerevisiae RNA (Sigma) at 58°C for 10 minutes. Hybridisation was carried out in TMAC hybridisation solution for 5 hours or overnight at 48°C with the addition of random-primed labelled probe. Washes were carried out in TMAC wash solution (3M TMAC,
0.6% SDS, 1mM EDTA pH 8.0, 10mM Na$_3$PO$_4$), with three 5 minute washes at 48-49°C and one final 15 minute wash at 50°C. Membranes were then rinsed in 3X SSC before mounting and exposing to Amersham Phosphor Screen.

2.10.3 DSN overhang assay

Pre-hybridisation was performed in TMAC hybridisation solution (3M TMAC, 0.6% SDS, 1mM EDTA pH 8.0, 10mM Na$_3$PO$_4$, 5X Denhardt’s (see Appendix A), 4ug/ul yeast RNA) at 58°C for 10 minutes. Hybridisation was carried out overnight with the addition of random-primed labelled telomeric oligonucleotide probes at 48°C for 18-21mer probes, 25°C for the 12mer probe and end-labelled probes LMW marker (NEB). Three washes were carried out at 48-50°C in TMAC wash solution (3M TMAC, 0.6% SDS, 1mM EDTA pH 8.0, 10mM Na$_3$PO$_4$) for 18-21mer probes, 28°C for 9-12mer probes. Final 15 minute washes were at 52°C for 18-21mer probes, 30°C for 9-12mer probes. Membranes were then rinsed in 3XSSC before mounting and exposing to Amersham Phosphor Screen.

2.11 Analysis and quantification of hybridisation assays

2.11.1 STELA

Blots were exposed to Amersham Phosphor Screens and imaged using the Typhoon 9400 Variable Mode Imager (GE Healthcare). Image Quant Gel Imager 2D gel software (GE Healthcare) analysis calculated lengths of individual bands from each STELA according to size markers. Flanking sequence length was deducted before further analysis using GraphPad Prism 6.0.

2.11.2 Dot Blots

Blots were exposed to Amersham Phosphor Screens and imaged using the Typhoon 9400 Variable Mode Imager (GE Healthcare). Gel Imager array software analysis
provided intensity readings for each dot. These values were internally normalised to a single dot from the input series before comparison.

2.11.3 DSN overhang assay

Standardising to a commercially available size marker, quantification was carried out as previously described (Chai et al., 2005; Zhao et al., 2008). An example experiment is shown for reference in Figure 2.11:1. After exposure to a Phosphor Imaging Screen, the gel image was analysed using Image Quant array analysis software (GE Healthcare). A column containing 100 boxes was overlaid on each of the experimental and control lanes, as well as the marker lane, and numbered 0 to 100 (Figure 2.11:1a). Marker positions were manually assigned to a box position (to 1/10th of a box) and the size at the centre of each box was interpolated using Graphpad Prism6 (Figure 2.11:1b).

Each box was then assigned this size and was plotted against the signal intensity as recorded by Image Quant in each box for the control and experiment lanes (Figure 2.11:1c). The intensity of the control lane was subtracted from the intensity of the experiment lane for each box to give an ODi value for each box (Figure 2.11:1c). As proportionally more probe binds to larger fragments, each value was divided by the size assigned to that value, giving an OD/Li, that is a relative value normalised to size.

Due to background at larger sizes, Zhao et al. suggested excluding larger fragments above 400nt, and an upper limit of 500nt was initially assigned. As sizing became impractical at the smallest sized fragments, a lower limit of 20nt was also assigned. Results of this example are shown in Figure 2.11:1d.
Figure 2.11: Quantification of DSN method

a) Example gel image with and without the sizing grid overlay (C=control, E=experiment), LMW marker is shown with high and low contrast to facilitate visualisation.

b) Standard curve used for sizing each grid-box.

c) Control, experiment, and experiment minus control results (ODi).

d) ODi/Li results for signal between 50 and 500 nt. Line is Gaussian fit ($r^2 = 0.8163$, mean=49.30).
2.12 Subtelomere genotyping

2.12.1 12q

Fragments from the 12q telomere-adjacent region were first amplified from genomic DNA using the 12q sub-telomere specific primer pair 12qA and 12qB (see Table 2.14-1 for primer sequences). PCR reactions contained 0.9μl 1X PCR buffer (see Appendix A), 3μM each primer, 2.5U Taq (KAPA Biosystems). PCRs were carried out in Applied Biosystems Veriti 96-well thermal cyclers. These products were then sized and quantified on a 1.5% gel before re-amplification in a secondary PCR containing the same amounts of PCR reagents.

**Cycling conditions**: 1 cycle of 1 minute at 96°C. 32 cycles of 15 seconds at 96°C, 30 seconds at 61°C, 1 minute at 68°C. 1 cycle of 5 minutes at 68°C.

Restriction digests were carried out as described by Baird et al. (2000) with each of the restriction enzymes illustrated in Figure 2.12:1. Restriction digests were incubated for one hour with the appropriate 1X restriction enzyme buffer and at the temperature indicated in Table 2.12-1. Restriction enzymes and buffers supplied by NEB. Products were separated by gel electrophoresis in 1.5% LE agarose gels (BsmI and KpnI) or 3.5% NuSieve agarose (AciI). In addition to the restriction analyses, a larger fragment was amplified using primers KSRV2.D and 12qB under the same

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**Figure 2.12:1** Position of informative restriction sites and the 1440bp deletion at the 12q subtelomere. Starting position of primers are also shown (bp)
conditions to confirm the presence or absence of a large deletion reported to be present in 6% of the population (Baird et al., 2000). All digests were first carried out on CEPH DNA controls whose haplotypes were already established to confirm experimental procedure.

2.12.2 Xp/Yp

PCR reactions for XpYp genotyping were set up as described for 12q, using the primers Tsk8C and Tsk8G (Table 2.14-1).

**Cycling conditions:** 1 cycle of 1 minute at 96°C. 32 cycles of 40 seconds at 96°C, 50 seconds at 63°C, 1 minute at 68°C. 1 cycle of 5 minutes at 68°C.

Restriction digests were carried out at described by Baird et al. (1995) using the appropriate 1X NEBuffer and incubation temperature indicated in Table 2.12-1 and resolved on 3.5% NuSeive agarose gels. All digests were first carried out on CEPH DNA controls whose haplotypes were already established to confirm experimental procedure.

![Diagram of Xp/Yp subtelomere with restriction sites and primer positions](image)

**Figure 2.12.2** Position of informative restriction sites at the XpYp subtelomere. Starting position of primers are also shown (bp).

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<td>37°C</td>
<td>37°C</td>
<td>37°C</td>
<td>65°C</td>
<td>37°C</td>
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</table>

*BSA added to FC 100μg/ml

**Table 2.12-1** Respective NEBuffers and temperatures used for genotyping assay restriction digests.
2.13 Western Blotting

2.13.1 Gel preparation

SDS-PAGE gels were prepared by hand using Bio-Rad plates and manifolds (10 wells per gel), and the percentage of acrylamide used was adapted for size of protein to be analysed (Figure 2.13:1). Gels were prepared as follows: Separating gel: 6-15% Acrylamide (Protogel (30%), 37.5:1 Acrylamide:Bisacrylamide, National Diagnostics), 0.21M Tris-HCl pH8.8, 0.1% SDS, 0.001% ammonium persulphate (APS); stacking gel: 4% Acrylamide, 0.24M Tris-HCl pH6.8, 0.1% SDS, 0.003% APS. Tetramethylethylenediamine (TEMED) was added to a final concentration of 0.0008% to polymerise gels.

\[
\begin{align*}
\text{α-tubulin (50)} & & \text{β-tubulin (55)} \\
10 & 50 & 100 & 150 & 200 & 250
\end{align*}
\]

- 12% HP1-α (25)
- 15% H3K9me3 (17)
- 7.5% Lamin C (62) SUN2 (80)
- 10% RAP1 (45)
- 6% Lamin B (67) SUN1 (90)
- 53BP1 (220-250)

*Figure 2.13:1* Diagram indicating the percentage of acrylamide used in SDS-PAGE gels for proteins within the range of sizes indicated. Sizes of proteins used in this study are indicated after the protein name in brackets. α- and β-tubulin were electrophoresed on gels containing 7.5-12% acrylamide. Details of antibodies used may be found in Table 2.16-1

2.13.2 Electrophoresis conditions and transfer

Lysates were diluted in Laemmlli loading buffer, FC: 63 mM Tris HCl, 10% Glycerol, 2% SDS, 0.0025% Bromophenol Blue, 2.5% β-mercaptoethanol. Typically 10ul of
sample was loaded per well with 5ul PageRuler Plus Prestained Protein Ladder (Fermentas). Samples were boiled for 10 minutes for the first use, 2-5 minutes on subsequent uses. Electrophoresis was carried out at 100V until loading dye passed the stacker:running gel interface then at 150V until migration of the Protein Ladder bands indicated adequate separation at the desired size. Amersham Hybond ECL Nitrocellulose membrane (GE Healthcare) was pre-soaked for 10 minutes in 1X transfer buffer (25mM Tris, 192mM glycine, 20% methanol), or in the case of 53BP1, membrane was pre-soaked in transfer buffer excluding methanol and with the addition of 0.01% SDS. Gels were removed from plates and soaked in transfer buffer for a further 10 minutes. Sandwiches were constructed as follows: 3 layers of 3MM Whatmann paper (soaked in 1X transfer buffer), Nitrocellulose membrane, SDS-PAGE gel, 3 layers of soaked 3MM Whatmann paper. Transfer was carried out using the BioRad Trans-Blot SD Semi-Dry transfer cell at constant ampage of 1mAmp/cm². Membranes were stained with Ponceau S solution (Sigma) to verify transfer before air-drying. Membranes that were not to be used immediately were stored at 4°C.

2.13.3 Immunoblotting

Membranes were blocked in 5% Marvel skimed milk diluted in 1X PBS with 0.1% Tween 20 (PBST) for one hour with persistent agitation. The primary antibody was diluted appropriately in 5% Marvel/PBST (see Table 2.16-1) and added to the membrane for a minimum of 1 hour. After an initial rinse in PBST, membranes were washed 3 times for 5 minutes in PBS/Tween. Secondary antibodies were diluted 1/10,000 in Marvel/PBST and added to the washed membrane for a minimum of 45 minutes. Washes were repeated and blots developed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) in accordance with manufacturer’s instructions. Blots were immediately exposed to Kodak X-ray film and developed.
2.13.4 Quantification

Films were scanned in full colour and converted to 8-bit in ImageJ. ImageJ software was used to derive a histogram of the intensity of each band and a relative intensity value. Values of target proteins were normalised to the values of a control on the same blot wherever possible. In order to accurately compare the normalised values across different blots, initial values were first normalised to the total signal contained in one blot for each protein immunoblotted.

2.14 Immunofluorescence

Cells were grown to a confluency of 50-70% (cell line-dependent) on 13mm glass coverslips previously acid treated in 1M HCl and sterilised. Cells were then fixed in cold 100% methanol (-20°C) and incubated at -20°C overnight.

Coverslips were washed 3 times in PBS and incubated for 1 hour in 3% BSA in PBS. Coverslips were then incubated for 1 hour in 3% BSA-PBS containing primary antibody at antibody-specific concentration (see Table 2.16-1). Coverslips were then washed three times in PBS with a final wash with agitation for 5 minutes before addition of secondary antibody at a dilution of 1/1000 in 3% BSA-PBS. Washes were repeated as before and coverslips were mounted on microscope slides with Prolong Gold mountant (Life Technologies). DNA was stained with the addition of DAPI to either the secondary antibody or to Prolong Gold. Fluorescence detection screening and analysis was carried out on the ScanR platform. Nuclear periphery was defined by ± 3 pixels from the edge of the DAPI stain as detected by the software.
### 2.15 Primers

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<tr>
<th>Name</th>
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<td>Teltail</td>
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<td>Tel2</td>
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*Table 2.14-1* Sequences of primers used in all PCRs
## 2.16 Antibodies

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Table 2.16-1 Primary antibodies used in Western blotting (WB), immunofluorescence (IF) and chromatin immunoprecipitation (ChIP). Dilutions used for WB and IF, and amount of antibody used in each ChIP are given.

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Table 2.16-2 Secondary antibodies used in Western blotting (WB) and immunofluorescence (IF). IgG=immunoglobulin G, HRP=horseradish peroxidase.
PART A • Telomere shortening in progeria cell lines

Chapter 3   Telomere shortening rate in progeroid laminopathy cell lines established by STELA

3.1 Background

The relationship between ageing and telomeres, and the occurrence of accelerated telomere shortening in non-laminopathy based progeria syndromes, has prompted several studies aiming to identify telomere abnormalities in HGPS cells and models. These studies have yielded promising, if limited, results. Telomere length is shorter in HGPS patients compared to controls (Allsopp et al., 1992; Decker et al., 2009) but as yet, no study has directly investigated telomere shortening rate in patient cells. This is most likely because of the small amounts of genetic material available due to the difficulties involved in culturing HGPS cells.

The objective was therefore to establish telomere shortening rate in HGPS dermal fibroblast cells. In addition to a classical HGPS line, telomere attrition rate was also recorded in three other progeroid laminopathy fibroblast lines in order to identify any correlation between altered telomere dynamics and the nature and positioning of differing lamin A mutations. The lengths of two telomeres (12q and XpYp) were measured at various time-points throughout the proliferative lifespan of each cell line. In order to establish an accurate rate of telomere shortening, the mean number of population doublings (PDs) between each passage was counted. Telomere shortening rate was then calculated relative to PD.
3.1.1 Measuring telomere length: STELA

Several methods exist to record telomere length in cultured cells. The first to be established, and the most commonly employed, is Terminal Restriction Fragment (TRF) analysis (Moyzis et al., 1988). TRF was the first method used to demonstrate telomere shortening in cultured fibroblasts (Harley et al., 1990). The technique involves restriction digestion of relatively large amounts of genomic DNA followed by gel electrophoresis and Southern blot hybridisation to a telomeric probe. This identifies telomere fragments as a smear which may be analysed to give a mean telomere length. Telomere lengths are extremely heterogeneous, however, and large variability can be seen even within a single cell type from a single individual (Harley et al., 1990; Moyzis et al., 1988; Weng et al., 1997). Although a robust technique, this bulk measurement of telomeres precludes the identification of any subtle population dynamics that may be present. It is also not possible to accurately identify short telomeres that may be responsible for senescence induction.

A similar problem arises with the use of Q-PCR. Due to the repetitive nature of telomeric DNA, it is necessary to employ a specialised approach to allow PCR amplification. The method described by Cawthon (2002) uses a primer pair with enough homology to telomeric sequence to anneal to telomeres, but differing from one another to such an extent as to avoid primer-dimer formation (Cawthon, 2002). Q-PCR only allows quantification of telomeric sequence relative to a control locus, rather than length, and results are not comparable between separate PCR runs.

An alternative that allows for individual measurements of telomeres is quantitative fluorescent in situ hybridisation (Q-FISH), which measures telomere length on metaphase spreads (Egholm et al., 1993; Nielsen et al., 1991). By employing a fluorescently labelled peptide nucleic acid (PNA) specific for telomere repeats, a highly stable PNA:DNA hybridisation (Lansdorp et al., 1996)
allows visualisation of all telomeres for quantification. Because Q-FISH identifies single telomeres, individual chromosome dynamics have been revealed (Martens et al., 1998), and the technique is ideal for documenting telomeric aberrations (Hande et al., 1999). Q-FISH is a highly informative approach that generally requires small amounts of starting material, however the starting cells must have the potential to form metaphase spreads. Q-FISH quantification may also be carried out through the use of fluorescence-activated cell sorting (FACS). However, due to the high-throughput nature of this flow-FISH technique, similar issues arise as with Q-PCR, in that relative telomeric sequence, rather than length is measured. Although standard Q-FISH may be highly informative, HGPS cells have an extremely high level of senescent and slowly-dividing cells, excluding the possibility of harvesting metaphase spreads, particularly at the end of proliferative lifespan.

As such, Single Telomere Length Analysis (STELA), a highly sensitive method that is well-established in the Royle laboratory, was selected. STELA exploits known and unique sequences from the subtelomeric regions of a limited number of chromosome arms (2p, 11q, 12q, 17p and XpYp (Britt-Compton et al., 2006) - so restricted due to the large sections of homology between

Figure 3.1.1 The distal end of the telomere. A STELA chromosome-specific primer anneals to a unique subtelomeric sequence at a specific chromosome arm. Telorette 2 anneals in line with the 5' strand and is the template for the first round of PCR. This product contains the complementary sequence of the second primer, Teltail. In subsequent PCR cycles, Teltail engages the template and initiates elongation.
subtelomeres (Mefford and Trask, 2002). This allows telomere-specific amplification. Figure 3.1:1 is a schematic representation of STELA-PCR. The forward primer anneals within the subtelomeric region of the specified chromosome arm. In lieu of a single reverse primer, a set of two partially complementary primers are used. Initially, the first primer, Telorette, anneals to the 3’overhang at its complementary 3’ end sequence. The 5’ end of the Telorette contains a unique sequence, identical to a second primer, Teltail. At the first round of PCR the G-rich 3’ end products are essentially tagged with this sequence and in subsequent rounds of PCR the Teltail primer may initiate elongation (Baird et al., 2003).

The products of STELA therefore represent individual telomeres from a population. Gel electrophoresis of PCR products and Southern blot hybridisation with a telomere-specific probe allows a direct comparison of PCR products with size markers in order to establish length. The method may also be used to amplify specific alleles in instances where single nucleotide polymorphisms (SNPs) have been identified within regions of chromosome-specific sequence, allowing allele-specific primer design.

STELA does have drawbacks and is a labour-intensive technique. As a PCR-based approach, extremely large telomeres (>15-20kb) may not be as effectively amplified. Although STELA requires only small amounts of DNA, the DNA must be of extremely high quality to ensure retention of an undamaged 3’ overhang as it is the required substrate for the Telorette primer. Therefore the use of columns and centrifugation is omitted from DNA extraction to avoid mechanical force shearing, and to avoid co-precipitation of contaminants which may affect PCR efficiency. The DNA pellet must therefore be visible and manually isolated after precipitation. The minimum number of cells required for a successful STELA DNA extraction was therefore established as ~5x10^5 cells prior to growing patient cells. Despite this, STELA provides extremely high
resolution, allowing visual identification of allelic subpopulations without the use of allele-specific primers (Baird et al., 2003), and so has the potential to distinguish subpopulation dynamics, including short telomeres that may be responsible for senescence induction.

As such, STELA provides a sensitive and informative assay for the measurement of telomere length from early and late proliferative stages, as well as insight into individual telomere dynamics at the single cell level.

### 3.1.2 Telomere length in progeria cell lines

It has been previously reported that the telomeres of HGPS lines are shorter than those of age-matched control lines (Allsopp et al., 1992). The first study into HGPS telomere length, carried out in 1992 by Allsopp et al., employed TRF analysis. This study gave the mean telomere length of five patients in comparison with age matched controls. A similar comparative approach using Q-FISH was carried out in 2009 (Decker et al., 2009) on three HGPS cell lines. This study demonstrated that two out of three patient lines contained shorter telomeres than normal controls. Although this study had the benefit of identifying specific telomeres, the length analysis was only carried out at one time point for all lines, and at greatly varying PDs (PD 17, 35 and 7). The HGPS line that did not show significantly differently sized telomeres from controls (AG11498) was analysed at PD 7. If the rate of telomere erosion is affected in HGPS cells, longer telomeres at early sampling could represent the norm, and the abnormally short telomeres recorded at later PDs in the other patient lines may be a result of accelerated telomere erosion.

Because both these studies were carried out at only one time point, it is not possible to determine whether short telomeres are the result of a developmental setback, i.e. telomere length is initially short in these children, or whether telomere shortening rate is increased in HGPS cells. Huang et al. (2008)
developed a fibroblast line with an excisable hTERT construct, in order to transfect mutant and wild-type lamin A forms and measure shortening rate in a normalised background lacking the disadvantages of patient cells. Cells over-expressing mutant forms of lamin A displayed an increased rate of shortening as measured by average telomere length from Q-PCR. Although this approach benefited from an isogenic starting point, ectopic expression of a transfected mutant form of lamin A in the presence of endogenous lamin A resulted in considerable over-expression which could affect the representativeness of the results derived from this study.

3.1.3 Cell lines

A HGPS fibroblast line derived from a 14 year old male, with the classical G608G, 1824C>T mutation was grown in culture. This line expresses progerin in a comparable amount to WT LA (Figure 3.1:2), in accordance with previously published data on this and several other classical HGPS cell lines (Rodriguez et al., 2009). In order to elucidate relationships between lamin A mutations and potential telomeric abnormalities, four more fibroblast lines derived from patients with various progeria-associated laminopathies were also grown.

The first, T623S, is derived from a 10 year old female with a 1868C>G mutation in LMNA that activates a cryptic splice site, as well as an amino acid change at codon 623, although the amino acid change is unlikely to be causative as the silent G608G results in a considerably more severe phenotype. The T623S mutation results in similar processing problems as classical HGPS but is located 15 amino acids further along exon 11, resulting in a 35 amino acid deletion (Δ35) which is located within the Δ50 deletion in RNA transcripts. As in classical HGPS, the resultant protein remains farnesylated due to deletion of the ZMPSTE24 recognition site, but an additional 15 amino acids are retained (see section 1.1.6 and Figure 3.1:2).
The T623S patient began to manifest minor symptoms at 6 years of age (Shalev et al., 2007) and a similarly late onset was observed in a previous 1868C>G case. This previous male patient lived to 45 years of age, by which time, after gradual deterioration, his symptoms were identical to those of classical HGPS (Fukuchi et al., 2004). These differences in onset may be due to some function conserved by the 15 amino acids missing in classical HGPS, or due to lower expression of the T623S mutant isoform (section 1.1.6).

The retention of the pre-lamin A farnesyl group has been implicated as a major contributor to the pathology of classical HGPS (Capell et al. 2005; Toth et al. 2005; Fong et al. 2006; Constantinescu et al. 2010). In order to compare a non-farnesylated mutant, a line derived from a biopsy of a 13 year old female with a heterozygous 1945A>T missense mutation at the beginning of exon 11 was also

---

**Figure 3.1.2** Position and resultant protein expression of LMNA mutations used in this study. 
(a) Western blot of lamin A/C in patient and control lines. HH is a normal human fibroblast cell line. Arrows indicate fully processed lamin A and C as well as incompletely processed forms found in the HGPS, T623S and RD lines. 
(b) Schematic of lamin A indicating position of amino acid changes in the cell lines used in this study. Regions encoding the N-terminal (NT) and C-terminal (CT) domains are indicated.
examined. This mutation results in a single amino acid change of glutamic acid to valine at position 578 and as such the line is referred to as E578V. Aberrantly spliced farnesylated products do not accumulate (Csoka, 2004; Toth et al., 2005), however, an increased proportion of E578V cells have misshapen nuclei compared to normal lines (Csoka 2004; Toth et al. 2005). FTI treatment reduces nuclear deformity and peripheral lamin A (Toth et al., 2005), supporting lamin A accumulation in the nuclear matrix as a key contributor to the HGPS phenotype. This patient exhibits relatively mild symptoms of progeria and atypical Werner’s syndrome.

A second non-farnesylated line, S143F was selected and the growth rate and senescence information was recorded. However, the limited number of PDs this line completed in culture before reaching senescence precluded it from the STELA analysis. The S143F line is derived from a 6 year old patient with early onset myopathy who developed progeria symptoms at 5 years of age (Kirschner et al., 2005). The heterozygous mutation is located in the second exon (428C>T) and does not result in aberrant splicing (Figure 3.1:2 and Kirschner et al. 2005). Although not apparent in Western blots carried out here (Figure 3.1:2), pre-lamin A may be present at low amounts in this line due to an interference with lamin A processing (Capanni et al., 2005).

A Restrictive Dermopathy (RD) line was also included in the analyses. RD is a genodermatosis caused by mutations in the ZMPSTE24 gene (see section 1.1.6). Mutations are homozygous or compound heterozygous and usually completely eradicate ZMPSTE24 proteolytic activity (Moulson et al., 2005; Navarro et al., 2013, 2004). The posttranslational processing carried out by ZMPSTE24 is essential for the formation of mature lamin A and as such, RD cells produce only pre-lamin A (Figure 3.1:2a). RD is categorised with other laminopathies for this reason, and is commonly studied as a more severe manifestation of HGPS due to overlapping symptoms. Two children with LMNA mutations, including one
with the classical G608G mutation, have been diagnosed with RD (Navarro et al., 2004).

RD was first described in 1986, characterised by an extremely severe cellular and physiological phenotype and neonatal death (Witt et al., 1986). After pregnancy issues and premature birth, babies exhibit growth retardation, tight, thin skin that is easily eroded and limited joint manoeuvrability. It is possible that the differential symptoms of RD that contribute to the severity of the disorder arise due to the complete absence of mature lamin A or a deficiency in ZMPSTE24 activities not related to lamin A processing. The cell line used in this study was derived from a patient carrying a homozygous 1085-1086insT in the ZMPST24 gene. This mutation is the most common in RD (Moulson et al., 2005; Navarro et al., 2005) and results in a premature transcriptional stop and no enzyme functionality.

Three normal skin fibroblast lines derived from healthy individuals were used as controls: Normal Human Diploid Fibroblast (NHDF), 155-BR and 161-BR, see Table 2.1-1).

3.2 Results

3.2.1 Growth rates and senescence levels

Cell lines were grown under ambient O₂, 5% CO₂ and with high humidity. All the patient cell lines were grown in DMEM media supplemented with 20% FCS and 100U/μg penicillin/streptomycin per ml media. The NHDF line was cultured in 10% FCS-supplemented DMEM, with 100U/μg penicillin/streptomycin per ml media. The 151-BR and 161-BR line were grown without antibiotics, with 15% FCS supplementation in DMEM, and 1% non-essential amino acids were added to the 155-BR medium. Patient cells generally grew very slowly (in some cases less than 1 PD every 5 days at early PDs) and splits were limited to 1:2. Faster
growing cell lines which divided roughly once every 24 hours, such as NHDF and E578V, were split 1:2-1:4. Cell counting was employed to establish population doublings (PDs). All cell lines were cultured from the lowest available PD from lab stocks and were grown to senescence where possible (i.e. cell number did not significantly increase after one week and/or senescence was recorded at >95%). Trypan Blue staining was employed to establish viability levels of all cell lines. Non-viable cells were counted and then discounted from all calculations.

Patient cells exhibited a correlation between replicative capacity and phenotypic severity (Figure 3.2:1a). All cell lines maintained a level of 95-100% viability throughout their proliferative lifespan, excluding 155-BR and HGPS which displayed a sudden drop in viability at their final passage (80.5% and 84.5% respectively), accounting for the variability in viability in these lines (Figure 3.2:1b). These data suggest that LMNA mutations do not have an effect on overall cell viability. Cell lines derived from patients with the most severe phenotypes tended to grow more slowly throughout their cultured lifespan than

Figure 3.2:1 Final PD and viability of each cell line a Final PD reached by each cell line cultured, excluding NHDF, which did not reach senescence. The HGPS value given is the average of two separate rounds of subculturing b Mean viability of patient and control lines which were carried forward to the STELA analysis, derived from Trypan Blue assays carried out throughout the proliferative lifespan of all cell lines.
cells derived from patients with milder symptoms (Figure 3.2:2). The normal cell lines that were passaged alongside the patient lines showed varying rates of growth. The NHDF line was abandoned prior to its reaching senescence due to contamination. However, senescence would be anticipated to halt proliferation at around 60 PDs (based on previous work in the lab as well as Suzuki et al. 2012), and at 35PDs the line still retained a largely actively dividing population of ~80% (Figure 3.2:3). Unexpectedly, 161-BR reached senescence at only 15.5 PDs (Figure 3.2:3b&c). As 161-BR is derived from a healthy 49 year old male, it is likely that at the time this cell line went into culture, the number of PDs recorded was greatly underestimated, perhaps as a result of the initial derivation of the line or erroneous record-keeping. The 155-BR also fell short of its anticipated growth potential, reaching only ~30PDs before reaching senescence. The HGPS, RD and T623S cell lines failed to divide beyond around 20 PDs, in accordance with previous reports (Benson et al., 2010; Goldman et al., 2004; Liu et al., 2006; Shumaker et al., 2006). The S143F cell line (mild progeria) grew to 35PDs, while E578V (very mild progeria symptoms), continued to divide to around 50 PDs, which is within the range of the expected lifespan of a normal fibroblast line (Hayflick, 1965).

Throughout the cultured lifespan of the patient and control cell lines, β-galactosidase senescence assay was carried out. This method exploits the specific increased abundance of β-galactosidase associated with an increased lysosomal function present only in senescent cells. Upon the addition of X-gal substrate in sub-optimal conditions (pH6 rather than pH4), the excessive amount of β-galactosidase present in senescent cells is able to cleave X-gal, which appear blue. Presenescent and quiescent cells remain unstained (Dimri et al., 1995).

In all cell lines, an increase in β-galactosidase staining correlated with a decrease in rate of division (Figure 3.2:2 and Figure 3.2:3). As expected, the
HGPS line displayed a high proportion of senescent cells. Even while growth rate remained steady, the HGPS population had a consistently higher proportion of β-galactosidase positive cells, with ~40% of cells senescent between the first PD analysed and ~PD 20 (Figure 3.2:2a). Despite the severity of RD, up to 89% of the cell population were not senescent in earlier passages (Figure 3.2:2b). However, this level drops to ~60% at around PD 13 – a similar time-point to the starting PD for HGPS. As the RD cell line was available at an earlier PD than HGPS, and the first recordable senescence data for the HGPS line was at roughly PD13, it is possible that younger HGPS cells may also have have a lower initial proportion of senescent cells.

**Figure 3.2:2** Patient cell lines were cultured from the lowest PD available to senescence except in the case of E578V which was initially cultured to around 80% senescence, then another frozen stock was recovered and grown to complete senescence. Growth experiment was repeated in full for HGPS. The second experiment is indicated on for both E578V (e) and HGPS (a) by a dotted line.
The other patient lines exhibited low initial numbers of senescent cells (~20%), similar to levels in the normal lines NHDF and 155-BR. The high proportion of β-galactosidase positive cells present in 161-BR confirm the impression that 161-BR was in fact much closer to senescence than was recorded prior to the cells being thawed. Due to the small number of pellets that it was possible to harvest from 161-BR over less than five PDs, it was excluded from STELA analysis. The 155-BR line grew unusually slowly in culture for almost 15 PDs before the proportion of senescent cells began to increase (Figure 3.2:3). Although it is possible that the divisional “age” of the cell line was also underestimated at the point of thawing, the senescence data, in combination with the number of PDs completed by 155-BR before senescence levels elevated, indicate that the slow growth rate, and potentially the early entry into senescence, genuinely reflect the biology of this line. This implies a potential similarity to the laminopathy lines in terms of growth capacity (discussed in section 3.3.3).

β-galactosidase assay is not without pitfalls. The required pH of exactly 6.0 proves to be difficult to confirm due to the limitations of equipment accuracy.
Also, differences between cell lines may affect the optimal pH for observing only senescence-associated activity. As such, data accrued from cell passaging experiments is slightly erratic in places and if it is assumed that β-galactosidase activity decreases in a linear or exponential manner, from the data acquired it can be inferred that the error rate is as much as ±10%. However, the β-galactosidase data collected does demonstrate observable trends for all cell lines.

3.2.2 Telomere shortening in laminopathy and control fibroblasts

STELA was carried out on DNA derived from samples taken at various time-points for four patient lines and two normal lines. Representative Southern blots for each cell line are shown in Appendix B.1. Each gel was run with a control DNA (extracted at one time point from a single cell pellet of HT1080 cells) in order to establish technical reproducibility of PCR and size separation. HT1080 bands were sized on each gel and the distributions were statistically tested in order to confirm no significant difference before pooling data collected from separate gels for each cell line. Data for all HT1080 bands is given in Appendix B.2. STELA primers are positioned at a set distance from the beginning of the telomere and so the flanking sequence was subtracted from sized bands prior to regression analysis using Graphpad Prism6 to establish a shortening rate at each end. A reduction in the median length of telomeres over time was observed in all but the NHDF line.

3.2.2.1 NHDF

The Normal Human Diploid Fibroblast cell line, NHDF, is derived from a healthy adult male. Problems in tissue culture precluded growing this line to senescence; however cell pellets were collected for over twenty PDs and used for STELA analysis. Five Southern blots were prepared on NHDF STELA products
and PCR efficiency and consistent gel running conditions were confirmed by ANOVA of the HT1080 control (Appendix B.2). The telomeres of NHDF cells have an extremely large length distribution at 12q and XpYp. The median values for each time point analysed did not decrease regularly as would be expected (Figure 3.2.4a, Table 3.2-1). This caused difficulties in deriving a significant shortening rate at the 12q telomere, which regression analysis assigned a rate of 

![Figure 3.2.4](image)

**Figure 3.2.4** Telomere length analysis on the NHDF cell line. **a** 12q STELA results after subtraction of 544bp. **b** Regression analysis on 12q STELA data. Shortening rate is \( 16.32 \pm 14.77 \text{bp/PD} \) \( (p = 0.2579) \). Dotted line indicates 95% confidence band. 

**c** Regression analysis on 10th and 90th percentiles, shortening rates -50.18±5.006bp/PD \( (p<0.0001) \) and -7.42±16.66bp/PD \( (p=0.6576) \) respectively. Dotted line indicates 95% confidence band. 

**d** XpYp STELA results after subtraction of 406bp. **e** Regression analysis on XpYp STELA data, excluding PD 28.0. Shortening rate is \( -90.98 \pm 18.35 \text{bp/PD} \) \( (p<0.0001) \). Dotted line indicates 95% confidence band.
of \(-16.94\pm14.96\text{bp/PD}\) \((p=0.2579)\).

A distribution histogram identifies two distinct peaks within the 12q STELA data (Figure 3.2:5a). This clustering implies the presence of one large and one short subpopulation, with some telomeres of intermediate length. Allelic length differences between maternal and paternal chromosomes are a possible source of this population anomaly. Unfortunately the NHDF line was genotyped to be homozygous at the 12q sub-telomere (Appendix C.1), precluding the use of allele-specific STELA to isolate a single subpopulation and alleviate noise. In order to eliminate the possibility of a contaminating concomitant cell population in the NHDF population, a minisatellite analysis was carried out. The MS32 minisatellite is a highly variable region, and amplification of a single individual’s genome results in one or two products only, indicating

<table>
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<tr>
<th>NHDF 12q</th>
<th>Population Doublings</th>
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<tr>
<td></td>
<td>6.4</td>
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<tr>
<td>Minimum</td>
<td>1505</td>
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<tr>
<td>Median</td>
<td>7079</td>
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<td>Maximum</td>
<td>12200</td>
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Table 3.2-1 Statistics for NHDF cell line telomere length data, all values given in bp.

Figure 3.2:5 Smoothed histogram of NHDF 12q STELA data shown \(a\) in entirety and \(b\) expanded to highlight short telomere length distribution (500-5000bp). Excluding PD6.4, the proportion of shorter telomeres decreases over time. Relative frequency in PD 28.0 is especially low for short telomeres, suggesting a loss of cells with short telomeres at this PD. Bin width set at 500bp.
homozygosity or heterozygosity at the locus. The analysis was carried out on all cell lines, which all proved to have either one or two alleles at MS32, confirming no admixture of cell lines in any of the cultures, including NHDF. Results are shown in Appendix D.

In the absence of any compounding non-native subpopulation, this data highlights two foreseen concerns regarding the use of STELA analysis, and likely any technique used for determination of telomere length of the same population over time. Firstly, as with any PCR-based method, there remains a limit to the size of fragments that may be amplified, thus imposing a ceiling on the upper limit of recordable telomere size. It is not possible to circumvent this outcome by limiting the use of STELA data to later passages as large telomeres that may be present in the population would begin to phase into the PCR product pool. This could also mask any change in telomere dynamics throughout the proliferative lifespan of cell lines. Also, when considering the patient lines, exclusion of time-points from the analysis would be unfeasible due to the limited proliferation of these cells.

Secondly, cells with short telomeres may be lost from the population prior to PCR. With serial passaging, and even at early PDs when the vast majority of cells are actively dividing and a large proportion of cells have long telomeres, a continually increasing proportion of cells will enter senescence due to telomere shortening (Martin-Ruiz et al., 2004). Senescent cells have many biological and morphological differences to actively dividing cells (Campisi, 2013), including their ability to re-adhere after trypsinisation. If this is the case, the result would be a loss of a proportion of senescent cells at each passage. If the proportion of cells activating senescence remains low, relatively steady gain and loss of senescent cells from the population could explain the steady-state levels of senescence observed initially in cell lines, or indeed the peaks and troughs around ~80%, rather than the expected continually increasing proportion if
senescent cells were not lost. As cells with short telomeres at one end are more likely to be cells that have doubled more than the average number of times in this population, it is likely that telomeres on other chromosomes are shorter in these cells, and that many of these cells are senescent. Cells with short telomeres may therefore be lost from the population in a biased manner, resulting in the telomeres of these cells to be disproportionally lost from the pool of telomeres in the STELA reactions.

Both these factors appear to have manifested in the 12q data for NHDF. The maximum length at the final three time-points confers a decrease in length, with the largest telomere to be amplified being 14904bp at PD 18.7 (Table 3.2-1). No fragments amplified above this size are present in the products of the first two time points. Distribution analysis reveals a trend towards an increase in the proportion of long telomeres, suggesting that additional long telomeres are being added into the recorded population (Figure 3.2:5).

This suggests that the largest telomeres are failing to be amplified by STELA at PD 6.4 and 12.3 (Figure 3.2:4a). Conversely, the minimum length recorded at each PD is smaller as the population ages, with the exception of the final time point (PD 28.0). The proportion of telomeres that are below 4kb decreases at later PDs (Figure 3.2:5). STELA is able to detect even the smallest fragments, and so the likelihood of amplification failure of short telomeres is extremely low. As telomere length is not anticipated to increase in primary cell lines, this reduction in smaller PCR products supports the loss of senescent cells from the population. These factors will result in an observed reduction in telomere length at early PDs and the number of shorter telomeres at later PDs, and therefore a tendency to underestimate telomere shortening rate.

Taking these factors into account, sensitivity analysis was carried out by regression analysis of the 10th and 90th percentiles and one-factor-at-a-time (OFAT) style exclusion of time points with uncertainty. By isolating the 10th and
90th percentiles, the shortest and longest telomeres are selected for analysis, which gives significantly different shortening rates of -50.18 ± 5.006bp/PD (p<0.0001) and -7.416 ± 16.66bp/PD (p=0.6576) respectively. An extremely significant p value for the shortest telomeres indicates that this rate is the more representative as a linear rate of change is anticipated, particularly in the exponential growth phase (see section 3.3.2). Removing PD 28.0 from the 10th percentile data due to the presumed loss of small ends, gives a shortening rate of -54.41 ± 5.957 (p<0.0001). This rate, though not significantly different from the rate including PD 28.0 (p=0.588), is likely the more representative. Next, time points were excluded OFAT from the 90th percentile data. None of the regression lines derived from this method provided a significant p value. This may be mainly attributable to the loss of longer telomeres at earlier PDs (see section 3.3.1).

The distribution of XpYp telomere lengths appears to have been within the upper limit of STELA amplification, with possible drop out of the longest ends at PD 6.4 (Figure 3.2:4d&e). A shortening rate of -99.37±16.77bp/PD was calculated from all data (p<0.0001), while exclusion of PD 6.4 unexpectedly led to a reduction in rate to -81.65 ± 23.65bp/PD, as well as a drop in significance (p=0.0006). Due to the small number of points collected for PD 28.0 (n=35), this time-point was also excluded from the complete dataset resulting in an insignificant change in rate to -90.98 ±18.35 bp/PD (p<0.0001).

3.2.2.2 155-BR

A second normal fibroblast line derived from a healthy 55 year old male, 155-BR, was passaged to senescence but grew notably slowly for a normal line (Figure 3.2:3b), and at a similar rate to patient lines. STELA reactions and Southern hybridisations for 12q were carried out by both Kim Fitzpatrick (KF) and Rachel Turner (RT) as indicated in Figure 3.2:6b across seven gels. Sizing was carried out by RT. HT1080 distribution confirmed no significant difference
between those STELAs carried out by KF and those by RT (p=0.9780) and so data was pooled to give a resultant shortening rate of -55.96±15.51bp/PD (p=0.0003, Figure 3.2:6b). 12q STELA results demonstrated a large telomere size distribution, including very small molecules. As with NHDF, the proportion of shorter telomeres is reduced over time (Figure 3.2:7). Repeating the regression analysis excluding PD 28.9 increases the shortening rate to -79.72±18.14bp/PD (p<0.0001).

At XpYp, a faster shortening rate of -111.4 ± 21.06bp/PD (p<0.0001) was
recorded. Results at PD 16.0 have a lower median and distribution than at the next time point. Again, at this early PD it is quite possible that the longest telomeres would not be within the amplification range of STELA. The distribution at this time point also seems to have shifted in that the main body of telomere lengths is shorter than would be expected. This may be indicative of a shift in PCR efficiency, as, due to the presence of long telomeres, polymerase may be preferentially elongating shorter telomeres in a compensatory manner. This time point was excluded in sensitivity analysis and the resultant shortening rate of \(-194.3\pm29.28\) bp/PD, \((p<0.0001)\) (Figure 3.2:6d) is significantly higher than at 12q \((p=0.0037)\), even with the exclusion of PD 28.9 from the 12q analysis \((p=0.0152)\). Differences in erosion rate at different ends have previously been recorded (Mendez-Bermudez and Royle, 2011). However, the XpYp shortening rate obtained is faster than even the fastest shortening rates previously recorded in normal fibroblasts (Baird et al., 2003; Vaziri et al., 1993). It is possible that this line has a faster telomere shortening rate than would be expected, and the similarity between growth rates of 155-BR versus patient lines may have implications for interpreting the patient results (see discussion).

![Figure 3.2:7](image-url)  
**Figure 3.2:7** Histogram of telomere length distributions for 155-BR at 12q shown a in entirety and b expanded to highlight short telomere length distribution (500-3000bp). Bin width set at 500bp.
3.2.2.3 HGPS

Due to the extremely slow growth rate of HGPS cells, and their rapid decline to senescence (Figure 3.2:2a), it was only possible to collect a limited number of cell pellets. These pellets were also very low in cell number (e.g. 2-6 x 10^5 per 25cm^2 flask) and upon reaching around 20PDs even lower numbers of cells were collected. As with all cell lines approaching senescence, this was in part due to the increased size of senescent cells, but also the impaired ability of senescent cells to adhere to tissue culture plastics after passaging. In the case of HGPS, the window for collecting cells was very narrow (Figure 3.2:2), and only a limited number of pellets contained enough cells to extract a visible DNA pellet.

As a consequence, STELA was performed on four pellets taken less than three PDs apart. This proved to be too short a window to derive an accurate shortening rate and regression analysis failed to produce a statistically significant slope in the case of 12q (-165.1±145.9bp/PD, p=0.2583). To eliminate noise, regression was attempted on the 10th and 90th percentiles, yielding the respective significant rates of -240.7±105.6bp/PD (p=0.0264) and -540.9±172.0bp/PD (p=0.0026). As these rates are not significantly different from one another (p=0.1389), it is possible to derive a pooled rate of -390.7bp/PD. However, as the range of these rates is large due to the inaccuracies of performing a regression analysis across such a short timeframe, these results are only indicative of an increased rate of telomere shortening. Notably, extremely short telomeres are present (<400bp), and telomeres are highly variable in length in accordance with previous findings using Q-FISH (Decker et al., 2009).

XpYp shortening rate was also not significantly different from zero (-215.8±119.1bp/PD, p=0.0704). Analysis of the 10th and 90th percentiles was attempted and the longer ends provided a significant and rapid shortening rate of -393.8±170.7bp/PD (p=0.0252). The 10th percentile regression did not yield a
significant result (-56.25±169.0bp/PD, p=0.7406), most likely due to varying amounts of smaller products present in the STELAs.

Figure 3.2: Telomere length analysis on the HGPS cell line a 12q STELA results after subtraction of 544bp b Regression analysis on 12q STELA data. Shortening rate is -165.1±145.9bp/PD, p=0.2583. Dotted line indicates 95% confidence band c Regression lines for 10th and 90th percentile telomeres. Shortening rates -240.7±105.6bp/PD (p=0.0264) and -540.9±172.0bp/PD (p=0.0026) respectively d XpYp STELA results after subtraction of 406bp e Regression analysis on XpYp STELA data. Shortening rate is -194.3±29.28bp/PD (p=0.0704). Dotted line indicates 95% confidence band f Regression lines for 10th and 90th percentile telomeres. Shortening rates -56.25±169.0bp/PD (p=0.7406) and -393.8±170.7bp/PD (p=0.0252) respectively.
Excluding a single outlier from the final time-point, the range of HGPS 12q telomere length changes from 15269-849.9 to 11841-413.6. Taking the median at these time points gives a rate change of -504.6bp/PD. Identical treatment at XpYp gives a rate of -245.8bp/PD (range change: 12675-1073bp/PD to 11074-3040bp/PD, excluding outlier at PD7.7). Again, these rates are indicative of an increased telomere shortening rate in HGPS cells.

3.2.2.4 RD

The RD cell line was available from PD 6 and as such it was possible to extract DNA from pellets taken across a longer time-frame than the HGPS line, and to expand the cells so that at senescence a sizeable pellet could be acquired, despite the severity of the effects of the mutation. As such, telomeres spanning 12.8 PDs were sized and a significant shortening rate ($p<0.0001$) was observed at both 12q (-210.5±24.67bp/PD) and XpYp (-183.5±34.40bp/PD). STELA reactions and Southern blots for XpYp were carried out by Kim Fitzpatrick. Due to apparent loss of large telomeres at PD7.5, as indicated by the median and distribution, regression analysis was repeated for XpYp excluding PD 7.5 resulting in a rate of -221.8±49.13bp/PD ($p<0.0001$).

For further insight into shortening rates at different chromosome ends in a severe phenotype, STELA was also carried out on RD at 17p. STELA results yielded a shortening rate of -196.8 ± 20.28 ($p<0.0001$). Products derived from the DNA sample taken at PD 7.5 appear to have failed to amplify efficiently, most likely due to the large distance between the annealing site of the 17p STELA primer available and the start of the telomere (3078bp). Excluding this data point results in a shortening rate of -223.8±26.85bp/PD ($p<0.0001$). All three ends demonstrated a shortening rate considerably faster than that previously recorded in normal cells, and are not significantly different from one another ($p=0.9515$).
Figure 3.2.9 Telomere length analysis on the RD cell line a 12q STELA results after subtraction of 544bp. b Regression analysis on 12q STELA data. Shortening rate is \(-210.5\pm24.67\text{bp/PD}\) (p<0.0001). Dotted line indicates 95% confidence band. c XpYp STELA results after subtraction of 406bp. PCR products and Southern blots generated by KF for XpYp telomere. d Regression analysis on XpYp STELA data excluding PD7.5. Shortening rate is \(-221.8\pm49.13\text{bp/PD}\) (p<0.0001). Dotted line indicates 95% confidence band. e 17p STELA results after subtraction of 3078bp. f Regression analysis on 17p STELA data excluding PD7.5. Shortening rate is \(-223.8\pm26.85\text{bp/PD}\) (p<0.0001). Dotted line indicates 95% confidence band.
Interestingly, a distinct subpopulation with longer telomeres is visible at 12q. This individual has inherited the same 12q allele from each parent (Appendix C.1), and so it is not possible to state whether this subpopulation is due to an allelic difference. In this instance, this subpopulation does not appear to be interfering with the regression analysis as it has been amplified efficiently at every time point.

3.2.2.5 T623S

The T623S phenotype is considered a milder form of classical HGPS, with the same symptoms but later onset and slower disease progression. In culture, T623S cells did not continue to PDs much beyond HGPS cells (approximately 22 PDs vs 20), however, cells of low PD were available for this line and STELA was carried out at six time-points across 13.3 PDs. 12q STELA PCRs and Southern blots were generated by both RT and KF.

Kruskall-Wallis analysis of HT1080 data at 12q demonstrated significant differences between two sets of gels, each set containing three out of six time points (gels 1,2 and 3 carried out by KF, gels 4 and 5 carried out by RT). Tukey’s multiple comparisons test revealed significant differences were only present between gel 1 and gel 4, and gel 2 and gel 4. Differences in HT1080 sets were due to increased efficiency of large fragment amplification in the PCR reactions carried out by RT (Appendix B.2). As larger fragments are not absent from the T623S PCRs carried out by KF, both datasets were considered for analysis. Initially, the two datasets were analysed separately to give similar shortening rates of 219.2±27.27bp/PD (KF, p<0.0001) and 191.9±21.34bp/PD (RT, p<0.0001), and comparison of the slopes revealed no significant difference (p=0.4236). As such the data was considered as a whole to give the largest insight across the longest timespan and repeating the regression analysis with all data pooled gives a shortening rate of -213.7±16.75bp/PD (p<0.0001) at 12q.
In the STELA data for XpYp it is apparent that a subpopulation of very long telomeres is present in these cells (almost 20kb at PD 15.6). Due to their length, this subpopulation is only amplifiable by STELA at later PDs, once shortening has reduced their length. As these points are only present in the final three PDs (although one long end appears to have been amplified at PD 10.5), it is not possible to easily ascertain distinct groupings using 90\textsuperscript{th} percentile data. XpYp data was sensitive to these bands, as removing outliers by ROUT analysis in Prism changed the shortening rate from -85.33±40.48bp/PD to -171.7±38.38bp/PD (p<0.0001). It should be noted that apparent outliers at PD

Figure 3.2:10 Telomere length analysis on the T623S cell line a 12q STELA results after subtraction of 544bp b Regression analysis on 12q STELA data excluding PD 21.3 (see main text). Shortening rate is 225.3±18.48bp/PD (p<0.0001). Dotted line indicates 95% confidence band c XpYp STELA results after subtraction of 406bp, yellow dots indicate outliers identified by ROUT analysis (Q=1%) d Regression analysis on XpYp STELA data excluding outliers. Shortening rate is 171.7±38.38bp/PD (p<0.0001). Dotted line indicates 95% confidence band.
15.6 were not identified by the ROUT analysis with the parameters used, and may have some bearing on the regression analysis, though the central position of this PD in the dataset would most likely effect the elevation and y-intercept of the slope, rather than the gradient itself. XpYp failed to amplify at PD 21.3, indicating contamination or quality issues with this sample. As such, PD 21.3 was also excluded from the 12q dataset to give a rate of $-225.3 \pm 18.48$ bp/PD.

### 3.2.2.6 E578V

The E578V line is derived from a patient with a comparatively very mild phenotype. During cell culture, these cells behaved similarly to the NHDF line, doubling approximately every 24 hours, and reached a high number of PDs before reaching senescence. However, median telomere length was quite short at even low PDs (4508bp median length at PD23.4 for 12q versus 6719bp and 6405bp at equivalent time points for 155-BR and NHDF), though this could be attributable to normal inter-individual differences. Shortening at 12q displayed a normal rate of $-53.92 \pm 4.54$ bp/PD, whereas XpYp exhibited a faster attrition of $-120.9 \pm 10.76$ bp/PD, though this is potentially still within normal rates.

Unusually, the difference between 12q and XpYp was very large despite quite well defined distributions at each time point in both cases resulting in very small ranges and tight confidence bands in regression analyses. Repeating the regression analysis after removal of the first time point resulted in slightly faster shortening rates, as would be expected, with no significant difference from the rates derived prior to sensitivity analysis. 12q gave a rate of $-61.69 \pm 6.768$ bp/PD, while XpYp increased to $-155.8 \pm 15.48$ bp/PD.

A summary of all shortening rates may be found in Table 3.3-3 (p124).
Discussion

Statistically significant shortening rates were acquired for all cell lines except NHDF at 12q and HGPS at both 12q and XpYp. Due to population dynamics, the sensitivity of several cell line shortening rates was tested to establish the effects of senescent cell detachment and inefficient amplification of large telomeres and extremely significant rates of loss (p<0.0001) were obtained at all ends except HGPS at XpYp. Trends suggest that telomere shortening rate is accelerated in the cell lines derived from patients with more severe phenotypes.

Figure 3.2:11 Telomere length analysis on the E578V cell line a 12q STELA results after subtraction of 544bp b Regression analysis on 12q STELA data. Shortening rate is \(-53.92\pm4.538\text{bp/PD}\) (p<0.0001). Dotted line indicates 95% confidence band c XpYp STELA results after subtraction of 406bp d Regression analysis on XpYp STELA data. Shortening rate is \(-120.9\pm10.76\text{bp/PD}\) (p<0.0001). Dotted line indicates 95% confidence band.
3.3.1 Exploring the effects of population dynamics and long molecule amplification efficiency on shortening rate

The increasing proportion of senescent cells in the population should result in a biased and continual loss of those cells with shorter telomeres from the population. With increased divisions, the proportion of cells containing shorter telomeres should also increase. It appeared from the data collected that a proportion of these cells was being lost, in some cases increasing the median telomere length recorded by STELA at later PDs, impacting the overall observed shortening rate. This apparent loss of cells also has implications for the accuracy of PD counts. As the PD calculation is made based on the number of cells counted when cells are plated out, if cells are lost after this time, and before the next count, additional divisions by the dividing population would be contributing to the PD count but go undetected. This would result in the observed PD to be an underestimation of the actual mean PD rate. In extreme instances, as was the case with HGPS, senescence levels were so high at late PDs that after passaging, less cells were counted at the final time-point than in the penultimate, resulting in a negative effect on cumulative PD at the final time-point (Figure 3.2:2a). If growth rate is recorded more slowly than is the case, faster telomere erosion could be seen as an artefact of this process. In other words, if shortening rate is presumed constant, and the number of PDs between time-points is underestimated, this would result in an observation of the same amount of telomere shortening occurring over fewer PDs, giving an increase in the observed shortening rate relative to PD. As the number of senescent cells increases over time, the greater the loss of cells, potentially leading to a continually increasing observed rate of shortening.

Two main factors indicated the loss of senescent cells from cell populations, firstly, a reduction in the proportion of shorter telomere ends in length analysis over time. Secondly, as a consistent gain of senescent cells would be anticipated
over time, the relatively steady proportion of senescent cells (~20%) observed in exponentially growing populations indicates that some senescent cells must be detaching after replating. At this stage in proliferation it could be anticipated that the same proportion of cells are entering senescence with each passage, however, the magnitude of this proportion, as well as the proportion of senescent cells detaching, cannot be inferred and would need to be determined empirically, and may vary between cell lines.

Potentially, cell counting at the point of passaging could be followed by counting cells that detach and become free in the media. It would be essential to determine the proportion of senescent cells in parallel, as well as distinguish between senescent and dead detached cells. It would be provident to use multiple markers for senescence, as some markers are more reliable than others and combinations of markers have been found to be highly informative (Lawless et al., 2010). It would also need to be determined how quickly the viability of detached senescent cells is affected, so as not to affect counts by misrepresenting the proportion of senescent versus dead cells. FACS would seem an ideal platform for distinguishing live from dead cells, however this experiment would potentially have to be carried out on a huge scale in order to provide the cell numbers required for FACS. However, if this issue could be addressed, it would be possible to utilise DAPI or propidium iodide staining to identify non-viable cells with sub-G1 DNA content, while simultaneously identifying a senescence marker, such as ki67 negativity combined with high γ-H2AX levels (Lawless et al., 2010). Once senescence levels in the population, and the number of detached senescent cells was established, it would be possible to derive a rate at which senescent cells detach.

By monitoring senescence levels in the patient and control lines, it is possible to interpret population dynamics to some extent. The β-galactosidase assay was carried out after re-plating cells, meaning a proportion of senescent cells could
be lost prior to β-galactosidase assay, potentially causing the irregularities observed in some cell line senescence data, and complicating interpretation of the data. The discontinued contribution of senescent cells to the dividing population further complicates accurately assigning the mean population doubling value. PD was calculated using the following formula: \[\log_{10}(\text{final count}) - \log_{10}(\text{initial count})/\log_{10}2.\] Senescent cells that have re-adhered are included in both the initial and final count, although as time passes, a larger and larger proportion of the cell population is not contributing to division (senescent cells). If half the cell population is doubling normally, and half is senescent, a count of 1 PD count would represent a mean of 2 PDs carried out by the dividing population. As the proportion of senescent cells increases beyond 50%, this effect would be amplified and cell populations will contain an

Figure 3.3:1 Illustration of population dynamics in an ageing cell line. Each column represents observations each time the population is passaged, counted, and assayed for β–galactosidase activity. Yellow background indicates cells that are included in cell counts, grey indicates cells that are lost prior to counting. The percentage of senescent cells, according to counting, is given at the bottom of the figure. Initially, cells with the longest telomeres (top left), while included in counts, may not contribute to STELA results. This does not affect the accuracy of PDs, but has implications for interpreting STELA data. Over time, more cells enter senescence, ~50% of which are lost and not counted. Dividing cells continue to divide, compensating for cells lost from counts. These cells should also continue to enter senescence at the same rate, which may influence the proportion of senescent cells.
increasing proportion of cells that are misrepresented by the observed cumulative PD. This is the likely cause of previous reports of observed increases in telomere shortening rate, rather than a genuine increase. In order to compensate for the discontinued contribution of senescent cells to the dividing population, the senescent cell count may not be simply excluded from cell counts, as with dead cells, because of their perpetuation in the population. To rectify this, the difference between the proportion of senescent cells from one passage to the next could be deducted at the following passage as this new senescent population is not contributing to doubling, however the inaccuracies of the β-galactosidase assay preclude this. As such, accounting for these population dynamics when establishing PD in a primary cell population would require a mathematical model to be devised which takes into account the presence of senescent cells and their contribution to cell counts, but their inability to divide. A basis for this model is illustrated in Figure 3.3:1.

In order to further dissect the effects of losing cells, and therefore a proportion of telomeres, on the accuracy of telomere shortening rate, a set of example data was created. A bimodal population is represented, with one subpopulation consisting of a broad distribution of short to medium-length telomeres, and the other a smaller subpopulation composed of long telomeres (Figure 3.3:2). For the purposes of observing the effect on regression analysis, short telomeres were allowed to fall below zero base pairs in the initial example data. In this example, the telomeres of this population range from 400-12,000bp and shorten at a rate of 100bp/PD, and a window of 15PDs is observed. In a scenario where all telomere lengths are measurable, regression analysis in Graphpad Prism 6 calculates the telomere shortening rate of -100bp/PD as significant, with an error of ±43.99 (Example A1, Figure 3.3:2a). Taking into account that telomeres cannot shorten perpetually, a lower threshold of 600bp was applied, and any example telomeres shortening beyond this were set at 600bp (Example A2, Figure 3.3:2b). This had a small but observable effect on the shortening rate,
Figure 3.3.2: Example dataset A. Example data containing two subpopulations. This data was manipulated in figures b-f in order to predict the effects of excluding various subpopulations, see main text and Table 3.3-1 for more detail. g regression comparison of each scenario from figures b-f.
reducing it to -89.48±42.83 bp/PD. This rate remained significantly different from zero. To compare the effect of the loss of long telomeres due to amplification failure, an upper threshold was set at 10kb. Ends exceeding this length were removed from the dataset, causing a dramatic shift in shortening rate to -22.89±37.40 bp/PD (Example A3, Figure 3.3:2c). Combining these scenarios results in a shortening rate of -34.13±38.94 bp/PD (Example A4, Figure 3.3:2d). In order to reflect the loss of short telomeres from the population, as was evident from the STELA data, roughly 50% of the “senescent” population (the 600bp telomeres) was removed. This combination of loss of long and short telomeres resulted in the least accurate shortening rate (4.435±37.49 bp/PD, Example A5, Figure 3.3:2e). As the results from Examples 2 and 3 suggested that the effect of losing longer ends has more serious consequences for the accuracy of the regression analysis, a final scenario was explored. Conditions were repeated as for Example A5 (i.e. telomeres will not shorten below 600bp and approximately 50% of these telomeres are lost at each time point), but all long ends were included (Example A6, Figure 3.3:2f). This resulted in a shortening rate of -60.90±43.24 bp/PD. A comparison of regression lines may be found in Figure 3.3:2g, and a summary in Table 3.3-1.

This analysis points to the loss of long telomeres as having the greatest impact on shortening rate accuracy. In order to exclude the possibility that this was due to the bimodal distribution being skewed, with fewer long telomeres, the process was repeated with another theoretical population, containing a small subpopulation of short telomeres and a large population of medium-length to long telomeres with the same shortening rate (Figure 3.3:3a, Example 1). This new distribution has the same range and the same error in shortening rate (±43.99bp/PD). Using the same parameters as above, the “senescent” population was assigned to telomeres under 600bp, and ~50% of these were removed from the analysis at each time point. This results in only a slight drop in accuracy when compared to the equivalent Example A6 (-55.51±41.52bp/PD).
**Figure 3.3:3** Example dataset B a example data containing two subpopulations, skewed towards longer telomere. This data was manipulated in figures b to predict the effects of excluding senescent cells from a population and in c to predict the effects of excluding long telomeres, reflecting a loss of PCR efficiency of longer molecules d regression comparison of a-c.

**Table 3.3-1** Summary table of the exclusions from the example data, including shortening rate and p values as derived from Graphpad Prism 6.
As would be expected, removing long telomeres (>10kb) from this population has a slightly less deleterious effect on the regression analysis than its equivalent Example A3 (-46.96±43.10bp/PD).

These analyses confirm that a loss of senescent cells from the population, as well as PCR amplification failure of long telomeres, will affect the telomere shortening rate derived from STELA data. While a failure to amplify larger telomeres remains a PCR-specific issue, any technique used to establish telomere shortening rate will be affected by the loss of senescent cells. As anticipated, all the scenarios tested lead to a reduction in telomere shortening rate, suggesting that any published rates to date may be underestimated. It would seem that the non-amplification of long telomeres has a greater influence on shortening rate than the effects caused by senescent cell loss.

Deviation from the actual shortening rate, and the significance of regression analysis are more greatly affected when long telomeres drop-out, as is slope elevation. This can be seen by direct comparison of Examples A5 and A6. This effect would seem to be amplified by the distance of the longest telomeres from the median and the main distribution of lengths, as a dataset with a long subpopulation is affected to a greater degree than a dataset that contains long telomeres within the main distribution (Example A3 versus Example B3). A subpopulation of short telomeres does not affect regression analysis to the same extent when the effects of senescence (i.e. telomeres do not shorten below a set length) are taken into account (Example B2 versus A6), and this may be due to the retention of a proportion of the telomeres. Still, the presence of isolated long and short subpopulations had the most impact on shortening rate, as was highlighted by the XpYp data for T623S. Extreme care should therefore be taken when interpreting any dataset that contains an isolated short or long subpopulation. Excluding T623S at XpYp, none of the cell lines appeared to contain subpopulations that may have been susceptible to the effect described.
above. Therefore, in this study, the effect of losing senescent cells may not have resulted in a large impact on shortening rate, however, the effect of senescent cell detachment on PD accuracy could be affecting the accuracy of shortening rate to an unknown degree. However, with the exception of the NHDF line which did not approach senescence, all cell lines experienced an increase in the proportion of senescent cells, which could minimise this effect. Additionally, any effect on shortening rate would become more apparent towards the end of the cell lines’ proliferative lifespan. This possibility is explored below.

### 3.3.2 Telomere shortening rate over lifespan

A drop-off in telomere length towards the end of replicative lifespan has been previously recorded in fibroblasts (Britt-Compton et al., 2009b, 2006). However, if, towards the end of proliferative lifespan, PD rate is recorded as slower than is the case, this increase in shortening rate will be observed in error. In order to identify changes in shortening rate over the lifespan of each cell line, the median values of each time-point were plotted (Figure 3.3:4a-f), and the rate of change between each median value was calculated (Table 3.3-2). Telomere length and cell division rate were also plotted over time in order to observe any interactions between these two factors (Figure 3.3:4g-l). Figure 3.3:5 is provided as a reference for senescence levels at the time points where DNA was harvested for STELA.

The 155-BR line has a particularly low $r^2$ value when a regression line is derived from the median values for both 12q and XpYp (Table 3.3-2). At several time points, the median varies greatly from the trend, causing difficulty interpreting rates of change, and as such does not provide reliable information. The NHDF line conforms well to a linear model, with high $r^2$ values at XpYp and at 12q when using the 10th percentile data. Taking the change in rate between each time-point shows a steady rate that fluctuates marginally around the rate established by regression of the raw data. The NHDF data was collected while
the cell line was still in its exponential growth phase, and as NHDF maintained a steady level of senescent cells (Figure 3.2:3a), the rate of telomere loss would not be expected to be affected by counting inaccuracies caused by senescent cell loss. This consistent growth rate is confirmed by the high $r^2$ value derived by regression analysis of growth rate over time ($r^2=0.9990$ (Figure 3.3:4g)). The slight decrease in rate between the final two time-points at XpYp could therefore be due to experimental fluctuation.

The patient lines show varying degrees of linearity. Due to the short period of time between points for HGPS it is not feasible to analyse the intervening rates. Slow initial rates of shortening in most cell lines are most likely attributed to amplification failure of the longest telomeres at the first time-point. This ceiling on amplification limited the detection of telomeres that exceed approximately 15kb for 12q and between 18-20kb for XpYp. The T623S data demonstrates this at the first two rates for XpYp, and the first rate for 12q. After this time, the rates do not vary greatly. Disregarding the first value for both telomere ends of E578V, a similar linearity is observed, however the shortening rate appears to decrease between the final time-points. Conversely, the RD shortening rate increases at the final rate-change at all three ends tested, suggesting that the efficiency of senescent cell re-adherence may be poorer in RD cells, consistent with the severe nature of the cellular phenotype. Comparing the time difference between the final three time-points for RD shows a marked decrease in doubling rate. 32 days passed between the final 3.8 PDs, in contrast to the previous 5.1 PDs which took place over only 14 days. High levels of senescent cells at this final stage could be skewing counting and creating an artificial observed increase in shortening rate as has been discussed. As the shortening rate is more constant when plotted against time rather than PD ($r^2$ values for regression on final 3 time points increase from: 12q=0.9658, XpYp= 0.9002, 17p= 0.9649 to: 12q=0.9856, XpYp=0.998, 17p=0.9861), doubling time could directly impact the rate of telomere shortening (see next section).
Figure 3.3: Shortening rates of each cell line over time. Values given for NHDF 12q are the 10th percentile data. Graphs a-f show the median telomere length (derived from complete datasets prior to sensitivity testing) at each time-point, plotted against PD. Dotted lines indicate range. Graphs g-l show median (derived as above) telomere length and PD plotted against time (days). Dotted lines indicate range.
Figure 3.3:5 Growth rates as determined by cell counting and senescence levels as determined by β-galactosidase assay. Time points where genomic DNA was derived and used as a template in STELA reactions are indicated by pink dots for ease of interpretation.
It has been shown that the rate of telomere erosion is independent of the length of the telomere (Baird et al., 2003). However, at 12q in the RD line, a subpopulation of long telomeres is distinguishable, roughly correlating with the 75th percentile, and appears to be shortening more quickly than the main body of telomeres (Figure 3.3:6). This was confirmed by normalising the data of each time point according to its median, so that they may be overlaid on a histogram plot (Figure 3.3:7a&b). This effect could be due to allelic differences between the two subpopulations and so regression analysis of the shortening rate of all cell lines was carried out.

Median telomere length of each line, at each telomere, was plotted against the sensitivity-corrected shortening rate. As shortening rate for HGPS XpYp was not reliable, this data was excluded from this analysis. Regression analysis reveals a statistically significant, if minor, effect of telomere length on shortening rate (p=0.0079 (Figure 3.3:7c)). Direct comparison of XpYp and 12q shows a non-

**Figure 3.3:6** Differential shortening of subpopulations in RD at 12q. a Regression analysis of the upper quartile versus the remaining data points. Shortening rates are shown on the figure. Slopes are significantly different (p=0.0059). UQ=upper quartile b Smoothed histogram of 12q data, the median of each time point has been set to zero to allow comparison c Blow-up of the longer telomere subpopulation. Over time this subpopulation peak shifts towards the median, indicating a faster rate of telomere shortening.
significant trend for XpYp to shorten more rapidly, though if longer telomere lengths result in faster shortening, this may be explained by the longer median telomere lengths in this group (Figure 3.3:7d). A similar difference was observed in the comparison of patient and control cells (Figure 3.3:7e), suggesting that 12q and/or patient telomeres are less susceptible to this length-dependent rate change. If long telomeres shorten more quickly this would cause a gradual decrease over time in the rate of shortening and could explain the gradual decrease in rate observed at the final point in the E578V line. If longer telomeres are in fact shortening more rapidly, this suggests that there is an inherit mechanism by which telomeres shorten gradually and processively at different rates. This mechanism may be the source of natural variation in telomere erosion rates, and potentially the differences between control and patient rates.

**Figure 3.3:7** Regression analyses of shortening rates against median telomere length. 

- **a** Regression analysis of median telomere length over shortening rate of each telomere analysed, excluding HGPS XpYp ($r^2=0.5224, p=0.0079$) 
- **b** Comparison of shortening rates versus median telomere length regression between 12q ($r^2=0.7748, p=0.0488$) and XpYp ($r^2=0.7659, p=0.0224$) 
- **c** Comparison of shortening rates between patient ($r^2=0.9731, p=0.00136$) and control lines ($r^2=0.5900, p=0.0260$)
3.3.3 Cell line differences in telomere shortening rate

A summary of the shortening rates at 12q and XpYp for all cell lines tested is given in Table 3.3-3a and Figure 3.3:8. Comparison of all shortening rates was carried out in Graphpad Prism6 and the resultant p values are given in Table 3.3-3b-e. Initial observations show considerably less variation in telomere shortening rate between cell lines at XyYp than 12q. The shortening rates of both control lines were significantly slower than T623S and RD at 12q (Table 3.3-3) and the data was not sensitive, having significantly faster shortening rates at 12q both before and after sensitivity testing. Previous reports for fibroblast shortening rate record a loss of between ~30-85bp/PD (Allsopp et al., 1992; Counter et al., 1992; Harley et al., 1990; Levy et al., 1992), with some studies reporting up to ~160bp/PD (Baird et al., 2003; Vaziri et al., 1993). The RD rate (-210.5 ± 24.67 bp/PD), T623S (-225.3 ± 18.48) and provisional HGPS (-390.7 bp/PD) shortening rates all exceed normal rates.

The shortening rate data for HGPS, although not significant in itself, was found to be significantly different from the NHDF control line and the E578V line, but not RD or T623S at 12q after sensitivity testing (Table 3.3-3). At 12q, the E578V line did not differ significantly from the normal cell lines, but did differ from the other patient lines significantly in all cases except HGPS prior to sensitivity analysis. Sensitivity-corrected testing of XpYp reveals a significant difference between the E578V shortening rate and NHDF. However, this rate (-155.8 ± 15.48 bp/PD) is still within normal parameters. This similarity to normal lines reflects the milder nature of the symptoms accompanying E578V and its normal rate of cell division in culture, and could indicate that any effect on telomere shortening due to lamin A mutation is either minimal or absent in this line.

The failure to record a significant rate of telomere shortening in the HGPS line was due to the short window in which samples were available. Crucially, the aberrantly high shortening rates recorded for the RD line are observed between
<table>
<thead>
<tr>
<th>a</th>
<th>12q</th>
<th>Shortening rate (Raw data) bp/ PD</th>
<th>p value</th>
<th>Shortening rate (Post-sensitivity testing) bp/ PD</th>
<th>p value</th>
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</thead>
<tbody>
<tr>
<td>NHDF (M)</td>
<td>-16.32 ± 14.77</td>
<td>0.2696</td>
<td>-54.41 ± 5.957*</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>155-BR (M)</td>
<td>-52.66 ± 15.18</td>
<td>0.0005</td>
<td>-72.86 ± 18.13</td>
<td>&lt;0.0001</td>
<td></td>
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<tr>
<td>E578V (F)</td>
<td>-53.92 ± 4.54</td>
<td>&lt;0.0001</td>
<td>-61.69 ± 6.768</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>T6235 (F)</td>
<td>-203.3 ± 15.90</td>
<td>&lt;0.0001</td>
<td>-225.3 ± 18.48</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>HGPS (M)</td>
<td>-168.0 ± 147.5</td>
<td>0.2552</td>
<td>-390.7</td>
<td>n/a**</td>
<td></td>
</tr>
<tr>
<td>RD (U)</td>
<td>-210.5 ± 24.67</td>
<td>&lt;0.0001</td>
<td>-221.8 ± 49.13</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>a</th>
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<th>Shortening rate (Raw data) bp/ PD</th>
<th>p value</th>
<th>Shortening rate (Post-sensitivity testing) bp/ PD</th>
<th>p value</th>
</tr>
</thead>
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<tr>
<td>NHDF (M)</td>
<td>-99.37 ± 16.77</td>
<td>&lt;0.0001</td>
<td>-90.98 ± 18.35</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>155-BR (M)</td>
<td>-111.4 ± 21.06</td>
<td>&lt;0.0001</td>
<td>-194.3 ± 29.28</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>E578V (F)</td>
<td>-120.9 ± 10.76</td>
<td>&lt;0.0001</td>
<td>-155.8 ± 15.48</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>T6235 (F)</td>
<td>-85.33 ± 40.48</td>
<td>0.0358</td>
<td>-171.7 ± 38.38</td>
<td>&lt;0.0001</td>
<td></td>
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<tr>
<td>HGPS (M)</td>
<td>-199.3 ± 123.8</td>
<td>0.1081</td>
<td>-393.8 ± 170.7***</td>
<td>0.0252</td>
<td></td>
</tr>
<tr>
<td>RD (U)</td>
<td>-183.5 ± 34.40</td>
<td>&lt;0.0001</td>
<td>-221.8 ± 49.13</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
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</table>

Table 3.3-3 a Shortening rates at 12q and XpYp for each cell line, before and after sensitivity analysis. The sex of each individual is indicated as Male, Female or Unknown in brackets after the cell line name. Non-significant p values are given in red *10th percentile data ** Pooled from 10th and 90th percentile data (p=0.0264 and p=0.0026 respectively) *** 90th percentile data. Letters in brackets after cell lines indicate Male or Female b-e p values from regression comparisons carried out pair-wise across all cell lines. Values are shown for 12q b before and c after sensitivity testing and XpYp d before and e after sensitivity testing.
PD 11.4 and 16.5 for XpYp and 17p, and from PD 7.5 at 12q (Figure 3.3:4d). At these lower PDs, the cell population is dividing rapidly and senescence levels are relatively low (Figure 3.3:5f), which would minimise the effects of cell loss on artificially increasing the observed telomere shortening rate, thus confirming a genuine accelerated telomere shortening rate in this line. Accelerated shortening is also observed at early PDs in the T623S line while senescence levels are minimal in. This is apparent at 12q, while confirmation of this pattern at XpYp is made difficult by the “drop-in” subpopulation with long telomeres (Figure 3.3:4e and Figure 3.3:5d). These rates from early time points are consistent with the hypothesis that an inherent mechanism of increased telomere attrition

**Figure 3.3:8** Comparison of telomere shortening rates in patient and control cell lines, with error. Shortening rates of patient and control cell lines at 12q **a** before and **b** after sensitivity testing (HGPS: circles indicate the rate of shortening of the 90th and 10th percentiles and error bars here indicate the combined ranges of the 90th and 10th percentile rates) and at XpYp **c** before and **d** after sensitivity testing.
at replication in these lines. As the T623S cells are derived from a patient with a disorder comparable to classical HGPS, except for slower onset and disease progression rate, the similarity of the molecular mechanisms behind both disorders, data from other studies, and the sensitivity tested HGPS data at 12q, it is likely that the rate of HGPS shortening is comparable to or faster than T623S and RD.

At XpYp, in a comparison of severe phenotypes and NHDF, a significant difference was only observed between the NHDF line and RD line (Table 3.3-3c), despite faster shortening rates than would expected in normal fibroblasts in T623S (-171.7 ± 38.38 bp/PD), and implied by sensitivity-corrected HGPS data (-393.8±170.7 bp/PD). After sensitivity testing, the T623S shortening rate was not significantly different from the NHDF rate of -90.98 ± 18.35 bp/PD despite no overlap in the ranges of each line (p=0.05582).

The most unexpected result is that of 155-BR at XpYp, which exhibits a shortening rate faster than would be expected of a normal fibroblast line (194.3±29.28bp/PD) between PDs 18.4 and 28.9. This unusually elevated erosion rate could be attributable to loss of senescent cells, and perhaps the longer telomeres at XpYp in comparison to 12q contribute to this increase. Alternatively, viewing the similarities between the 155-BR line and the severe-phenotype patient cell growth rates, it is possible that long division times may exact an effect on telomere length. Previous work has shown that slowly dividing clonally-derived fibroblast populations have exhibited accelerated shortening rates compared to their more quickly dividing counterparts, and this was attributed to an acceleration of telomere shortening of cells approaching senescence (Britt-Compton et al., 2006). However, as the XpYp shortening rate was relatively stable at earlier PDs when senescence levels were low, this suggests that slowly growing populations may in fact incur accelerated telomere shortening. It would be possible to determine if this is the case by
imposing growth restrictions on a normal line and comparing shortening rate to a control. This could be achieved by chemical inhibition, however it would be crucial not to expose the cells to any toxic substance that may affect the integrity of the telomere. Although potentially toxic to a certain degree, media starvation could provide a low-impact alternative to chemical arrests.

The sex-related nature of the XpYp causes other complications when interpreting the XpYp shortening rates. X-inactivation in females results in an increase of telomere shortening rate at the inactivated X (Xi) (Surrallés et al., 1999). This will produce a direct effect on telomere length distribution in females, in this data set E778V and T623S, and could explain the faster shortening rate of the E578V at XpYp versus 12q, however, the T623S shortening rate at XpYp is slower than at 12q, though not significantly (p=0.5734). However, significant increases in shortening rate at XpYp versus 12q may be observed for both NHDF and 155-BR, despite both being male. X-inactivation is marked by condensed chromatin across the Xi. Although the X chromosome is not relocated within the nuclei of HGPS cells (Mehta et al., 2011), patients lose histone 3 trimethylation on lysine residue 27 (H3K27me3), a marker for facultative chromatin on the Xi (Shumaker et al., 2006). This may

Figure 3.3:9 Last recorded 12q telomere length for each line at a 12q and b XpYp. PD at final measurement: NHDF=28.0 (not senescent), 155-BR=28.9 E578V=46.5, T623S=22.1, HGPS=20.3, RD=20.3

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accelerate or influence the rate of shortening of Xi in patient cells in an unpredictable manner, and the 12q data could be a more reliable source of information than the XpYp data. Expression of sex-related genes could also be altered in patient cells.

Finally, the last recordable median telomere length in each cell line was compared for 12q and XpYp (Figure 3.3:9). At both ends there is a trend for cell lines with the most severe phenotypes to have a higher median length at the point of senescence. However, the range of telomere lengths of the RD line at senescence included very short telomeres. The distribution of telomere length in RD is also very similar to that of control line 155-BR at both ends. Given this, and the natural variation in telomere length distribution in humans, it is possible that this reflects natural variation not associated with a senescence-induction mechanism.

3.3.4 Potential sources for increased telomere shortening in laminopathy patient cells

Because the 155-BR cell line did not behave as a normal cell line, using NHDF as a controlled comparison, and in comparison to previously published telomere shortening rates relative to PD (Allsopp et al., 1992; Baird et al., 2003; Counter et al., 1992; Harley et al., 1990; Levy et al., 1992; Vaziri et al., 1993), it would appear that the T623S and RD cell lines are shortening at an accelerated rate. This is especially clear at 12q, which is more representative of the autosomal genome. It is most likely that telomeres of the HGPS line are also shortening more rapidly. The patient cell lines demonstrate a correlation between increased senescence and short telomeres confirming that patient fibroblasts have a shorter lifespan than normal cells as a direct consequence of accelerated telomere shortening. This is in agreement with a previous study in which
expression of the telomerase subunit TERT abolished senescence in HGPS and progerin-expressing cells (Benson et al., 2010).

The major commonality between the T623S, HGPS and RD lines is the accumulation of farnesylated forms of lamin A. Farnesylated lamin A aggregates at the periphery, forming a thickened lamina and dysmorphic nuclear membrane. Farnesylated lamin A is also associated with slow cell cycle progression, DNA damage accumulation and signalling and chromatin organisation disruption. Therefore, increased telomere shortening may be an effect of farnesylation. Given that E578V exhibits limited nuclear blebbing in the absence of farnesylated lamin A (Csoka, 2004; Toth et al., 2005), and a relatively high rate of shortening at XpYp, it is possible that accumulation of lamin A at the nuclear periphery and/or the resultant physical aberrations could be influencing telomere shortening. However, as shortening rates at both 12q and XpYp were both within the normal range of telomere shortening, it would seem that retention of the farnesyl group is necessary for accelerated telomere shortening.

DNA damage levels are highly elevated in HGPS and other laminopathy and laminopathy-model cells (Csoka, 2004; Gonzalez-Suarez et al., 2009; Li and Zou, 2005; Liu et al., 2008, 2006), and current understanding could attribute this to accelerated proteasomal degradation of DNA damage factor 53BP1 (Gonzalez-Suarez et al., 2011). The repercussive slow response of 53BP1 to recruit to γ-H2AX foci provides one explanation for the slow cell cycle progression of HGPS cells (Liu et al., 2005). Additionally, the PCNA replication factor is re-localised upon truncation of lamin A (Spann et al., 1997), increasing the likelihood of fork stalls, and increased fork-stalling has been recorded in Lmna−/− MEFs (Singh et al., 2013). In the presence of progerin, telomeres exhibit increased γ-H2AX damage-signalling (Decker et al., 2009). If damage is concentrated at telomeres, failure to repair that damage could result in loss of telomeric repeats. However,
one would expect this damage to be distributed randomly throughout telomeric sequences. DNA breaks and subsequent loss of DNA would be initiated at various points along the telomere, resulting in telomeres of a range of sizes and potentially telomere fusions and other aberrations. STELA amplifies telomeres of all sizes under a certain range, and sudden large deletions are readily detectable (Benson et al., 2010). None of the patient lines show signs of such jumps in product size on STELA blots, suggesting that sudden loss is not a cause of increased shortening. It is possible that some forms of damage could result in the loss of the 3’-overhang, the substrate required for STELA PCR. Consequently, STELA may not be able to identify all damaged telomeres.

Using Q-FISH, Benson et al. (2010) found chromosomal aberrations at higher levels at telomeres in HGPS cells compared to controls, consisting of a wide variety of abnormalities, including sister telomere fusions and losses, telomere doublets, chromosomal breaks and extra-chromosomal telomeres observed at only 3.5% of HGPS chromosomes (approximately three anomalies per cell) versus 0.8% of controls (approximately one anomaly per cell) in accordance with findings in Lmna-/- MEFs, despite an absence of sister-chromatid exchange (Gonzalez-Suarez et al., 2009). This marginal difference suggests that although repair of telomeric lesions is impeded in HGPS cells, it is unlikely to impact telomere shortening rate to the degree observed here. It would seem that cells are undergoing a continually increased loss of telomeric DNA. Additionally, a previous study using STELA has identified a frequency of large-scale or catastrophic deletion events of ~3% in normal cells (Baird et al., 2003), suggesting that STELA may in fact be a more sensitive analysis for the identification of lesions. Although it remains possible that a specific route of DNA damage is activated in HGPS cells that nullifies the effectiveness of STELA, the variety of aberrations recorded by Benson et al. suggest that this is not the case.
HGPS cells exhibited a large distribution of telomere lengths, in accordance with previous findings (Benson et al., 2010), and it is likely that a proportion of the shorter telomeres are the result of catastrophic events. In normal cells, short telomeres that have suffered major deletions do not perpetuate in the population, as their numbers do not accumulate over time (Baird et al., 2003). In the case of the HGPS results, it would be difficult to separate this fraction of small telomeres from the senescent population, even if a longer divisional history could be examined, and so it is not possible to identify whether these telomeres are lost from the population, or contributing to accelerated senescence. By observing the smallest telomeres of the remaining patient cell lines, and the distribution of lengths at each time point, neither appreciably alters in any way over time other than has been already described. This further supports the notion that, while catastrophic events may occur at higher rates in patient cells, the effect of this is minimal in terms of a long-term effect on telomere length.

There are three possible scenarios for the fate of a telomere that has been damaged. Firstly, damage may be repaired, and resultant shorter telomeres that acquire or retain an overhang would remain detectable by STELA, but were not observed. Damage resulting in loss of overhangs is not detectable through this method, but if overhangs are left unrepaired, this may initiate a second scenario, in which damage at telomeres or anywhere in the genome cannot be repaired and apoptosis is initiated, however a reduction in viability was not observed in the patient lines according to Trypan Blue analysis. An undetectable effect on viability during proliferation could be attributed to only a small number of cells exhibiting fatal damage, and as such probably would not impact telomere shortening rate to any detectable degree. Thirdly, prolonged DNA damage signalling would activate entry into damage-induced senescence. Although this may not be accurately inferred from the data available, the consistent distribution of telomere lengths throughout the STELA analyses,
most visible in instances where discreet subpopulations exist, as well as the size and distribution of telomere at senescence do not suggest a strong tendency to enter premature cell arrest. Although a combination of these minor effects may potentially be creating the observed effect on shortening rate, another mechanism is more likely at work which elevates the rate of continual telomere shortening in patient cells. An ongoing, increased erosion fits well with the STELA data, and it is possible that this may be a result of slowed cell cycle progression (see previous section).

### 3.3.5 Summary

Five progeroid laminopathy fibroblast lines were grown in culture to senescence, four of which were carried forward for a telomere shortening rate analysis using data collected from STELA and cell division rates as derived from cell counts. In parallel, three control fibroblast lines were cultured, and telomere shortening analysis was carried out on two of these lines, only one of which reached senescence. This cell line (155-BR) grew slowly, resembling the laminopathy lines, although one patient line (E578V) grew at the same rate as the second normal line (NHDF), which grew at a normal rate (~1PD/day). E578V exhibited a normal shortening rate of ~60bp/PD at 12q but a borderline rate of ~155bp/PD at XpYp, perhaps indicative of the mild nature of symptoms associated with the E578V mutation, but could be within normal inter-individual variance in shortening rate. The RD cell line exhibited a significantly elevated rate of telomere shortening at both XpYp and 12q compared to controls. T623S also had a significantly elevated shortening rate at 12q compared to controls, however, despite the rapid rate of shortening at XpYp (~170bp/PD), failed to reach significance compared to controls. A very limited number of PDs was analysed for the HGPS line, causing difficulty in deriving a shortening rate, however results implied an elevated rate, similar to that of T623S and RD.
Due to the chromosomal specificity and high resolution of STELA, it was possible to perform in-depth analyses of the changing telomere population in a dividing primary cell line. Loss of senescent cells from the population was confirmed and found to effect the accuracy of regression analysis to derive telomere shortening rate by affecting the representativeness of PD counts. PCR amplification failure of extremely long telomeres could have a major effect on shortening rate accuracy if long telomere subpopulations drop in during the course of shortening rate experiments. These two factors have several implications for deriving an accurate telomere shortening rate, and for any time-course experiments carried out in primary lines that rely upon accurate PD counts.

In the datasets presented here, only T623S and NHDF seemed dramatically affected by amplification failure at XpYp and 12q respectively. Also, the effect of senescent cell loss proved to be minimal, implying that loss of senescent cells, while an issue, did not appear to have had a major effect on the calculated shortening rates.
Chapter 4  Exploring mechanisms behind accelerated telomeres shortening in laminopathy patient cells

4.1 Background

The STELA data demonstrates an accelerated telomere shortening rate in patient lines containing mutations that result in lamin A forms that retain the farnesyl modification. As none of the patient lines appeared to exhibit sudden loss of large sections of telomeric DNA, it is most likely that the acceleration is caused by a processive and continual increased loss of telomere repeats.

Therefore, lagging strand synthesis and post-replication end-processing are potential sources of increased telomere loss. Control of telomere overhang processing is heavily regulated by the cell cycle both in yeast (Frank et al., 2006; Vodenicharov and Wellinger, 2006) and humans (Dai et al., 2010). Dai et al. (2010) discovered that human overhangs increase in size during the cell cycle, peaking at late S and early G2 phases, when, they concluded, the 5’ strand is extended to reduce overhang length. If telomere end processing is cell cycle dependent, it is possible that extended stays at the various checkpoints could result in abnormally-processed telomere overhangs. As the length of the 5’ end accompanying the 3’ overhang dictates the maximum replicable length of one of the two daughter strands of any replicated telomere, overhang length drives telomere shortening in a proportional manner (Huffman et al., 2000). If processing is occurring in an uncontrolled manner and forming longer overhangs in patient cells, the shorter length of the 5’ strand will drive accelerated telomere attrition.

Sfier and de Lange found that in the absence of 53BP1, overhangs were ten times longer in MEF cells (Sfeir and de Lange, 2012). Upon serial culturing of 53BP1 null MEFs, Q-FISH revealed a 20-30% reduction in telomere length after
only five days. 53BP1 is present at lower levels in lamin null MEFs (Gonzalez-Suarez et al., 2009) and is slow to be recruited to sites of DNA damage in HGPS fibroblasts, as well as Zmpste24−/− MEFs (Liu et al., 2005). Mutated lamin A is able to up-regulate CTSL activity (a major endosomal/lysosomal pathway component), which results in 53BP1 export from the nucleus and its subsequent proteosomal degradation (Gonzalez-Suarez et al., 2011). If the absence of 53BP1 results in dramatically longer overhangs, it is quite feasible that the altered biology of 53BP1 in patient cells could lead to increased overhang length, and the accompanying telomere shortening acceleration.

Although several approaches have been published describing quantification of telomere overhangs, most of these techniques are extremely labour-intensive and require specialist equipment. As the telomeric overhang makes up a miniscule fraction of total genomic DNA, quantification is extremely difficult. So limiting is the amount of DNA that is to be measured, many approaches aim only to establish a relative volume measurement of overhang DNA. That is, the amount of telomeric overhang DNA may be quantified relative to the amount of telomeric or genomic DNA, but the overhang lengths are unknown. The methods of in-gel hybridisation, in which terminal restriction fragments are run on a native gel and hybridised to a telomeric probe in order to detect single stranded telomeric DNA (Dionne and Wellinger, 1996; McElligott and Wellinger, 1997), and the G-tail telomere hybridization protection assay (HPA) which anneals chemiluminescent probes in native conditions to the overhang for direct quantification (Tahara et al., 2005), offer such results. HPA was developed as a high-throughput alternative to the overhang protection assay (OPA). In OPA, overhangs are coated in UP1 and gp32 proteins which protect the overhangs from restriction digest by DNasel, before proteinase K treatment and native gel analysis and size quantification (Chai et al., 2005). In a similar approach, oligonucleotides may be ligated to the overhang in the telomere nucleotide ligation assay (T-OLA) (Cimino-Reale et al., 2001). The method
chosen to carry forward for analysis of overhangs in patient cells, the duplex-specific nuclease (DSN) method, is less reliant on stringency and specialist reagents than these methods. The method exploits the nuclease activity of the DSN enzyme, derived from the Red King crab. This enzyme shows high specificity for double-stranded DNA, and extremely low activity on single stranded DNA (Shagin et al., 2002). Upon complete digestion with DSN, remaining double-stranded products have been reported to be of 6 nucleotides or less, (Shagin et al., 2002). The undigested single-stranded DNA may be run on a native gel and probed for G-rich repeats.

As a negative control for the DSN digest, an ExoI digest is set up in parallel. The 3′→5′ activity of ExoI should fully digest telomeric overhangs. Products are run on a denaturing polyacrylamide gel, Southern blotted and detected by radiolabelled CCCTAA(n) probe specific for the G-rich telomere repeats. Products in the experimental sample appear as a smear which may be normalised to the control lane. The experimental process is outlined in Figure 4.1:1, and the quantification method is detailed in Materials and Methods section 2.11.3.

**Figure 4.1:1** DSN digests are carried out on genomic DNA derived from cultured cells. **a** The experimental sample is digested with DSN alone, leaving the 3′ overhang intact. **b** Control sample is incubated with ExoI prior to DSN digestion. Exonuclease activity of ExoI digests 3′ overhang, releasing deoxyribonucleoside 5′-monophosphates. **c** Experimental and control sample are run side-by-side with a size marker on a denaturing polyacrylamide gel, Southern blotted, and hybridised with radiolabelled C-rich probe (see section 2.9.1.1).
4.2 Results

4.2.1 Initial observations of DSN method

In order to establish the method and optimise conditions, the experiment was carried out on control DNA from HT1080 cell-line stocks. Parallel experimental and control digests were run side-by-side. Smears were observed in the experimental lane, and absent from the control lane when probing with a C-rich labelled oligo (Figure 4.2:1a). Preliminary experiments also showed no signal when probed with a G-rich oligo (Figure 4.2:1c), indicating that single-stranded

![Figure 4.2:1 Preliminary results from DSN overhang assay. A DSN digest and a DSN + Exol digest were carried out on HT1080 DNA (5µg/reaction), run on a polyacrylamide denaturing gel and transferred to a positively charged nylon membrane. Marker sizes are indicated in nt on the left-hand side of the figure. The membrane was probed with a CCCTAA\textsubscript{(n)} radioactively end-labelled oligonucleotide (TelC, see Table 2.9-1) and exposed to a Phosphor Imaging screen (a). The probe was then removed and the membrane re-exposed to confirm probe removal (b). Finally, the membrane was re-hybridised to a TTAGGG(n) end-labelled oligonucleotide oligonucleotide (G1, see Table 2.9-1) to demonstrate overhang specificity (c).]
DNA in the experimental digest consisted of ExoI-sensitive single stranded DNA composed of G-rich 3’overhang.

Standardising to a commercially available size marker, quantification was carried out as previously described (Chai et al., 2005; Zhao et al., 2008) (see section 2.11.3). However, several problems with quantification arose. As the smallest markers of commercially available ladders are either above 20nt or are so small as to result in undetectable signal after hybridisation, extrapolation of the low end of the size range was necessary. As such, a method for quantification of small molecules was devised to ensure accurate sizing of the smallest fragments (see below).

4.2.2 Troubleshooting

It was necessary to develop low molecular markers beyond the range of commercially available ladders and so several oligos consisting of differing numbers of G-rich telomere repeats were generated. The possibility of deriving a ssDNA ladder in the lab was explored but oligonucleotides containing G-rich telomere sequence were chosen to reflect the size and migration of overhang fragments (sequence rich in cytosine runs considerably faster than guanine-rich sequence in polyacrylamide (Frank and Köster, 1979)), and following a personal communication with Professor Woody Wright. However, initial electrophoresis of these oligonucleotides resulted in unexpected sizing relative to the LMW marker. In order to correct this, high-performance liquid chromatography (HPLC) purified oligonucleotides were purchased, however the results were similar (Figure 4.2:2), and the smallest oligonucleotide (G4, 12nt) did not bind the radiolabelled probe efficiently enough to emit a detectable signal.

A more fundamental issue arose at the gel electrophoresis stage. It was apparent from the loading buffer dye front that the migration of the digested samples was retarded in comparison to the size markers (Figure 4.2:3a&b). This
caused major problems with quantification as the relative migration of markers appeared to vary with each gel electrophoresis, and it was clear that the sizes derived were not representative. In order to identify and resolve the cause of the migration problems, several additives were supplemented to a commercial marker, including inactivated enzymes, glycerol and BSA equivalent to that added to digest reactions (Figure 4.2:3d). SDS was added to one digest in an attempt to correct the migration issues, however this was extremely disruptive of migration. It was apparent from agarose gel electrophoresis that small molecules were abundant in DSN digests (Figure 4.2:3c). In order to confirm whether these small fragments were the source of the migration issues, λ and Φ markers were added to digested and undigested genomic DNA (Figure 4.2:3e). When markers were added to undigested DNA, the marker bands migrated unhindered through the gel, however, upon digestion with DSN, migration was seriously affected. Ethanol precipitation was carried out on one digest prior to the addition of marker in order to identify whether small fragments and/or mononucleotides were the cause of the migration problems, as well as a proteinase K treatment to determine whether the protein content of the digests

Figure 4.2:2  HPLC purified oligonucleotides electrophoresed under DSN overhang conditions. Three oligonucleotides of various sizes (G3=21nt, G4=12nt, G5=30nt, see Table 2.5-1) were size separated alongside a low molecular weight marker as indicated. LMW marker sizes are indicated in nt at the far left of the image. Size of each oligonucleotide marker was estimated through extrapolation of the LMW marker data, via the method described in Section 2.11.3. In order to effectively detect the small-sized oligonucleotides, a C-rich 12mer (ssOc12 - Table 2.9-1) was radiolabelled and used in the hybridisation after optimisation.
Figure 4.2:3 Results of DSN overhang optimisation experiments a DSN assay carried out on HT108 DNA with control (DSN+ExoI) and experiment (DSN alone) reactions. Dotted blue line indicates the migration of the bromophenol blue dye front b Photograph of an acrylamide gel during electrophoresis. Lanes contain: 1: LMW ladder, enzyme buffers and glycerol, 2= HT1080 control digest, 3= HT1080 experiment digest, 4= H2O, 5= KK control digest, 6=KK experiment digest c 1% agarose gel containing genomic DNA from HT1080 and KK cell lines. DNA was digested with DSN and and with an without ExoI treatment d LMW ladder resolved on a denaturing polyacrylamide gel with the following additives: 1= none, 2 = heat inactivated enzymes, 3 = control digest products, 4 =BSA, 5 = control digest products + SDS. (e) e λ and Φ markers were mixed with genomic DNA and digested with DSN. Ethanol precipitation (EtOH) and proteinase K (Prot. K) treatment were carried out as indicated.
was an issue. As can be seen in Figure 4.2:3e, the addition of ethanol precipitated DNA allowed similar migration and expected size resolution to the addition of undigested DNA, while proteinase K treatment had no effect.

After completing this process of elimination, it was apparent that the digest process itself was the source of the migration issues. If average overhang length is considered to be 65nt long (Zhao et al., 2008), a conservative estimate for the amount of overhang DNA in a 5µg sample would be ~2pg. Assuming that the completed 5µg digests consisted of almost entirely of ≤6nt fragments, it appeared that overloading of the lanes at this size range interfered with electrophoresis of the very small quantity of overhang products. As current DNA clean-up methods inescapably result in sample loss, and given the closeness in size between the digest bi-products and smaller overhangs, a clean-up method that would discriminate between the two could not be devised.

In order to tailor the DSN assay to the use of patient DNA, digests were attempted using lower amounts of DNA. Signal from hybridisations of digests carried out on less than 5µg DNA was imperceptible. Ultimately, it was not possible to resolve the problems highlighted above, and so it was concluded that this technique is not suitable for use with the smaller amounts of DNA available from patient cells lines.

4.2.3 Native dot blots

As the amount of genomic DNA available from patient cells was a major limiting factor, development of a volume-based method to quantify telomeric single-stranded overhangs was attempted. Although this precluded direct analysis of overhang length, it was apparent from the shortcomings of the DSN method that length analysis would not be possible with the amounts of DNA available from patient cells. Native dot-blotting was selected as a simple and direct method for relative overhang quantification. In order to minimise any pipetting
Figure 4.2:4 Results of native dot blot optimisation experiments. 

**a** T7 digestion timecourse carried out on U2OS cell DNA and hybridised to the radiolabelled probes indicated. 

**b** Quantification of (a) 

**c** Example dot blot showing relative signal before (1) and after (2) Exo I digest from U2OS DNA. Samples were divided into two and loaded onto two separate membranes before probing each with either a C-rich or G-rich telomere-specific probe (contrast levels shown for TelC- and G3-probed membranes not are not equivalent). 

**d** As (c) but with Hirt extracted DNA. 

**e** Quantified C-rich probe signal from three independent replicas (rep 1-3). Hirt extracted DNA was left undigested (1) or Exol digested (2). Error bars represent SEM from three dots, values normalised to undigested DNA. au=arbitrary units.
error and normalise the amount of DNA loaded onto membranes, all samples were partially digested with AluI prior to further digestion and loading.

In order to test the sensitivity of this method, a time-course was carried out on U2OS genomic DNA with T7 exonuclease. T7 is able to resect the 5’ strand of dsDNA, effectively extending overhangs. Digest products were split across two dot blot membranes and then hybridised with either a C-rich telomere probe, TelC, in order to detect the G-rich overhang, or a G-rich probe, G3 as a negative control (see Table 2.9-1 for probe sequences). The relative signal from hybridisation with the TelC probe increased over time but unexpectedly hybridisation with the G-rich probe resulted in a quantifiable signal which was affected by T7 digestion in a similar proportion to the C-rich probe results (Figure 4.2:4a).

Once acquired, DNA samples were split in half. One half was treated with ExoI to remove overhangs while the other half was left undigested. ExoI digested and undigested DNA was then loaded side-by-side in triplicate onto a membrane under native conditions (see section 2.8.2). Membranes were hybridised to G- and C-rich probes (Figure 4.2:3b), and the amount of overhang signal (undigested sample) could then be quantified relative to the signal from the control sample (ExoI digested). Digestion of DNA with ExoI should eliminate overhang signal, however C-rich probe was still bound to dots (Figure 4.2:3b). This signal, and the signal from G-rich probe may have been attributable to large amounts of single stranded telomeric DNA present in ALT cells (Nabetani and Ishikawa, 2009). To counter this effect, modified Hirt extractions were carried out on DNA samples. The Hirt extraction procedure (Hirt, 1967) is normally employed in order to isolate extrachromosomal DNA, such as plasmids, from mammalian cells. A centrifugation step is carried out which results in two fractions – genomic DNA in the pellet and extrachromosomal
DNA in the supernatant. In this case, the supernatant was discarded and the pellet resolubilised in order to purify chromosomal DNA (see section 2.2.2).

Experiments were repeated with Hirt extracted DNA, and although background signal improved, signal was still detectable with the C-rich probe on ExoI digested DNA still, and on G-probed DNA. This was also the case for telomerase positive cell lines (data not shown), which do not contain extra-chromosomal telomeric sequences (Cesare and Griffith, 2004). Large telomere molecules are observed when hybridising with G- and C-rich probes using the DSN method, and this is attributed by the method authors to an unknown fraction of DSN-inaccessible single stranded DNA (Zhao et al., 2008). Either Hirt extraction was not able to separate this fraction from chromosomal DNA, perhaps due to its size, or the stringency of this method is not adequate.

Several identical experiments (with Hirt extraction) were carried out in order to determine whether this background signal from extrachromosomal telomeric DNA in the ALT U2Os cell line caused issues for quantification (Figure 4.2:3d). Results showed some variation in signal from one experiment to another. The signal from ExoI digests was considered as baseline background, and a relative value for each experiment was derived by a ratio of digested:undigested DNA. In the three experiments shown in Figure 4.2:3d, the resultant ratios were as follows: replica 1 = 0.358, replica 2 = 0.531, replica 3 = 0.432. Due to this variation it was decided to abandon this method for quantifying telomere single-stranded overhangs.

4.3 Discussion

4.3.1 Alternative methods for overhang analysis

As neither method above provided consistent or robust results, and given the limited amount of genomic material, it is unlikely that any of the well-defined
techniques that measure overhang directly would be suitable for measurement of overhangs in DNA from patient cell lines, in particular those that measure length, rather than relative measurements.

Cellular levels of POT1 are relative to the amount of single-stranded telomeric overhang (Loayza and De Lange, 2003). Immunofluorescence using a POT1 primary antibody was evaluated as a method for quantification of overhangs. However, the quality of currently available commercial antibodies was not high enough to derive quantifiable data. An alternative application of POT1 antibody would be as an immunoblotting method by quantification of POT1 in lysates derived from patient cells. Again, quantification was not possible due to the quality of the available antibody.

Although every attempt was made to carry out the analysis directly on patient DNA, as the quantity of DNA holds a crucial barrier to accurate quantification it would be more feasible to express mutant lamin A forms ectopically in immortalised cells and harvest large quantities of DNA, and it would be necessary to inactivate telomerase in order to eliminate extension of overhangs. Alternatively, several other possible causes for accelerated shortening were considered and could be explored. These are discussed and some preliminary data presented in section 7.1.1.
PART B • The nuclear lamina and telomere biology in
ALT and Telomerase positive cancer cells

Chapter 5  Differential expression of lamins and related proteins in ALT and telomerase positive cell lines

5.1 Background

An essential stage in cancer cell development is the acquisition of a TMM in order to escape senescence and continue division. Around 85% of cancers carry out telomere extension via activation of telomerase (Shay and Bacchetti, 1997). The remainder of cancers activate an Alternative Lengthening of Telomeres (ALT) mechanism (Bryan et al., 1997), which utilises recombination-based processes to maintain telomeres (Dunham et al., 2000; Varley et al., 2002). As mutations in A-type lamins have been shown to influence telomere maintenance in Chapter 3, A-type lamin function may differ within the context of differential maintenance mechanisms.

Several aspects of nuclear organisation and chromatin status are affected in both lamins-deficient and ALT cells. HGPS cells display a global reduction of condensed heterochromatin, including the constitutive heterochromatin markers found commonly at telomeres (Csoka, 2004; Shumaker et al., 2006). Loss of telomere chromatin condensation also occurs in ALT cells (Episkopou et al., 2014), coupled with increased motility of shortened and de-protected telomeres (Jegou et al., 2009). This increased motility is dependent on 53BP1 (Dimitrova et al., 2008), whose nuclear abundance is modulated by the presence of A type lamins (Gonzalez-Suarez et al., 2011). Therefore, levels of lamin A
could be crucial for 53BP1 activity in ALT cells, and its associated effect on telomere motility.

As well as their role in 53BP1 regulation, lamins may play a more direct role in the mobility of nuclear factors. Telomeres are relocated towards the nuclear periphery in senescent cells and this change in localisation and increased aggregation of telomeres is associated with increasing lamina deformity with continuing cell divisions (Raz et al., 2008, 2006). Lmna-/- MEFs also display a redistribution of telomeres towards the periphery (Gonzalez-Suarez et al., 2009; Uhlírová et al., 2010). Telomeric DNA-containing ALT-associated PML bodies (APBs) are an important marker of ALT activity (see section 1.2.4.2). Although evidence suggests that APBs are resident to recombinatorial processes of ALT (Draskovic et al., 2009), their role remains mysterious as ALT processes may occur in their absence (Jeyapalan et al., 2008). PML bodies associate with the nucleoskeleton (Stuurman et al., 1990) and move more rapidly and freely in lamin A deficient cells, aggregating and localising to the nuclear periphery more frequently (Stixová et al., 2012). APBs may also accumulate outside the nuclear membrane following rupture in laminopathy cells (Houben et al., 2013). Absence of lamin A in MEFs reduces telomere association with PML bodies (Uhlírová et al., 2010), suggesting that an increase in lamin A could aid APB formation. As PML bodies are associated with intranuclear lamins, and a subset of ALT-associated PML bodies are able to mobilise and co-localise with telomeres (Jegou et al., 2009), altered lamin biology in ALT cells could be contributing to APB:telomere interactions.

Previously, no investigation into the status of lamins in ALT cells has been carried out. As such, investigation began with the quantification of lamins, a selection of lamina associated factors, and potential linkers between the lamina and chromatin in a panel of ALT and telomerase positive cell lines (summarised in Table 5.1-1). Three lines with a common derivation but differing telomere
maintenance status were included (JFCF.6T.5K, JFCF.6T-1J/11C and JFCF.6T/11E, hereafter referred to as 5K, 11C and 11E, respectively) in order to observe potential differences between ALT and telomerase lines in the context of as close an isogenic background as possible.

<table>
<thead>
<tr>
<th>ALT</th>
<th>Telomerase positive (Tel+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>Origin/derivation</td>
</tr>
<tr>
<td>WI38ALT</td>
<td>Lung fibroblast (foetal)</td>
</tr>
<tr>
<td>SUSM-1</td>
<td>Liver fibroblast (embryonic), transformed</td>
</tr>
<tr>
<td>JFCF6T.5K</td>
<td>CF jejunal fibroblast (post crisis), transformed</td>
</tr>
<tr>
<td>JFCJ.6T-1J/11E</td>
<td>CF jejunal fibroblast, transformed</td>
</tr>
<tr>
<td>WV</td>
<td>Werner syndrome fibroblast, transformed</td>
</tr>
<tr>
<td>Saos</td>
<td>Osteosarcoma</td>
</tr>
<tr>
<td>U2OS</td>
<td>Osteosarcoma</td>
</tr>
<tr>
<td>Illcf/2a</td>
<td>Li-Fraumeni breast fibrosarcoma</td>
</tr>
</tbody>
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| **Table 5.1-1** ALT and telomerase positive cell lines used in this chapter. Further information, including culturing conditions may be found in Table 2.1-1. CF=cystic fibrosis, HNPCC=Hereditary non-polyposis colon cancer |

## 5.2 Results

### 5.2.1 Protein quantification by Western blot

Western blotting was employed to establish endogenous levels of lamins and related proteins in ALT and telomerase cells. Initially, the telomerase positive line NT2D1 was included in the cell line panel. However, this line displayed
unusual characteristics, such as absence of A-type lamins and SUN proteins. Statistically, protein levels differed significantly from the other telomerase positive lines for all but 53BP1, SUN1 and SUN2 content, despite the apparent absence of SUN from NT2D1 (Appendix E). As A-type lamins have key roles in differentiation and development, these differences were most likely attributable to the cell line's derivation from pluripotent embryonic cells, and as such NT2D1 was excluded from the final panel.

Lamins A and C were quantified using an antibody raised against an N-terminal orientated epitope capable of identifying both splice forms. Lamin A was present in telomerase positive cells at almost three times the amount in ALT cells (p<0.0001), and lamin C was also in significantly greater abundance in

**Figure 5.2:1** Representative Western blot of each protein and cell line in the panel. Whole cell lysates were derived from cell pellets of each line by the addition of Laemmli buffer. Lysates were size separated on SDS-PAGE gels containing the appropriate percentage of acrylamide with Page Ruler Plus protein marker (see section 2.13). Control immunoblotting was carried out on the same membrane wherever possible. Bands were normalised within each blot in order to allow comparison across blots. ALT (A) or telomerase positive (T) status is indicated at the bottom of the figure.
telomerase positive cells, but at only a 1.4-fold increase (p=0.0016). Lamin B was significantly elevated in telomerase positive cells (p=0.0007), by a factor of 1.2 (Figure 5.2:1 and Figure 5.2:2a-c). Loss of A-type lamins has been shown to activate the degradation pathway of 53BP1 in MEFs (Gonzalez-Suarez et al., 2011). To assay whether levels of 53BP1 were affected by the differential levels of A-type lamins in ALT and telomerase positive cells, 53BP1 levels were also quantified. However, 53BP1 levels were not significantly different between the two sets of cell lines, nor did lower levels of lamin A in individual cell lines

![Graphs showing protein content](image)

**Figure 5.2:2** Total protein content as quantified by Western blot in ALT and telomerase positive cell lines, normalised to β-tubulin. Error bars show SEM from three independent biological replicas. p values are derived from t tests on ALT versus telomerase positive cells. au=arbitrary units  

- **a** Lamin A levels are significantly higher in telomerase positive cells (p<0.0001)  
- **b** Lamin C levels are significantly higher in telomerase positive cells (p=0.0016)  
- **c** Lamin B1 levels are significantly higher in telomerase positive cells (p=0.0007)  
- **d** 53BP1 levels are not significantly different between ALT and telomerase positive cells.
correlate with lower 53BP1 levels (compare Figure 5.2:2a&d). SUSM-1, a DSB repair deficient line derived from embryonic tissue, showed extremely low levels of 53BP1, likely contributing to its DNA repair status.

The HP1 protein family was identified as a candidate linker for lamina and

![Figure 5.2:3](image_url) Total protein content as quantified by Western blot in ALT and telomerase positive cell lines, normalised to either α-tubulin or β-tubulin as indicated in brackets in legend. Error bars show SEM from three independent biological replicas. p values are derived from t tests on ALT versus telomerase positive cells. au=arbitrary units a HP1-α results (β) b HP1-γ results (β) c SUN1 results (α) d SUN2 results (β) e RAP1 results (α)
chromatin interactions (see sections 1.1.3.3 & 1.1.5.3). HP1-α is capable of binding LBR (Ye et al., 1997), pre-lamin A and LAP2α (Lattanzi et al., 2007). HP1-α levels are reduced in HGPS cells (Scaffidi and Misteli, 2005), while APB formation is dependent on the presence of HP1 proteins (Jiang et al., 2009). Therefore levels of HP1-α were investigated but were not significantly different between ALT and telomerase lines (Figure 5.2:3a). Subsequently, HP1-γ was considered due to its role in telomere maintenance (Canudas et al., 2011) and were found to be almost two-fold higher in telomerase positive cells (p<0.0001, Figure 5.2:3b).

SUN proteins are integral membrane proteins associated with the lamina, and SUN1 is required for chromatin detachment from the nuclear membrane in mitosis (Patel et al., 2014; Turgay et al., 2014). SUN1 also plays a role in tethering telomeres to the nuclear membrane, aiding post-mitotic re-assembly (Crabbe et al., 2012), and so may have a role in telomere anchorage in ALT. SUN1 levels were investigated and proved to be present in telomerase cells at almost 4.5 times the level in ALT cells (p<0.0001, Figure 5.2:3c). SUN2 was also examined and no significant differences were found between SUN2 levels in ALT and Telomerase positive cells. Interestingly, SUN2 levels were so low in the telomerase positive lines SW480 and LoVo as to be virtually absent (Figure 5.2:1 and Figure 5.2:3d). As both these lines are derived from colon tissue, this is most likely indicative of an underlying biology specific to this cell type. Upon exclusion of these cell lines from the SUN2 data, differences remain non-significant (p=0.5891). As SUN1 may link chromatin to the lamina via RAP1 (Crabbe et al., 2012), levels of RAP1 were quantified and found to be more than twice as abundant in telomerase positive cells (Figure 5.2:3e).
5.2.2 Protein quantification and identification of lamin localisation by immunofluorescence microscopy

In order to confirm the Western findings, and verify any differences in protein localisation between ALT and telomerase positive cell lines, immunofluorescence (IF) microscopy was carried out on three representative ALT and three representative telomerase positive lines, including 11E (ALT) and 11C (Tel+). Lamin A/C, lamin B, 53BP1 and HP1-γ were chosen for further investigation by IF. The Olympus Scan R platform was used to gather the IF data. Several hundred cells were scanned for each protein in each cell line, and several parameters were recorded, including the total signal intensity of each.

Figure 5.2:4 Comparison of Western blot and IF total protein results. IF values are normalised internally to allow comparison to Western data. t tests were carried out to compare ALT and Tel+ cell line total protein results for IF and did not reveal significant differences. Error bars indicate error from two biologically independent repeats for IF, and three independent repeats for Westerns. au=arbitrary units.
protein per nucleus, and the size-normalised signal per nucleus, derived by normalising to DAPI signal. DAPI staining was also used to select for single nuclei, and exclude nuclei displaying extreme abnormalities, such as very low circularity.

In order to compare total protein as assayed by Western blot and IF, the total signal intensity values provided by the Scan R software were internally normalised to sit within the range of the Western total protein values (arbitrary units). As lamin A and lamin C were both quantified using a single antibody, it was not possible to discriminate between the two when analysing the IF data, and so total lamin A and C Western values were combined prior to comparison. The results for IF and Westerns differed for relative total protein content (Figure 5.2:4), most likely due to the differential availability of epitopes to the primary antibodies between the two techniques. IF total signal of each protein was not found to differ significantly between ALT and telomerase cells (see below), despite close parity between the total protein results for IF and Western results for lamin B and HP1-γ (Figure 5.2:4b&d).

This could be due to the automated counting system, as optimal parameters for nucleus recognition is not possible between different cell lines. This may result in cells with certain morphologies to be discounted from the data pool. For example, the relative size of nuclei did not differ significantly between ALT and telomerase cells (data not shown). ALT cell nuclei were observed to be more variable in size through manual screening, potentially due to increased amounts of DNA resulting from extrachromosomal telomeric DNA. This implies that large nuclei were not included in the ALT data, although every care was taken to allow inclusion of these cells. Circularity also did not differ significantly between telomerase and ALT cells (data not shown) despite the presence of considerable numbers of misshapen nuclei observed by manual screening particularly in WI38ALT and U2OS cells (Figure 5.2:5-Figure 5.2:8). However, the screening did
**Figure 5.2:5** IF results for lamin A/C.  
**a** Microscope images showing DAPI and lamin A/C immunostaining as indicated.  
**b** Mean total intensity of lamin A/C in each cell line.  
**c** Mean size-normalised intensity, defined as the total amount of lamin A/C relative to DAPI staining in each cell, as derived by Scan R software.  
**d** Mean total intensity of lamin A/C located at the nuclear periphery.  
**e** Mean ratio of intranuclear lamin A/C relative to peripheral lamin A/C. Error bars represent error from two independent biological replicas.  
au=arbitrary units.
Figure 5.2: IF results for lamin B

a) Microscope images showing DAPI and lamin B immunostaining as indicated.
b) Mean total intensity of lamin B in each cell line.
c) Mean size-normalised intensity, defined as the total amount of lamin B relative to DAPI staining in each cell, as derived by Scan R software.
d) Mean total intensity of lamin B located at the nuclear periphery.
e) Mean ratio of intranuclear lamin B relative to peripheral lamin B. Error bars represent error from two independent biological replicas. au=arbitrary units.
reveal differences between lamin localisation in ALT and telomerase cells.

Nuclear levels of Lamin A/C did not differ between ALT and telomerase positive cells when measured according to total lamin A/C (Figure 5.2:5b), or relative to size as indicated by DAPI staining (Figure 5.2:5c). In order to determine whether lamin A/C localisation at the nuclear periphery varied between ALT and telomerase cell lines, Scan R was programmed to identify the nuclear perimeter according to the edge of the DAPI staining and quantify the amount of lamin within 3 pixels either side of this boundary. Total peripheral lamin A/C was not significantly different between ALT and telomerase positive cells (Figure 5.2:5d), however intranuclear levels of Lamin A/C were significantly higher relative to peripheral levels in ALT cells (p=0.0164, Figure 5.2:5e), although this effect was minimal (1.1 fold higher).

These measurements were repeated for lamin B (Figure 5.2:6). Again, nuclear levels of lamin B were not significantly different between ALT and telomerase positive cells (Figure 5.2:6b&c), although total lamin B was almost significantly higher in ALT cells (p=0.0532), counter-intuitive to the Western data. Examination of total peripheral lamin B again revealed no significant difference (p=0.2704, Figure 5.2:6d), while the proportion of intranuclear lamin B was 1.4 times higher in ALT cells (p=0.0087, Figure 5.2:6e).

Due to the extremely large size of 53BP1 (>250kDa), a specialised transfer procedure was developed for Western blotting, however, variability between replicas was relatively high. Due to this level of uncertainty, IF was partly carried out in order to verify the Western findings. 53BP1 results correlated with Western findings, in that no significant difference was found between the total 53BP1 in ALT and telomerase cells (Figure 5.2:7b&c), although IF also exhibited more variation between replicas than the rest of the proteins examined in the panel. Automatic foci counting was carried out on 53BP1 immunostained cells.
Figure 5.2:7 IF results for 53BP1. 

a) Microscope images showing DAPI and 53BP1 immunostaining as indicated. Green channel has been enhanced post-capture in order to aid visualisation.

b) Mean total intensity of 53BP1 in each cell line. 

c) Mean size-normalised intensity, defined as the total amount of 53BP1 relative to DAPI staining in each cell, as derived by Scan R software.

d) Percentage of cells with zero, 1-5 or >5 53BP1 nuclear foci as detected by Scan R in ALT and telomerase positive cells.
and foci counts were found to differ despite no overall difference in 53BP1 levels. Telomerase positive cells were three times more likely to contain no 53BP1 damage foci than ALT cells (p=0.0230), and ALT cells were more than twice as likely to contain more than five foci (p=0.0059, Figure 5.2:7d.). This is in accordance with elevated damage levels and rearrangements in ALT cells. The comparable amounts of 53BP1 between ALT and telomerase cells may have
been due to a higher quantity of diffuse 53BP1 in telomerase positive cells (Figure 5.2:7a). Finally, although total HP1-γ levels were not found to be significantly different between ALT and telomerase positive cells, which may have been due to highly variable amounts of HP1-γ, primarily in the telomerase positive cells, size-normalised values were approximately 1.5 times higher in ALT cells (p=0.0379, Figure 5.2:8).

5.3 Discussion

Several differences in lamina-associated factors were identified between ALT and telomerase positive cells through protein quantification and localisation assays. Lamins A, B and C and SUN1 were found in higher abundance in telomerase positive cells, compared to ALT cells, as well as HP1-γ and RAP1, candidate linkers for chromatin:lamina interactions. The panel of ALT and telomerase positive cells that were investigated with Western blots included three cell lines derived from a single individual which have adopted different telomere maintenance mechanisms during establishment in culture. As these three lines share the same origin, differences observed between them are more likely to reflect differential mechanistic properties between the ALT and telomerase maintenance programs, rather than inter-individual differences. Some divergence will have occurred in the derivation of these lines, as may be seen in the occasional variation between 11E and 5K despite both being ALT, although this may be as a result of differing ALT pathways (alternative modes of ALT-based recombination have been reviewed by Cesare & Reddel 2010).

Direct comparison of the JFCF.6T cell Western results supports most of the findings from the broader panel (Figure 5.3:1). Levels of A-type lamins were found at higher concentration in the telomerase positive 11C line, while no difference was found between the ALT and telomerase positive JFCF.6T lines for lamin B. As lamin B levels were found to be extremely consistent in telomerase
cell lines (Figure 5.2:2c), this is possibly due to the greater variability in the 11E replicas. The results are sensitive to an outlier in the 11E data-set and exclusion of this point gives a significant difference between the ALT and telomerase cells (p=0.032). Excluding this outlier, all proteins examined followed the trend of increased protein levels in the telomerase positive line, although the differences for SUN1 and RAP1 failed to reach significance (Figure 5.3:1). Interestingly, SUN2 and HP1-α levels were found to be significantly lower in the ALT lines 11E and 5K lines, compared to 11C, whereas no significant difference was identified in the full panel. 11E and 5K differed significantly in their SUN2 content, despite both being ALT positive (p=0.0310), and levels were highly variable between both the different ALT lines and telomerase lines in the full panel. This supports the initial finding from the complete panel that there is no relationship between SUN2 levels and TMM. No such difference existed between 11E and 5K for HP1-
α (p=0.6184), putting the panel analysis for HP1-α into question. In a similar fashion to SUN2, HP1-α levels were dramatically lower in the colon-derived SW480 and LoVo telomerase positive lines. Exclusion of these lines from the analysis results in a significant difference in HP1-α levels (p=0.0125), marking it as potential candidate for involvement in ALT and telomerase differential activity.

Lamin A, B1 and C were found at significantly higher levels in telomerase positive cells compared to ALT cells according to the complete panel data, although this finding was not repeated with the immunofluorescence data. The reduced and denatured conditions of Western blotting allow for greater accessibility to antibodies, and is therefore a more reliable reference for total protein content. Also, IF signal may not be linear in relation to the amount of protein in the cell due to steric hindrance in this respect. This discrepancy alludes to the existence of a subset of lamins in telomerase positive cells that is less accessible, and could be explored via solubility assays. At least one epitope of lamin A located between residues 171 and 246 is accessible to an antibody characterised by Jagatheesan et al. (1999) on intranuclear lamin A, but not the peripheral fraction (Jagatheesan et al., 1999). Again, this supports the hypothesis that intranuclear lamin A forms alternate structures compared to peripheral lamin A, and may reflect the alternate functions of the two lamin A pools (Dechat et al., 2010). If intranuclear lamin A plays a role in APB formation or localisation, this may explain how a decrease in lamin A that would be anticipated to inhibit APB formation (Uhlírová et al., 2010) is present in ALT cells.

The recorded data from the IF screen for area and circularity did not reflect the observed variable size or increase in nuclear deformity of ALT cells, and it is possible that large and/or misshapen nuclei may have been excluded from the screen. If this is the case, and the Western data is the most representative, it is possible that the amount of lamin could remain constant, while becoming more
diffuse within enlarging ALT nuclei. As lamins quantitation was relative to tubulins, in this scenario tubulin levels would need to increase to compensate for the increased nuclear size. This correlation could be identified via the use of fluorescence-activated cell sorting, which would be able to size each nucleus according to DNA content, while quantifying lamin signal.

ALT cells that were included in the IF screen contained a lower proportion of lamin A, B1 and C at the nuclear periphery compared to telomerase positive cells. Increased relative levels of lamin A at the nuclear periphery is a marker of HGPS, resulting from the increased integration of lamins into the nuclear matrix, and is associated with a reduction of lamin A mobility (Dahl et al., 2006; Scaffidi and Misteli, 2005). In normal cells, lamins are continually produced and integrated into the lamina (Gerace et al., 1984). Two distinct fractions of lamin A are distinguishable through their differential motility — approximately 15% of lamin A is able to move freely and rapidly throughout the nucleoplasm, while the remaining fraction, as well as periphery lamin A is more stable, indicating a higher order of lamin structure (Dahl et al., 2006). On the other hand, the presence of progerin eliminates almost all mobility of both WT lamin A and progerin, resulting in the sequestration of both at the nuclear periphery, and the typical thickened lamina observed in HGPS (Dahl et al., 2006; Goldman et al., 2004; Scaffidi and Misteli, 2005). In FRET assays, fluorescence recovery is absent from the lamina after normal levels of lamin A have recovered within the nucleoplasm even in WT cells (Dahl et al., 2006), underlining the stable and immobile nature of lamin A that is integrated into the lamina. Additionally, once integrated, mature lamin A does not return to the intranuclear space, but reforms solely at the periphery after mitosis (Goldman et al., 1992), further supporting the existence of alternative structure formations with differential solubility and localisation properties.
These data support the existence of a less soluble and proportionally larger fraction of peripheral A-type lamins in telomerase cells compared with ALT cells. The capacity of progerin to impair nuclear mobility, including telomere movement (De Vos et al., 2010) suggests that alterations in lamina organisation may directly influence telomere mobility. Indeed, loss of lamin A results in an increase in telomere mobility in fibroblasts (De Vos et al., 2010). Given that telomere motility is abnormally elevated in ALT cells (Jegou et al., 2009), it is most likely that the properties of lamin A in telomerase cells reflect those of normal cells, while in ALT cells higher order organisation of A-type lamins is limited in order to maintain a more motile nuclear environment, conducive to the ALT mechanism. The differential motility of lamins in ALT, telomerase positive and normal cells could be established through the application of FRAP, however introduction of exogenous, fluorescently-tagged lamin A into ALT cells could result in a reversal of increased nuclear motility, as this phenotype is could be due to the reduced amount of lamin A in these cells, as lamin organisation is concentration dependent (Guo et al., 2014).

53BP1 promotes increased telomere mobility (Dimitrova et al., 2008), however levels were not found to be affected in ALT cells. In the absence of progerin, lamin A deficiency results in an increase in telomere motility in fibroblasts (De Vos et al., 2010) despite the reduction in 53BP1 that this would be expected to incur (Gonzalez-Suarez et al., 2011). It is therefore possible that the observed reduction in lamin A/C is adequate to facilitate nuclear motility without activating 53BP1 degradation, or that 53BP1 function is altered in a way that cannot be observed through quantification alone, which may be the case in HGPS patient cells (see preliminary data in section 7.1.1.1).

A-type lamins are capable of stabilising DNA repair foci in the nucleus (Mahen et al., 2013). Positional immobilisation of chromatin in the nucleus restricts DSB repair, while increased mobility results in increased genomic instability through
induction of inappropriate exchanges (Chiolo et al., 2011; Soutoglou et al., 2007), which would benefit ALT cells. Depletion of macromolecular lamin structures responsible for increased nuclear stability could contribute to increased mobility of telomere ends to promote recombination in ALT cells. Loss of lamin A results in decondensation of heterochromatin in MEFs (Gonzalez-Suarez et al., 2009), and decondensation of telomeres enables enhanced telomere elongation through recombination in mouse cells (Benetti et al., 2007; Blasco, 2007; García-Cao et al., 2004). The observed reduction in HP1-γ is consistent with this mode of action. If loss of A-type lamins contributes to the loss of heterochromatin in ALT cells, this presents another potential mechanism by which reduced nuclear lamin could facilitate the ALT telomere maintenance program.
Chapter 6 Interactions between chromatin and lamins in ALT and telomerase positive cells

6.1 Background

As A-type lamins are at a lower level in ALT cells and this may be contributing to increased telomere motility, it is possible that direct or indirect interactions between the lamina and chromatin would be altered in ALT cells. Differential distribution of peripheral lamins and intranuclear lamins suggests a conformational change in higher order lamin A structure, which in turn could affect steric interactions with DNA binding factors. Lamin A is able to engage chromatin through γ-H2AX in order to stabilise DNA damage repair foci (Mahen et al., 2013), and binds and associates with several factors that bind DNA directly such as BAF, LBR (Dechat et al., 2004), lamin B (Dechat et al., 2004; Martin et al., 2010), HP1-α (Lattanzi et al., 2007) and LAP2α (Dechat et al., 2004; Lattanzi et al., 2007) as well as indirectly, for example LBR may bind to chromatin through HP1-α (Ye et al., 1997). Although many of these interactions are transiently involved in mitosis, differences in nuclear matrix components were clearly detectable in asynchronous cells (see Chapter 5), suggesting a role for the lamina in telomere organisation outside of the mitotic cycle.

Chromatin immunoprecipitation (ChIP) was therefore carried out on ALT and telomerase positive asynchronous populations in order to identify differential telomere binding to A-type lamins. These experiments were carried out prior to the publication of a study identifying SUN1 as a linker between the lamina and telomeres via its binding to RAP1 (Crabbe et al., 2012). As SUN proteins had already been identified as chromatin interactor candidates, ChIP was attempted with both SUN1 and SUN2 antibodies but no telomeric DNA was pulled down (data not shown). As such, chromatin immunoprecipitation (ChIP) was carried
out using a lamin A/C antibody with the aim of co-immunoprecipitating interacting partners of lamin A that were responsible for telomere binding, along with any bound telomeric DNA. As HP1 proteins were also promising candidates identified in the quantification stage, ChIP was also performed using HP1-α and HP1-γ antibodies.

6.2 Results

6.2.1 Optimisation and verification of ChIP

To investigate a direct or indirect interaction between A-type lamins and telomeric chromatin, ChIP was undertaken on a selection of ALT and telomerase positive cell lines. The goal of ChIP is to isolate genomic DNA sequences associated with proteins of interest. In order to do so, proteins and chromatin must first be cross linked with the use of formaldehyde before shearing the chromatin to a suitable size, in this case via sonication. Fragmented chromatin is then immunoprecipitated with an antibody specific to the protein of interest, purified and quantified. Large numbers of cells are generally required, although several relatively new techniques are available which require lower amounts of starting material (Acevedo et al., 2007; Adli and Bernstein, 2011; Dahl and Collas, 2008; Gilfillan et al., 2012).

Due to the complexity of the ChIP technique, several stages required optimisation and verification. Firstly, choice of antibody is imperative to the success of IP. A polyclonal Lamin A/C antibody previously used in ChIP experiments in MEFs (Gonzalez-Suarez et al., 2009) was chosen as no previous work had published the use of an A-type lamin antibody in ChIP of human chromatin. To confirm specificity of the IP, a “ChIP-validated” H3K9me3 antibody was selected as an experimental control for ChIP due to its common application in this role and its well established association with
heterochromatin. A normal rabbit IgG was used as a negative control. ChIP was also attempted with commercially available “ChIP-validated” antibodies against HP1-α and HP1-γ.

Figure 6.2:1  

**a** Immunoblot for H3K9me3 ChIP validation using two lots of ab8898 (lot number indicated at top of blot) showing differential IP efficiency. E1 indicates a mild elution, E2 is a harsh elution (see section 2.6.7).  

**b** H3K9me3 ChIP validation using Active Motif H3K9me3 antibody 3916, Immunoblotted with Active Motif 3916 and ab8898  

**c** Immunoblot validating HP1-α and –γ antibodies for use in ChIP  

**d** Immunoblot validating lamin A/C sc-6215 antibody for use in ChIP  

**e** ChIP chromatin after sonication (see section 2.6.3.1). IP=immunoprecipitation, IB=immunoblotted protein
The specificity of all antibodies was tested via IP under ChIP conditions (see section 2.6.7) and Western blotting (Figure 6.2:1). Repeated rounds of verification on the same “ChIP-verified” H3K9me3 antibody purchased from the same company at different times revealed a high degree of batch-to-batch variation in IP efficiency (Figure 6.2:1a). Ultimately, to avoid this variation, only one batch of each antibody was used for full-scale ChIP after verification. The H3K9me3 antibody subsequently purchased from Active Motif and used in ChIP was specific for H3K9me3 in IP but was not suitable for Western. This IP was therefore verified by use of the previous H3K9me3 antibody purchased from Abcam (Figure 6.2:1b). Verification via this method gave specific and consistent results for the remaining antibodies used (Figure 6.2:1c&d).

Another essential step in ChIP optimisation is the shearing treatment of chromatin to ensure acquisition of fragments of a suitable size for capture during the IP steps. A standard size range for input chromatin is 400-600bp (Schoppee Bortz and Wamhoff, 2011) allowing for at least two nucleosomes (usually spaced 147bp apart (Richmond and Davey, 2003)) to be bound to individual fragments. However, when investigating indirect chromatin interactions, increasing the size of this range may aid in IP of target sequence due to increased inefficiency (Prof Rob Klose - personal communication). Shearing efficiency was initially optimised for each cell line and then median size of chromatin fragments was confirmed for input chromatin for each replica (Figure 6.2:1c&d). Lysis was also optimised after it was found that cells were not lysed in standard ChIP lysis buffers. As a result, nuclei isolation took place in buffer containing SDS at room temperature (see section 2.6.3).

The length of time that lysates and antibody-bead conjugates were incubated was optimised in parallel with the amount of antibody for optimal noise to signal ratio. As small amounts of short DNA fragments are isolated by ChIP, a commercially available kit specifically developed for ChIP purification was used.
The IPure kit (Diagenode) exploits the negative charge of the DNA backbone and uses magnetic beads for purification in order to minimise DNA loss. ChIP output of telomeric sequence was quantified by dot blot and the specificity of ChIP verified by PCR. Primer sets were chosen for regions known to be composed of open or closed chromatin to verify heterochromatin specificity for H3K9me3, HP1-α and HP1-γ. The tandem repeat locus D4Z4 has previously been reported to associate with lamin A, and previously published primer sequences were used as a positive control for lamin A interactions (Ottaviani et al., 2010).

6.2.2 A-type lamins do not associate differently with telomeric chromatin in ALT versus telomerase positive cells

The results for each replica of ChIP are shown in Figure 6.2:2, Figure 6.2:3 and Figure 6.2:4. The GAPDH promoter primer set was used as an indicator of open chromatin (designed by Jon Williams, Royle laboratory), due to its constitutive expression. An unidentified, non-specific small product was sporadically amplified with this primer set (see Figures 6.2:2-3). The IgX primers (designed by Abcam) anneal at a gene desert, and were used to indicate and confirm closed chromatin. D4Z4 is a tandem repeat and therefore also represents closed chromatin. The results of the semi-quantitative PCR assays for IgX suggest that the H3K9me3 ChIP was highly inefficient as considerable binding of this marker for closed chromatin would be expected at this gene desert. Despite the PCR analysis being only semi-quantitative, the dot blots carried out confirmed low efficiency as low levels of telomeric DNA was also pulled down. Initial optimisations of ChIP were able to IP telomeric DNA and the IgX region at a level that far exceeded the final results, while pulling down minimal amounts of open chromatin as confirmed by the GAPDH promoter PCRs. However these results could not be reproduced. It is possible that the optimised conditions arrived at in order to quantify lamin A/C interactions became sub-optimal for
Figure 6.2:2 First ChIP replica a Results of semi-quantitative PCR on ChIP samples with the primer sets indicated. Products were run on a 2.5% NuSieve agarose gel b H₂O control samples for semi-quantitative PCR c Diagram illustrating sample locations in each section of d d Dot blot of ChIP samples including input references. 10μl of a 50μl reaction was loaded for each ChIP.
Figure 6.2:3 Second ChIP replica a Results of semi-quantitative PCR on ChIP samples with the primer sets indicated. Products were run on a 2.5% NuSieve agarose gel b H2O control samples for semi-quantitative PCR c Diagram illustrating sample locations in each section of d d Dot blot of ChIP samples including input references. 10μl of a 50μl reaction was loaded for each ChIP.
Figure 6.2:4 Third ChIP replica a & b Results of semi-quantitative PCR on ChIP samples with the primer sets indicated. Products were run on a 2.5% NuSieve agarose gel c H2O control samples for semi-quantitative PCR d Diagram illustrating sample locations in e e Dot blot of ChIP samples including input references. 10µl of a 50µl reaction was loaded for each ChIP, except in the case of HeLa where all quantities were doubled.
direct interactions with chromatin. Due to the ChIP inefficiency of HP1-γ at low antibody concentrations that would allow for the use of a single batch of antibody, this IP was discontinued, although this initial ChIP indicated elevated association of HP1-γ with telomeres in HeLa and HT1080 cells (Figure 6.2:5e).

The final ChIP data for telomeric IP achieved from each antibody is given in duplicate only in Figure 6.2:5 as the third replica showed high levels of background in IgG ChIPs in both the PCRs and dot blots, as well as an apparent absence of IP in the JFCF.6T lines and HT1080 (Figure 6.2:4b&e). In accordance with published data comparing ALT and telomerase lines derived from a
common source (Episkopou et al., 2014), H3K9me3 has a higher association with telomeres in telomerase positive cell lines than in ALT lines \((p=0.008, \text{ Figure 6.2:5a})\). In order to allow comparison with total H3K9me3, Western blotting was carried out on each line (Figure 6.2:5b). This did not reveal a significant difference between a selection of ALT and telomerase positive cell lines from the panel. No significant difference was observed between ALT and telomerase cells for lamin A and HP1-α ChIPs (Figure 6.2:5c&d).

### 6.2.3 Discussion

Although the efficiency of ChIP was low, results for H3K9me3 were in agreement with previously published ChIP data (Episkopou et al., 2014). Telomeres were found to be associated with H3K9me3 at higher levels in telomerase positive cells compared with the ALT lines, despite no difference in global H3K9 trimethylation levels. No significant difference was found between the JFCF ALT and telomerase lines \((p=0.1333)\).

Episkopou et al. found that ALT telomere chromatin is packaged more loosely at telomeres, that is, a greater distance between nucleosomes was observed. The proportion of H3K9 residues that were trimethylated remained constant, regardless of telomerase activation status and the reduced abundance of histones at ALT telomeres. Also, heterochromatin status was found to be unchanged at ALT centromeres, and micrococcal nuclease access to genomic chromatin was unaffected, making it very unlikely that nucleosome redistribution or H3K9 methylation status would be significantly altered in the rest of the genome.

It is quite possible that telomere histone content is relatively constant between ALT and telomerase positive nuclei, and that nucleosomes are merely spread more diffusely across the additional telomeric DNA present in ALT cells. A relative increase in telomeric content in ALT versus telomerase cells is apparent
in the signal differences in the dot blots shown in Figures 6.2:2-4, in agreement with previously published Q-PCR findings (Lau et al., 2013). The ALT cell lines reproducibly exhibited up to ten times the amount of telomeric DNA in input dilutions despite similar cell numbers in each sample. Indeed, to maintain a comparable range, double loading was eventually used on the HeLa ChIP extracts (Figure 6.2:4e).

Total H3K9me3 levels were not reported in the study by Episkopou et al., but together these data suggest that the number of H3 histones at telomeres remains unaltered upon a cell adopting an ALT pathway, as does the trimethylation status of the H3K9 residue. This implies that distribution of nucleosomes is affected only at telomeres, which become elongated in an unregulated fashion in ALT, and the level of histone binding does not compensate for this additional length. This is consistent with the suggestion that shelterin binding may be reduced at ALT telomeres, and with evidence of differential composition of binding factors at ALT telomeres (Conomos et al., 2013). Alterations in ALT telomere structure result in the loss of association of several known telomere-binding proteins, as well as ALT-specific binding of orphan receptors to telomeres (Déjardin and Kingston, 2009). As longer telomeres in telomerase positive cells have a higher density of H3K9me3 (Arnoult et al., 2012; Déjardin and Kingston, 2009), the process by which telomere repeats are gained must dictate whether or not additional nucleosome units are formed, and could therefore be processively linked to the action of telomerase.

As no previous investigation has been carried out into differential association of HP1-α and –γ at telomeres in ALT and telomerase cells it is not possible to compare these data to any published work. The initial results from HP1-γ ChIP correlate with increased telomeric binding to heterochromatin proteins in telomerase positive cells, however this increase aligns with an increased
presence of HP1-γ in telomerase cells (although 11C is not in accordance with this trend (Figure 6.2:6d)). HP1-γ binds chromatin through the H3K9me3 residue (Lachner et al., 2001), and so reduced H3K9me3 binding to ALT telomeres could be anticipated to affect HP1-γ binding relative to telomeric content in this manner. Hp1-γ levels were found to be almost two-fold higher in telomerase positive versus ALT cells from Western analysis (section 5.2.1). Given that global levels of H3K9me3 do not differ between the two TMM types, this could indicate an additional role for HP1-γ besides chromatin compaction in telomerase positive cells.

Figure 6.2:6 Comparison of Western and ChIP results. ChIP results have been internally normalised to facilitate comparison. a H3K9me3 b lamin A/C c HP1-α d HP1-γ
There was no observable trend between levels of HP1-α and levels of bound telomeric DNA, and increased levels of HP1-α in telomerase positive cells did not reflect an increase in HP1-α association with telomeres (Figure 6.2:6c). This difference may be due to the differential roles of HP-1γ and HP1-α at heterochromatin. While HP1-α is primarily associated with centromeric chromatin (Frydrychova et al., 2008; Hayakawa et al., 2003; Perrini et al., 2004), HP1-γ may locate to both heterochromatin and euchromatin (Luke and Lingner, 2009; Schoeftner and Blasco, 2010).

TERRAs are Telomeric repeat-containing RNA transcribed at telomeres and may have a role in epigenetic remodelling of telomeres and telomerase expression (Minc et al., 2000). Arnoult et al. (2012) found HP1-α expression is required to repress TERRA expression, while TERRA expression is elevated in ALT cells (Episkopou et al., 2014). Lower expression of HP1-α in ALT cells could therefore contribute to TERRA up-regulation. Reduced methylation of H3K9 and other histone residues associated with closed chromatin also results in up-regulated TERRA expression (Arnoult et al., 2012), which is again consistent with the findings here. The authors of these studies suggest that the increased level of TERRA expression in ALT cells is conducive to homologous recombination, as was found to be the case in yeast (Balk et al., 2013). Increased levels and association of HP1-γ could also play a role in TERRA expression as, in coordination with H3K9me3, HP1-γ is able to induce transcription (Vakoc et al., 2005).

In the time since these experiments were carried out, one group has attempted to carry out ChIP on human telomeric DNA with lamin A, and also experienced low chromatin recovery (Crabbe et al., 2012). During validation and optimisation, ChIP runs using the lamin A/C antibody pulled down considerably more telomeric chromatin (>8% input) than in the final data presented. This drop in efficiency reflects the difficulties of ChIP, which are mentioned by the
authors of both the Crabbe et al. and Episkopou et al. studies, and these problems are enhanced when attempting IP with a non-direct chromatin interactor such as lamin A. The study by Crabbe et al. moved away from traditional ChIP, employing a more typical protein IP method after protein-chromatin formaldehyde cross-linking, by assaying protein rather than chromatin IP levels. Under these conditions, the complex structures and interactions at the nuclear membrane would most likely result in the majority of protein IP to be as a result of protein-protein interactions, As such, co-IPs would be a comparable approach, and further experiments utilising lamin A antibodies could be attempted in ALT and telomerase positive cells. As the variation in A-Type lamin levels could reflect an altered mobility in the ALT nucleus, it is possible that interactions between telomeric chromatin (and genomic chromatin in general) and lamins or their chromatin linkers are not altered, and are merely more motile due to increased intranuclear lamin mobility.
Chapter 7 Final Discussion

7.1 Accelerated telomere shortening in patient cells expressing farnesylated lamin A forms

The first aim of this project was to establish whether the short telomeres previously observed in HGPS cell lines are a result of an increase in the erosion rate of telomeres. The STELA results presented in chapter three indicated an elevated telomere shortening rate in patient cell lines with mutations resulting in retention of C-terminal farnesylation in lamin A products. Closer analysis of STELA results indicated clear differences in shortening rates between the 12q and XpYp telomeres in some cell lines. The ranges of shortening rates at each telomere did not overlap for the cell lines NHDF, 155-BR and E578V, and a marginal overlap of 3bp/PD was observed at T623S. This may have been attributable to unusual telomere dynamics related to senescence or X-inactivation at XpYp (Martens et al., 2000; Surrallés et al., 1999).

As STELA provides a high-resolution profile of single telomere dynamics, an in-depth analysis of telomere dynamics was carried out to identify any issues that may arise when deriving shortening rate relative to population doublings. This highlighted several problematic factors regarding the acquisition of an accurate mean PD rate. Although the proportion of senescent cells increases over time, potentially causing an increase of cells lost from cell counts, models indicated that this may have a minimal effect on telomere shortening rate relative to PD. It could be proposed that the deficiencies of HGPS cells, which may result in loss of the 3’ overhang and therefore not be recordable by STELA, could be causing an increase in cell death which could reduce the proliferative capacity of the population. However, this was not reflected in a cell viability assay.
Models also revealed that amplification failure of subpopulations of extremely long telomeres (>15-20kb), may have an impact on deriving an accurate shortening rate, and that both this and the loss of senescent cells will lead to a tendency to underestimate telomere shortening rate. By understanding these subpopulation dynamics, it is possible to account for PCR efficiency issues through exclusion of any subpopulations that drop in through the analysis, although these should only arise in instances where telomeres in some cells are extremely long. It was not possible to identify through the data accumulated whether increased numbers of cells enter senescence due to telomere damage signalling arising from damage rather than critical shortening, although very short telomeres (<400bp) were observed in the HGPS cell line.

Another issue that could not be addressed through modelling based on STELA results is the effect of accumulating numbers of senescent cells on the representativeness of the mean PD measurement. High levels of senescent cells, or even a subpopulation that is slow growing due to high levels of DNA damage, will result in an overall delay in the time it takes for a population to complete a mean doubling. That is, cells that are actively dividing will double more times than is evident in the mean PD measurement. Although the purpose of calculating a mean PD measurement is to account for variations within the population, the increasing proportion of mitotically static senescent cells which are not contributing to division but remain in the population, will continually skew the mean PD towards a lower value than represents the dividing population in which telomeres are shortening. The resulting underestimation of PD number results in an overestimation of telomere shortening relative to PD. The analysis of shortening rates in the cell lines T623S and RD over time, which were available at early enough PDs to afford telomere length measurements at time points with high and low levels of senescence, indicated that telomere shortening rate is elevated in these cell lines, and does not vary greatly between early and late time point measurements. As an increase in senescent cells would
be expected to increase the shortening rate, this implies that this effect is either minor enough to not impact the telomere shortening rate relative to PD, or it is cancelled out by the anticipated observed reduction in shortening rate due to senescent cell accumulation described above. In any future studies on primary cell lines, it would be essential to observe telomere shortening rate from as early a PD as possible. Ideally, clonal populations with less telomere length variability should be derived, and it is unfortunate that the limited doubling capacity of HGPS and other laminopathy cell lines precludes the option of isolating clonal cultures with longer telomeres before senescence levels interfere.

7.1.1 Future work

Efforts were made to isolate the cause of accelerated telomere shortening in patient cells. Methods to measure the length of the 3’ overhang were attempted but failed to yield reliable results (see Chapter 4). Other potential pathways resulting in accelerated telomere shortening in patient cell lines were also considered, and several experimental options are out-lined below.

7.1.1.1 The role of 53BP1

53BP1 affects overhang length (Sfeir and de Lange, 2012), but also has key roles in DNA damage repair. Telomeres in HGPS cells suffer increased DNA damage compared to the rest of the genome (Benson et al., 2010), while 53BP1 is recruited more slowly to sites of DNA damage in HGPS cells (Liu et al., 2005), and is degraded in the absence of lamin A in MEFs (Gonzalez-Suarez et al., 2011). Therefore, 53BP1 could play other roles in telomere integrity, and rescue of 53BP1 levels in patient cells may result in elimination of increased erosion. Low levels of 53BP1 in BRCA1 deficient breast tumour cells may be restored by treatment with vitamin D via its interruption of CTSL activity (Gonzalez-Suarez et al., 2011; Grotsky et al., 2013). In order to test the feasibility of vitamin D
treatment in patient cells, attempts were made to down-regulate activation of CTSL in U2OS cells with vitamin D, with an ultimate goal of repeating the experiment in patient and control cells for as many PDs as possible in order to derive a telomere shortening rate. U2OS cells were exposed continually to vitamin D for several days before CTSL and 53BP1 levels were assayed by Western blot analysis. Unfortunately, preliminary results were unreliable (data not shown). As increased levels of 53BP1 could have consequences for the cell, and CTSL alone is not sufficient to eliminate 53BP1 degradation (Gonzalez-Suarez et al., 2011), it is possible that up-regulation of 53BP1 is tightly regulated through another pathway. It would therefore be necessary to repeat these experiments in 53BP1-deficient cells, ideally in a patient line or BRCA1-deficient line.

Direct quantification of 53BP1 in laminopathy cell lines has not been published previously. Preliminary results gained from Western blots of patient cell lysates indicated a trend for 53BP1 levels to decrease with the number of cell divisions in normal and patient cell lines. Unexpectedly, relative levels of 53BP1 in HGPS (and RD) were higher than controls and cell lines from patients with milder phenotypes at final passages (Figure 7.1:1). This is somewhat at odds with the observation that 53BP1 is slow to recruit to sites of DNA damage in patient cells, and the low levels of 53BP1 in Lmna<sup>-/-</sup> mouse models, although the complete absence of lamin A in these mice could account for the discrepancy. It is possible that the constant state of DDR activation, and the potential continual degradation of 53BP1 in HGPS and RD cells could result in 53BP1 upregulation, compensating for proteasomal action, while still effecting the efficient recruitment of 53BP1.

Toxicity of vitamin D has been shown to be tolerable for use in human patients, however, vitamin D may cause a reduction in proliferation rate of cells (Chen et al., 2000). If slow growth rate is a factor in the accruement of telomere loss, and
vitamin D did have a negative effect on cell growth rate, appropriate controls would need to be considered. Culturing the cells under normoxic conditions is one potential measure that may compensate for a reduction in growth rate and a side-by-side comparison against cells grown under standard hyperoxic conditions could reveal a time-dependent mechanism by which telomeres shorten. If 53BP1 were found to play a role in telomere shortening, overhang length would ultimately still need to be confirmed as 53BP1 is involved in a broad range of events and processes that could impact telomere biology (see section 1.3.1).

Figure 7.1: 53BP1 levels in control and laminopathy cell lines a Whole cell lysates were derived from cell pellets harvested at various PDs and immunoblotted against a 53BP1 antibody. Due to the size and number of modifications present on 53BP1, several bands are visible for each sample, of which all were quantified b Due to the large size of 53BP1 (250kDa) it was necessary to run a control (GAPDH) on a separate gel c 53BP1 signal was normalised to GAPDH. au=arbitrary units.
7.1.1.2 Cohesion

Shortened telomeres have been observed in dyskeratosis congenita (DC) patients with mutations in the Shelterin component TIN2 (Walne et al., 2008). Overexpression of TIN2 with DC mutations results in telomere shortening in tumour cells (Yang et al., 2011). TIN2 is also required for sister-telomere cohesion (Canudas and Smith, 2009) through the recruitment of HP1-α and –γ (Canudas et al., 2011). Mutations in the region of the HP1 binding motif in TIN2 disrupt this interaction and result in telomere shortening in association with a loss of cohesion (Canudas et al., 2011).

HP1-α is reduced in HGPS cells, and preliminary data suggests a trend towards a reduction in HP1-γ levels in cells with age, and in HGPS cells (Figure 7.1:2). If HP1 proteins are required for cohesion, laminopathy cells may suffer a drop in sister telomere cohesion, and resultant effects on telomere length. Interestingly, Canudas et al. (2011) found that in the presence of mutated TIN2, lymphoblasts proved less susceptible to cohesion loss. Lamin A is found at considerably low concentration in lymphoblastoid lines (Figure 7.1:3) in accordance with previous reports of low A-type lamin expression (Guilly et al., 1987; Rober et al., 1990).

**Figure 7.1:2** HP1-γ levels in control and laminopathy cell lines a Whole cell lysates were derived from cell pellets harvested at various PDs and immunoblotted against a 53BP1 antibody b Signal was normalised to GAPDH signal (run on the same gel) used for quantification in Figure 7.1:1. au=arbitrary units
Additionally, telomere length in lymphocytes of HGPS patients is not reduced relative to controls, in contrast to telomere length in fibroblasts from the same patients (Decker et al., 2009).

If HP1 is reduced by the presence of mutant lamin A, sister-telomeres in HGPS cells may not be able to maintain normal levels of cohesion, which could result in accelerated telomere shortening through a similar mechanism to DC. Utilising the techniques from the Canudas et al. study, it would be possible to record sister telomere cohesion in patient cell lines via FISH on specific chromosome ends in combination with a telomere-specific PNA probe in interphase cells. As such, BACs containing 12q and 16p terminal sequences were purchased in order to produce telomere-specific probes, and these are currently in development in the Royle laboratory. As lymphoblastoid cells contain very low levels of lamin A, lymphoblasts could be used as an ideal control to identify whether farnesylated and mutated lamin A forms or if over-accumulation of A-type lamins alone are the major contributors to HGPS cellular phenotype, without the need for ectopic overexpression. A role for HP1 could be verified by recording HP1 levels in lymphoblasts.

![Western blot of whole cell lysates derived from lymphoblastoid lines KK, KUK and GM1229 and various fibroblast-derived lines. Lysates were immunoblotted against a lamin A/C antibody (LA) Loading control GAPDH is shown for comparison. Short=short exposure, long=long exposure](image)

**Figure 7.1.3** Western blot of whole cell lysates derived from lymphoblastoid lines KK, KUK and GM1229 and various fibroblast-derived lines. Lysates were immunoblotted against a lamin A/C antibody (LA) Loading control GAPDH is shown for comparison. Short=short exposure, long=long exposure
7.2 Altered lamin state in ALT cells may enable the ALT phenotype

Investigation of lamin levels and localisation in ALT and telomerase positive cancer cells revealed an association between lower levels of lamins and less lamin integration into the nuclear matrix in ALT cells. Previous reports would indicate that the mobility of nuclear factors and telomeres in ALT cells is elevated (Jegou et al., 2009), and that this plays a role in the biology of ALT telomere maintenance. The organisation of chromatin within the nucleus is highly dependent on lamina-associated factors (Zuleger et al., 2013). Intranuclear lamins mediate and stabilise DNA and repair factors (Mahen et al., 2013), and are required for efficient replication (Spann et al., 1997). The data presented here suggest further evidence for a physical connection between intranuclear lamins, telomeres and associated factors required for repair and replication, as well as alternative conformation of macromolecular structures within the nucleus compared to the periphery, although an intranuclear network remains to be reproducibly visualised to a satisfactory level.

7.2.1 Future work

The immunofluorescence results are indicative of a shift in the stability of the secondary structure of peripheral and intranuclear lamins in ALT cells. Visualisation of altered lamin mobility in ALT cells with the use of FRAP would require the introduction of fluorescently-labelled lamin proteins. Unfortunately, a contributing factor to the altered lamin organisation in ALT cells is likely due to the reduced amount of lamins recorded in these cells, as the subnuclear organisation of lamins is concentration dependent (Guo et al., 2014). To avoid interfering with lamin levels, solubility assays may be carried out instead, using telomerase positive and ALT cells without any alteration in expression. This would confirm whether the lamina is adopting a less complex and insoluble
structure in ALT versus telomerase positive cells. In order to observe whether this change in nuclear environment is able to affect telomere dynamics, it would be necessary to record telomere movements in live cells through the introduction of a fluorescently labelled telomere tag.

Once this is established, it may be possible to make use of the concentration-dependent nature of lamin organisation. Lamin B has been shown to have a role in post-mitotic organisation of chromatin and is required for chromatin compartmentalisation (Dechat et al., 2004; Martin et al., 2010). Although lamin B is not dependent on lamin A for its organisation within the nucleus (Shimi et al., 2008), lamin B may be excluded from blebs and lobulations in HGPS cells that still contain lamin A (Goldman et al., 2004) and recent findings suggest that coordination of lamina organisation is dictated by the total concentration of lamins, and more specifically lamin A (Guo et al., 2014). It is therefore highly likely that re-introduction of lamin A could result in redistribution of both A- and B-type lamins, and an increased formation of higher-order complexes. This could potentially restore local telomere stabilisation, at least in part, and subsequently may reduce the heterogeneity of ALT telomere length through repression of recombination. Live microscope imaging recording telomere movement could be undertaken before and after ectopic introduction of A-type lamins or progerin (which also promotes aggregation and stabilisation of lamins) into ALT cells. Various aspects of ALT biology in these cells could then also be undertaken. In order to determine the effects of lamins on recombination, co-FISH could be carried out and rates of sister-chromatid exchange compared before and after increased lamin expression. In order to explore the putative link between lamins and APB organisation, the dynamics and association of APBs and telomeres could be recorded by recreating live cell imaging experiments carried out by Jegou et al. (2009). Stable expression and extended tissue culture of ALT cells would provide the opportunity to carry out STELA to confirm whether telomere length and distribution are effected by the
gain of lamins. Also, alteration of chromatin mobility could impact instability levels at the MS32 minisatellite locus, which could be assayed as described in section 2.4.

SUN1 has roles in mitosis, as does lamin B, and specifically binds telomeres during post-mitotic assembly (Crabbe et al., 2012). It is possible that nuclear re-assembly is different in ALT and telomerase cells, as the comparatively increased amount of SUN1 in telomerase positive cells could provide increased telomere anchorage through increased RAP1 at re-assembly, in manner undetectable by ChIP in unsynchronised cells (Crabbe et al., 2012). Nuclear disassembly could also be affected by SUN1 levels (Patel et al., 2014; Turgay et al., 2014). As these interactions are cell cycle-dependent, cell synchronisation prior to quantification by Western blot as in section 5.2.1 could reveal more dramatic differences between the cell lines. As was suggested in section 6.2.3, protein co-IP may be a more robust method for identification of differential levels of lamin A or SUN1 interaction with chromatin components, as well as with one another in ALT and telomerase positive cells. Cell synchronisation and sampling at different points in the cell cycle prior to IP may provide more insight into these interactions.

7.3 Concluding remarks

In the case of HGPS and related laminopathies, the existence of a telomere:lamin connection has great implications for the telomere biology of laminopathy cells. The retention of the farnesyl group at the C-terminus of some mutated forms of lamin A associates with an increase in telomere shortening rate, and could therefore contribute to the elevated levels of senescent cells found in patient tissues and the accelerated ageing phenotype. In these cell lines lamin A is integrated more readily into the nuclear matrix, greatly depleting the intranuclear fraction. This could be related to a sliding scale whereby ALT cells represent a highly mobile internal nuclear structure in
which telomeres move rapidly and may be recruited to sites of recombination and replication. Telomerase positive cells represent an intermediate state, retaining some lamins within the nucleus which may perform key nuclear functions, while progeroid laminopathy lines that retain farnesylation, in which reorganisation of lamins inhibits the motility of intranuclear lamin structures (as well as the amount of intranuclear lamin), exhibit replication and repair deficiencies. It is likely that the telomerase positive lamin state represents something similar to a normal cell line, and further experiments into telomere motility in cancer cells would require the inclusion of normal cell lines to verify this. Results of chromatin immunoprecipitation suggest that these alterations in lamina composition do not impede the degree to which lamins interact with chromatin and it is the physical relocation and flexibility of lamins integrated into the intranuclear lamin network versus the periphery which dictate chromatin organisation and motility, and therefore relative frequency of telomere co-localisation with replication, repair and recombination factors.

In conclusion, nuclear lamina composition is linked to the telomere maintenance mechanism adopted by a cell line. The reduction in A-type lamins in ALT cells could have multiple knock-on effects including increased mobility, and subsequently increased recombination events of ALT telomeres. The reduced presence of lamins and their associated factors may be necessary to facilitate telomere exchange in ALT cells by allowing freer movement and recombination of telomeres, while in progeroid laminopathies telomere motility is likely impeded, diminishing the ability of the cell to repair DNA damage at telomeres. A series of experiments investigating telomere motility could confirm this theory. Interestingly, although lamin A deficiency in mice results in a reduction in 53BP1 levels, preliminary data suggest this is not the case in HGPS and RD cells. Also, reduced lamin A/C in ALT cells does not correlate with reduced 53BP1. This could support a major role for telomere organisation and
motility in the telomere biology of laminopathy cells that is not dependent on 53BP1.
Appendices

Appendix A  Materials and Methods supplemental

**PCR buffer:** Prepared as 11.1X mixture by Dr Nicola Royle and diluted to 1X in PCR reactions

<table>
<thead>
<tr>
<th>Reagent</th>
<th>11.1X concentration</th>
<th>1X concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCl pH 8.8</td>
<td>500mM</td>
<td>45mM</td>
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<tr>
<td>Ammonium Sulphate</td>
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<tr>
<td>dTTP</td>
<td>11.1mM</td>
<td>1mM</td>
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<tr>
<td>BSA</td>
<td>1.25mg/ml</td>
<td>113µg/ml</td>
</tr>
</tbody>
</table>

**Denhardt’s solution:** Prepared a as a 50X stock and diluted 1 in 10 for a final 5X concentration in hybridisation solution.

FC: 0.5% Ficoll 400, 0.5% BSA, 0.5% polyvinylpyrrolidone (all w/v)

**SSC:** Taken from Departmental 20X stock solution and diluted as indicated in Materials and Methods section.

20X: 3M NaCl, 0.3M Na₃C₆H₅O₇

**TBE:** Taken from Departmental 10X/5X stock and diluted to 0.5-1X as indicated in Materials and Methods section.

10X: 0.89M Tris, 0.89M Boric acid, 20mM EDTA
**Oligonucleotide labelling buffer (OLB)**

OLB was prepared as a 6X in the department by preparation of 3 separate solutions which were then combined:

**Solution A (10µl):**

1.25ml 2M Tris HCL pH 8.0, 50µl 5M Magnesium Chloride, 700µl Elga water (double deionised/distilled), 36µl β-mercaptoethanol

10µl each (100mM stock): dATP, dGTP, dTTP

**Solution B (25µl):**

2M Hepes pH 6.6

**Solution C (15µl):**

Pd(N)6 Random Hexamer Sodium Salt (Fisher/Fermentas #SO142). Provided as a 120µl stock containing 24ug of Hexamer.
Appendix B  STELA

B.1 STELA Southern blots

Representative images of STELA hybridisations for each telomere assayed for each cell line. Population doubling (PD) is indicated at the top of each gel image, as well as the HT1080 technical control, and H$_2$O PCR controls. All PCRs, gel electrophoresis and Southern hybridisations carried out by Rachel Turner unless otherwise stated.
RD XpYp PCR, gel electrophoresis and Southern hybridisations carried out by Kimberly Fitzpatrick (KF).
T623S 12q PCR, gel electrophoresis and Southern hybridisations carried out by both Rachel Turner (RT) and Kim Fitzpatrick (KF).
B.2 HT1080 control data
Quantified sizes of HT1080 products from STELAs for each gel carried out in the STELA analysis.
Appendix C  Confirmation of cell line sub-telomere genotypes

C.1 12q genotyping

Due to the presence of sub-populations in the STELA data, it was theorised that these populations could derive from two parental alleles. Given the difficulties interpreting particularly the NHDF and HGPS 12q data, allele-specific STELA could be employed to isolate a single subpopulation for analysis, reducing the data distribution. Genotyping was undertaken as described in (Baird et al., 2000).

Firstly, PCR was conducted to identify the presence or absence of a ~2.2kb deletion in each of the sample DNAs. PCR was then conducted on a small region of the 12q sub-telomere containing SNPs within each of three restriction sites that are fully informative for 12q subtelomere genotype, as well as a 29 base insertion/deletion (see table C.1-1 and section 2.12.1). Results of the restriction digest are shown in Figure C.1:1 and Table C.1-2.

<table>
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<th>Position</th>
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<th>KpnI</th>
<th>Ins/del</th>
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<td>C</td>
</tr>
<tr>
<td>D</td>
<td>T</td>
</tr>
<tr>
<td>Δ</td>
<td>T</td>
</tr>
</tbody>
</table>

Table C.1-1 12q subtelomere genotypes and relative positions of informative restriction sites.

*(Baird et al., 2000)
Figure C.1:1

12q genotyping a PCR products from the deletion assay run on a 1% agarose gel. 133101 and 134602 are CEPH DNAs previously characterised as negative and positive, respectively, for the 2.2kb deletion b & c PCR products were digested with indicated restriction enzyme and run on a 3% NuSieve agarose gel.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Acil (C/T)</th>
<th>Bsm1 (A/G)</th>
<th>Kpn1 (A/G)</th>
<th>Ins/del (+/-)</th>
<th>Genotype</th>
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<td>B/B</td>
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<td>T</td>
<td>G</td>
<td>A</td>
<td>-</td>
<td>B/B</td>
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Table C.1-2 SNP typing and haplotypes from results in Figure C.1:1
C.2 XpYp genotyping

Genotyping was carried out on XpYp for all patient and control cells to further characterise the cell lines. In a similar approach to 12q, a region of the XpYp sub-telomere was amplified and restriction analysis carried out as described in (Baird et al., 1995)

Details of XpYp sub-telomere haplotypes as defined by informative SNPs within restriction sites (Table C.2-1 and section 2.12.2). Results from the restriction analysis are shown in Figure C.2:1 and summarised in Table C.2-2.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Mbol</th>
<th>TaqI</th>
<th>AvoII</th>
<th>Ddel</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>C</td>
<td>G</td>
<td>G</td>
<td>C</td>
</tr>
<tr>
<td>B</td>
<td>T</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>C</td>
<td>T</td>
<td>G</td>
<td>A</td>
<td>A</td>
</tr>
</tbody>
</table>

*Population frequency *(Baird et al., 1995)

Figure C.2:1 XpYp genotyping. PCR products were digested with indicated restriction enzyme and run on a 3% NuSieve agarose gel.
Table C.2-2 SNP typing and haplotypes from results in Figure C.2:1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MboII (C/T)</th>
<th>TaqI (G/A)</th>
<th>Avall (G/A)</th>
<th>Ddel (C/A)</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHDF</td>
<td>C</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>A/A</td>
</tr>
<tr>
<td>115-BR</td>
<td>C/T</td>
<td>G/A</td>
<td>G/A</td>
<td>C/A</td>
<td>A/B</td>
</tr>
<tr>
<td>HGPS</td>
<td>C</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>A/A</td>
</tr>
<tr>
<td>RD</td>
<td>C/T</td>
<td>G/A</td>
<td>G/A</td>
<td>C/A</td>
<td>A/B</td>
</tr>
<tr>
<td>E578V</td>
<td>C/T</td>
<td>G/A</td>
<td>G/A</td>
<td>C/A</td>
<td>A/B</td>
</tr>
<tr>
<td>T623S</td>
<td>C/T</td>
<td>G/A</td>
<td>G/A</td>
<td>C/A</td>
<td>A/B</td>
</tr>
</tbody>
</table>

Appendix D  Confirmation of cell line purity with MS32 analysis

Results of MS32 screening. PCR products were resolved in acrylamide gels before Southern blotting and hybridisation with MS32-specific probe.
Appendix E  NT2D1 exclusion

Results from Western blotting and quantification of telomerase cell lines for each of the proteins indicated. NT2D1 was separated from the telomere groups and t tests between NT2D1 values and the remaining telomerase lines were carried out. * indicates level of significance, ns=not significant, au=arbitrary units.
Appendix F  ChIP

Figures F1-3: Intensity measurements taken from dot blot input signals from each replica. Four dilutions were loaded onto the membrane in duplicate for each ChIP. Intercept was set at zero and the slope derived from line of best fit was used to normalise the percentage input for each ChIP. au=arbitrary units.
Table F-1 slope gradients and $r^2$ values derived from input dilution series' in Figures F1-3.
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