The effects of overexpression of lamin a and two mutants associated with premature aging on stem cell differentiation and proliferation.

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The Effects of Overexpression of Lamin A and Two Mutants Associated With Premature Aging On Stem Cell Differentiation And Proliferation.

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Abstract

The nuclear lamina is composed of A and B-type lamins and performs a number of functions within the nucleus. Mutations within lamins give rise to a group of diseases called laminopathies including autosomal dominant Emery-Dreifuss muscular dystrophy and lipodystrophy. Hutchinson Gilford Progeria Syndrome (HGPS), or so-called premature aging, is a rare and devastating laminopathy disorder caused by mutations in *LMNA* that leads to the production of a truncated mutant form of prelamin A, called progerin or LAΔ50, that cannot undergo proteolytic processing to yield mature lamin A. In an atypical form of HGPS, a point mutation at amino acid 644 alters the Zmpste24 endoproteolytic cleavage site, causing the production of mutant prelamin A. Why and how these prelamin A mutants cause such devastating phenotypes is not fully understood. It has been suggested that the mutant prelamin A may affect the ability of adult stem cells to self-renewal and differentiate, which are essential processes in order to replace damage of old tissues, critical for organismal longevity. Herein, expression plasmids containing two mutations found in classical and atypical HGPS were transiently overexpressed in clonal rat dermal papilla (DP) 9 cells, which, *in vivo*, reside at the base of the hair follicle and play an important role in hair follicle cycling. DP9 cells showed an increased number of nuclear abnormalities compared to control cells, which have previously been shown to be characteristic of cells from HGPS patients. DP9 cell lines stably overexpressing either FLAG-prelamin A, FLAG-prelamin AΔ50 or FLAG-prelamin A(R644C) were created. To assess the ability of the stem cells to self-renew, stable cell lines were routinely passaged and counted. Results indicate that stable overexpression of FLAG-prelamin AΔ50 and FLAG-prelamin A(R644C) inhibits the ability of stem cells to self-renew *in vitro*. Previous reports have shown that DP9 cells may be directed towards both adipogenic and osteogenic lineages. Therefore, in this study, stable DP9 cell lines expressing FLAG-prelamin A, FLAG-prelamin AΔ50 or FLAG-prelamin A(R644C) were exposed to an adipogenic medium for six days. Results showed that overexpression of FLAG-prelamin A, FLAG-prelamin AΔ50 and FLAG-prelamin A(R644C) inhibited the accumulation of

intracellular lipids, reflecting a decreased ability to differentiate *in vitro*. Taken together, these results suggest the lipodystrophy and alopecia associated with HGPS may be due to the failure of adult stem cell populations within each tissue to both self-renew and differentiate, and may underpin the disease pathogenesis. Furthermore, these results have implications for other laminopathies that produce mutant prelamin A.

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Contents

List of Figures

Figure Page

Chapter 1. Introduction

The Nucleus

The nucleus is the hallmark of all eukaryotic cells and served as the first evolutionary step towards multicellularity (Verstraeten *et al.*, 2007). This complex dynamic organelle is built around a three-dimensional interphase chromatin structure that serves as the building block of its own environment, i.e. a functional nucleus, and is responsible for a vast array of functions (Qumsiyeh, 1999). During interphase these functions include RNA production, DNA replication and the bi-directional transport of macromolecules (Lamond and Earnshaw, 1998; Cardoso *et al.*, 1999; Qumsiyeh, 1999). As the nucleus is built around chromatin, the inner nuclear membrane provides a physical attachment point to segregate chromatin into highly specific and defined chromosomal territories (Marshall *et al.*, 1997). Furthermore, the nucleus provides specific structural platforms to aid regulatory factors to control the transcription of genes. The nuclear envelope (NE) also acts as a physical barrier between the nucleoplasm and the cytoplasm, thus separating various metabolic processes and also preventing the translation of unspliced mRNA; this compartmentalisation adds a level of regulation required for complex organisms (Pennisi, 2004; Mans *et al.*, 2004; Jékely, 2005). The nucleus is perforated by protein channels that allow the import and export of molecules into and out of the nucleus (Gorlich and Kutay, 1999). This has important consequences in terms of signalling mechanisms that serve to either activate or repress gene transcription, and thus the nucleus also functions to control these signalling cascade events that result in an information exchange between nucleus and cytosol (Hansson *et al.*, 2004; Hayden and Ghosh, 2004).

The structure of the nucleus comprises an internal nucleoplasm surrounded by a double membrane, the two components of which being the inner nuclear membrane (INM) and the outer nuclear membrane (ONM) (Figure 1) (Maidment and Ellis, 2002; Worman and Courvalin 2005).

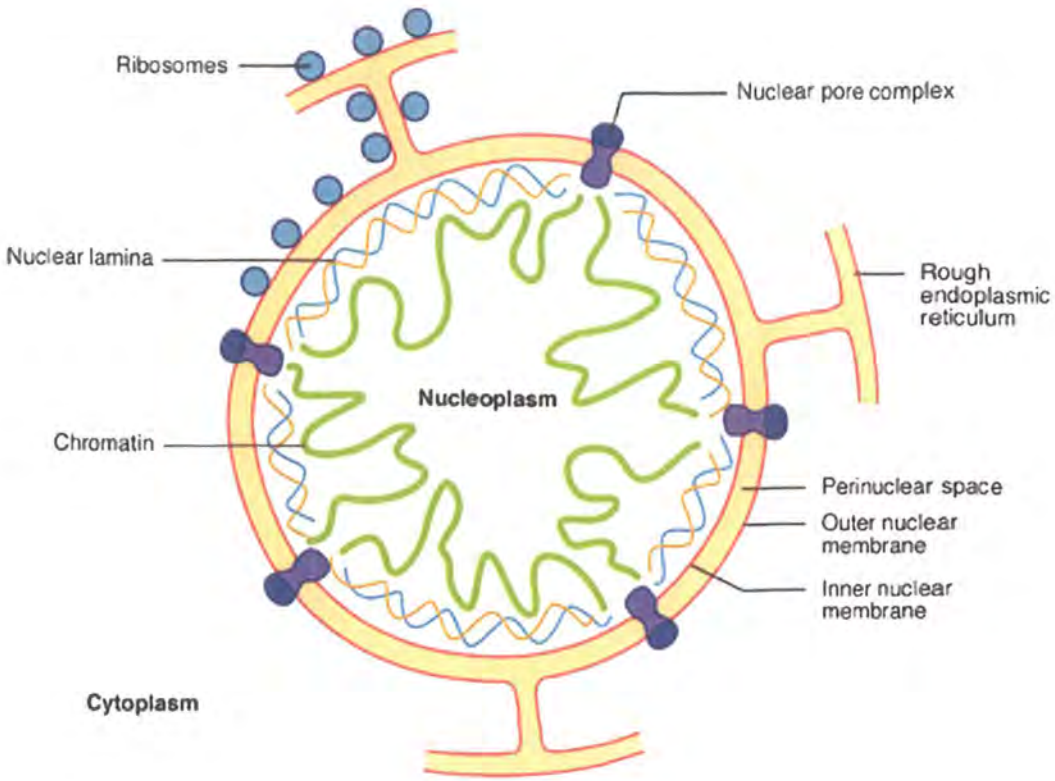


Figure 1. Overview of the structure of the nuclear envelope. (See text for details, Maidment and Ellis, 2002)

Whilst the INM contains a unique set of proteins, the ONM is contiguous with the rough endoplasmic reticulum (RER) and thus shares a similar protein content. This double membrane creates a perinuclear space that is an extension of the ER lumen (Burke and Stewart, 2006). The double membrane is punctuated by a multi-protein complex called the nuclear pore complex (NPC) that tightly controls transport between the nucleoplasm and cytoplasm (Gerace and Burke, 1988; Gorlich and Kutay, 1999). Each NPC is evenly distributed around the nuclear envelope and consists of approximately 30 proteins called nucleoporins that are collectively assembled in a modular fashion (Cronshaw *et al.*, 2002; Lim and Fahrenkrog, 2006). As well as integral nucleoporins such as grp210 and POM121, there are nucleoporins that are unique to both the nucleoplasmic and cytoplasmic face of the NPC. On the nucleoplasmic face, the Nup107-160 complex binds

to the basket nucleoporin, Nup153, whose incorporation upon nuclear envelope re-assembly is dependent on a functional nuclear lamina (Smythe *et al.*, 2000; Maeshima *et al.*, 2006). Transport through the NPC is the subject of some debate whilst the NPC has also been shown to be involved in gene activation/repression (Bayliss *et al.*, 2000; Rout *et al.*, 2000; Ribbeck and Gorlich, 2001; Dilworth *et al.*, 2001; Rout *et al.*, 2003). Underlying the inner nuclear membrane is a filamentous meshwork called the nuclear lamina, containing A and B-type lamins and lamin-binding proteins (Burke and Stewart, 2002; Hutchison, 2002; Hutchison and Worman, 2004; Gruenbaum *et al.*, 2005; Broers *et al.*, 2006; Burke and Stewart, 2006).

Outer Nuclear Membrane and Perinuclear Space

Previously, it had long been believed that since the ONM and RER both share ribosomes and are continuous then it follows that they should have an identical protein composition (Broers *et al.*, 2006). However, several lines of evidence suggest that they are quite distinct in protein composition with proteins found in the ONM that are not found in the bulk ER. Furthermore, the proteins in the ONM and INM have been shown to interact and this has led to the proposal of a novel nucleo-cytoskeletal link via the perinuclear space (PNS), with repercussions for nuclear position and anchorage (Figure 2) (Zhang *et al.*, 2001; Zhen *et al.*, 2002; Starr and Han, 2003; Padmakumar *et al.*, 2004).

The precedent for a mammalian nucleo-cytoskeletal link via the PNS was based largely on research into *Caenorhabditis elegans*. Mutation within the *C.elegans* UNC-84 protein identified it as having a non-specific effect on both neural and non-neural cell lineages during development and being responsible for correct nuclear migration and anchoring (Horvitz *et al.*, 1983; Malone *et al.*, 1999). The same group then showed that this protein was located at the INM, with its location dependent on the single *C.elegans* lamin for correct retention at the INM (Malone *et al.*, 1999; Lee *et al.*, 2002). The C-terminal of UNC-84 extends into the PNS and shares a degree of similarity with the *Schizosaccharomyces pombe* Sad1 protein that is required for functional spindle

architecture and anchors the spindle pole body to the nuclear envelope (Hagan and Yanagida, 1995; Malone *et al.*, 1999). Comparable mammalian proteins that shared similarity to this ~200 amino acid C-terminal region were termed SUN proteins (Sad/UNC-84 homology) (Malone *et al.*, 1999). That an INM protein could have effects on nuclear positioning and anchorage led to the suggestion that the INM and ONM were connected via the PNS, and indeed evidence for this was first reported for defects in the ANC-1 protein of *C.elegans* (Starr and Han, 2002). ANC-1 is a conserved type II ONM protein responsible for anchoring the nucleus to the actin cytoskeleton as well as being responsible for the correct position of mitochondria, and is dependent on UNC-84 for correct localisation at the ONM (Starr and Han, 2002; Starr and Han, 2003). Further work evidenced that another ONM protein, UNC-83, which is spatially and temporally controlled through development, was also required for proper nuclear anchoring and migration and interacts with UNC-84 via a conserved domain in the PNS and may act to connect microtubules to the nucleus (Starr *et al.*, 2001; Starr and Han, 2003; McGee *et al.*, 2006). Taken together, this is the first evidence showing that resident proteins of the ONM and INM may interact via lumenal domains across the PNS, providing a structural link between the nucleus and the cytoskeleton.

ANC-1 is homologous to a group of proteins that are conserved within mammals and are now collectively referred to as nesprins (Starr and Han, 2003). Although nesprins may vary in size with alternatively spliced variants ranging from 50kDa to 1MDa, they share some notable characteristic features. Firstly, they contain multiple spectrin repeats sequences, which have been previously described for other large structural proteins such as dystrophin and α-actinin (Apel *et al.*, 2000; Zhang *et al.*, 2001; Zhen *et al.*, 2002; Young and Kothary, 2005; Zhang *et al.*, 2005). These spectrin repeat sequences are distributed throughout the central core domain of the protein, enabling self-interaction between polypeptides (Mislow *et al.*, 2002a). The N-terminal contains an actin-binding domain (ABD) composed of two calponin homology domains, whilst the C-terminal contains a conserved 60 amino acid functional domain (Worman and Gundersen, 2006). This C-terminal domain has been termed a KASH (Klarsicht, ANC-1, Syne Homology)

domain and contains a transmembrane segment as well as functioning in intermediate filament binding and microtubule binding (Starr and Han, 2003).

Nesprins-1,2 and 3 are coded for by three genes in mammals and have a variety of names within the literature: CPG2, syne-1, myne-1, ENAPTIN (nesprin-1) and syne-2, NUANCE (nesprin-2) (Apel *et al.*, 2000; Zhang *et al.*, 2001; Zhen *et al.*, 2002; Wilhelmsen *et al.*, 2005; Zhang *et al.*, 2005; Burke and Stewart, 2006; Worman and Gundersen, 2006). Nesprin-1 and 2 are extremely large proteins, of ~976 kDa and ~764 kDa respectively, and are located at the ONM. Nesprin-3 is similarly located at the ONM, but smaller at ~110 kDa and is distinct from both nesprin-1 and 2 as it binds plectin at its N-terminal rather than actin (Wilhelmsen *et al.*, 2005). Nesprins-1 and 2 also express a large number of splice variants, some of which do not contain the actin-binding domain and/or the KASH domain (Mislow *et al.*, 2002a; Cottrell *et al.*, 2004; Padmakumar *et al.*, 2004; Zhang *et al.*, 2005). One variant, nesprin-1α is localised to the INM where it binds both lamin A and C and emerin *in vitro* and *in vivo*, with a possible function in helping to organise the nucleoskeleton via binding to nuclear actin (Mislow *et al.*, 2002a,b; Libotte *et al.*, 2005; Wilhelmsen *et al.*, 2005; Zhang *et al.*, 2005; Broers *et al.*, 2006; Wilhelmsen *et al.*, 2006).

The localisation of nesprins to either the INM or ONM is largely dependent on the size of the splice variant because whilst the smaller isoforms (< 60kDa) are able to passively diffuse from the bulk ER past the NPC and localise to the INM, the larger isoforms are exempt from this membrane due to the restricted size of the lateral channels of the NPC and instead reside within the ONM (Soullam and Worman, 1995; Worman and Gundersen, 2006). Tethering of the larger nesprin-1 and 2 isoforms and nesprin-3 at the ONM occurs through SUN1/2, mammalian homologues of UNC-84 via binding to the KASH domain (Fridkin *et al.*, 2004; Hodzic *et al.*, 2004; Padmakumar *et al.*, 2005; Crisp *et al.*, 2006; Padmakumar *et al.*, 2006). That both SUN1 and SUN2 are able to carry out this tethering function of giant nesprins suggests a redundant functionality of both proteins, with the interaction complex now being termed the LINC (Linker of Nucleoskeleton and Cytoskeleton) complex (Figure 2) (Crisp *et al.*, 2006).

The various nesprin isoforms have multiple functions including regulating myotubal nuclear clustering at neuromuscular junctions, targeting the muscle A-kinase anchoring protein (MAKAP) to the nuclear envelope in cardiomyoctes and regulating the endocytosis of glutamate receptors in the brain (Apel *et al.*, 2000; Cottrell *et al.*, 2004; Pare *et al.*, 2005; Zhang *et al.*, 2007) (reviewed in Wilhelmsen *et al.*, 2006). Additionally, it has recently been shown that mutations within nesprin-1 lead to an autosomal recessive form of cerebellar ataxia (OMIM 610743) (Gros-Louis *et al.*, 2007).

The perinuclear space (PNS) between the INM and ONM has been considered identical to the continuous ER lumen. However, hypothetically, as the RER and ONM differ in protein composition then logically it may be suggested that the composition of the PNS could differ from the bulk ER lumen as portions of INM proteins localized to the INM and NPCs within this lumenal region could bind to resident non-membrane proteins (Worman and Courvalin, 2005). Indeed, mutations in an ATPase called torsinA that causes early-onset AD dystonia, causes the protein, normally a lumenal ER protein, to concentrate in the PNS where it has been found to bind preferentially to the INM protein LAP1 (OMIM 605204) (Senior and Gerace, 1988; Gonzalez-Alegre and Paulson, 2004; Goodchild and Dauer, 2004; Naismith *et al.*, 2004; Goodchild and Dauer, 2005). The disease is characterized by the involuntary twisting movements of the patient as a result of neuronal dysfunction. How the altered protein-protein interaction between torsinA and LAP1 contributes to the disease phenotype is not yet understood, however, some have suggested that it may alter protein-protein interactions in the PNS, therefore, having a more global effect on nuclear positioning, a suggestion reinforced by work in both *Drosophila* and *C.elegans* (Basham and Rose, 2001; Gerace, 2004; Koh *et al.*, 2004).

Inner Nuclear Membrane

The constituent proteins of the inner nuclear membrane are responsible for a variety of essential cellular functions ranging from cell signalling, gene regulation and enzymatic activity (Ye and Worman, 1996; Imai *et al.*, 1997; Markiewicz *et al.*, 2006). A full

characterisation of the nuclear envelope composition in different tissues was deemed necessary in order to fully understand tissue-specific diseases that arise from mutations within nuclear envelope proteins (Figure 2) (Schirmer *et al.*, 2003) A comparative proteomics approach had previously identified 7 out of 10 known nuclear envelope transmembrane proteins (NETs) (Dreger *et al.*, 2001). A more refined subtractive proteomics approach using purified rat liver tissue identified all 13 known NETs and a further putative 67 NETs (Schirmer *et al.*, 2003). This approach revealed significant differences in NE composition between different tissue types, with some new NETs exhibiting a 10-fold difference in expression between tissues (Schirmer *et al.*, 2003; Schirmer and Gerace 2005). As yet it is unclear as to the function of each of these proteins but future work will determine this. Furthermore, it would seem that mutations in these proteins are more than likely to contribute to human disease given the nature of currently known INM proteins and their role in disease and thus elucidating the function of these 67 proteins may provide novel insights into helping solve the underlying pathogenesis of diseases that arise through mutations in nuclear envelope proteins (discussed later) (Schirmer and Gerace, 2005; Wilkie and Schirmer, 2006). One clue comes from the finding that some NETs are differentially upregulated during myogenesis, suggesting that this may be the case for differentiation of other cells and though this remains to be tested is an intriguing possibility (Chen *et al.*, 2006).

INM proteins are firstly synthesised in the RER with at least one transmembrane (TM) domain (Worman and Courvalin, 2005). Thereafter they are subject to INM localisation due to the 'selective retention model' of protein targeting whereby proteins of less than 60 kDA may laterally diffuse past the NPC into the INM where they can bind to the underlying nuclear lamina or chromatin (or both) components (Powell and Burke, 1990; Soullam and Worman, 1993; Soullam and Worman, 1995; Ellenberg *et al.*, 1997; Östlund *et al.*, 1999; Wu *et al.*, 2002; Ohba *et al.*, 2004). This model is non-selective and discriminates only against the size of the protein. Furthermore, it has been suggested that a degree of remodeling of the NPC may be necessary in order to allow the passage of the proteins from the ONM to the INM as the process is energy dependent (Ohba *et al.*, 2004). This study also disqualified other hypotheses including vesicle fusion and soluble

transport mechanisms (Ohba *et al.*, 2004). Entry of yeast INM orthologues to the nucleus directly through NPC channels has recently been reported (King *et al.*, 2006). These proteins contained basic sequence motifs that resembled nuclear localisation sequences (NLSs) with their import reliant upon karyopherin-α, karyopherin-β1 and the Ran-GTPase cycle, drawing obvious similarities between this and the import of soluble nuclear proteins (King *et al.*, 2006; Lusk *et al.*, 2007). Since most mammalian INM proteins contain an NLS-like sequence it may prove that an active NPC-mediated import of INM proteins works alongside the selective-retention model (Horton and Nakai, 1997; Vaughan *et al.*, 2001; Wu *et al.*, 2002; King *et al.*, 2006; Östlund *et al.*, 2006). Phosphorylation of INM proteins at the start of mitosis causes them to detach from the underlying nuclear lamina and chromatin and diffuse to the ER, allowing for correct concomitant NE disassembly. However, INM proteins are selectively targeted to the decondensing chromatin in daughter cells to form the new NE prior to lamina assembly (Courvalin *et al.*, 1992; Chaudhary and Courvalin, 1993; Foisner and Gerace, 1993; Buendia and Courvalin, 1997; Ellenberg *et al.*, 1997; Yang *et al.*, 1997; Ellis *et al.*, 1998; Haraguchi *et al.*, 2000).

Lamina B Receptor (LBR)

The lamin B receptor (LBR) is a 58 kDa INM protein identified by a biochemical screen for lamin-binding proteins in a lamin-depleted NE fraction (Worman *et al.*, 1988). It was found to preferentially bind lamin B1 over lamin A, with antibodies raised against it revealing a nuclear envelope staining (Worman *et al.*, 1988). Subsequent analysis revealed that LBR has a basic N-terminal domain of ~200 amino acids that faces the nucleoplasm, which interacts with B-type lamins, LAP2β, the heterochromatin proteins 1 (HP1) α and γ, histones H3/H4 and heterochromatin (Worman *et al.*, 1988; Ye and Worman, 1994; Ye and Worman, 1996; Ye *et al.*, 1997; Holmer *et al.*, 1998; Duband-Goulet and Courvalin, 2000; Polioudaki *et al.*, 2001; Makatsori *et al.*, 2004). This is followed by a hydrophobic region containing eight putative TM domains (Worman *et al.*, 1990; Schuler *et al.*, 1994; Ye and Worman, 1994). The C-terminal domain is ~400 amino acids and shares sequence similarity to conserved sterol reductases, an enzyme that catalytically converts cholesta-8, 14-dien-3β-ol, to cholesterol, with expression of hLBR in yeast confirming this C14-streol

reductase activity (Holmer *et al.*, 1998; Silve *et al.*, 1998; Waterhan *et al.*, 2003). LBR is essential for fetal development and has an important role in targeting membranes to the NE assembly post-mitosis (Pyrpasopoulou *et al.*, 1996; Ellenberg *et al.*, 1997; Haraguchi *et al.*, 2000; Waterham *et al.*, 2003). Mutations in LBR give rise to two distinct diseases, Pelger-Huet anomaly (PHA; OMIM 169400) and Hydropsectopic calcification-moth eaten (HEM) or Greenberg skeletal dysplasia (OMIM 215140) (Hoffmann *et al.*, 2002; Best *et al.*, 2003; Waterham *et al.*, 2003). PHA is characterized by abnormal nuclear shape and chromatin organisation in blood granulocytes and may be inherited either as an AD or AR trait (Hoffmann *et al.*, 2002). HEM/Greenberg skeletal dysplasia is an AR chondrodystrophy with a lethal course, characterized by short limbs and abnormal bone calcification (Waterham *et al.*, 2003). Cultured fibroblasts exhibit increased levels of cholesta-8, 14-dien-3β-ol in line with the sterol reductase ability of LBR and it has been proposed that the nature of the mutation, ether heterozygous and homozygous, is the ultimate determinant of the disease phenotype (Waterham *et al.*, 2003; Worman and Courvalin, 2005).

Emerin

Emerin is a 34 kDa type II integral INM protein encoded by the gene *EMD* (initially called *STA*) located by positional cloning to the X chromosome (Bione *et al.*, 1994; Manilal *et al.*, 1996; Nagano *et al.*, 1996). Emerin has a nucleoplasmic N-terminal domain of 220 amino acids, a single TM domain and a short C-terminal domain in the PNS (Manilal *et al.*, 1996; Nagano *et al.*, 1996). Sequences in the nucleoplasmic domain mediate its inner nuclear membrane retention (Östlund *et al.*, 1999; Tsuchiya *et al.*, 1999). The nucleoplasmic domain is responsible for binding lamins A and C, interactions that are either directly or indirectly responsible for its INM localisation (Ellis *et al.*, 1997; Fairley *et al.*, 1999; Sullivan *et al.*, 1999; Clements *et al.*, 2000; Raharjo *et al.*, 2001; Sakaki *et al.*, 2001; Vaughan *et al.*, 2001; Libotte *et al.*, 2005). Emerin is one of a number of proteins that contains a conserved 43 amino acid folded motif called an LEM (LAP2, Emerin, MAN1) domain near its N-terminus facing the nucleoplasm (Lin *et al.*, 2000; Zheng *et al.*, 2000; Cai *et al.*, 2001; Laguri *et al.*, 2001; Wolff *et al.*, 2001). It is believed

that this domain allows certain overlapping functions between all LEM-domain containing proteins, for example, RNA*i* of either Ce-MAN1 or Ce-emerin allows 85% viability in *C.elegans*, whilst RNA*i* of both leads to 100% lethality (Liu *et al.*, 2003). Similarly, emerin is dispensable for cell survival and normal development (Harborth *et al.*, 2001; Gruenbaum *et al.*, 2002). The LEM-domain binds to a variety of proteins: the chromatin-associated protein barrier-to-autointegration factor (BAF); the transcription factor germ cell-less (GCL), which binds to the DP3 subunit of E2F-DP heterodimeric transcription factors thus blocking E2F-DP-dependent gene expression; the death promoting transcriptional repressor Btf; the transcriptional regulator Lmo7; the splicing factor YT521-B (Lee *et al.*, 2001; Nili *et al.*, 2001; Holaska *et al.*, 2003; Wilkinson *et al.*, 2003; Bengtsson and Wilson, 2004; Haraguchi *et al.*, 2004; Mansharamani and Wilson, 2005; Holaska *et al.*, 2006; Margalit *et al.*, 2007). The C-terminal domain of emerin also contains a conserved adenomatous polyposis coli (APC) consensus sequence capable of binding β-catenin, a developmentally important downstream effector in the Wnt-signalling pathway (Markiewicz *et al.*, 2006). Together, these interactions suggest a diverse role for emerin as a scaffold protein capable of regulating chromatin structure and gene transcription.

Emerin is mutated in X-linked Emery-Dreifuss muscular dystrophy, which is characterised by progressive muscle wasting, shortening of tendons, and heart and conduction system defects (X-EDMD; OMIM 310300) (Bione *et al.*, 1994; Manilal *et al.*, 1996; Nagano *et al.*, 1996). Most mutations cause a reduction or complete loss of emerin with mislocalisation to the ER in some cases (Nagano *et al.*, 1996; Manilal *et al.*, 1998; Fairley *et al.*, 1999; Ellis *et al.*, 2000; Niebroj-Dobosz *et al.*, 2003). Furthermore, this alters the interaction between emerin and A-type lamins, causing in increased solubility of both A and B-type lamins (Markiewicz *et al.*, 2002a).

Recent proteomic analysis of binding partners for emerin in HeLa cell confirms that it exists in distinct protein complexes (Holaska and Wilson, 2007). One of these is a nuclear myosin I-containing complex that might sense and regulate mechanical tension at the nuclear envelope (Holaska *et al.*, 2007). Indeed, emerin null cells have impaired

mechanotransduction ability (Lammerding *et al.*, 2005). Additionally, emerin null cells have abnormally shaped nuclei with membrane and chromatin protrusions and exhibit accelerated growth rate with increased rates of apoptosis (Fidzianska *et al.*, 1998; Markiewicz *et al.*, 2006; Meaburn *et al.*, 2007). The increased growth rate of emerin null cells is due to increased accumulation of active nuclear β-catenin (Markiewicz *et al.*, 2006). Overexpression of emerin restricts β-catenin to the cytoplasm inhibiting the activation of the Wnt-pathway (Markeiwicz *et al.*, 2006). Further to this, gene-profiling of cells from both patients and emerin null mice have shown that myoblast differentiation is delayed as compared to control cells, explained by a disruption to the transcriptional pathways regulated by MyoD and Rb (Bakay *et al.*, 2006; Melcon *et al.*, 2006; Ozawa *et al.*, 2006). Gene expression profiling of hearts taken from *EMD* null mice shows an activation of MAPK signalling pathway and downstream targets implicated in the disease pathogenesis (Muchir *et al.*, 2007). Taken together, these results provide a basis for understanding the phenotypic characteristics of X-EDMD.

Lamina-Associated Proteins (LAPs)

Another member of the LEM-domain containing group of proteins is the increasingly diverse lamina-associated proteins (LAPs) (Senior and Gerace, 1988; Foisner and Gerace, 1993). LAPs were initially identified based on their resistance to biochemical extraction and identified as INM proteins by monoclonal antibodies (Senior and Gerace, 1988; Foisner and Gerace, 1993; Gerace and Foisner, 1994). The LAP1 family of proteins are derived from alternative splicing of the *LAP1* gene, which codes for LAP1A, B and C (Foisner and Gerace, 1993). LAP1 is a 75 kDa type II integral INM protein, whilst LAP1B and C are 68 and 57 kDa respectively, and all share the same TM and lumenal domains (Foisner and Gerace, 1993; Martin *et al.*, 1995). All three isoforms are expressed in a developmentally regulated manner and bind both A and B-type lamins (Martin *et al.*, 1995; Maison *et al.*, 1997).

In contrast, there are six isoforms of the alternatively spliced *LAP2* gene, namely LAP2α, β, γ, δ, ε, and ζ. LAP2β, γ, δ and ε are all type II integral INM proteins that contain a long

N-terminal nucleoplasmic domain followed by a single TM domain and a short C-terminal lumenal domain. In contrast, LAP2α and ζ do not contain TM domains, and while LAP2ζ does share the first 219 N-terminal amino acids of LAP2β, γ, δ and ε, LAP2α contains a unique C-terminal region (Harris *et al.*, 1994; Furukawa *et al.*, 1995; Harris *et al.*, 1995; Berger *et al.*, 1996; Yang *et al.*, 1997; Gant *et al.*, 1999; Dechat *et al.*, 2000a,b). LAP2β is the largest TM LAP2 that binds preferentially to lamin B1 via a lamin B1 binding domain between amino acids 298 and 370, with the interaction between LAP2β and lamin B1 responsible for its INM localisation (Foisner and Gerace, 1993; Furukawa *et al.*, 1995; Furukawa *et al.*, 1998). The lamin B binding domain is also present in the LAP2δ and ε isoforms but is partially absent in LAP2γ and ε, suggesting that the LAP2δ and ε isoforms may bind lamin B1 (Furukawa *et al.*, 1995; Furukawa *et al.*, 1998; Dechat *et al.*, 2000b). However, it has also been shown that XLAP2β exists in a protein complex with both A and B-type lamins in *Xenopus*, though this has yet to be shown for human LAP2β (Lang and Krohne, 2003).

LAP2β has been suggested to be important for correct chromatin-NE interactions post-mitosis as a basic domain within LAP2β initiates DNA docking (Yang *et al.*, 1997; Gant *et al.*, 1999; Lang *et al.*, 1999; Ulbert *et al.*, 2006). Consistent with this, LAP2β also contains an LEM domain that mediates the interaction an indirect interaction with DNA via the cross linking protein BAF, which binds DNA in a non-sequence-specific manner (Zheng *et al.*, 2000; Cai *et al.*, 2001; Shumaker *et al.*, 2001; Bradley *et al.*, 2005; Margalit *et al.*, 2007). Furthermore, there is an additional LEM-like domain at the N-terminal of LAP2 proteins that interacts directly with DNA and LAP2β can bind the chromatin protein HA95 via its C-terminal region (Cai *et al.*, 2001; Martins *et al.*, 2003; Ulbert *et al.*, 2006). This data suggests that LAP2β is involved in gene expression by regulating higher order chromatin structures and, moreover, that this may be of a repressive nature as LAP2β binds the transcriptional repressor GCL and also histone deacetylase (HDAC), which has been linked to transcriptionally inactive heterochromatin (Sadoni *et al.*, 1999; Nili *et al.*, 2001; Somech *et al.*, 2005). That LAP2β binds to GCL implies that LAP2-lamin complexes have a role in transcriptional control, as GCL is known to inhibit the

DP3 subunit of the E2F-DP3 complex independently of Rb (Nili *et al.*, 2001; Gruenbaum *et al.*, 2005; Broers *et al.*, 2006).

Another LAP2 family protein LAP2α also suggests that this protein family is involved in chromatin structure and transcriptional regulation (Cohen *et al.*, 2001). Although LAP2α lacks a TM domain it may still reside at the nucleoplasmic face of the INM, held there by interactions with lamin B1 at the nuclear periphery (Foisner and Gerace, 1993; Dechat *et al.*, 1998). However, LAP2α also resides in the internal nucleoskeleton, where it preferentially binds and forms stable complexes with intranuclear A-type lamins during interphase, whilst it is located to telomeric chromosome regions in a phosphorylation dependent manner during mitosis along with LAP2β, BAF and emerin (Dechat *et al.*, 1998; Vlcek *et al.*, 1999; Dechat *et al.*, 2000a; Markiewicz *et al.*, 2002b; Vlcek *et al.*, 2002; Dechat *et al.*, 2004; Margalit *et al.*, 2007). As with other LAP2 family members, LAP2α contains a LEM and an N-terminal LEM-like domain, mediating interactions with DNA indirectly (via BAF) and directly, respectively (Dechat *et al.*, 2000b). This data, together with FRAP (fluorescence recovery after photobleaching) studies showing that LAP2α and BAF are very mobile during interphase, suggests that lamin A/C-LAP2α-BAF complexes are involved in a dynamic ordering of chromatin during both mitosis and interphase (Moir *et al.*, 2000; Sengura-Totten and Wilson, 2004; Vlcek *et al.*, 2001; Dechat *et al.*, 2004; Shimi *et al.*, 2004; Margalit *et al.*, 2007; Vlcek and Foisner, 2007a,b). Further evidence for the role LAP2 proteins in transcriptional regulation comes from the findings that lamin A-LAP2α complexes interact with the tumour suppressor retinoblastoma (Rb) (Mancini *et al.*, 1994; Ozaki *et al.*, 1994; Dechat *et al.*, 2000; Markiewicz *et al.*, 2002b). Lamin A-LAP2α acts to tether hypophosphorylated Rb to the nucleoskeleton where it binds to E2F-DP heterodimers and blocks E2F-dependent gene transcription via a variety of methods including the recruitment of SWI/SNF chromatin remodeling complex, HDAC, polycomb group protein complexes, and histone and DNA methyltransferases (Markiewicz *et al.*, 2002b; Chau and Wang, 2003; Stevaux and Dyson, 2002 Frolov and Dyson, 2004; Korenjak and Brehm, 2005; Dorner *et al.*, 2006; Galderisi *et al.*, 2006). During G1, Rb is phosphorylated by cyclin D-CDK heterodimers allowing progression to S phase of the cell cycle, thereby making Rb an important regulator of

proliferation, and it has also been implicated in various differentiation processes (Chen *et al.*, 1996; Novitch *et al.*, 1999; Classon *et al.*, 2000; Thomas *et al.*, 1999; Fajas *et al.*, 2002; Cole *et al.*, 2004; Hansen *et al.*, 2004; Huh *et al.*, 2004; Markiewicz *et al.*, 2005; Dorner *et al.*, 2006). The LAP2α C-terminal binds to the pocket C domain of Rb and can inhibit E2F-genes by directly binding to them in an Rb-dependent manner (Dorner *et al.*, 2006). Overexpression of LAP2α inhibits cell cycle progression and favors cell cycle exit and differentiation, a process that appears to be regulated upstream by the LAP2α – binding protein LINT-25 (Dorner *et al.*, 2006; Naetar *et al.*, 2007). Also, a functional lamin A-LAP2α-Rb complex is required to maintain a proliferative state within fibroblasts, with knockdown of either lamin A or LAP2α leading to faster progression through G1 and S phase inducing cell cycle arrest (Pekovic *et al.*, 2007). Consistent with this, *LMNA*-null MEFs show enhanced cell cycle arrest in response to DNA damage and mislocalisation of Rb with its subsequent proteasomal degradation (Johnson *et al.*, 2004). Moreover, cell lines depleted of functional lamin A/C are resistant to $p16^{INK4A}$-mediated G1 arrest, reflecting the need for a functional lamin A-LAP2α-Rb complex for effective cell cycle control (Nitta *et al.*, 2006). Based on these findings, the cell cycle and differentiation consequences of this lamin A-LAP2α-Rb complex have been implicated in the control of adult stem cell self-renewal and differentiation (Hutchison and Worman, 2004; Gotzmann and Foisner, 2005). Mutations within the C-terminus of LAP2α cause a rare form of dilated cardiomyopathy by affecting its interaction with lamin A/C (CMD1T; OMIM 188380) (Taylor *et al.*, 2005).

MAN1

MAN1 is encoded by the human gene *LEMD3* and is a 97 kDa protein implicated in transforming growth factor β/bone morphogenic protein (TGFβ/BMP) signalling (Lin *et al.*, 2000; Lin *et al.*, 2005; Pan *et al.*, 2005; Worman, 2005; Worman, 2006; Bengtsson, 2007; Wagner and Krohne, 2007). It was first identified as the largest (97 kDa) of three autoantigens recognized by autoantibodies from a human subject with collagen vascular disease (Paulin-Levasseur *et al.*, 1996; Lin *et al.*, 2000). Structurally, MAN1 consists of a nucleoplasmic N-terminal domain, two transmembrane segments and a nucleoplasmic C-

terminal domain (Lin *et al.*, 2000). Deletion mutants and FRAP studies have shown that MAN1 localises to the INM via the 'selective retention model' and its INM position is dependent on the N-terminal TM and nucleoplasmic domains (Wu *et al.*, 2002). The N-terminus has a conserved LEM-domain and interacts with emerin, GCL, Btf and BAF *in vitro*, as well as both A and B-type lamins via their tail domains (Lin *et al.*, 2000; Liu *et al.*, 2003; Mansharamani and Wilson, 2005). Several lines of evidence from various organisms have started to reveal the function of this protein. In *C.elegans*, Ce-MAN1 is essential for viability and normal chromosome segregation and is dependent upon a direct interaction with Ce-lamin for localisation to the INM (Liu *et al.*, 2003). Whilst RNA*i* of Ce-MAN1 only resulted in 15% embryonic lethality, removal of Ce-emerin as well as Ce-MAN1 led to 100% lethality, indicating that these two proteins share a degree of overlapping functionality (Liu *et al.*, 2003). In *Xenopus laevis*, introduction of an antisense morpholino oligonucleotide to disrupt endogenous XMAN1 leads to developmental complications (Osada *et al.*, 2003). In humans, mutations in MAN1 lead to three autosomal-dominant skeletal diseases: osteopoikilosis, Buschke-Ollendorff syndrome and melorheostosis, which are characterized by increased bone density (OMIM 166700) (Hellemans *et al.*, 2004; Hellemans *et al.*, 2006). These diseases may be explained by the finding that the C-terminal globular nucleoplasmic domain adopts a winged-helix fold involved in receptor-regulated SMAD (rSMAD) binding and antagonizes TGFβ/BMP signaling (Osada *et al.*, 2003; Raju *et al.*, 2003; Hellemans *et al.*, 2004; Lin *et al.*, 2005; Pan *et al.*, 2005; Caputo *et al.*, 2006). Through this C-terminal motif, MAN1 associates with rSMADS 1-3, in particular SMADs 2 and 3, but not SMAD4, in a ligand-independent manner. Overexpression of MAN1 inhibits SMAD2/3 phosphorylation, heterodimerization with SMAD4, thereby possibly sequestering them at the NE and thus causing repression of transcriptional activation of the TGF-β/BMP-responsive promoters (Osada *et al.*, 2005; Lin *et al.*, 2005; Pan *et al.*, 2005). Furthermore, MAN1 may possibly bind to both rSMADS and their targeted DNA sequences using the winged-helix fold motif and therefore directly influence gene expression (Caputo *et al.*, 2006; Worman, 2006). The exact mechanisms of TGF-β/BMP antagonisation remain to be elucidated, but it has been recently suggested that MAN1 may function to either disrupt R-SMAD–co-SMAD complexes and/or induce dephosphorylation of R-SMADs (Bengtsson,

2007). Regardless of the mechanism, the pathogenesis of the diseases associated with MAN1 mutations may be explained by the increased TGF-β/BMP-signalling after regulation of rSMADs is abolished by the mutations (Worman, 2005). It has also been shown that TGF-β/BMP singalling activates protein phosphatase 2A (PP2A), which in turn dephosphorylates rSMADs. Furthermore, PP2A dephosphorylates pRb in a cell-cycle-dependent manner, with functional A-type lamins required for this dephosphorylation event (Van Berlo *et al.*, 2005). In *LMNA*-null MEFs PP2A is unable to effectively dephosphorylate rSMADs, therefore allowing the perpetuation of the TGF-β signal by activating collagen I and III promoters (Van Berlo *et al.*, 2005).

Recently, a MAN1-like protein has been identified called LEM2 (or NET-25) (Schirmer *et al.*, 2003; Lee and Wilson, 2004). LEM2 contains a LEM-domain at its N-terminal, but lacks the specific C-terminal DNA-binding region of MAN1, necessary for TGF-β/BMP-signalling (Brachner *et al.*, 2006). It is a ubiquitously expressed 56 kDa protein localised to the INM, is dependent on a C-terminal interaction with A-type lamins for retention and behaves similarly to other LEM-domain containing proteins during mitosis (Haraguchi *et al.*, 2001; Dechat *et al.*, 2004; Brachner *et al.*, 2006). Depletion of functional LEM2 by RNA*i* leads to nuclear abnormalities with these defects occurring independently of postmitotic NE reassembly, suggesting that LEM2 has a critical role in maintaining nuclear envelope integrity, possibly by interactions with proteins and/or chromatin on the nucleoplasmic side (Ulbert *et al.*, 2006).

SUN proteins

Mammalian cells contain at least four SUN domain containing proteins which share C-terminal homology with three *C.elegans* proteins, UNC-83, UNC-84 and the germ-line specific Matefin (for role at the ONM, see page 12) (Malone *et al.*, 1999; Fridkin *et al.*, 2004; Hodzic *et al.*, 2004; McGee *et al.*, 2006). Mammalian SUN1 and SUN2 have been shown to localise to the INM with their C-terminal SUN domain extending into the PNS (Hodzic *et al.*, 2004; Padamukmar *et al.*, 2005). Evidence suggests that SUN1 and SUN2 may form both homodimers and heterodimers (Crisp *et al.*, 2006; Wang *et al.*, 2006).

Whilst in *C.elegans*, INM localisation of UNC-84 is dependent on its N-terminal interaction with Ce-lamin, SUN1 and SUN2 are not dependent on the interaction with lamins for their retention at the INM, although they may bind to A-type lamins (Lee *et al.*, 2002; Crisp *et al.*, 2006; Haque *et al.*, 2006; Hasan *et al.*, 2006; Wang *et al.*, 2006). Indeed, reintroduction of A-type lamins into *LMNA*-null MEFs does not lead to the correct localisation of SUN2, which is mislocalised in these cells and thus interactions, possibly with chromatin, have been suggested for SUN1 and 2 retention (Crisp *et al.*, 2006). However, interestingly, SUN1 has been found to have a high binding affinity for prelamin A and due to the short-half of prelamin A, it has been suggested that SUN1 may have a role in the incorporation of prelamin A into the nuclear lamina rather than prelamin A functioning to locate SUN1 to the INM (Crisp *et al.*, 2006). SUN3 is a testis-specific isoform that conforms to the general topology of SUN1 and 2 (Crisp *et al.*, 2006).

Functionally, it has recently been shown that SUN1 specifically associates with telomeres during meiotic prophase I and that overexpression of SUN1 mutants leads to a failure of mammalian gametogenesis (Ding *et al.*, 2007). This result was also conformed in an independent study of SUN2, suggesting a conserved mechanism of telomere attachment during meiosis (Schmitt *et al.*, 2007). Recent evidence also implicates SUN1, but not SUN2, in helping to organize and distribute NPCs across the nuclear surface (Liu *et al.*, 2007). More widely reported has been the association of SUN1 and SUN2 with nesprin-2 in the PNS between their SUN domains and KASH domains respectively, forming a physical connection between the actin and microtubule cytoskeletons and the nucleus (Padamakumar *et al.*, 2004; Crisp *et al.*, 2006). Furthermore, it has been proposed that the SUN-nesprin link and subsequent ability to allow correct nuclear anchorage and migration may be mediated by the strength of the interaction between the two in the PNS via a pocket domain formed by the linker domain between the TM and the SUN domain (McGee *et al.*, 2006).

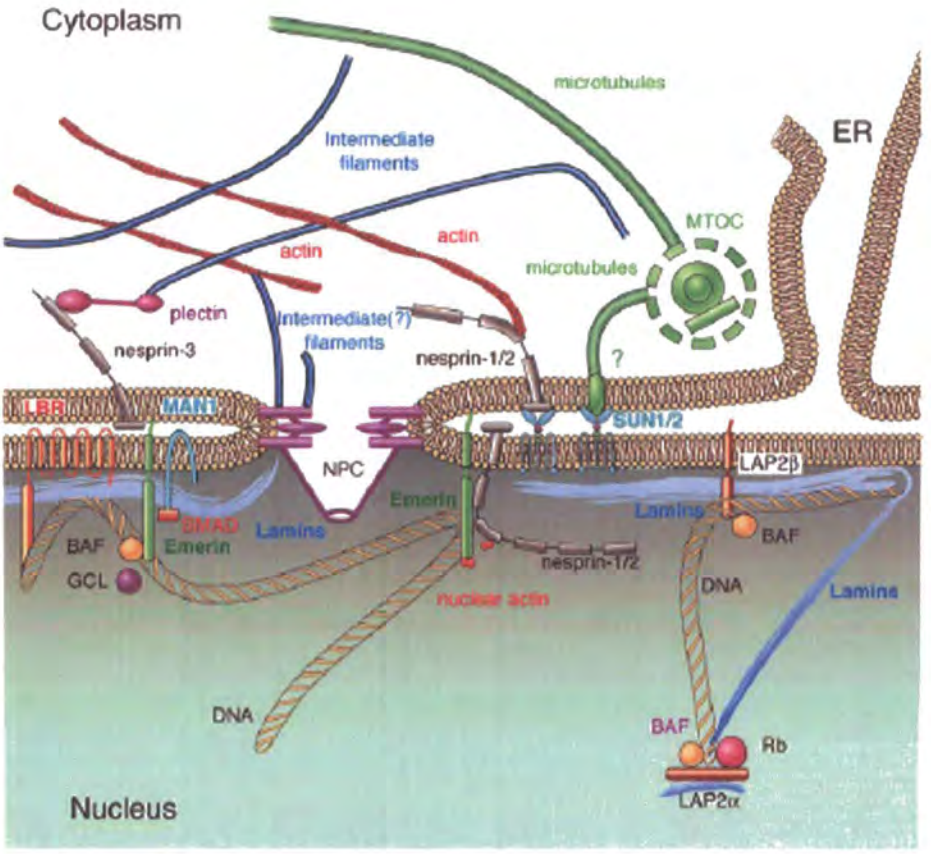


Figure 2. Schematic diagram of interactions at the nuclear envelope. The nucleo-cytoskeletal link is via the LINC complex. INM proteins interact with the underlying nuclear lamina. LEM-domain containing proteins all interact with BAF and thus interact indirectly with DNA. Lamin A/C is also found within the nucleoplasm where it is involved in a complex with a LAP2 isoform LAP2α and the hypophosphorylated form of Rb. (Adapted from Broers *et al.*, 2006)

Structure, Function and Dynamics of the Nuclear Lamina

Lamin structure and regulation of expression

The nuclear lamina is a fibrous structure composed of lamin polypeptides underlying the INM and largely determines the overall structure of the nucleus during interphase (Fawcett, 1966; Burke and Stewart, 2002). The lamins are classified as either A or B-type depending on their protein structure, behavior during mitosis, biochemical properties, and their tissue expression specificity (Dwyer and Blobel, 1976; Gerace and Blobel 1980; Krohne and Benavente 1986; Broers *et al.*, 1997; Broers *et al.*, 2006; Mattout *et al.*, 2006). Lamins are type V intermediate filaments (IFs) and as such they share a comparative tripartite domain structure, yet are distinct due to the presence of a NLS (Figure 3) (Hutchison and Worman, 2004; Smith *et al.*, 2005; Mattout *et al.*, 2006). Structurally, lamins consists of globular head and tail domains that border the central α-helical rod domain, which is divided into four coiled-coil domains (1A, 1B, 2A, and 2B), separated by flexible linker regions, and characterised by the presence of heptad repeats and an additional 42 amino acids (Hutchison and Worman, 2004). Lamins are targeted to the nucleus by the presence of a NLS in the tail domain that interacts with importins and permits nuclear import (Loewinger and McKeon, 1988; Monteiro *et al.* 1994). Additionally, with the exception of lamin C, there is a CAAX motif at the C-terminal domain that is involved processing of the prepeptide to the propeptide (see page 32) (Sinensky *et al.*, 1994; Rusinol and Sinensky, 2006). Furthermore, lamins also contain an S-type immunoglobulin fold in their C-terminal (Dhe-Paganon *et al.*, 2002; Krimm *et al.*, 2002).

The assumed formation of lamin polymers is based largely on understanding from the formation of cytoplasmic IFs. However, lamins polymerize by forming obligate parallel homodimers of 50nm that interact via the coiled-coil domain and are then assumed to assemble into longer chains or filaments via head-to-tail interactions that then associate laterally to form a higher order network (Sasse *et al.*, 1997; Strelkov *et al.*, 2004;

Herrmann and Aebi, 2004; Hutchison and Worman, 2004). Although all of the different mammalian lamin proteins interact in a yeast two-hybrid assay and *in vitro* biochemical assays, it remains to be elucidated whether the complex nuclear lamina of mammalian somatic cells contains heteropolymers or only homopolymers of lamins that form filaments *in vivo* (Gieffers and Krohne, 1991; Moir *et al.*, 1991; Heitlinger *et al.*, 1992; Ye and Worman, 1995; Schirmer and Gerace, 2001; Schirmer and Gerace, 2004). However, a recent study using quantitative FRET (fluorescence energy resonance transfer) analysis has suggested lamins may exist as homopolymers with subsequent interactions between both A and B-type lamin homopolymers (Delbarre *et al.*, 2006).

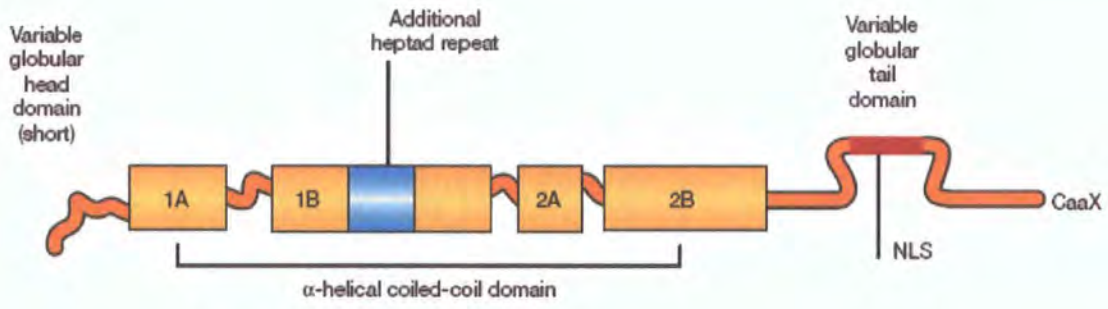


Figure 3. General structure of lamin polypeptides. (See text for details, adapted from Hutchison and Worman, 2004)

Humans have three distinct genes that encode seven different lamin polypeptides (Hutchison and Worman, 2004). A-type lamins arise by alternative splicing of RNA encoded by *LMNA*, a 12 exon gene which is localised to chromosome 1q21.2 (Fisher *et al.*, 1986; Lin and Worman, 1993; Wynder *et al.*, 1996). There are four known splice isoforms of *LMNA*, lamin A, lamin C, lamin AΔ10 and lamin C2 (Fisher *et al.*, 1986; Lin and Worman, 1993; Furukawa *et al.*, 1994; Alsheimer and Benavente, 1996; Machiels *et al.*, 1996). Lamins A and C are identical for their first 566 amino acids but vary in their C-terminal, with lamin C containing five unique C-terminal amino acids and lamin A an

additional 98 amino acids (Burke and Stewart, 2006). Lamin AΔ10 is an isoform lacking the amino acids encoded by exon 10, whilst lamin C2 is a male germline-specific isoform arising from alternative splicing of mRNA initiated from a different start site (Furukawa *et al.*, 1994; Alsheimer and Benavente, 1996; Machiels *et al.*, 1996). Two unlinked genes encode three separate B-type lamins. Lamin B1 is encoded by *LMNB1* and is located on chromosome 5q23.3–q31.1 (Lin and Worman, 1995; Wydner *et al.*, 1996). Whilst *LMNB2*, located on chromosome 19p13.3, encodes two alternatively spliced isoforms, lamin B2 and lamin B3 (Höger *et al.*, 1990; Biamonti *et al.*, 1992; Furukawa and Hotta, 1993).

Both A and B-type lamins are developmentally regulated (Broers *et al.*, 2006). B-type lamins are ubiquitously expressed in all cell types with lamins B1 and B2 required for cell survival in both embryos and adult tissues as well as for maintenance of nuclear shape and development (Lehner *et al.*, 1987; Harborth *et al.*, 2001; Steen and Collas, 2001; Vergnes *et al.*, 2004). Lamin B3 is a male germline-specfic lamin B2 splice isoform, conferring some characteristics to the shape of the gamete nuclei, suggesting that lamins have tissue-specific effects (Furukawa and Hotta 1993; Furukawa *et al.*, 1994).

In contrast, A-type lamins have been implicated in differentiated cells types with a pattern of expression primarily driven by the developmental program controlling embryogenesis and organ differentiation (Rober *et al.*, 1989; Holaska *et al.*, 2002). Essentially, although there are some discrepancies amongst the reported data, early embryo and embryonic stem cells do not express A-type lamins but are usually induced during development after cells have committed to a differentiation pathway (Schatten *et al.* 1985; Stewart and Burke 1987; Houliston *et al.* 1988; Röber *et al.* 1989; Pugh *et al.*, 1997; Gruenbaum *et al.* 2003; Constantinescu *et al.* 2006). Further to this, *LMNA*-null mice progress quite normally during early stages of development but then display severely retarded postnatal growth characterised by the appearance of muscular dystrophy and cardiomyopathy, suggesting A-type lamins are not essential for development (Sullivan *et al.*, 1999). However, in humans, haploinsufficiency for LMNA, due to the presence of a nonsense mutation at codon 6, or the presence of a homozygous mutation leading to a premature stop codon in

LMNA causes postnatal death or death *in utero* (Bonne *et al.*, 1999; van Engelen *et al.*, 2005).

Lamins A and C are the major two gene products of *LMNA*, with lamin AΔ10 expressed in tumour cell lines as well as several normal cell types (Machiels *et al.*, 1996). The minor *LMNA* splice variant, lamin C2, is specific to male gametes and is the only A-type lamin detected there (Furukawa *et al.*, 1994; Alsheimer and Benavente 1996). Tissue levels of lamin A and C are dependent on the differentiation state of the tissue, whilst ratios of lamin A to C may also differ amongst tissues (Worman *et al.* 1988; Lanoix *et al.* 1992; Martelli *et al.* 1992; Hamid *et al.* 1996, Broers *et al.*, 1997; Lattanzi *et al.* 2003, Constantinescu *et al.* 2006). Thus, that A-type lamins are expressed most highly amongst differentiated cells has led to the hypothesis that A-type lamins maintain a differentiated cell state, although they do not directly influence differentiation (Stewart and Burke 1987; Röber *et al.* 1989, Peter and Nigg, 1991; Pugh *et al.*, 1997; Constantinescu *et al.* 2006).

As well as embryonic stem cells, notable exceptions where cells do not express A-type lamins or do so at lower levels include some immune system stem cells, some hematopoietic stem cells, some neuroendocrine system cells and some forms of cancer where it is associated with cancer subtypes and aggressiveness (Guilly *et al.*, 1987; Lebel *et al.*, 1987; Paulin-Levasseur *et al.* 1988, Worman *et al.*, 1988; Röber *et al.* 1989; Röber *et al.* 1990, Stadelmann *et al.* 1990; Broers *et al.* 1993; Machiels *et al.* 1997; Moss *et al.* 1999; Venables *et al.* 2001; Mounkes *et al.* 2003; Tilli *et al.*, 2003; Agrelo *et al.* 2005; Coradeghini *et al.*, 2006).

A-type lamins are presumed to be primarily regulated at the level of transcription (Mattia *et al.* 1992, Alsheimer and Benavente 1996, Hamid *et al.* 1996; Pugh *et al.*, 1997); however, evidence suggesting a role for post-transcriptional regulation also exists (Lanoix *et al.* 1992). The proximal promoter of rat *LMNA* has been characterised, with various motifs important in determining the overall activity of the lamin A/C promoter (Muralikrishna and Parnaik 2001; Janaki Ramaiah and Parnaik, 2006). The GC box at –101 has the ability to bind Sp1/Sp3 transcription factors, and the AP-1 motif at −7 is able

to bind c-Jun and c-Fos (Nakajima and Abe 1995; Tiwari *et al.* 1998; Muralikrishna *et al.*, 2001). Moreover, there is a GT-rich motif at –55 that binds to the Sp family of transcription factors and has been shown to interact with the transcriptional coactivator CREB-binding protein (CBP) via ChIP (chromatin immunoprecipitation) analysis (Janaki Ramaiah and Parnaik, 2006). Furthermore, a 420 base pair regulatory region was identified within intron 1 of rat *LMNA*, with a suggested function to control that it may control cell-type-specific transcription of A-type lamins (Arora *et al.* 2004). A retinoic acid responsive element within the lamin A/C promoter (termed L-RARE) has also been identified in P19 embryonic carcinoma (EC) cells between position –54 and –36 that promotes lamin A/C transcription upon retinoic acid treatment (Okumura *et al.*, 2000; Okumura *et al.*, 2004). The study of lamin A/C transcription has proved important as silencing of lamin A/C expression has been correlated with hypermethylation of promoter CpG islands in human leukaemia and lymphoma cells and provides a mechanistic epigenetic basis for some malignancies (Agrelo *et al.* 2005).

Processing of prelamin A

Lamin A is synthesised as a precursor prelamin A molecule before insertion into the nuclear lamina (Gerace *et al.*, 1986; Dagenais *et al.*, 1985; Lehner *et al.*, 1986; Beck *et al.*, 1990). Proteins that undergo lipid modification, called protein prenylation, can be drawn into two groups depending on their lipid modification (Young *et al.*, 2005). One group consists of Rab GTPases that are geranylgeranylated at terminal cysteine residues (Young *et al.*, 2005). The other is a group of proteins (prelamin A, lamin B1, lamin B2, Ras, Rho) that contain a CAAX motif (C = cysteine, A = any aliphatic amino acid, X = S, M, C, A, Q, L) at the C-terminal (Sinensky *et al.*, 1994). Where X is S, M, C, A or Q then the protein is first farnesylated (15-carbon lipid), whereas if X is L then it is geranylgeranylated (20-carbon lipid) (Seabra *et al.*, 1992; Armstrong *et al.*, 1995). These proteins are then further modified, first by an endoproteolytic processing event and then by methylation of the C-terminal prenylcysteine (Clarke, 1992; Zhang and Casey, 1996; Young *et al.*, 2005). Prelamin A is unique to this group as it undergoes an additional endoproteolytic step that removes the last 15 amino acids, including the farnesylcysteine

methyl ester, from the C-terminal to produce mature lamin A (Figure 4) (Beck *et al.*, 1990; Lutz *et al.*, 1992).

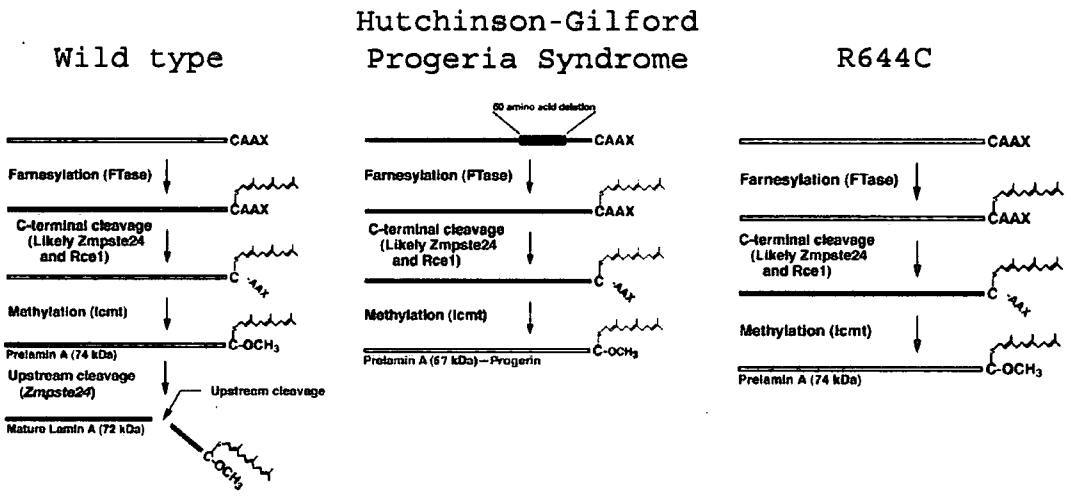


Figure 4. Processing pathway of wild type prelamin A and in disease. Wild type prelamin A undergoes a series of post-translational processing steps before mature lamin A is formed. Prelamin A transcripts in HGPS contain an internal deletion of 50 amino acids which removes the second endoproteolytic cleavage site, resulting in prelamin A that is both farnesylated and carboxymethylated, with the mutant product termed progerin. In an atypical form of HGPS, arginine is mutated to cysteine at position 644, resulting in an alteration to the Zmpste24 recognition site required for the last processing step. Again, the mutant prelamin A is expected to be both farnesylated and carboxymethylated. Although not shown here, in Zmpste24$^{-/-}$ cells there is in an accumulation of a prelamin A transcript that is found in cells that harbor the lamin A R644C mutation, with the earlier proteolytic step carried out by Rce1. (Adapted from Young *et al.*, 2005)

The first step of prelamin processing is the farnesylation by protein farnesyl transferase and involves the addition of a 15-carbon farnesyl lipid via a thioester linkage to the thiol group of the cysteine within the CAAX motif (Sinensky *et al.*, 1994). Lamin farnesylation assists in targeting the proteins to the nuclear envelope (Holtz *et al.*, 1989; Kitten and Nigg, 1991). Evidence suggests that farnesylation is an absolute requirement for the assembly of lamin A into the nuclear lamina as cells incubated with the isoprenoid biosynthesis inhibitor, lovastatin, causes nonfarnesylated prelamin A to accumulate into nucleoplasmic aggregates (Holtz *et al.*, 1989; Beck *et al.*, 1990; Lutz *et al.* 1992). It has been suggested that farnesylation occurs at distinct nucleoplasmic foci (Lutz *et al.* 1992, Moir *et al.* 1995). Supporting this, NARF (nuclear prelamin A recognition factor) is a nuclear membrane associated protein also found at nucleoplasmic foci that binds the C-terminal tail of prelamin A with prenylation dependent specificity, and although its function is unknown, it may be involved in properly presenting the substrate to the active site of Zmpste24 (Barton and Worman 1999). Recent studies have shown that when a prelamin A mutant (L647R), which cannot undergo its finally endoproteolytic step cleavage step, was co-transfected with a SUN1 N-terminal domain construct into *LMNA*-null MEFs, the SUN1 N-terminal construct was relocalised away from the nucleoplasm to the NE, revealing a strong interaction between farnesylated carboxymethylated prelamin A and the N-terminal of SUN1 (Crisp *et al.*, 2006). The authors have suggested that rather than targeting SUN1 to the NE, SUN1 may function to target prelamin A to the NE for incorporation into the nuclear lamina (Crisp *et al.*, 2006). However, these hypotheses, for both NARF and SUN1 and their relationship to prelamin A, require further work.

After farnesylation, the last three peptides of the protein (the –AAX of the CAAX motif) are cleaved either by the endoproteinase Zmpste24 (alternatively called FACE1 but herein referred to solely as Zmpste24), a multi-spanning membrane zinc metalloproteinase localized to the endoplasmic reticulum and nuclear envelope, or Rce1 (Ras-converting enzyme 1) (Bergo *et al.* 2002, Pendás *et al.* 2002; Corrigan *et al.*, 2005). The newly exposed farnesylcysteine is methylated by isoprenylcysteine carboxy methyltransferase (ICMT; also a membrane protein in the ER and nucleus) (Sinensky *et al.*, 1994). The presence of this farnesylated and carboxymethylated peptide acts to block the

polymerisation of lamin A into the nuclear lamina, although these steps are fundamental for processing of prelamin A to lamin A (Lutz *et al.*, 1992; Kilic *et al.*, 1997).

Finally, the last fifteen amino acids of prelamin A are then endoproteolytically removed by Zmpste24, which recognises the conserved hexapeptide RSY↓LLG, producing mature lamin A which then integrates into the nuclear lamina (Weber *et al.*, 1989; Lutz *et al.*, 1990; Kilic *et al.*, 1997; Corrigan *et al.*, 2005). Although Zmpste24 is responsible for the cleavage of -AAX and the later cleavage of the terminal 15 amino acids in insect cells, it is not known whether this is true for mammalian cells (Bergo *et al.* 2002; Corrigan *et al.*, 2005). However, overexpression of a Zmpste24 expression plasmid is sufficient to restore the prelamin A processing in *Zmpste24*$^{-/-}$ MEFs and produce mature lamin A, suggesting a role for both cleavage events (Pendás *et al.* 2002).

A functional CAAX motif seems to be essential for prelamin A incorporation into the nuclear lamina, with each modification highly specific and dependent on the previous modification (Hennekes and Nigg, 1994; Monteiro *et al.*, 1994; Izumi *et al.*, 2000; Corrigan *et al.*, 2005; Rusinol and Sinensky, 2006). It has been suggested that the multitude of steps involved in the processing of prelamin to mature lamin A confers the ability to reversibly associate with membranes via a hydrophobic interaction with the phospholipid bilayer (Dalton and Sinensky, 1995; Rando, 1996; Kilic *et al.*, 1999; Corrigan *et al.*, 2005). Alternatively, the processing may aid in the interaction with a membrane receptor protein, such as the interaction between farnesylated lamin B with its INM anchor LBR or the interaction between NARF/SUN1 and farnesylated (and carboxymethylated for SUN1) prelamin (Marshall 1993; Ye and Worman 1994; Kisselev *et al.*, 1995; Kilic *et al.*, 1997; Barton and Worman 1999; Lin *et al.*, 2000; Crisp *et al.*, 2006).

However, the processing steps of prelamin A are not required for the incorporation of lamin A into the nuclear lamina, as microinjected lamin A is incorporated into the nuclear lamina (Pugh *et al.*, 1997; Goldman *et al.*, 2004). Furthermore, mature lamin A is disassembled and reassembled into the lamina during the course of mitosis (Gerace *et al.*,

1984). Indeed, it has been shown that A-type lamins are transiently localized to the nuclear interior during G1 and become incorporated into the lamina as G1 progresses (Bridger *et al.* 1993, Dechat *et al.* 2000a, Moir *et al.* 2000; Holaska *et al.* 2002). Lamin A also forms a nucleoplasmic veil during interphase although the nature of remains to be fully clarified (Hozak *et al.*, 1995; Broers *et al.*, 1999; Zastrow *et al.*, 2004). Whilst lamin A contains a CAAX motif and endoproteolytic site, lamin C does not and thus A-type lamins follow different pathways of incorporation into the lamina (Broers *et al.*, 2006). Although both proteins are usually incorporated in the nuclear lamina in roughly equivalent amounts, it has been reported that lamin C is dependent on lamin A for localization to the NE as well as for incorporation into the lamina (Pugh *et al.* 1997, Vaughan *et al.* 2001). Microinjected lamin C accumulates as nucleoplasmic foci before integration into the peripheral lamina (Pugh *et al.* 1997). Moreover, the presence of lamin C can cause lamin A to default to the lamin C pathway and become incorporated into intranuclear foci before transit to the peripheral lamina (Pugh *et al.* 1997). Similarly, microinjected lamin A is initially localized to the nucleoplasm before incorporation in the peripheral lamina (Goldman *et al.* 1992, Schmidt *et al.* 1994). Whilst mature lamin A may incorporate into the nuclear lamina it is clear that the evolutionary conserved prelamin A processing has a crucial, if not yet fully known, functional significance.

Lamina function

The functions of lamins have largely been based on their various interactions in the nucleus (Zastrow *et al.*, 2004). These functions can be broadly classified into the following groups: tensegrity element; chromatin scaffold; transcriptional partner; genomic caretaker (Hutchison and Worman, 2004; Zastrow *et al.*, 2004; Smith *et al.*, 2005; Verstraten *et al.*, 2007; Vlcek and Foisner, 2007b).

Several lines of evidence suggest that the lamina is essential in maintaining the structure of the nuclear envelope, by virtue of their ability to resist deformation and protect chromatin and as such has been described as a tensegrity element (Hutchison and Worman, 2004). Knockout mouse lines for both *LMNA* and *LMNB1* have yielded

important results underlining their structural importance. Lamin B1 and B2 are ubiquitously expressed in nearly all cells and mice homozygous for *LMNB1* die at birth whilst fibroblasts exhibit gross nuclear envelope defects (Vergnes *et al.*, 2004). Mice null for *LMNA* have decreased mechanical properties, leading to nuclear envelope defects and mislocalisation of INM proteins (Sullivan *et al.*, 1999; Harborth *et al.*, 2001; Muchir *et al.*, 2003; Broers *et al.*, 2004; Lammerding *et al.*, 2004; Nikolova *et al.*, 2004). Furthermore, cells in which disease-causing lamin A/C mutants are transiently overexpressed display increased nuclear defects (Östlund *et al.*, 2001; Raharjo *et al.*, 2001; Vigouroux *et al.*, 2001; Novelli *et al.*, 2002; Capanni *et al.*, 2003; Chen *et al.*, 2003; Eriksson *et al.*, 2003; Favreau *et al.*, 2003; Holt *et al.*, 2003; Goldman *et al.*, 2004; Muchir *et al.*, 2004; Navarro *et al.*, 2004). Lamins have been directly implicated in nuclear size as overexpression of CAAX-motif-containing lamins leads to increased nuclear membrane growth (Ralle *et al.*, 2004; Prufert *et al.*, 2004). Moreover, another CAAX-motif-containing protein in *Drosophila*, Kugelkern, has been suggested to be a rate-determining factor for nuclear size increase (Brandt *et al.*, 2006). Lamins interact with the nucleoporin Nup153 and are responsible for positioning and anchoring the NPC as *LMNA* null cells show asymmetric NPC distribution (Sullivan *et al.*, 1999; Smythe *et al.*, 2000). Evidence of a nucleo-cytoskeletal link suggests that lamins also have a role in maintaining cellular integrity via the LINC complex and that mutations in lamin A/C may cause muscular dystrophy serves to reinforce this (Bonne *et al.*, 1999; Mislow *et al.*, 2002a,b; Nikolova *et al.*, 2004; Libotte *et al.*, 2005; Zhang *et al.*, 2005; Houben *et al.*, 2007).

The suggestion that the lamina may act as a chromatin scaffold or anchor is based on evidence showing that lamins bind DNA, chromatin and core histones via the lamin A/C C-terminal domain (Luderus *et al.*, 1992; Glass *et al.*, 1993; Luderus *et al.*, 1994; Taniura *et al.*, 1995). Furthermore, given this interaction it would allow lamins to serve as scaffolds for multiprotein complexes associated with chromatin (Stierlé *et al.*, 2003). Moreover, lamin A may also bind chromatin via BAF, which also mediates the non-specific binding of LEM-domain containing proteins to chromatin (Zheng *et al.*, 2000; Lee *et al.*, 2001; Shumaker *et al.*, 2001; Holaska *et al.*, 2003; Liu *et al.*, 2003). Nucleoplasmic A-type lamins have been suggested to act as a scaffold for RNA-

polymerase II-dependent transcription, DNA synthesis and RNA splicing, although the latter may function independently of a lamin scaffold (Meier *et al.*, 1991; Spann *et al.*, 1997; Jagatheesan *et al.*, 1999 Moir *et al.*, 2000; Kumaran *et al.*, 2002; Spann *et al.*, 2002; Vecerova *et al.*, 2004).

Several transcriptional regulators have been found to interact with lamins such as MOK2 and Oct-1, which interact with lamin A/C and B, respectively (Imai *et al.*, 1997; Dreuillet *et al.*, 2002; Zastrow *et al.*, 2004). The expression of lamin A/C mutations in the MOK2 binding domain casues the relocalisation of MOK2 into nuclear aggregates, which has been hypothesized to deregulate MOK2 target genes (Dreuillet *et al.*, 2007). Another is Rb, and the lamin A/C-LAP2α-Rb complex functions to control cell cycle progression and differentiation, with loss of either lamin A/C or LAP2α causing abnormal cell cycle characteristics (See page 20) (Markiewicz *et al.*, 2002b; Johnson *et al.*, 2004; Dorner *et al.*, 2006; Nitta *et al.*, 2006; Pekovic *et al.*, 2007). Furthermore, lamin A/C is thought to act as an essential docking site for the control of Rb dephosphorylation by PP2A, which also acts to dephosphorylate rSMADs in two independent pathways and thus *LMNA*-null MEFs show increased proliferation and increased fibrosis (See page 23) (Van Berlo *et al.*, 2005). Recently, it has been suggested that the interaction between lamin A/C and the transcription factor c-Fos acts to sequester it at the NE, which results in the AP-1 mediated gene transcription (Ivorra *et al.*, 2006). Subsequent release form this repressive complex allows the formation of C-Fos/C-Jun heterodimers (AP-1 complex) and activation of target genes (Ivorra *et al.*, 2006). Prelamin A has been found to be a binding partner for the sterol regulatory element binding protein (SREBP1), a member of the bHLH transcription factor family that upregulates PPARγ during adipogenesis (Fajas *et al.*, 1999; Lloyd *et al.*, 2002; Capanni *et al.*, 2005; Fève, 2005; Rosen, 2005). Due to the interactions of lamin A/C with emerin at the INM, lamin A/C has been indirectly implicated in signal transduction and transcription as emerin has been shown to bind to β-catenin, involved in the canonical Wnt pathway (Markiewicz *et al.*, 2006).

Lamins have also been labeled 'genomic caretakers' as defective processing of prelamin A has been linked to increased DNA damage (Misteli and Scaffidi, 2005; Lees-Miller, 2006;

Liu *et al.*, 2006; Manju *et al.*, 2006; Verstraten *et al.*, 2007). HGPS fibroblasts show an increased amount of unrepaired DNA as judged by the presence of foci containing phosphorylated histone H2AX (Scaffidi and Misteli, 2006). Furthermore, the recruitment of DNA repair factors 53BP and Rad51 to H2AX-tagged sites of DNA lesions is delayed in human HGPS fibroblasts as well as in *Zmpste24*-null MEFs, and with transient overexpression of expression plasmids reversing these effects it draws a direct link to prelamin A as being the primary cause (Liu *et al.*, 2005). Additionally, H2AX-53BP complexes take longer to dissociate in *Zmpste24*-null MEFs, and these cells show increased sensitivity to DNA damaging agents with a resulting overall increase in levels of genomic instability (Liu *et al.*, 2005). Transcriptional analyses carried out on tissues from *Zmpste24*-null MEFs showed an upregulation of classical p53 target genes, which is involved in a pathway that induces cell and tissue senescence and eventually leads to accelerated aging (Campisi, 2005; Varela *et al.*, 2005). Lamin A/C is upregulated in a p53-dependent manner, suggesting that they may be linked and implicates p53 signaling in laminopathies (Rahman-Rohblick *et al.*, 2007). Subsequently, the existence of a 'structural cell cycle checkpoint' involved in the detection of nuclear envelope and/or chromatin abnormalities has been proposed (Cadinanos *et al.*, 2005; Varela *et al.*, 2005). Apart from DNA damage, mutations in lamins and alterations to prelamin A processing causes loss of heterochromatin and changes in epigenetic modifications that regulate heterochromatin (Sullivan *et al.*, 1999; Goldman *et al.*, 2004; Nikolova *et al.*, 2004; Columbaro *et al.*, 2005; Filesi *et al.*, 2005; Scaffidi and Misteli, 2005; Maraldi *et al.*, 2006a,b; Shumaker *et al.*, 2006; Lattanzi *et al.*, 2007; Maraldi *et al.*, 2007a,b).

Envelopathies

Diseases that arise due to mutations within proteins at the nuclear envelope have been collectively termed envelopathies (Burke and Stewart, 2006). These include an autosomal recessive form of cerebellar ataxia (caused by mutation in nesprin 1), PHA and HEM/Greenberg skeletal dysplasia (LBR), X-linked EDMD (emerin), dilated cardiomyopathy (LAP2α), and three skeletal diseases: osteopoikilosis, Buschke-

Ollendorff syndrome and melorheostosis (all MAN1) (Bione *et al.*, 1994; Manilal *et al.*, 1996; Nagano *et al.*, 1996; Hoffmann *et al.*, 2002; Best *et al.*, 2003; Waterham *et al.*, 2003; Hellemans *et al.*, 2004; Taylor *et al.*, 2005; Hellemans *et al.*, 2006; Gros-Louis *et al.*, 2007). A subset of nuclear envelopathies are those diseases caused by mutations in lamins, called laminopathies.

Laminopathies

Mutations in both A and B type lamins give rise to a wide spectrum of diseases collectively called laminopathies and reviewed extensively within the literature reflecting a growing interest in these key nuclear proteins (Gruenbaum *et al.*, 2005; Smith *et al.*, 2005; Worman and Courvalin, 2005; Broers *et al.*, 2006; Burke and Stewart, 2006; Gotzmann and Foisner, 2006; Maraldi *et al.*, 2006a,b; Mattout *et al.*, 2006; Parnaik and Manju, 2006; Rankin and Ellard, 2006; Young *et al.*, 2006; Verstraeten *et al.*, 2007; Worman and Bonne; 2007). Primary laminopathies arise from mutations within *LMNA*, *LMNB1*, or *LMNB2* and disease-causing mutations within *Zmpste24* are called secondary laminopathies. Whilst disease-causing mutations within A-type lamins were first identified in 1999, mutations within the two lamin B genes have only recently been discovered. Duplication of *LMNB1* causes adult-onset AD leukodystrophy, characterised by progressive loss of myelin in the central nervous system (ADLD; OMIM 169500) (Padiath *et al.*, 2006). Barraquer-Simmons syndrome is a sporadic acquired partial lipodystrophy resulting from mutation within *LMNB2* (APL; OMIM 608709) (Hegele *et al.*, 2006). The mechanistic basis for how mutations in these two genes cause two very distinct diseases remains unknown. Indeed, that there are now more than 250 mutations that have been documented within *LMNA* and how mutations within a single protein ubiquitously expressed in differentiated tissues can cause varying disease phenotypes is an intriguing question (Hutchison *et al.*, 2001; http://www.dmd.nl/lmna_home.html). Some have attempted to correlate laminopathy phenotypes with the genotype, however such is the homogeneity of mutations throughout gene that it proved impossible to predict a definite genotype-phenotype association (Hegele, 2005).

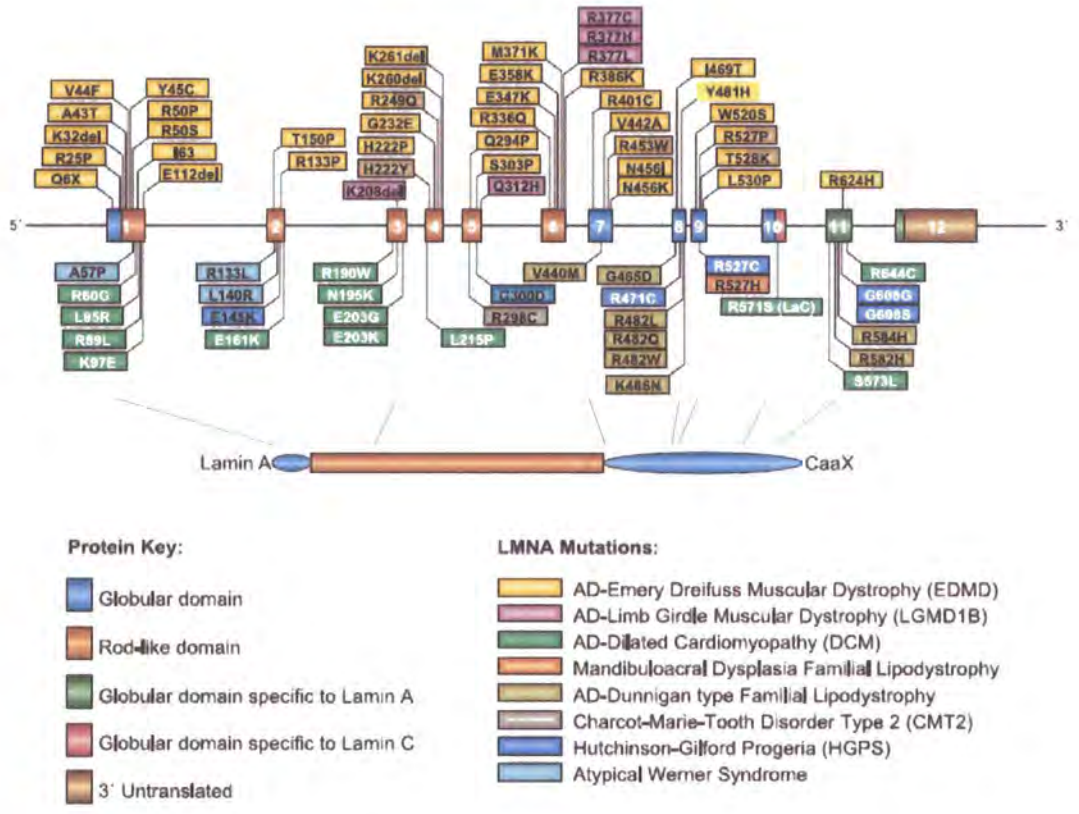


Figure 5. Disease-causing lamin A/C mutations. Comprehensive diagram showing the positions of mutations in *LMNA* and the diseases they cause. (See text for details of each laminopathy, adapted from Burke and Stewart, 2006).

Autosomal Dominant – Emery Dreifuss muscular dystrophy (AD-EDMD)

Whilst X-linked EDMD is due to a mutation within the INM protein emerin, mutations within lamin A/C may cause an autosomal dominant form of EDMD (OMIM 181350) (Bione *et al.*, 1994; Bonne *et al.*, 1999; Muchir *et al.*, 2006). AD-EDMD presents during childhood and leads to progressive wasting of muscle, initially of the humeroperoneal and

then limb girdle muscles, and frequently these are cardiac defects, notably dilated cardiomyopathy (DCM) (Bonne *et al.*, 2000). Mutations are primarily nonsense mutations and their location throughout the gene underline the absence of a genotype-phenotype relationship (Bonne *et al.*, 2000; Helbling-Leclerc *et al.*, 2002; van der Kooi *et al.*, 2002; Muchir *et al.*, 2004). Notably, there is also a rare autosomal recessive form of EDMD, which caused severe muscle wasting by 40 years of age (OMIM 604929) (Raffaele Di Barletta *et al.*, 2000).

Dilated cardiomyopathy with conduction system defect (CMD1A)

Dilated cardiomyopathy with conduction system defects is characterised by cardiac dilation and reduced systolic function, with an atrioventricular conduction block or atrial arrhythmias, frequently resulting in sudden death (OMIM 115200) (Fatkin *et al.*, 1999; Brodsky *et al.*, 2000; Charniot *et al.*, 2003; Sanna *et al.*, 2003; Sebillon *et al.*, 2003; Hermida-Prieto *et al.*, 2004). However, there is little or no skeletal muscle involvement as is the case for AD-EDMD (Fatkin *et al.*, 1999). Most mutations found for DCM-CD1 are located in the rod domains of lamins A and C (Fatkin *et al.*, 1999; Broers *et al.*, 2006)

Limb girdle muscular dystrophy 1B (LGMD 1B)

The autosomal dominant limb girdle muscular dystrophy type 1B has a range of clinical features, but is essentially characterized by progressive limb girdle weakness and an associated DCM (OMIM 155001) (Muchir *et al.*, 2000; Emery, 2002). Again, most mutations in *LMNA* that cause LGMD 1B were found to be missense mutations with analysis showing that these three striated muscle disorders are allelic variants and that members of the same family with the same mutations may present different phenotypes (Bonne *et al.*, 2000; Brodsky *et al.*, 2000; Muchir *et al.*, 2000).

Charcot-Marie-Tooth syndrome type 2b (CMT2B1)

CMT2B1 is an autosomal recessive axonal disease characterised by reduced motor nerve velocities with associated muscle wasting in the lower and upper limbs (OMIM 605588) (De Sandre-Giovannoli *et al.*, 2002; Chaouch *et al.*, 2003; Tazir *et al.*, 2004). Mice null for *LMNA* also show a similar axonal phenotype to human patients homozygous for the R298C mutation found in CMT2B1 (De Sandre-Giovannoli *et al.*, 2002).

Lipodystrophies

Lipodystrophies are a heterogeneous group of disorders characterised by complete or partial loss of adipose tissue and is often associated with insulin-resistant diabetes mellitus (Kobberling and Dunnigan, 1986; Hegele, 2000; Reitman *et al.*, 2000). One of these, Dunnigan-type familial partial lipodystrophy, is a rare disorder that presents at puberty and is characterised by progressive loss of subcutaneous adipose tissue from the extremities, trunk and gluteal region, with a concomitant accumulation of adipose tissue in the intra-abdominal region, face and neck (FLPD; OMIM 151660) (Garg *et al.*, 1999; Hegele *et al.*, 2000; Lelliott *et al.*, 2002; Agarwal and Garg, 2006). Furthermore, the disorder is associated with metabolic complications, with a likelihood of glucose intolerance, insulin resistance and type II diabetes mellitus, which is more prevalent in women than men (Rankin and Ellard, 2006). Three groups mapped the FPLD gene to chromosome 1q21-22 (Jackson *et al.*, 1998; Peters *et al.*, 1998; Anderson *et al.*, 1999), a region containing over 100 genes including the *LMNA*. A short time thereafter, *LMNA* was confirmed as the causative gene in FPLD (Cao and Hegele, 2000; Shackleton *et al.*, 2000; Speckman *et al.*, 2000). To date, the majority of mutations, some 75%, involve missense mutations of the arginine at codon 482 (R482Q, R482W, R482L), which is positioned within exon 8 in the globular carboxy- terminal of both lamins A and C (Haque *et al.*, 2003; Capeau *et al.*, 2005; Agarwal and Garg, 2006; Burke and Stewart, 2006; Jacob and Garg, 2006). Other mutations occur at codon 486 and in exon 11 at codons 582 and 584, with the latter two presenting as atypical cases of FLPD as the patients had mild

subcutaneous fat loss when compared to typical patients with a R482 mutation (Speckman *et al.*, 2000; Vigoroux *et al.*, 2000; Garg *et al.*, 2001; Garg *et al.*, 2002). Patients with FLPD have also been found to have N-terminal mutations in lamin A/C (R28W and R62G) with the patients suffering from both muscle weakness and cardiac dysfunction, which are notably absent from typical cases of FLPD (Garg *et al.*, 2002; Jacob and Garg, 2006).

Werner's Syndrome (WRN)

Typical Werner's syndrome is an autosomal recessive premature aging disease caused by mutations within *RECQL2*, a gene that encodes a RecQ DNA helicase, and is characterised by scleroderma-like skin changes, cataract formation, subcutaneous calcification, premature arteriosclerosis, *diabetes mellitus*, short stature and predisposition to cancer (WRN; OMIM 277700) (Goto *et al.*, 1997; Yu *et al.*, 1997; Mohaghegh and Hickson, 2001). Atypical Werner's syndrome is a more severe than typical WRN and is caused by missense mutations in *LMNA*, specifically within both the rod domain and C-terminal globular domain (Chen *et al.*, 2003; Hegele, 2003).

Mandibuloacral dysplasia (MAD)

Mandibuloacral dysplasia type A is characterised by post-natal growth retardation, skeletal abnormalities such as hypoplasia of the mandible and clavicles, short stature and partial lipodystrophy, and premature aging, thus sharing phenotypes of two other laminopathies, HGPS and FPLD (MADA; OMIM 248370) (Novelli *et al.*, 2002; Simha and Garg, 2002; Afifi and El-Bassyouni, 2005). MADA is caused by a homozygous R527H mutation (Novelli *et al.*, 2002; Shen *et al.*, 2003; Simha *et al.*, 2003). This mutation is located in the C-terminal globular domain of lamin A and leads to the accumulation of prelamin A at the nuclear envelope, the formation of intranuclear prelamin A-containing structures and a loss of heterochromatin markers (Filesi *et al.*, 2005). Mandibuloacral dysplasia type B is similar to MADA but caused by mutations within *Zmpste24*, and is characterised by

generalised lipodystrophy affecting the extremities (OMIM 608612) (Agarwal *et al.*, 2003).

Restrictive Dermopathy (RD)

RD is an autosomal recessive fatal perinatal disorder characterised by severe intrauterine growth retardation, and tight skin with generalised contractures, resulting in death usually after a few days (OMIM 275210) (Navarro *et al.*, 2004). The disease is caused by mutations in either *LMNA* due to complete loss of exon 11 and subsequent loss of the second endoproteolytic cleavage site, or in *Zmpste24* due to the heterozygous insertion of 1bp causing a premature stop codon and therefore suppressing the protease activity of Zmpste24 (Navarro *et al.*, 2004; Levy *et al.*, 2005; Moulson *et al.*, 2005; Toth *et al.*, 2005).

Hutchinson-Gilford Progeria Syndrome (HGPS)

Hutchinson-Gilford Progeria Syndrome (HGPS) is a severe laminopathy, with a reported incidence of 1:4-8 million and since 1886 just over 100 cases have been reported with 40 known current cases worldwide (OMIM 176670) (Novelli and D'Apice, 2003; Pollex and Hegele, 2004; Martin, 2005). Death occurs at an early age, between 12-15 years, with at least 90% of HGPS subjects dying from cardiovascular causes, with atherosclerosis being the predominant pathology (Hasty *et al.*, 2003; Al Shali and Hegele, 2004). HGPS patients appear normal at birth, but within a year begin to show accelerated aging (Pollex and Hegele, 2004). Patients then show typical symptoms, including micrognathia (small jaw), craniofacial disproportion, alopecia, prominent eyes and scalp, delayed growth, short stature, below average weight, a lack of subcutaneous fat, wrinkled skin, speckled hypopigmentation, reduced bone density and a 'horse riding' stance (Mounkes and Stewart, 2004; Pollex and Hegele, 2004). Thus, patients seem to age up to ten years per every year of their life (Pollex and Hegele, 2004). However, patients do not show all signs of aging, with the notable absence of any increase in tumour susceptibility, cataract

formation or cognitive degeneration, prompting the disease to be called a 'segmental' progeroid syndrome, as it does not fully recapitulate all symptoms of the normal aging process (Mounkes and Stewart, 2004). However, new evidence has implicated an association of osteosarcoma with an atypical form of HGPS (Shalev *et al.*, 2007)

Classical autosomal dominant HGPS arises from a *de novo* mutation in *LMNA* that results in a mutant lamin A product, it does not affect lamin C, causing disastrous affects on nuclear architecture and genomic stability (Eriksson *et al.*, 2003; De Sandre-Giovannoli *et al.*, 2003; Cao and Hegele, 2003). In one study, 18 out of 20 classical cases of HGPS harboured an identical *de novo* point mutation 1824C>T, causing a single-base substitution, G608G (GGC>GGT), within exon 11 (Eriksson *et al.*, 2003). The group also identified two other *de novo* mutations resulting in HGPS, a different heterozygous base substitution in the same codon G608S (GGC>AGC) and a heterozygous base substitution in exon 2, E145K (GAG>AAG) (Eriksson *et al.*, 2003). Analysis of the G608G mutation revealed that the single base substitution led to the activation of a cryptic splice site within *LMNA*, resulting in the production of a protein that lacks an internal (pVal607-Gln656del) 50 amino acid sequence near the C-terminus (Eriksson *et al.*, 2003). This deletion removes a potential phosphorylation site, but the CAAX motif remains intact and is not expected to interfere with farnesylation, release of –AAX or carboxymethylation (Eriksson *et al.*, 2003). However, the mutation does remove the second endoproteolytic cleavage site, RSY↓LLG, preventing the final processing step to mature lamin A, creating a mutant protein termed 'progerin' that is both carboxymethylated and farnesylated (Figure 4) (Goldman *et al.*, 2004). Progerin transcript levels are approximately 40% of the total lamin A transcripts, thus suggesting that use of the splice site is incomplete and that the mutant protein has a dominant negative effect (Reddel and Weiss, 2004). Fibroblasts from patients exhibit characteristic nuclear abnormalities such as blebbing, NPC clustering and even nuclear envelope ruptures with chromatin extrusions (Eriksson *et al.*, 2003; Goldman *et al.*, 2004). A range of other mutations, both autosomal dominant and recessive, have been found in *LMNA*, giving rise to atypical HGPS (Csoka *et al.*, 2004b; Fukuchi *et al.*, 2004; Plasilova *et al.*, 2004; Verstraeten *et al.*, 2006; Mazereeuw-Hautier *et al.*, 2007). One such mutation is a substitution at position 644 of arginine for a cysteine,

purported to alter the second endoproteolytic cleavage recognition site, and thus, like progerin, is both farnesylated and carboxymethylated (Figure 4) (Csoka *et al.*, 2004b). Notably, an autosomal recessive mutation, K542N, that causes atypical HGPS, with some characteristics of MAD, is located at the solvent-accessible Ig-like domain, suggesting different pathogenic mechanisms for the disease as compared to typical HGPS (Plasilova *et al.*, 2004).

Stem cells

Stem cells are at the forefront of research as they represent a potential source of cells that may be used to replace or regenerate damaged tissues (Gharzi *et al.*, 2003; Barrilleaux *et al.*, 2006). Stem cells are distinguished by their ability to divide asymmetrically, therefore producing progeny that allows for its self-renewal to retain 'stemness', whilst also committing to a defined set of progeny capable of differentiating into various cell types (Weissman *et al.*, 2001; Wobus and Boheler, 2005). Stem cells are usually classified according to their self-renewal and differentiation potential. Totipotent stem cells may form all embryonic and extra-embryonic cell types (Ciosk *et al.*, 2006). The less potent pluripotent stem cells may produce any cell of the three germ layers, whilst multipotent stem cells may produce a subset of cell lineages and the least potent, unipotent stem cells, give rise only to only one type of cell (Moore and Lemischka, 2006; Hoffman and Merrill, 2007). Human embryonic stem cells have been isolated and are subject to various functional regulations that allow them to self-renew, such as the expression of telomerase and the expression of certain transcription factors including Oct4, Nanog and SOX2, which actively bind promoters of genes required for growth and proliferation (Armstrong *et al.*, 2000; Chambers and Smith, 2004; Armstrong *et al.*, 2005; Boyer *et al.*, 2005; Gan *et al.*, 2007; Hiyama and Hiyama, 2007). Active repression of genes required for differentiation by Polycomb group complexes also occurs (Boyer *et al.*, 2006; Lee *et al.*, 2006). Somatic, or adult, stem cells also exist within specific niches and are capable of maintaining, generating and replacing terminally differentiated cells within their own specific tissue (Nystul and Spradling, 2006). The best-characterised of these are the

haematopoietic stem cells (HSCs) derived from bone marrow, which are multipotent and give rise to blood cells (Jiang *et al.*, 2002a,b). Furthermore, adult stem cells exist in a variety of adult tissues including mesenchymal stem cells (MSCs), which are also derived from the bone marrow stroma, multipotent adipose-derived stem cells (hMADs), skin-derived precursor (SKPs) stem cells, mammary stem cells, hepatic stem cells, pancreatic stem cells, skeletal muscle satellite cells and neural stem cells (Bjornsen *et al.*, 1999; Pitteneger *et al.*, 1999; Toma *et al.*, 2001; Zuk *et al.*, 2001; Jiang *et al.*, 2002a,b; Zuk *et al.*, 2002; Frockop *et al.*, 2003; Fernandes *et al.*, 2004; Ogawa *et al.*, 2004; Rodriguez *et al.*, 2004; Shackleton *et al.*, 2005; Toma *et al.*, 2005). Previous dogma stated that tissue-specific adult stem cells would only give rise to tissue-specific progenitor cells, however, it is now emerging that adult stem cells have a high degree of plasticity with contribution across germ layers (Wagers and Weissman, 2004; Nystul and Spradling, 2006).

Hair follicle stem cells

The hair follicle appendage results from invagination of the epidermis during development, which is regulated by a host of signalling cues, including BMP, FGF, TNF, Wnt and sonic hedgehog signalling (Shh) pathways (Schmidt-Ullrich and Paus, 2005; Waters *et al.*, 2007). Once matured, the hair follicle undergoes rounds of cycling, divided into three stages, namely anagen, catagen and telogen (Waters *et al.*, 2007). During anagen, the relatively undifferentiated matrix cells, also known as transit-amplifying cells, proliferate and differentiate to generate the hair shaft and the surrounding inner root sheath at the base of the growing follicle, which together are comprised of at least eight different cell lineages (Lavker *et al.*, 2003; Alonso and Fuchs, 2006; Cotsarelis, 2006a). Anagen duration is variable between species and body location (Cotsarelis, 2006b). Once this growth stage is complete, cells of the matrix undergo apoptosis bringing the hair follicle bulb into close proximity to the bulge compartment (Lavker *et al.*, 2003; Millar, 2002; Muller-Rover *et al.*, 2001). This is followed by a period of quiescence, telogen, before activation of cells within the hair follicle stem cell compartments initiate a new round of anagen (Alonso and Fuchs, 2006). Two distinct stem cell niches exist in the hair follicle that serves to reform the hair shaft and IRS during rounds of cycling (Jahoda, 2003;

Blanpain *et al.*, 2004). The bulge region contains label-retaining multipotent epithelial stem cells that express distinct cell surface markers identified by gene expression profiling (Cotsarelis *et al.*, 1990; Morris *et al.*, 2004; Ohyama *et al.*, 2006). At the onset of anagen, a population at the bottom of the stem cell bulge compartment is activated to divide rapidly which then migrate to the base of the follicle and differentiate to form the hair shaft and IRS (Oshima *et al.*, 2001). The cells in the bulge then return to a quiescent state, rarely undergoing mitosis (Cotsarelis *et al.*, 2006b). As well as a involvement in hair follicle cycling, bulge stem cells may participate in wound healing (Jahoda and Reynolds, 2001; Ito *et al.*, 2007) A second stem cell niche is the mesenchymal-derived dermal papilla, located at the base of the hair follicle and is essential for hair follicle production (Jahoda *et al.*, 1984; Kishimoto *et al.*, 2000). It has been suggested that at the telogen to anagen transition, the dermal papilla activates stem cells in the bulge niche, the so-called 'bugle activation hypothesis', with crosstalk between this epithelial-mesenchymal interaction likely due to a combination of Wnt, BMP and Shh signaling pathways (Kishimoto *et al.*, 2000; Jahoda, 2003; Alonso and Fuchs, 2003; Lowry *et al.*, 2005; Alonso and Fuchs, 2006; Cotsarelis, 2006a,b; Ohyama, 2007; Tiede *et al.*, 2007; Waters *et al.*, 2007). Both bulge and dermal papilla stem cells exhibit a great degree of plasticity both *in vitro* and *in vivo*, giving rise to adipogenic, osteogenic, neural and haemopoietic lineages (Lako *et al.*, 2002; Jahoda *et al.*, 2003; Blanpain *et al.*, 2004; Richardson *et al.*, 2005; Blanpain *et al.*, 2007).

Role of lamins in adult stem cells

Early findings that A-type lamins are differentially regulated during development have implicated their importance in somatic tissues, being described as the 'guardian of the soma' (Burke and Stewart, 1987; Hutchison and Worman, 2004). Consistent with this, A-type lamins are absent from human embryonic stem cells and *LMNA* knockout mice appear normal at birth, although with subsequent growth retardation (Sullivan *et al.*, 1999; Constantinescu *et al.*, 2006). As many of the tissues affected in laminopathies are of mesenchymal origin, it suggests that there may be a common disease pathogenesis that affects these tissues. It has therefore been proposed that laminopathic phenotypes may be

due to the failure of adult stem cells to self-renew and differentiate to replace damaged tissues, probably via the impairment of Rb-mediated pathways (Hutchison and Worman, 2004). Indeed, overexpression of laminopathy-causing mutations causes the failure of C2C12 myoblasts and 3T3-L1 preadipocytes to differentiate *in vitro*, and gene expression profiling has shown disruption to the Rb-MyoD pathway in muscle regeneration (Favreau *et al.*, 2004; Markiewicz *et al.*, 2005; Bakay *et al.*, 2006; Boguslavsky *et al.*, 2006; Frock *et al.*, 2006; Melcon *et al.*, 2006).

Current work and aims

The failure of adult stem cells to self-renew and differentiate in laminopathies has been suggested to be one of the major underlying pathogenic mechanisms governing the disease phenotypes (Hutchison and Worman, 2004). Due to the paucity of the material required to test this hypothesis, it has yet to be determined whether mutations found in typical and atypical HGPS cause adult stem cell self-renewal malfunction or an inhibition of cellular differentiation *in vitro*, or both. Therefore, this work sought to clarify this issue. To achieve this, rat dermal papilla 9 clonal adult stem cells were used to generate a stable line expressing FLAG-tagged prelamin A constructs harbouring mutations found in both typical and atypical HGPS. To assess self-renewal capacity, cells were routinely passaged and counted. To assay for differentiation potential, cells were exposed to adipogenic medium for six days. A Tet-On inducible gene expression system was also initiated so that future work may elucidate the role of wild type lamin A and lamin A mutants on adult stem cell self-renewal and differentiation in greater detail.

Chapter 2. Material and Methods

Transformation of E.coli with pSVK3-FLAG-prelamin A or pEGFP-prelamin A

All cloning procedures were carried out according to standard methods (Sambrook *et al.*, 1989). The ATG-FLAG-prelamin A cDNA has been previously cloned into the mammalian expression vector pSVK3 (Amersham Pharmacia Biotech Inc, Picastway, New Jersey, USA) which has a multiple cloning site downstream from an SV40 early promoter and an ampicillin resistance cassette (Östlund *et al.*, 2001). The construct was kindly provided by H.J. Worman (Departments of Medicine and of Anatomy and Cell Biology, Columbia University College of Physicians and Surgeons, New York, USA).

The pSVK3-FLAG-prelamin A construct was reconstituted in 50µl of ddH_20 and used to transform competent *E.coli* cells. Briefly, 100µl of *E.coli* cells were thawed on ice for 10 minutes. After which time, 10µl of pSVK3-FLAG-prelamin A was added to the cells and mixed carefully using a pipette, then left for up to 30 minutes on ice. The cells were then placed at 42°C for 90 seconds, then returned to ice for exactly 2 minutes. Next, 1ml of room temperature LB was added to the cells and then incubated at 37°C for 90 minutes with shaking at 225-250 rpm. Cells were then plated onto LB agar containing 100mg/ml ampicillin and allowed to grow for 16-20 hours at 37°C. From these plates, clearly isolated colonies were chosen and grown in LB with 100mg/ml ampicillin for 16 hours with shaking at 225-250 rpm before conducting small scale DNA purification of the culture.

Prelamin A cDNA has been previously cloned into pEGFP-C2 (Clontech Inc, Palo Alto, California, USA), which contains a multiple cloning site downstream from the cytomegalovirus early promoter and expresses the gene with a C-terminus EGFP fusion (Vaughan *et al.*, 2001). A vial of frozen *E.coli* cells previously transformed with pEGFP-C2-prelamin A were thawed on ice for 10 minutes, then a 20µl aliquot was transferred to

5ml liquid LB containing 50mg/ml kanamycin and grown for 16 hours with shaking at 225-250 rpm before conducting small scale DNA purification of the culture.

DNA purification

Small-scale DNA purification (<5µg purified plasmid DNA) was carried out according to the manufacturer's guidelines (Promega Corporation, Madison, Wisconsin, USA). Briefly, 2ml of cells from an overnight culture were centrifuged at 10,000 x *g* for 5 minutes at room temperature, resuspended (50mM Tris-HCl (pH 7.5), 10mM EDTA, 100µg RNase A) and then lysed (0.2M NaOH, 1% SDS). Endonucleases and other contaminating proteins were then inactivated by alkaline protease treatment, followed by neutralisation (4.09M guanidine hydrochloride, 0.759M potassium acetate, 2.12M glacial acetic acid) of the protease. The bacterial lysate was then centrifuged at 16,000 x *g* for 10 minutes at room temperature (all centrifugation in this method at room temperature unless stated otherwise). Cleared lysate was then transferred to a spin column and centrifuged at 16,000 x *g* for 1 minute. A wash solution (60% ethanol, 60mM potassium acetate, 8.3mM Tris-HCl, 0.04mM EDTA) was then added to the spin column and in turn centrifuged at 16,000 x *g* for 1 minute. This was followed by another wash but with a centrifugation time of 2 minutes. The plasmid DNA was then eluted by adding 100µl of nuclease-free water and centrifuging at 16,000 x *g* for 1 minute. Plasmid DNA was kept in a sterile 1.5ml microcentrifuge tube at -20°C. DNA was quantified using a Cecil GeneQuest CE 2301 spectrophotometer at a wavelength of 260_{nm}.

Large scale DNA purification (up to 1mg purified plasmid DNA) was carried out according to the manufacturer's (Promega). Initially, a 5ml culture of transformed *E.coli* was grown for 8 hours at 37°C with shaking at 225-250 rpm. The cells were then transferred to 200ml of LB and the appropriate antibiotic and grown for 16-21 hours at 37°C with shaking at 225-250 rpm. The cells were pelleted by centrifugation at 5,000 x *g* for 10 minutes using a room temperature rotor. Cells were then thoroughly resuspended (50mM Tris-HCl (pH 7.5), 10mM EDTA (pH 8.0), 100µg/ml RNase A), lysed (0.2M

NaOH, 1% SDS) and then neutralised (4.09M guanidine hydrochloride (pH 4.8), 795mM, 2.12M glacial acetic acid). The lysate was then centrifuged at 14,000 x *g* for 20 minutes in a fixed-angle rotor at room temperature. The lysate was then passed through a DNA binding column via a vacuum manifold. An endotoxin removal wash was passed through the column via by the vacuum before the addition of a column wash (60% ethanol, 60mM potassium acetate, 8.3mM Tris-HCl, 0.04mM EDTA), which was also drawn through the column by the vacuum. The column membrane was then air dried for 5 minutes by applying the vacuum. In order to elute the DNA, the column was placed in a 50ml disposable plastic centrifuge tube and then 1.5ml nuclease-free water was added to the column before centrifuging in a swinging-bucket rotor at 2,000 x *g* for 5 minutes at room temperature. The eluted DNA was transferred to a sterile 1.5ml microcentrifuge tube and stored at -20°C. DNA was quantified using a Cecil GeneQuest CE 2301 spectrophotometer at a wavelength of 260_{nm}.

Site-directed mutagenesis of pSVK3-FLAG-prelamin A and pEGFP-prelamin A

In order to produce plasmids that would express the two mutant proteins with the 150nt internal deletion or the R644C point mutation, the QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California, USA) was used to introduce mutations into the wild type pSVK3-FLAG-prelamin A and pEGFP-C2-prelamin A vectors. This method utilises the dsDNA prelamin A vectors and two synthetic oligonucleotide primers, which both contain the desired mutations to be introduced into the target insert-containing vectors. The complimentary primers are extended during thermal cycling by *PfuUltra* HF DNA polymerase, which eradicates unwanted second-site errors and produces high mutation efficiencies. The parental DNA template is then digested with *Dpn* I, an endonuclease that recognises methylated and hemimethylated DNA. The product vector containing the desired mutation is then transformed into competent *E.coli* cells for nick repair.

Mutagenic primers were designed according to manufacturer's guidelines (Sigma Ltd, Poole, Dorset, UK). The LAΔ50 forward primer used was 5'-GCGGCTCAGGAGCCCAGAGCCCCCAGAACTGCAGC-3', along with its complimentary sequence. The LA(R644C) forward primer used was 5'-GCCCAGGAGGTAGGAGCAGGTGACCAGATTGTC-3', along with its complimentary sequence. For thermal cycling, two different sample reactions were prepared for each vector mutation, with 10ng and 20ng of template dsDNA used with a constant primer concentration. Each sample reaction contained 125ng of both the forward and reverse primer and 2.5 U/μl *PfuUltra* HF DNA polymerase. A positive control reaction containing 10ng of pWhitescript was also prepared. Control primers and *PfuUltra* HF DNA polymerase concentrations were as above. Cycling parameters for the mutagenesis is dependent on both the vector size and the nature of the mutation to be introduced into the vector. A summary of temperatures, times and cycles is provided below.

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	*[1]	95°C	30 seconds
		55°C	1 minute
		68°C	*[2]

The number of cycles and the duration of the annealing temperature are shown below.

Vector	*[1]	*[2]
pSVK3-FLAG prelamin A Δ50	18	6.5 minutes
pSVK3-FLAG prelamin A (R644C)	12	6.5 minutes
pEGFP-C2-prelamin A Δ50	18	7.0 minutes
pEGFP-C2-prelamin A (R644C)	12	7.0 minutes

After thermal cycling, the samples were either left overnight at 4°C or cooled on ice for 2 minutes. Each sample was digested with 10 U/μl *Dpn* I and incubated for 1 hour at 37°C.

Transformation of *E.coli* cells with the digested sample was as previously described but with some notable exceptions. Supercompetent XL1-Blue cells were used for transformation, the heat shock step was 45 seconds, NZY+ broth was used instead of LB post-heat shock, and the transformation reactions were incubated for 1 hour at 37 °C with shaking at 225-250rpm. The transformation reactions were then plated on agar plates containing the appropriate antibiotic, 100mg/ml ampicillin for pSVK3-FLAG-prelamin A Δ50 and pSVK3-FLAG-prelamin A (R644C), and 50mg/ml kanamycin for pEGFP-C2-prelamin A Δ50 and pEGFP-C2-prelamin A (R644C). Plates were grown for 16-20 hours at 37°C, before single well-isolated colonies were picked and grown for 16 hours in LB plus the appropriate antibiotic. Small-scale DNA purification was then performed as described previously, followed by sequencing. All sequencing was performed by a 3730 DNA Analyser (Applied Biosystems, Foster City, California, USA). Large-scale DNA purification was then performed as described previously to ensure DNA was of a concentration appropriate for transfecting cells.

pTRE-Tight-ATG-FLAG-prelamin A-WT, Δ50 and R644C production

In order to excise the ATG-FLAG-prelamin A inserts from the pSVK3 vector, pSVK3-FLAG-prelamin A-WT, pSVK3-FLAG-prelamin A-Δ50 and pSVK3-FLAG-prelamin A-R644C were each digested with 10 U/µl *Acc*651 and 10 U/µl *Sal* I (Promega). The pTRE-Tight vector (Clontech) was digested with the same endonucleases at the same concentrations. The pTRE-Tight vector contains an MCS downstream from the Tet-responsive P_{tight} promoter, which contains a minimal CMV promoter; any cDNA cloned into the MCS will be responsive to the rtTA regulatory protein of the Tet-On Advanced system. Each sample was run on a 0.8% agarose gel and the correct band was excised and purified from the gel according to the manufacturer's guidelines (Qiagen Inc, Valencia, California, USA). The ATG-FLAG-prelamin A-WT, Δ50 and R644C inserts were each separately ligated into digested pTRE-Tight at a molar ratio of 3:1 (insert:vector) using 1 Weiss unit of T4 DNA Ligase (Promega) and incubated for 3 hours at 22°C, before transformation of competent *E.coli* and subsequent plating, as described previously.

Single well-isolated colonies were grown in LB plus 100mg/ml ampicillin for 16 hours at 37°C with 225-250 rpm shaking. After small-scale DNA purification, the insertion junctions were confirmed by sequencing using the following primers (Sigma), forward (located at 2577-2600): 5'–AGGCGTATCACGAGGCCCTTTCGT–3', and reverse (located at 683-660): 5'–TATTACCGCCTTTGAGTGAGCTGA–3'. Large-scale DNA purification was then performed as previously described.

Cell culture

Rat clonal dermal papilla (DP) 9 and rat clonal dermal sheath (DS) 7 cells were cultured in MEM (Sigma) supplemented with 10% FBS (Invitrogen Ltd, Paisley, Scotland or Clontech) with the addition of 50 μg/ml gentamycin (Invitrogen) and 250 μg/ml amphotericin (Invitrogen). U2-OS Luc Tet-On Advanced cells (Clontech) were cultured in D-MEM containing 4.5 mg/L glucose (Invitrogen) supplemented with 10% NCS (Invitrogen) with 50 μg/ml streptomycin (Invitrogen) and 10 U/ml penicillin (Invitrogen). Cells were maintained in $25cm^2$ or $75cm^2$ flasks (Nunc, Rochester, New York, USA) or 10cm dishes (Greiner-Bio-One, Stonehouse, Gloucestershire, UK).

Transfection and establishment of stable cell lines

Rat DP9 cells were transfected using Amaxa Nucelofector II Technology using Primary Endothelial or Normal Human Dermal Fibroblast-Adult kits according to the manufacturer's guidelines (Amaxa AG, Cologne, Germany). Briefly, cells were grown to 80-90% confluency, with cells passaged 3-5 days before transfection. On the day of transfection, cells were detached from culture flasks using 0.25% trypsin (Invitrogen), which was then inactivated using MEM plus 10% FBS supplemented with antibiotics. Cells were then thoroughly resuspended and counted using a haemocytometer to determine the cell density. The appropriate volume that contained 5×10^5 cells was centrifuged at 100 x *g* for 10 minutes at room temperature. Residual medium was removed

and the pellet resuspended in 100µl Nucleofector Solution. The solution was then mixed with 5µg of 1µg/µl DNA and immediately placed in the Nucleofector cuvette holder and electroporated using the program number U-23. The sample was removed and supplemented with MEM plus 10% FBS. For transient transfections, the sample was then placed in one well of a 6-well plate containing four glass coverslips and MEM plus 10% FBS and grown for 24 hours at 37°C in a humidified incubator with 5% CO2. The media was replaced 24 hours later with fresh MEM plus 10% FBS, and the cells allowed to grow for additional 24 hours before being processed for indirect immunofluorescence.

Alternatively, for the production of stable cell lines expressing FLAG-prelamin A WT, Δ50 or R644C, vector DNA was co-transfected with a linear hygromcyin B marker (Clontech) at a ratio of 10:1 (DNA:linear marker). Post-transfection, the sample was added to a 10cm dish containing MEM plus 10% FBS. The media was replaced after 24 hours, and after a further 24 hours 100µg/ml hygromycin B was added to the media. In all cases, the media was changed every 3-4 days and at all times contained 100µg/ml hygromycin B. Some 7-10 days post-transfection, isolated colonies had formed and plates were trypsinised. Cells were transferred to a 96-well plate (Nunc) at an average of 0.5 cells per well to increase the probability of single colony production. Remaining cells were re-plated and grown under selective conditions until colonies had formed in the 96-well plates. Heterogeneous stable cells that had not undergone single-cell cloning were then frozen down. After 3-5 days 96-well plates were examined for single colonies and those wells containing one colony were noted and propagated further. After the colonies had reached 70-80% confluency in one well of a 24-well plate, they were re-plated equally into one well of a 24-well plate containing a glass coverslip and into a single well of a 24-well plate without a glass coverslip. Once the cells had reached approximately 70-80% confluency, the glass coverslip was processed for indirect immunofluorescence and judged for the presence of the FLAG signal, whilst the other 'stock' well was propagated. Three clones were then identified for each construct and cells were then grown further before freezing aliquots of cells before beginning adipogenic assays and determining clonal growth rates.

Rat DP9 cells were transfected as above with the pTet-On Advanced vector (Clontech) and were allowed to adhere for 24 hours before the growth media was replaced, and after a further 24 hours 350μg/ml geneticin (Invitrogen) was added to the media. Cells underwent selection, single-cell cloning and propagation, however, they were not processed for indirect immunofluorescence, but were instead grown to 70-80% confluency in one well of a 6-well plate and then re-seeded equally again into two single wells of a 6-well plate. One well was then processed for immunoblotting to assess the level of the rtTA protein expressed. Aliquots of cells were then frozen down due to time constraints.

Indirect immunofluorescence microscopy

Rat DP9 cells were grown on glass coverslips and fixed for 15 minutes at room temperature with 4% formaldehyde (AnalaR) in PBS. Cells were then permeabilised with 0.5% Triton-X100 (Sigma) in PBS for 5 minutes at 4°C. Primary antibodies were applied for 1 hour at room temperature in various combinations and were diluted in 1% NCS in PBS. Primary antibodies used, their dilutions and sources are: mouse monoclonal LAP15 against LAP2α [1:10, Dechat *et al.*, 1998], rabbit polyclonal RbSer780 against Rb phosphorylated at serine residue 780 [1:100, Cell Signalling, Danvers, Massachusetts, USA], rabbit polyclonal RbSer807/811 against Rb phosphorylated at serine residue 807/811 [1:100, Cell Signalling], mouse monoclonal Emerin [1:50, Novacastra Laboratories Ltd, Newcastle-upon-Tyne, UK], mouse monoclonal anti-FLAG M2 [1:500, Sigma], rabbit polyclonal anti-FLAG [1:500, Sigma]. Secondary antibodies were applied for 1 hour at room temperature and were a combination of donkey anti-mouse IgG or donkey anti-rabbit IgG (both Stratech Scientific Ltd, Newmarket, UK), each conjugated to either tetramethylrhodamine B isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) and diluted 1:100 in 1% NCS in PBS. After washing in PBS, cells coverslips were mounted on to glass slides in 30% (v/v) glycerol containing 12% (v/v) Mowiol (Sigma), 2mg/ml 4,6-diamindino-2-phenylindole (DAPI) and 2.5% (v/v) 1,4-diazobicyclo-[2.2.2]-octane (DABCO; Sigma). Images of stained cells were taken using a Bio-Rad Radiance 2000 confocal laser scanner (Bio-Rad Inc, Hercules, California, USA) attached to a Zeiss

Axioskop (Carl Zeiss GmbH, Germany), which was fitted with a 40X 1.4 NA Plan Neofluor lens. Images were viewed and saved using LaserSharp 2000 (Bio-Rad), before importing into ImageJ, where images were merged before subsequent collation using Adobe Photoshop 7.0. Laser power was adjusted to enable comparison of the fluorescence signal.

Immunoblotting

Samples were separated by one dimensional SDS-PAGE was carried out according to standard protocols (Laemlli, 1970). For immunoblotting, proteins were separated on a 12% gel and electrophoretically transferred to a nitrocellulose membrane (0.2mm, Whatman PLC, Brentford, UK) in 48mM Tris-HCl (pH 9.4) and 39mM glycine by using the Mini Transblot system (Bio-Rad). The membranes were saturated with 5% (wt./vol.) nonfat dry milk in TBST [50 mM Tris-HCl (pH 8.0), 138 mM NaCl, 2.7 mM KCl, 0.05% Tween 20] (Sigma), then incubated overnight with the primary antibody at 4°C. The membrane was then washed with TBST and then incubated with the secondary antibody for 1 hour at room temperature. The primary antibody used was mouse monoclonal TetR (Clontech), diluted 1:1000. The secondary antibody used was donkey anti-mouse IgG conjugated to horseradish peroxidise (Stratech). Immunological detection of proteins was performed with the Enhanced Chemiluminescence (ECL; GE Healthcare, Chalfont St. Giles, UK) kit and bands visualised using the LAS-1000 imaging system (FujiFilm, Tokyo, Japan).

Luciferase assay

U2-OS Luc Tet-On Advanced cells were plated at 0.5 x 10^5 per well of a 6-well plate in increasing concentrations of doxycycline and grown for 48 hours. Alternatively, DP9 Tet-On Advanced clones were transiently transfected with pTRE-Tight-Luc as described previously, and grown for 48 hours in 100ng/ml doxycycline. Luciferase activity was then assayed using the Dual-Luciferase Reporter Assay System according to the manufacturer's guidelines (Promega). Luminescence was measured using a Lumat LB 9507 luminometer (Berthold Technologies GmbH, Bad Wildbad, Germany).

Determination of cell growth

Each clone was plated at 70,000 cells per 60mm culture dish (Greiner-Bio-One) and passaged every 3 or 4 days, with cell growth calculated as population doubling potential, using the formula, (log N_1- log N_2) / log 2.0. Where N_1 is the cell number counted after 3 or 4 days and N_2 is the number of cells seeded after each passage, which was 70,000 per clone. Regression line analysis was carried by the 'line of best fit' function in Microsoft Excel.

Adipogenic assay

Rat DP9 and DS7 cells were subjected to two different methods that have previously been reported to differentiate either rat clonal DP and DS cells or 3T3-L1 preadipocytes *in vitro*. The first method was adapted from Jahoda *et al.*, 2003. Both DP9 (p21) and DS7 (p35) were plated in duplicate at 40,000 cells per 35-mm dish (Nunc) and allowed to adhere overnight in MEM plus 10% FBS at 37°C in a humidified incubator with 5% CO_2. The next day, adipogenic medium (MEM plus 15% rabbit serum, 2.07mM insulin and 100nM dexamethasone) was added to one of each pair of DP9 and DS7 clones, whilst the other pair was resupplemented with growth media, MEM plus 10% FBS, as a control.

Thereafter, the media was changed every two days with either growth media or adipogenic media as appropriate, and the cultures maintained for six days in total from the point of addition of the adipogenic media.

The second method that was employed was adapted from Boguslavsky *et al.*, 2006. Both DP9 (p21) and DS7 (p35) were plated in duplicate at either 40,000 or 100,000 cells per 35-mm dish and allowed to adhere overnight in MEM plus 10% FBS at 37°C in a humidified incubator with 5% CO_2. The following day, adipogenic medium (DMEM plus 10% FBS, 1mM insulin and 1mM dexamethasone) was added to one of each pair of DP9 and DS7 clones, whilst the other pair was resupplemented with MEM plus 10% FBS as a control. After two days, a secondary adipogenic medium (DMEM plus 10% FBS, 1mM insulin) was added to one of each pair, whilst growth media was added to the controls. Both sets of media were replaced after two days with respective fresh media for an additional two days thereafter, giving a total of six days under growth or adipogenic conditions.

Having established a method for the *in vitro* differentiation of untransfected DP9 cells, it was decided to pursue the first method when performing adipogenic assays with the DP9 prelamin A wild type, Δ50 and R644C stable cell lines. The only alteration from the method above is that 100mg/ml hygromycin B (Clontech) was present in the growth and adipogenic media at all times.

Lipid detection

After six days under culture conditions, cells were stained with oil red-O to detect intracellular lipid accumulation. The culture medium was removed and cells were washed once in PBS, and then fixed in calcium formol (4% formaldehyde, 1% calcium chloride) for 1 hour at room temperature. The cells were incubated for 15 minutes with a filtered solution of three parts of saturated oil red-O in isopropanol and two parts ddH_20. The stained cells were then washed once with 60% isopropanol, washed thoroughly with

ddH_20, and then overlaid with PBS. Plates were then scanned into Adobe Photoshop using a ScanJet 6300C (Hewlett Packard).

Semi-quantitative analysis of Oil Red-O

Oil red-O dye was extracted by submerging the plates in 4% SDS in 60% isopropanol for 15 minutes at room temperature. The optical density of the oil red-O was measured in a Cecil GeneQuest CE 2301 spectrophotometer at a wavelength of 370_{nm}. Percentage of differentiation of each clone was expressed as a percentage of the OD_{370nm} of wild type DP9 after 6 days under adipogenic conditions. Results were assessed for significance using a Student's t-test.

Chapter 3. Results

Introduction

Structural defects to the nuclear envelope have been well documented in cells containing lamin mutations, although the extent is somewhat variable, depending on the position of the mutation (Broers *et al.*, 2006). Fibroblasts from HGPS patients show symptomatic nuclear abnormalities as do both human fibroblasts and HeLa cells transiently transfected with a GFP-tagged progerin construct (Eriksson *et al.*, 2003; Goldman *et al.*, 2004). These structural abnormalities are protein concentration dependent and include nuclear pore clustering, a loss of heterochromatin and lobulations of the nuclear envelope (Goldman *et al.*, 2004). At present, no study has addressed the effect of transiently overexpressing progerin in adult stem cells, presumably due to the difficulty in obtaining such material. Yet, it has been proposed that it is a necessary requirement that there should be functioning A-type lamins in maintaining the proliferative state and inductive potential of adult stem cells in order to retain the ability to repair old or damaged tissues (Hutchison and Worman, 2004). Therefore, constructs were made that expressed both FLAG- and GFP-tagged prelamin A and two mutants associated with typical and atypical HGPS, prelamin A Δ50 (LAΔ50) and prelamin A R644C (LA(R644C)) respectively. The LAΔ50 mutant has a 50 amino acid internal deletion V607 through Q656, whilst the LA(R644C) mutant has an arginine substituted at position 644 for a cysteine (R644C). These constructs were then transiently overexpressed in adult rat clonal DP9 cell and processed for indirect immunofluorescence.

Transient overexpression of two mutants associated with premature aging causes nuclear abnormalities in adult stem cells.

Nuclei were counted by observing those present in a randomly selected microscopic field of view (where n = 200). Nuclei were counted only if wild type or mutant lamin A was

expressed in cells, as judged by either anti-FLAG antibody or by fluorescent signal. Nuclei were deemed to be morphologically 'normal' if the nuclear envelope was ellipsoid in shape with no perturbation to the peripheral envelope and with only a faint internal signal. Contrastingly, nuclei showing structural abnormalities after 72 hours post-transfection could be further sub-classified according to the nature of the abnormality observed (Figure 6). Of these, nuclei were classified as having 'trans-nuclear' lamin structures if they presented at least two lamin tubule-like structure, which transverses the nuclear envelope. Those nuclei established as having 'intra-nuclear' structures showed prominent aggregates of lamin A protein within the nucleus. Small lamin positive ellipsoid microstructures were found next to 'parent' nuclei and these were termed 'micronuclei'. The last defect observed was an 'invagination' or 'blebbing' of the nuclear envelope, whereby the nuclear envelope showed a distinct abhorrent morphology with either lobulation of the envelope or indentations.

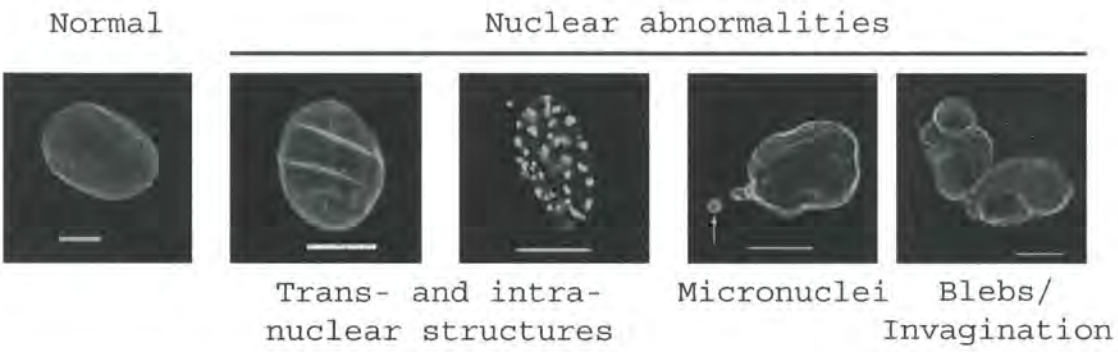
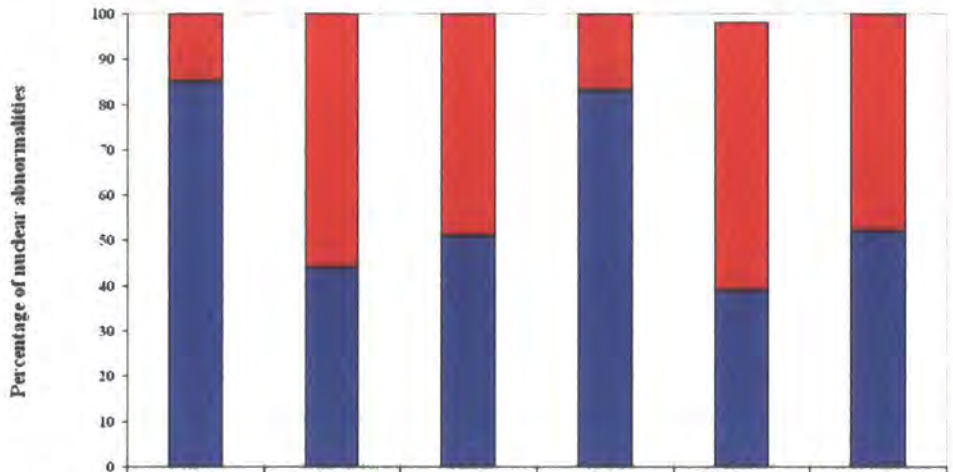


Figure 6. Micrograph of nuclear abnormalities found in transfected DP9 cells. Nuclei were classified as 'normal' if they had correct nuclear envelope staining. Nuclei classified as 'abnormal' presented one of four major nuclear abnormalities, found from overexpressing mutant lamin A in both FLAG- and GFP-tagged expression constructs. (Scale, 10μm)

Each nucleus scored as 'abnormal' was noted for its most prominent abnormality. If a nucleus presented more than two of the above abnormalities, then its classificiation was decided by the following order: micronuclei > blebbing/invagination = intranuclear aggregates > transnuclear. As micronuclei appeared to be the most severe abnormality this was placed first, whilst the blebbing/invagination and intranuclear aggregates were placed joint second as they were never seen in the same cell.

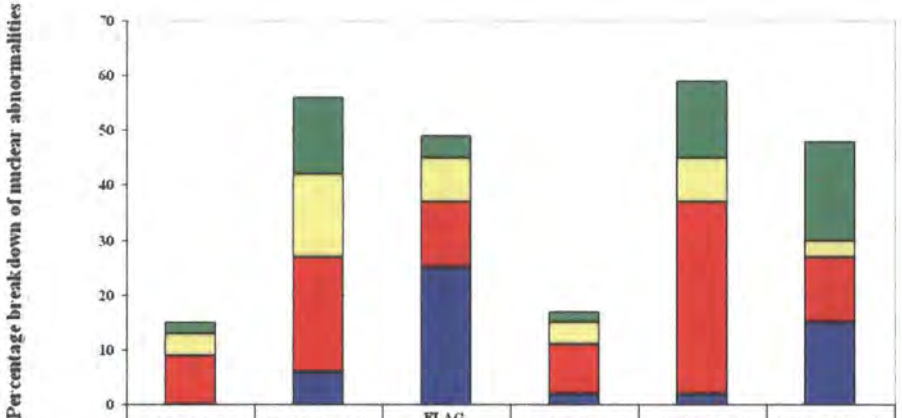
Upon transfection with both FLAG-tagged and GFP-tagged LAΔ50 and LA(R644C), there was a notable increase in the percentage of nuclear abnormalities in the DP9 cells after 72 hours as compared with DP9 cells transfected with either FLAG- or GFP-tagged wild type lamin A (Figure 7*A*). The highest percentages of abnormalities were found in both FLAG- and GFP- tagged LAΔ50 cells, with both showing similar numbers of abnormalities, 56% vs. 59% (Figure 7*A*). Although DP9 cells showed less abnormalities when overexpressed with LA(R644C), comparison between the FLAG- and GFP-tagged constructs yielded similar results, 49% vs. 48% (Figure 7*A*). These results confirm earlier reports that overexpression of two mutants associated with premature aging cause structural defects in the nuclear envelope, although these are the first observed in adult stem cells (Goldman *et al.*, 2004; Csoka *et al.*, 2004b). However, even for DP9 cells transfected with the wild type FLAG- and GFP-tagged lamin A protein, there were nuclear envelope abnormalities detected in high numbers of cells, at 15% and 17% respectively, indicating that the high concentrations of exogenously expressed wild type lamin A may have toxic effects upon nuclear envelope structure (Figure 7*A*). Taken together, these results suggest that not only is a functional lamin A protein essential for maintaining nuclear envelope integrity, but also the concentration at which it is present in the nucleus is important to maintain nuclear envelope stability. Despite this, it should be noted that the majority of cells expressing either the FLAG- and GFP-lamin A mutants still retained a normal nuclear envelope structure with the prelamin A being processed into the nuclear envelope, based on microscopic study, which is consistent with previous reports (Goldman *et al.*, 2004).

A



	FLAG-LA	FLAG-LAΔ150	FLAG-LA(R644C)	GFP-LA	GFP-LAΔ150	GFP-LA(R644C)
Abnormal	15	56	49	17	59	48
Normal	85	44	51	83	39	52

B



	FLAG-LA	FLAG-LAΔ150	FLAG-LA(R644C)	GFP-LA	GFP-LAΔ150	GFP-LA(R644C)
Blebs/Invaginations	2	14	4	2	14	18
Micronuclei	4	15	8	4	8	3
Transnuclear	9	21	12	9	35	12
Intranuclear	0	6	25	2	2	15

Figure 7. Analysis of nuclear abnormalities found in DP9 cells transfected with mutant lamins associated with premature aging. Cells were classified as 'normal' or 'abnormal' based on nuclear envelope structure (*A*). Notice that overexpression of both FLAG- and GFP-tagged mutant lamins associated with premature aging caused an increase in the number of nuclear abnormalities in DP9 cells. Nuclei classified as 'abnormal' from the results in (*A*) were then categorized according to their nature (*B*).

Of the nuclei classified as 'abnormal' during the initial count of 200 nuclei, further detailed analysis was performed using confocal microscopy and categorized according to their nature as set out in Figure 6 (Figure 7*B*). The progerin protein is both carboxymethylated and farnesylated and is therefore expected to integrate into the nuclear lamina, as has been shown here and in other studies (Figure 6) (Dechat *et al.*, 2007). Furthermore, the characteristic blebbing of the nuclear envelope was present in DP9 cells transfected with both FLAG and GFP-tagged LAΔ50 (Figure 7*B*). As this defect has already been noted in previous studies, further attention was made to the other nuclear abnormalities observed.

Cells transfected with FLAG- or GFP-tagged wild type LA that were noted as having a nuclear abnormality predominantly contained transnuclear structures (both 9% of total cells), as has been shown previously for overexpression of chimeric GFP-lamin A constructs in CHO-K1 cells (Figure 7*B*) (Broers *et al.*, 1999). Similarly, these structures were also prominent, albeit to a greater extent, in cells overexpressing LAΔ50, either with a FLAG or GFP-tag (21% vs. 35% of all cells, respectively) (Figure 7*B*). In order to investigate further the nature of the transnuclear structures, *Z*-series were generated using 15 frames with a step size of 0.25μm in the *Z*-direction. Images were then split and assembled into sequential *Z*-sections (Figure 8). The transnuclear lamin structures extend from the top of the cell and penetrate deep into the core of nucleus (Figure 8, *A-C*), and also from the bottom to the core of the nucleus (Figure 8, *D-F*), suggesting that these are deep extensions of the nuclear envelope rather than accumulations of lamin protein within the nucleoplasm.

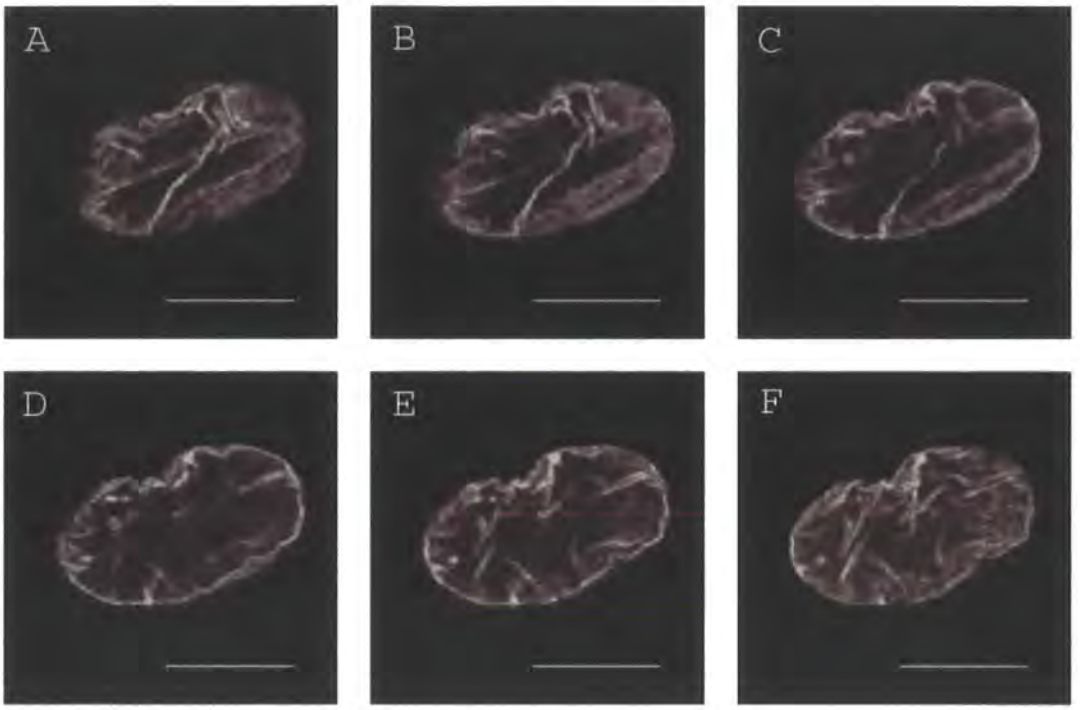


Figure 8. Transnuclear lamin structures. A series of images were taken along the *Z*-direction revealing that tubule-like lamin A structures penetrate deep within the centre of the nucleus with overexpression of lamin AΔ50, from both the top (*A-C*) and the bottom (*D-F*) of the cell. (Scale 10μm)

In contrast, fewer transnuclear lamin structures were found in DP9 cells transfected with the LA(R644C) constructs, with the predominant nuclear abnormality being intranuclear aggregates of lamin A protein (Figure 7*B*). Nuclear aggregates of lamins have been widely reported previously for various mutations (Schmidt *et al.*, 1994; Izumi *et al.*, 2000; Ostlund *et al.*, 2001; Raharjo *et al.*, 2001; Vaughan *et al.*, 2001; Markiewicz *et al.*, 2002b; Holt *et al.*, 2003). A montage of *Z*-series images revealed that the intranuclear aggregates were present throughout the entire nucleus, and whilst some of the aggregates localised to the nuclear envelope, there was an absence of mutant lamin A protein assembled into most parts of the nuclear periphery (Figure 9).

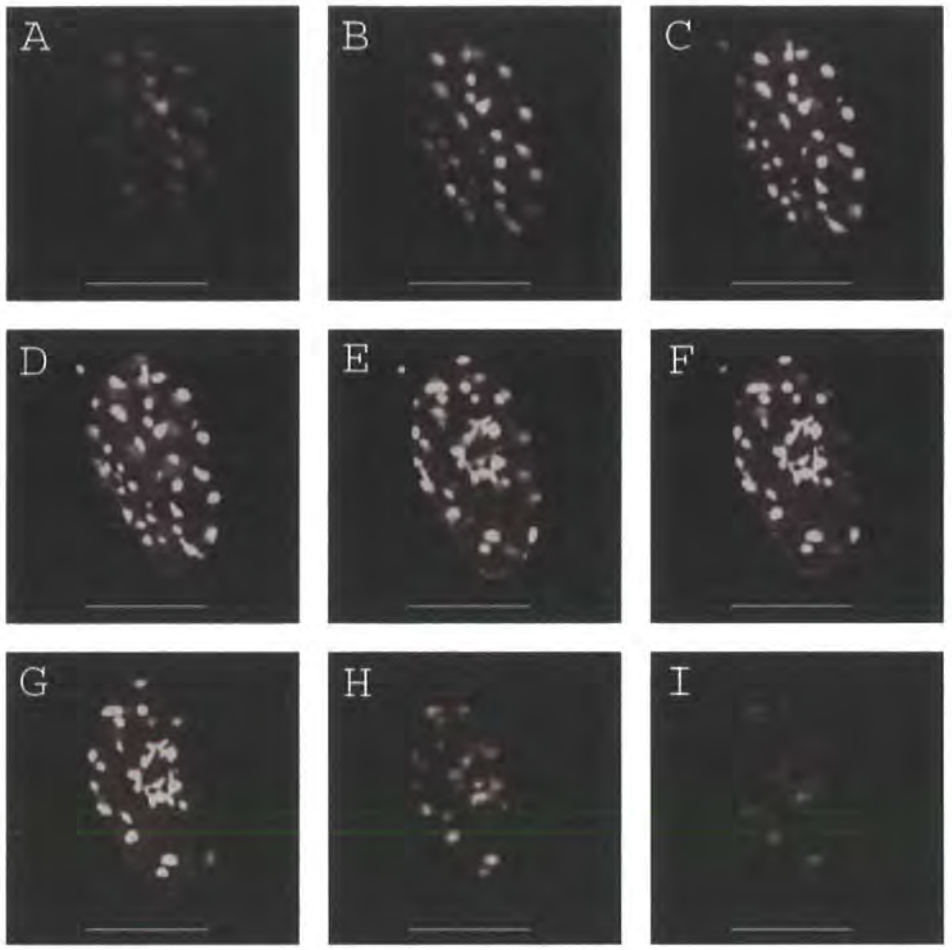


Figure 9. Intranuclear lamin A aggregates. A series of images taken in the *Z*-direction show that overexpression of lamin A(R644C) cause the formation of intranuclear aggregates that are not extensions of the nuclear envelope, but are discrete lamin A foci that prevent the correct incorporation of lamin A into the nuclear envelope. (Scale 10μm)

To help define further the nuclear abnormalities found in DP9 cells transfected with lamin mutants, cells were stained for emerin, which is a protein known to localise to the inner nuclear membrane (Nagano *et al.*, 1996). In cells transfected with FLAG-LA, emerin colocalised with the anti-FLAG signal at the INM as expected (Figure 10*A*). However, emerin did not colocalise fully with FLAG-LAΔ50 when found to accumulate in intranuclear aggregates (Figure 10*B*).

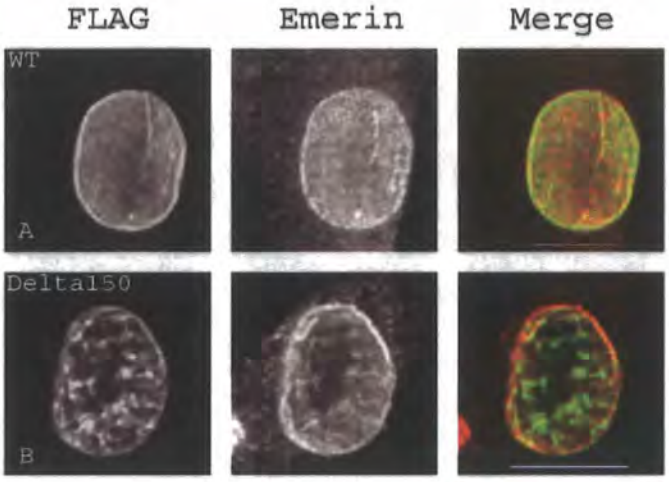


Figure 10. Emerin does not colocalise with intranuclear mutant lamin A. DP9 cells overexpressing FLAG-tagged LA show correct colocalisation with emerin, an INM protein (*A*). However, DP9 cells overexpressing FLAG-LAΔ50 that results in the presence of intranuclear lamin structures does not colocalise with emerin (*B*). (Scale 10μm)

This is reinforced by instances where overexpression of both GFP-LAΔ50 and GFP-LA(R644C) caused the accumulation of intranuclear aggregates; again, emerin stained the nuclear envelope, but did not colocalise with the lamin aggregates (Figure 11*C,E,F*). Nuclear rim staining with a concomitant presence of intranuclear aggregates for GFP-R644C was found to be variable (Figure 11*E,F,G*). Staining was either present at the nuclear rim (Figure 11*G*), clustered at some regions (Figure 11*E*) or largely absent or at low levels (Figure 11*F*).

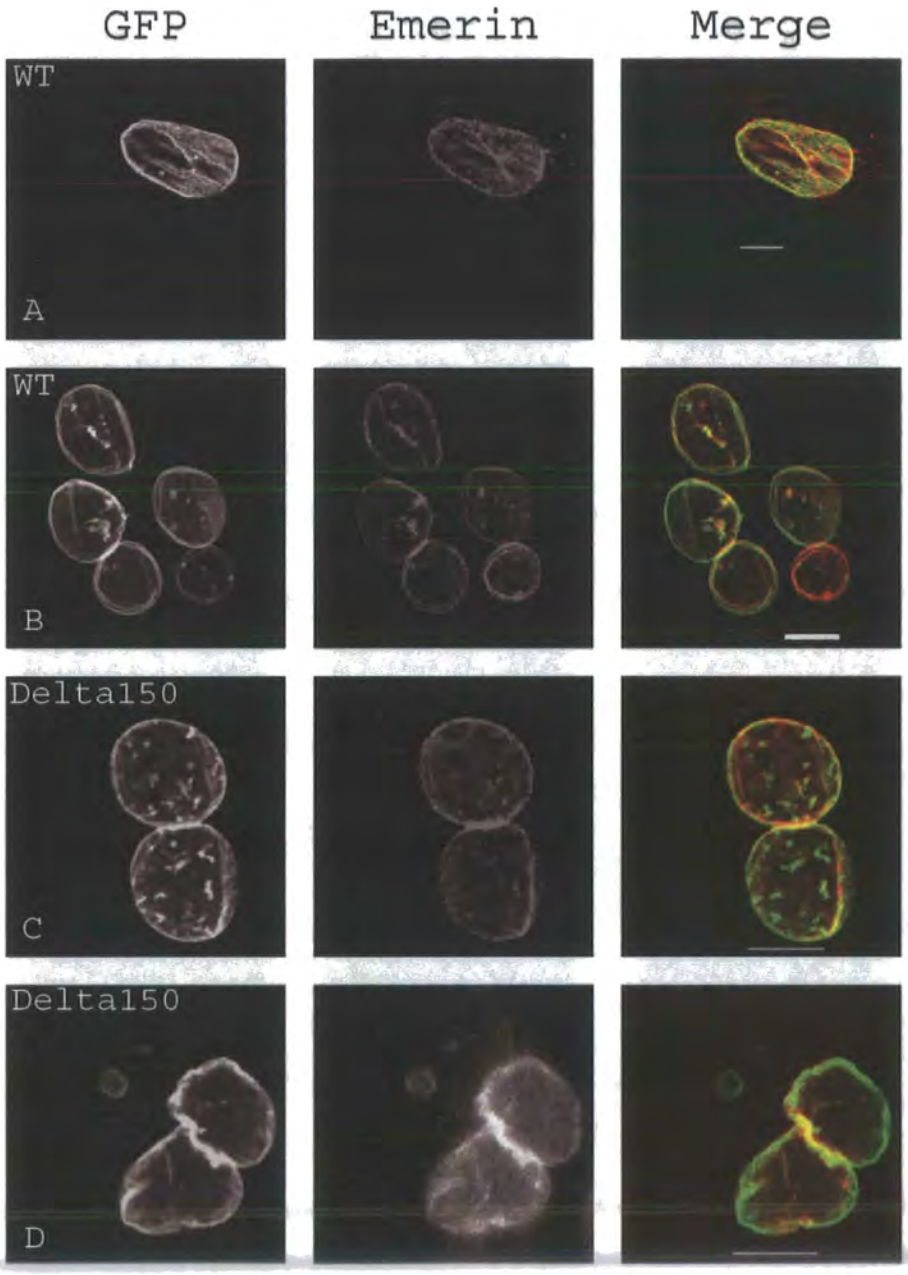
In contrast to intranuclear lamin aggregates, transnuclear lamin structures found in DP9 cells transfected with GFP-LA and GFP-LA (R644C) did colocalise with emerin, which suggested complete incorporation into the nuclear lamina (Figure 11*A,B,G*). Moreover, it suggested that transnuclear lamin structures are actually an extension of the nuclear envelope rather than stand-alone lamin structures within the nucleus and as such are distinct structures from the intranuclear aggregates, both in terms of morphology and the assembly pathway of the protein (Schmidt *et al.*, 1994; Pugh *et al.*, 1997). This further

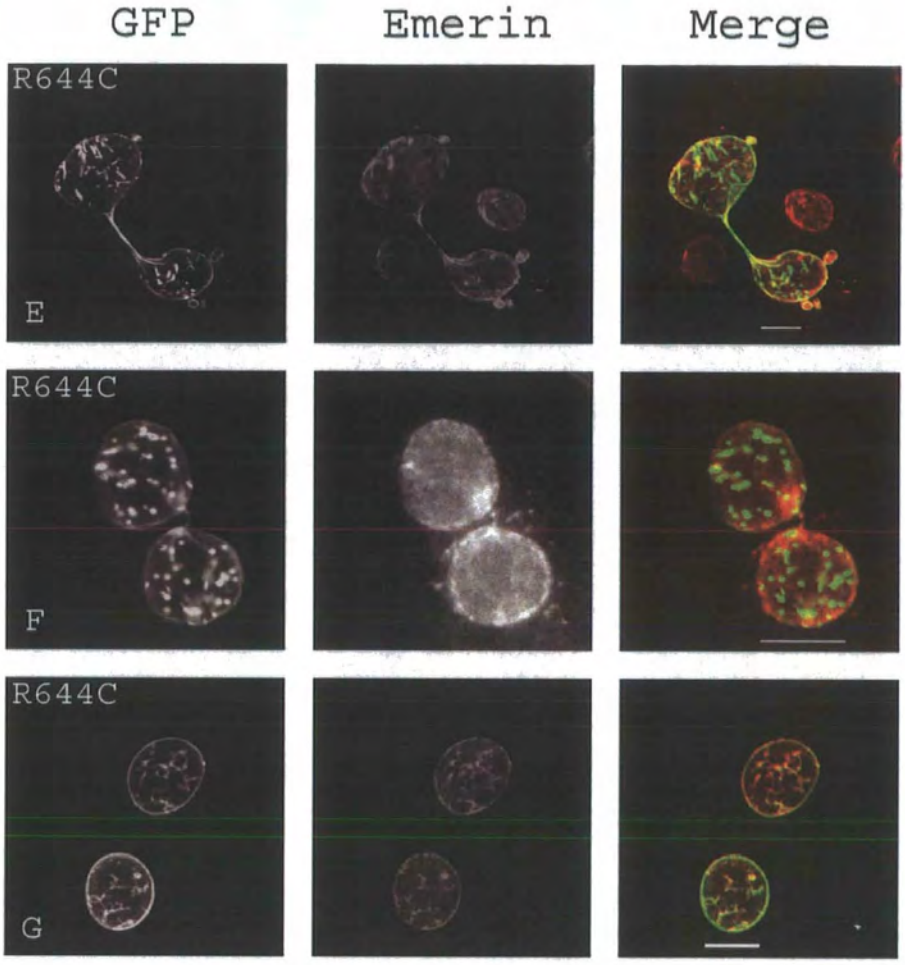
underlines the earlier result suggestion that these transnuclear lamin structures penetrate deep into the nucleus (Figure 8).

The nature of the formation of micronuclei was also analysed. In all circumstances, regardless of construct or tag, micronuclei were DAPI positive and contained colocalisation of an anti-FLAG or fluorescent signal with emerin (Figure 11*D*). The presence of these structures displayed a general trend of being greater in cells transfected with mutant constructs, although they were also present in cells transfected with wild type constructs (Figure 7*B*). The colocalisation of the lamin protein with emerin suggests that the lamin protein was first incorporated into the lamina, before subsequently being expelled out of the 'parent' nuclei.

Emerin is dependent on A-type lamins for INM localisation, therefore mutant lamin A colocalisation with emerin in micronuclei and transnuclear lamin structures suggests that the interaction between emerin and the mutant lamin A is not affected, at least at the microscopic level (Sullivan *et al.*, 1999; Vaughan *et al.*, 2001). Secondly, it draws a distinction between micronuclei and transnuclear lamin formation on one hand, and intranuclear aggregate lamin A formation on the other, as the latter does not colocalise with emerin, suggesting that the mutant protein does not incorporate into the lamina and remains within the nucleoplasm as an immobile aggregate. Interestingly, the nuclear lamin aggregates occurred more often in cells transfected with the LA(R644C) construct, than with the LAΔ150 constructs. It remains to be seen why this is so as the LA(R644C) mutant is expected to be both farnesylated and carboxymethylated, the same as the LAΔ50 mutant.

Figure 11. Overexpression of mutant lamins in DP9 cells causes redistribution of emerin. GFP-LA colocalised with emerin (*A,B*), whilst there was either no localisation with emerin when intranuclear lamin aggregates were present (*C,F*) or there was a redistribution of emerin into mutant lamin A positive micronuclei (*D,E*) when GFP-LA mutants were overexpressed in DP9 cells. Conversely, some GFP-LA mutant cells showed colocalisation (*G*). (Scale 10μm)



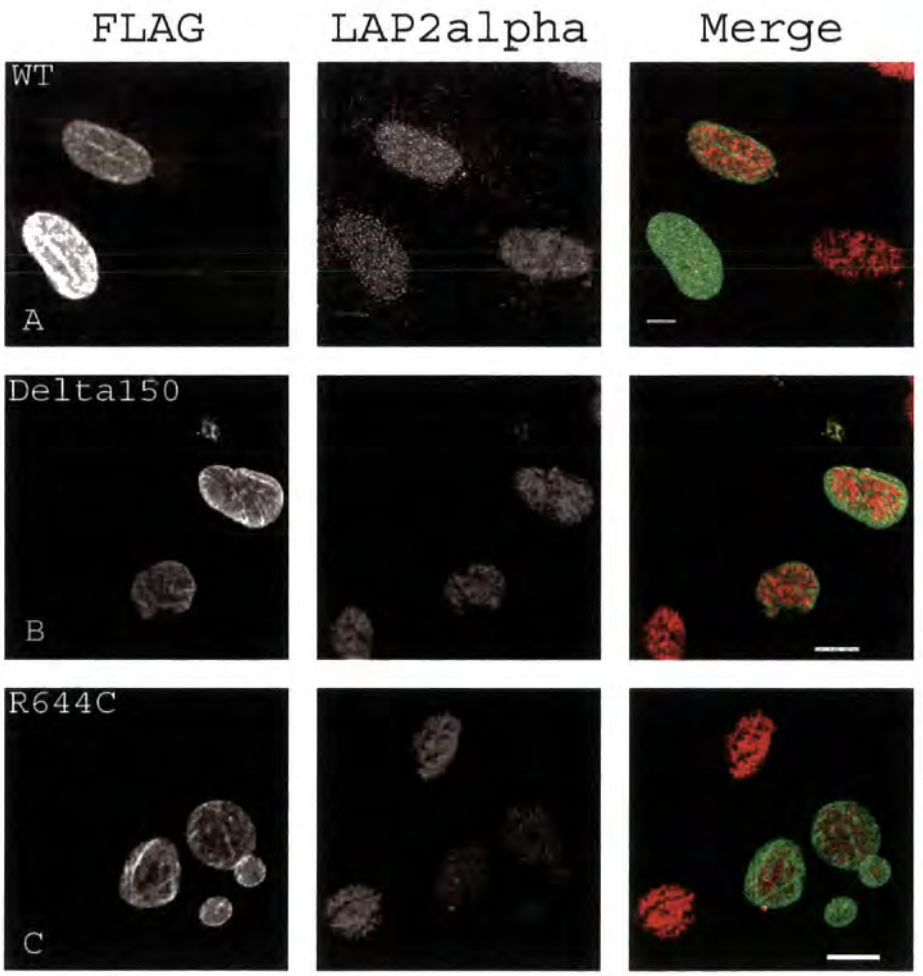

GFP
Emerin
Merge
R644C
E
R644C
F
R644C
G

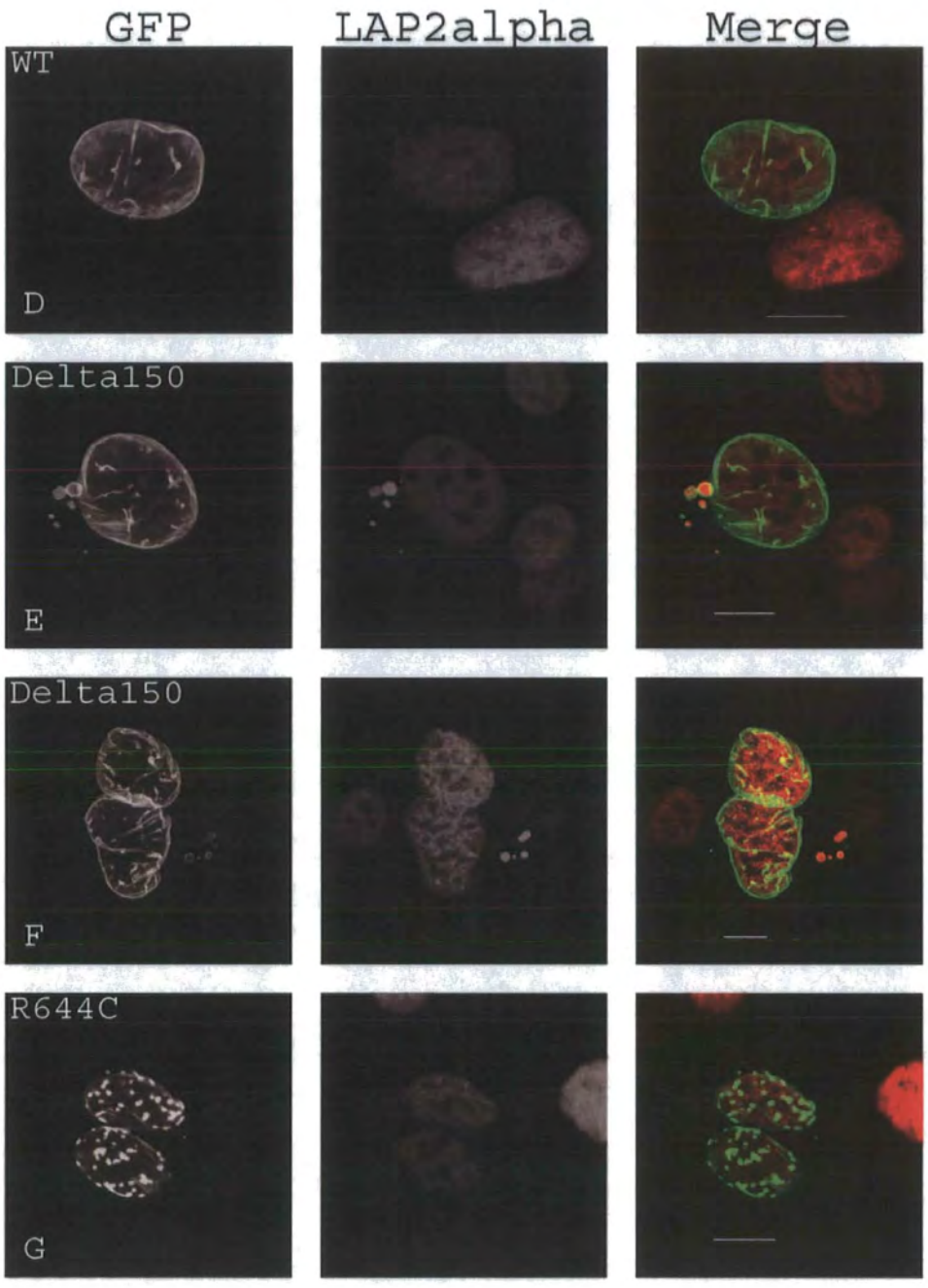
Lamin A has been reported to be a binding partner of hypophosphorylated Rb after *in vitro* binding assays, through the latter's large pocket domain (Mancini *et al.*, 1994; Ozaki *et al.*, 1994). Furthermore, lamin A interacts with an isoform of the LAP2 family of proteins, LAP2α (Dechat *et al.*, 2000a). Further characterisation of this complex of proteins revealed that LAP2α binds to Rb within a region spanning pockets B and C, with lamin A-LAP2α complexes required for Rb function (Markiewicz *et al.*, 2002b). Indeed, *LMNA* null mice show altered Rb dynamics, with mislocalisation of the protein and ultimate degradation by the proteasome (Johnson *et al.*, 2004). This has led to the suggestion that lamin A-LAP2α-Rb complexes may play a crucial role in laminopathies, where this complex may be disrupted with consequences for cell growth and division (Hutchison and Worman, 2004; Gotzmann and Foisner, 2005). Therefore, cells transfected with both FLAG- and GFP-tagged lamin A and two mutants were processed for immunofluorescence with LAP2α or Rb antibodies.

DP9 cells transfected with FLAG-tagged lamin A and two mutants did not reveal any differences in LAP2α localisation (Figure 12, *A-C*). Moreover, expression levels of the FLAG-tagged constructs did not have any immediate effect on localisation of LAP2α (compare the two FLAG-expressing cells in Figure 12*A*). Overexpression of GFP-tagged wild type and mutant lamin A constructs yielded similar results to those overexpressing FLAG-tagged constructs (Figure 12 *D-G*). Localisation of LAP2α in GFP-LA DP9 cells was uniform across the nucleus, with the exception of nucleoli, where it was absent (Figure 12*D*). Even in DP9 cells overexpressing GFP-LAΔ50 and presenting the characteristic blebbing of primary HGPS fibroblasts, LAP2α was localised across the nucleus in a uniform manner (Figure 12*B,E,F*). Confirming this, DP9 cells overexpressing GFP-LA(R644C), which contained intranuclear aggregates, did not mislocalise LAP2α into the aggregates themselves (Figure 12*G*). These results suggest that overexpression of lamin A and two premature aging mutants do not affect the interaction between LAP2α and endogenous lamin A/C, as implied from the correct localisation of LAP2α, or if they do then it is at a level that is subtler than can be assessed by microscopy. These results are in contrast to earlier reports that have shown that LAP2α is sequestered into intranuclear

aggregates in cells transfected with specific lamin mutants (Dechat *et al.*, 2000a; Markiewicz *et al.*, 2002b).

Figure 12. LAP2α distribution is not affected by transient overexpression of FLAG- or GFP-tagged lamin A mutants. DP9 cells displaying nuclear abnormalities caused by overexpressing LAΔ50 or LA(R644C) with either blebbing/invaginations (*B,F*), micronuclei (*C,E*) or intranuclear lamin aggregates (*G*), showed no unusual distribution of the nucleoplasmic protein LAP2α. (Scale 10μm)



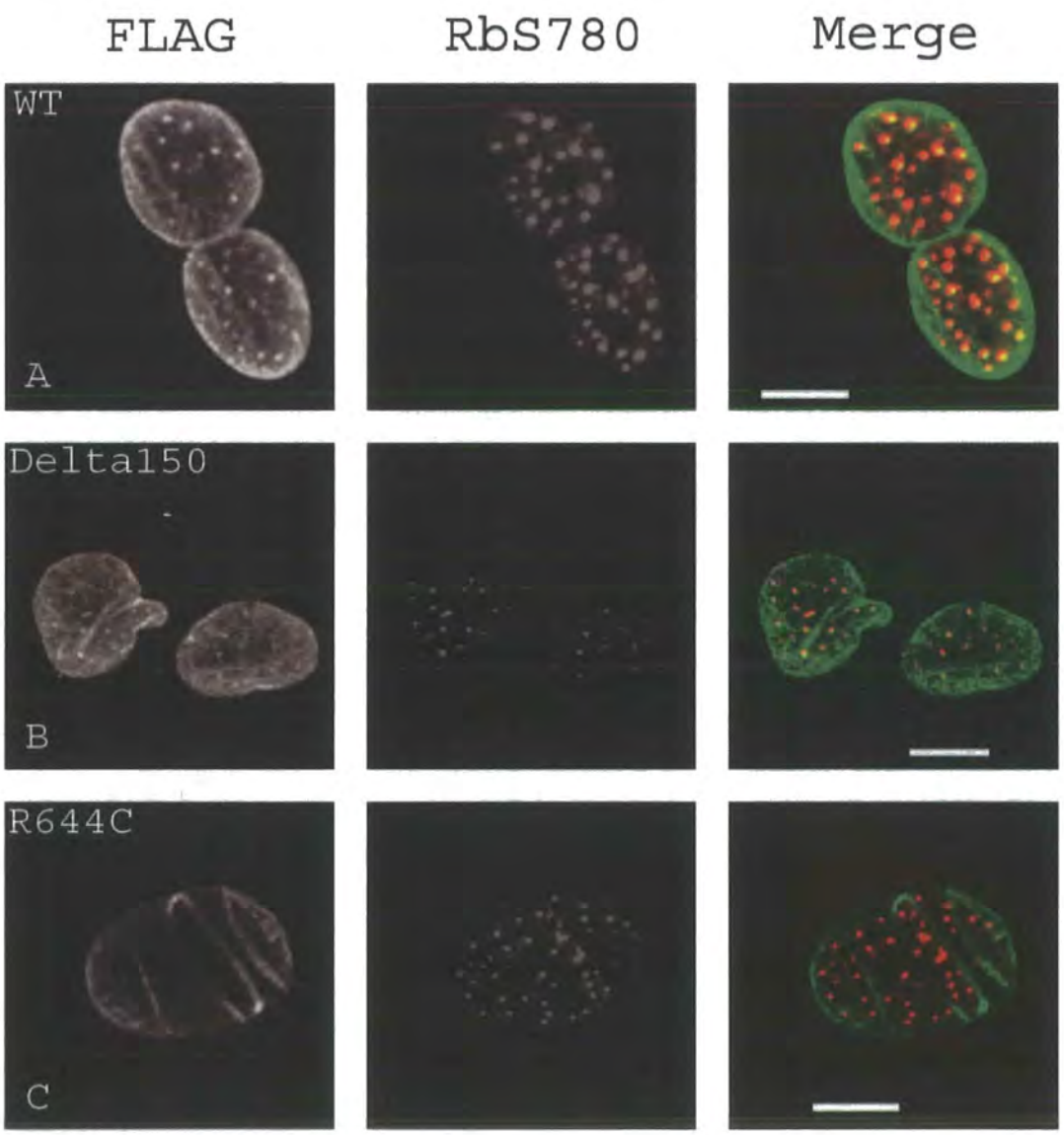

GFP
LAP2alpha
Merge
WT
D
Delta150
E
Delta150
F
R644C
G

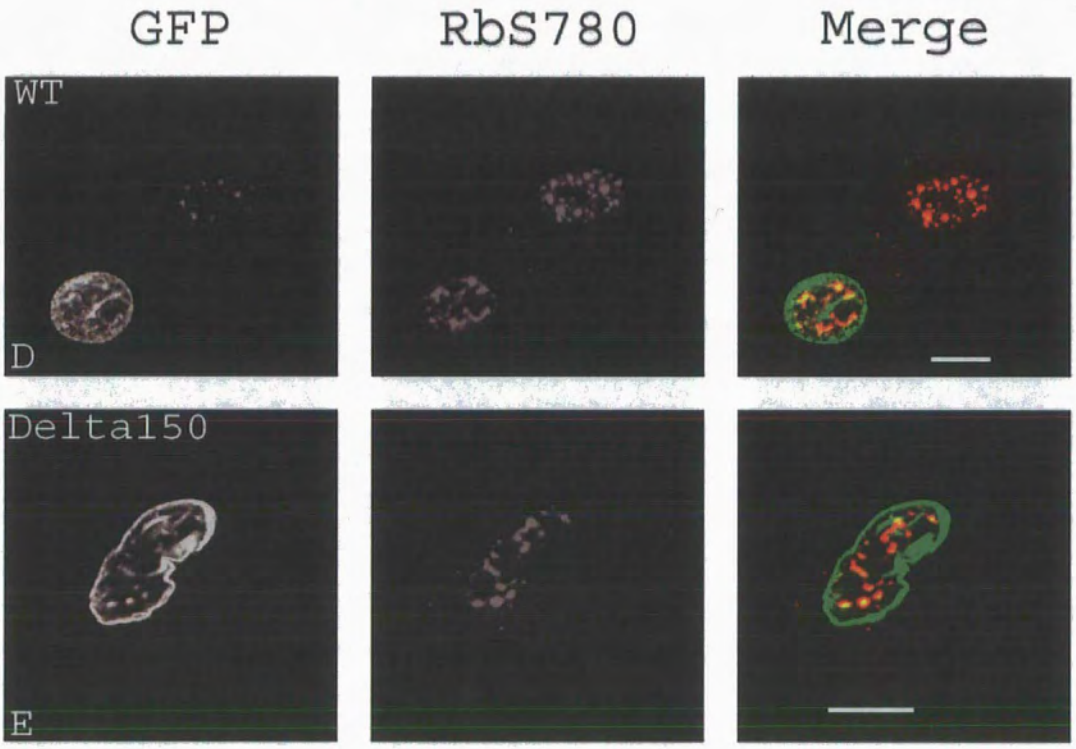
Cells transiently overexpressing FLAG- or GFP-tagged constructs were also costained for the lamin A-binding retinoblastoma susceptibility protein, Rb. A form of Rb phosphorylated at serine residue 780 was examined in cells transfected with FLAG- and GFP-tagged constructs. Rb is phosphorylated by cyclin D-CDK 4,6 complexes at the G_1-S phase transition, followed by further phosphorylation by cyclin E-CDK 2 heterodimers and remains phosphorylated until the end of M phase whereupon it is dephosphorylated by all three isoforms of protein phosphastase 1, allowing it to resume its repressive function (Mittnacht *et al.*, 1997; Vietri *et al.*, 2006). The serine residue 780 is known to be one of the first sites phosphorylated at the end of G_1 (Lundberg and Weinberg, 1998). In the current study discrete loci of RbS780 were found in cells, therefore suggesting that they were in S or G_2/M phase (Figure 13). Cells transfected with FLAG-LA constructs showing intranuclear (Figure 13*A,B*) or transnuclear (Figure 13*C*) lamin structures also showed a degree of colocalisation with the RbS780 foci. Additionally, RbS780 was also found in nuclear blebbing (Figure 13*C*). These observations were supported by transfection with GFP-tagged lamin constructs, as cells that contained nuclear abnormalities still contained RbS780 foci (Figure 13*E*). Analysis of the foci did not reveal any differences between the number of RbS780 foci and the level of expression of the constructs (Figure 13*D*), and also the construct used for transfection. However, the sizes of the foci were slightly smaller in DP9 cells expressing either of the two lamin A mutants as compared to DP9 cells expressing wild type lamin A (Figure 13).

These findings are somewhat surprising for a number of reasons. Firstly, that RbS780 should be found as foci within cells, as it has been reported that this phospho-isoform of Rb usually has a uniform distribution throughout the nucleus when cells are in S or G_2/M phase (Pevokic *et al.*, 2007). This discrepancy may be accounted for the by the difference in the origin of the cell type, as previous studies have utilised human or mouse fibroblasts, or the antibody may recognise different phospho-isoforms of Rb. Secondly, that lamin A should colocalise with a hyperphosphorylated form of Rb is also surprising, given that it has been reported that only a hypophosphorylated form of Rb is tethered to the nucleus by the lamin A/C-LAP2α complex (Markiewicz *et al.*, 2002b). This result suggests then that overexpression of lamin A may hinder the ability of phosphorylated Rb to be released

from this complex, or that the mechanistic basis of this process is different between species. Additionally, that the phosphorylated form of Rb should be found within the interior of the blebbing of the nuclear envelope underlines earlier results in this report suggesting that the these nuclear abnormalities are continuous with the nuclear envelope.

Figure 13. Overexpression of FLAG- and GFP-tagged lamin A mutants does not prevent RB phosphorylation of serine residue 780. DP9 cells showed discrete foci of retinoblastoma protein phosphorylated at serine residue 780, which appeared to show a degree of colocalisation with lamin A (*A,D*). This was irrespective of the construct used and they were also present in cells with nuclear abnormalities, either blebbing (*B,E*) or transnuclear lamin structures (*C*). The foci of Rb phosphorylated at serine residue 780 appeared to be smaller in cells expressing mutant lamin A (*B,C*). (Scale 10μm)




GFP
RbS780
Merge
WT
D
Delta150
E

Co-staining with an antibody that recognised a different phospho-isoform of Rb, phosphorylated at serine residue 807/811, gave a different pattern of localisation to the RbS780 isoform (Figure 14). Cells overexpressing GFP-LA showed a relatively uniform pattern of distribution localised across the nucleus (Figure 14*A*), as did DP9 cells overexpressing either the GFP-LAΔ50 or GFP-LA(R644C) constructs, regardless of the presence of nuclear envelope blebbing and intranuclear aggregates (Figure 14*B,C*). There was no difference in expression levels of RbS807/811 between GFP-LA and non-GFP expressing cells, showing that overexpression of wild type lamin A did not affect Rb phosphorylation (Figure 14*A*). Similar results were obtained from transiently expressing GFP-LAΔ50, but there was a reduced level of RbS807/811 in DP9 cells expressing GFP-LA(R644C) as judged by signal intensity (Figure 14*B,C*). However, as expected, phosphorylated Rb was absent from cells exiting M and entering G_1 phase (Figure 14*D*). This last result is significant as it shows that, although being a transformed cell line, the DP9 cells are still subject to regulatory cell-cycle-dependent events (Akagi, 2004; Ahuja *et al.*, 2005). Taken together, these results infer that transient overexpression of lamin A or LAΔ50 does not affect the process of Rb phosphorylation and therefore do not inhibit the G1-S phase transition in DP9 cells after 72 hours, whereas transient overexpression of LA(R644C) may cause a reduction in the cellular ability to phosphorylate Rb at serine residue 807/811.

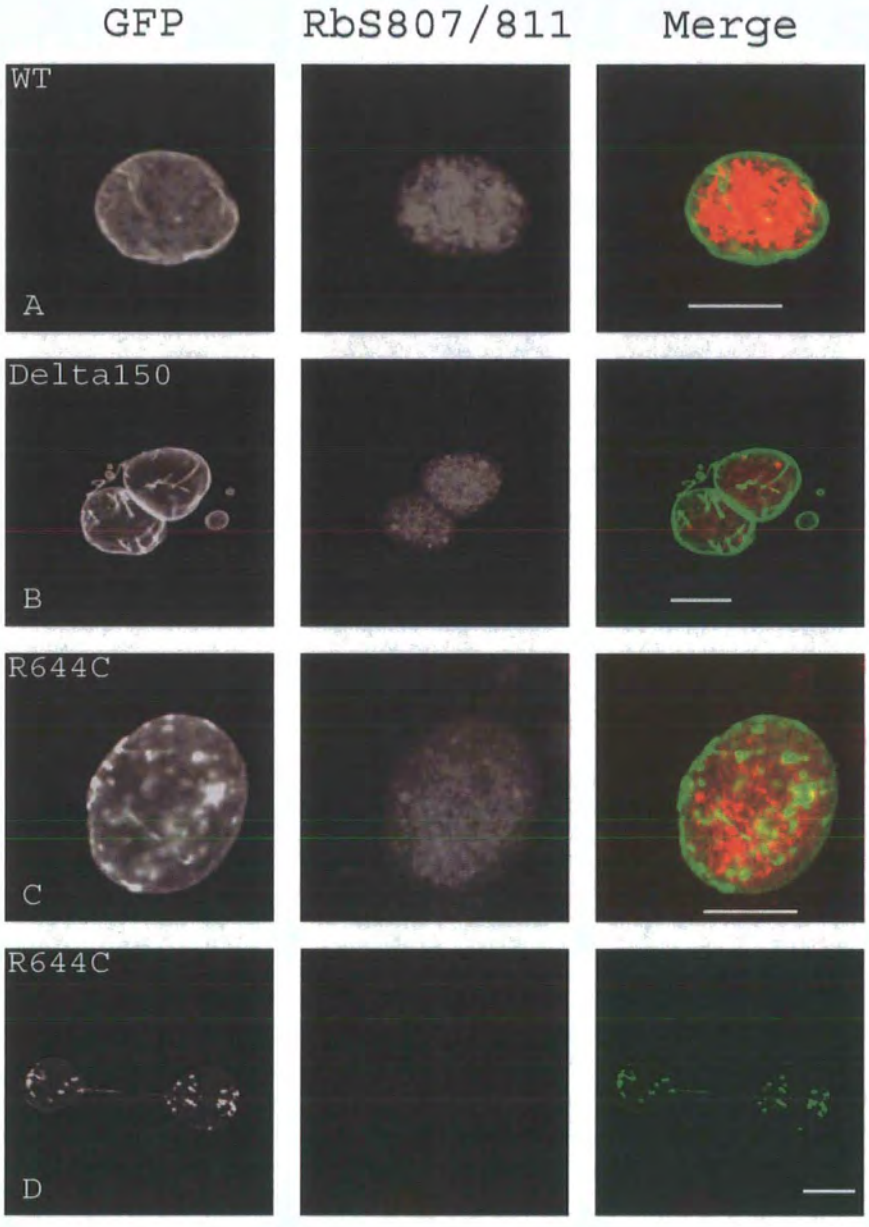


Figure 14. GFP-LA(R644C) may disrupt phosphorylation of Rb at serine 807/811. Rb is phosphorylated at serine residue 807/811 in a cell cycle-dependent manner (*A*), and overexpression of GFP-LAΔ50 (*B*) does not prevent this. In contrast, there is less Rb phosphorylated at serine residue 807/811 in DP9 cells expressing GFP-LA(R644C) (*C*). The absence of a phosphorylated form of Rb suggests transition into G_1 phase of the cell cycle and suppression of S-phase genes (*D*). (Scale 10μm)

Generation of a stable stem cell line overexpressing lamin A, lamin AΔ50 and lamin A(R664C).

In order to investigate the effect that continued overexpression of lamin A may have on the ability for adult rat clonal stem cells to differentiate and replicate *in vitro*, rat DP9 cell lines were created that stably express either FLAG-prelamin A WT, FLAG-prelamin A Δ50 or FLAG-prelamin A(R644C). Adult rat clonal DP9 cells have previously been well characterised. They are fibroblast-like in appearance and have been shown to be have the ability to be directed towards an both an osteogenic and adipogenic lineage (Jahoda *et al.*, 2003). Furthermore, human dermal papilla cells have been shown to have the ability to be directed towards both adipogenic and osteogenic lineages. Interestingly, this inductive potential is retained after serial passaging *in vitro* and is also retained regardless of the age of individual (Richardson *et al.*, 2005). Human dermal papilla cells would have been the preferred choice of cell type as the differences between mouse and human models with regards to studying laminopathies has been well documented (Stewart *et al.*, 2007). However, these have been known to senesce and/or undergo increased apoptosis early in culture and thus do not represent a robust cell model for the necessary adipogenic and growth assays required after transfection, single-cell cloning and subsequent expansion (Figure 15).

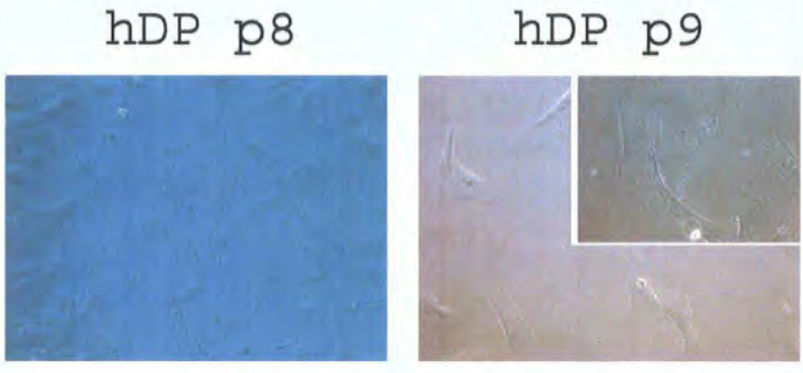


Figure 15. Human dermal papilla cells show reduced self-renewal potential with serial passage. The number of human dermal papilla cells, as viewed at x10 (inset, x20), is reduced with serial passage, indicating that they senesce early or undergo high rates of apoptosis under *in vitro* conditions.

In contrast, rat DP9 cells are transformed adult stem cells and therefore maintain the ability to self-renew with continued passaging *in vitro*. As a result, adult rat clonal DP9 cells were co-transfected with constructs expressing the WT, Δ50 or R644C prelamin A cDNA together with a linear hygromcyin B marker. After selection with hygromycin B, antibiotic-resistant cells were plated in 96-well plates, with single colonies expanded and then processed for indirect immunofluorescence. Expression levels of lamin A WT, Δ50 or R644C were slightly variable, but anti-FLAG signal was detected at the nuclear periphery (Figure 16). An aliquot of these nine clones were frozen down, and the remaining cells used for proliferation and differentiation assays.

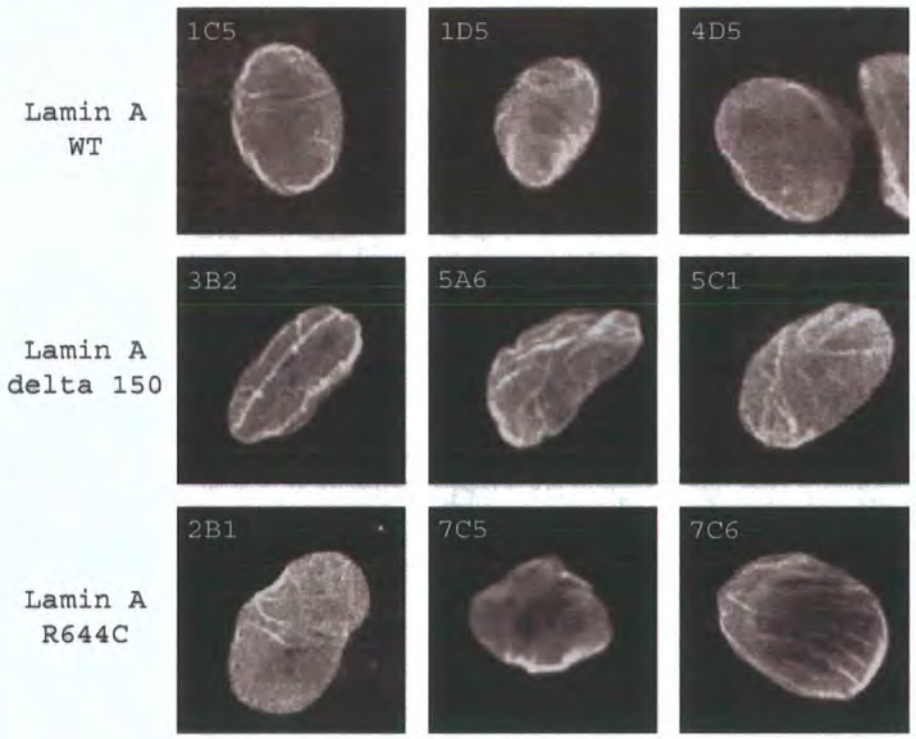


Figure 16. Clonal expression levels. Relative expression levels of three clones each of DP9 cells overexpressing either FLAG-LA, FLAG-LAΔ50 or FLAG-LA(R644C) as judged by indirect immunofluorescence. (Scale bar 10μm)

Overexpression of two lamin A mutants associated with premature aging in adult stem cells inhibits their growth potential.

Adult tissues contain stem cells capable of both self-renewal and differentiation, allowing for the maintenance of specific tissue lineages (Wagers and Weissman, 2004). As mitotic cells, these adult stem cells are susceptible to a cell-division-dependent increasing probability of acquiring a mutation that may drive them towards senescence or apoptosis (Campisi, 2005). It has already been reported that HGPS fibroblasts show a growth profile whereby cells have a hyperproliferative growth potential in earlier passages, followed by a swift dramatic decline and route into early senescence as compared with control fibroblast cultures (Bridger and Kill, 2004). This result has been used to suggest that adult stem cells in laminopathy patients might show reduced ability to self-renewal, therefore inhibiting the ability for the tissue to regenerate in response to damage (Hutchison and Worman, 2004). Thus, in order to investigate whether the self-renewal capacity or growth potential of adult stem cells overexpressing LAΔ50 or LA(R644C) is affected with serial passage, the nine clones created above were routinely passaged and the accumulated population doubling potential calculated (Figure 17*A,B*). The population doubling time is derived from the middle of the exponential growth phase and is an average figure that applies to the whole population, thus it includes a variety of cell division rates within the population and also cells that are undergoing apoptosis or senescence.

Untransfected DP9 cells showed a steady-state growth profile, as one might expect from a transformed cell line, whereby similar numbers of cells were counted after each passage, with the regression line almost linear (Figure 17*B Linear [DP9 Untransfected]*). Similarly, continued overexpression of FLAG-LA wild type resulted in an almost linear doubling potential, although perhaps the toxic effect of continued overexpression of wild type lamin A is reflected in the slightly decreasing regression line (Figure 17*B Linear [DP9 Wild Type]*). Continued passing of cells would clarify this question. Furthermore, the population doubling was at a lower level than the untransfected cells, implying that cell proliferation was slower than untransfected DP9 cells, which suggests that continued overexpression of wild type lamin A has a inhibitory effect on growth pathways.

In contrast, both DP9 cells stably overexpressing FLAG-LAΔ50 or FLAG-LA(R644C) showed a growth profile exhibiting a higher population doubling potential at an earlier passage number, with a notable decline in doubling potential with increasing passage number, which was greater for FLAG-LAΔ50 than it was for FLAG-LA(R644C) (Figure 17*A*). These results are consistent with previous reports showing that primary fibroblast from patients with typical HGPS exhibit a hyperproliferative growth profile followed by a decline in growth potential with increased rates of apoptosis (Bridger and Kill, 2004). At this stage, however, it is not clear whether the driving force behind this decline in population doubling potential for both mutants is due to increased rates of apoptosis or due to an increase in the number of senescent cells within the populations, further work is required. Taken together, these preliminary results indicate that continued overexpression of two lamin A mutants associated with premature aging inhibits the growth potential of adult stem cells.

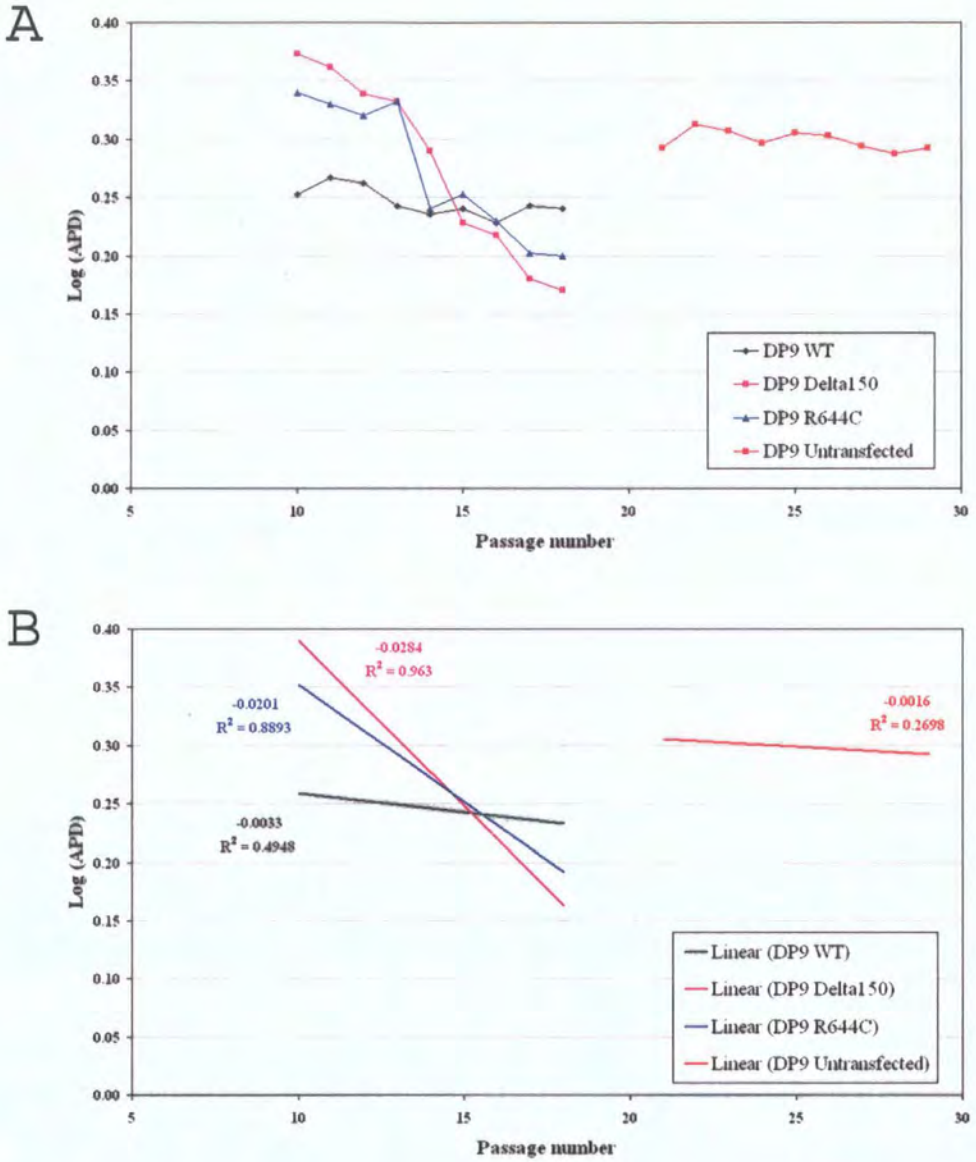


Figure 17. Overexpression of two lamin A mutants associated with premature aging inhibits stem cell growth potential. Untransfected DP9 cells have a constant accumulated doubling potential, whilst DP9 cells stably overexpressing wild type FLAG-LA have a similar profile (*A*). In contrast, DP9 cells stably overexpressing FLAG-LAΔ50 or R644C show a heightened proliferative capacity at earlier passage numbers with a notable decline with serial passaging (*A*). Regression line analysis of data from (*A*) is shown together with the gradient and R^2 values (*B*).

Establishment of an in vitro method for the differentiation of adult stem cells.

To investigate the effect of overexpressing prelamin A and two mutants associated premature aging on the ability of adult stem cells to differentiate, adipogenesis was chosen as a model for a number of key reasons. Firstly, rat clonal stem cells and human dermal papilla have been shown to have the capacity to differentiate *in vitro* towards adipogenic and osteogenic lineages, and thus provides an essential positive control (Jahoda *et al.*, 2003; Richardson *et al.*, 2005). Secondly, adipogenesis is a well-defined series of events coordinated by the regulation of transcription factors, which can be replicated *in vitro* by the addition of an "empirically derived cocktail" of chemicals that induces cells towards differentiation into adipocytes (Darlington, 1998; MacDougald and Mandrup, 2002; Fève, 2005; Rosen, 2005; Ailhaud, 2006; Bengoechea-Alonso and Ericsson, 2007).

In order to establish a method for the *in vitro* differentiation of adult stem cells overexpressing FLAG-tagged lamin A constructs, two different adipogenic media were used for untransfected rat DP9 and DS7 cells. Although it has been previously shown that rat clonal stem cells differentiated according to a defined adipogenic media (Jahoda *et al.*, 2003), another report has used a different adipogenic media to study the differentiation of 3T3-L1 preadipocytes overexpressing FLAG-tagged lamin A constructs (Boguslavsky *et al.*, 2006). Cells were and grown for six days in one of the two media, with a media change every two days, before being fixed and stained for intracellular lipid accumulation.

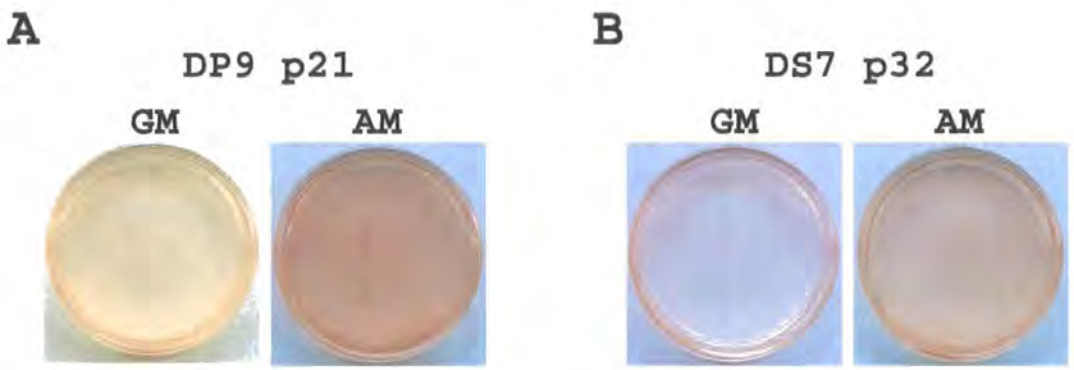
Both DP9 and DS7 cells showed no spontaneous differentiation after six days in growth media, as judged by the absence of oil red-O staining (Figure 18 *A,B [both GM]*). In contrast, both DP9 and DS7 cells exposed to an adipogenic media adapted from Jahoda *et al.* (2003) exhibited considerable accumulation of intracellular lipids after six days, with notably more oil red-O staining for DP9 cells than DS7 cells (Figure 18 *A,B [both AM]*).

DP9 and DS7 cells both reached confluency within the first 3 days under both growth and adipogenic conditions (Figure 18 *C,D [Day 3]*). Both DP9 and DS7 cells continued to undergo mitosis until day six in growth conditions, as judged by the presence of the

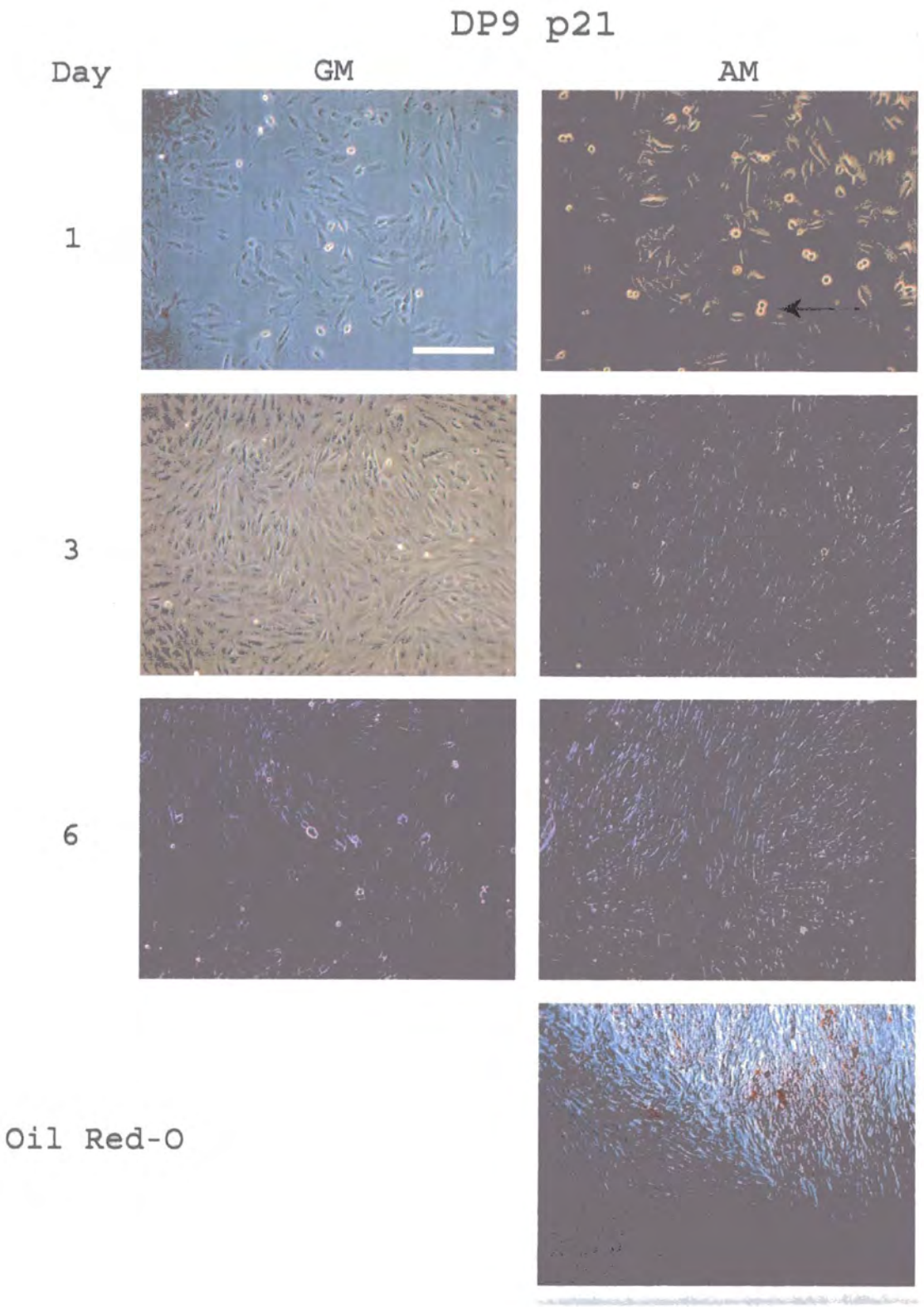
brightly emitting dividing cells (Figure 18 *C,D [GM,Day 6]*), whereas there was a cessation of mitosis from day 3 onwards under adipogenic conditions for both DP9 and DS7 cells (Figure 18 *C,D [AM,Day 6]*) as the cultures had presumably undergone clonal expansion and had entered the differentiation step of adipocyte development (Agarwal and Garg, 2006). Staining with oil red-O revealed that accumulation of intracellular lipids was not uniform in distribution but rather exhibited some localised areas that showed significantly more staining than surrounding cells (Figure 18 *C,D [Oil red-O]*).

In contrast, DP9 and DS7 cells exposed to the adipogenic media adapted from Boguslavsky *et al.* (2006) showed a distinct lack of intracellular lipids (Figure 19). Again, no spontaneous differentiation occurred, as reflected by the absence of intracellular lipids in cells after six days of growth conditions (Figure 19 *A,B(GM)*). DS7 cells failed to differentiate at all under adipogenic conditions at two different initial cell densities (Figure 19 *B(AM)*). However, DP9 cells did accumulate a small amount of intracellular lipids, although this was barely detectable (Figure 19 *A(AM)*). Taken together, both these results reaffirm previous reports that adult stem cells from the hair follicle have the capacity to differentiate *in vitro* and can be directed towards an adipogenic lineage. Furthermore, it reveals that differing culture conditions may impact on stem cell fate. Based on these results, the adipogenic media adapted from Jahoda *et al.* (2003) was employed for adipogenic assays involving clones overexpressing FLAG-tagged prelamin A constructs.

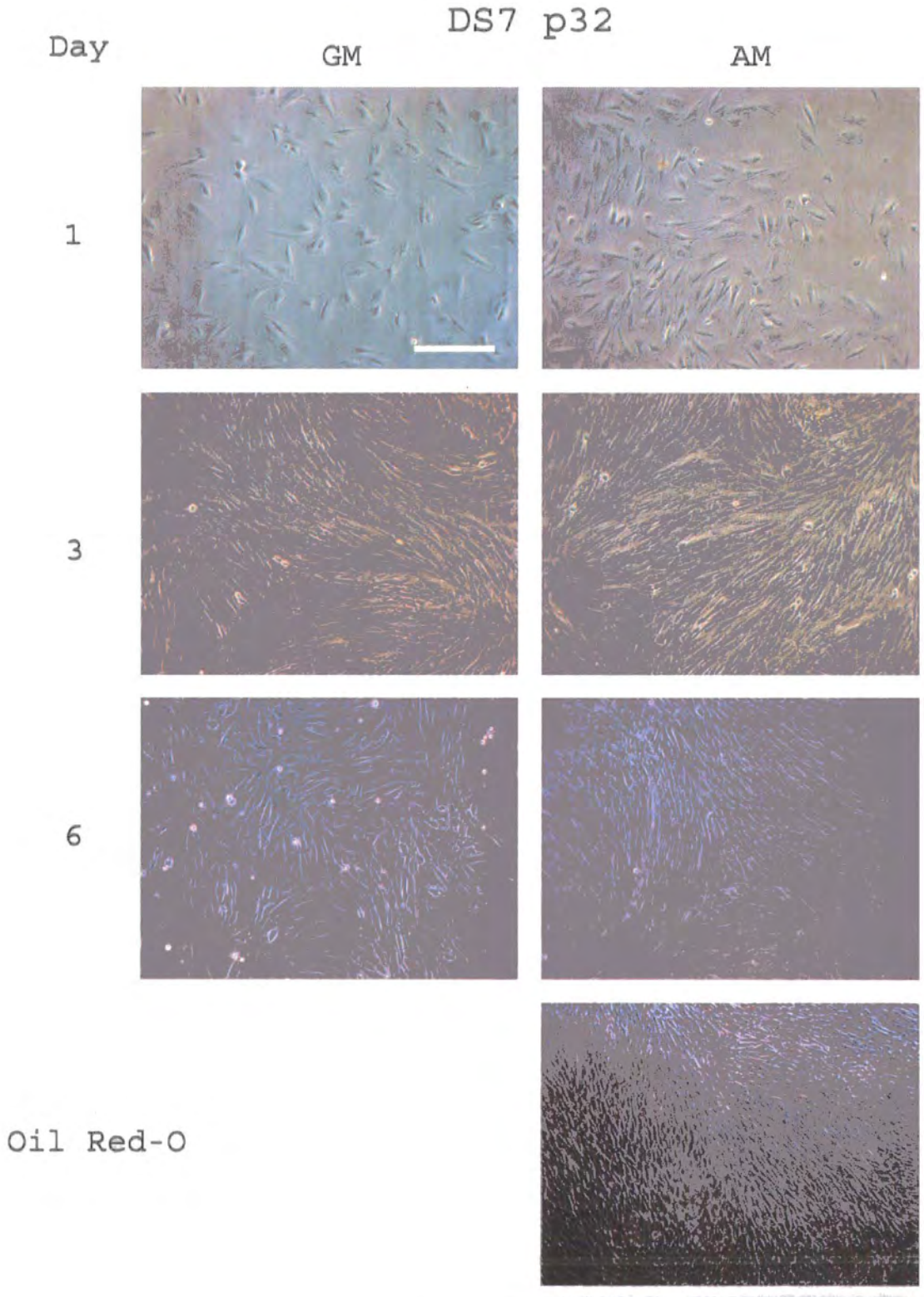
Figure 18. Hair follicle stem cells can be directed towards an adipogenic lineage *in vitro*. DP9 (*A*) and DS7 (*B*) cells accumulate intracellular lipids after six days in adipogenic culture conditions, indicating that they have undergone a program of differentiation. Note that no spontaneous differentiation occurred after six days in control growth medium. Phase contrast images reveal a halt in mitotic activity before a programme of differentiation, leading to intracellular accumulation of lipids in DP9 (*C*) and DS7 (*D*) cells after six days of adipogenic conditions. Mitotic cells are shown by an arrow (*C*). (Scale 200μm)



C



D



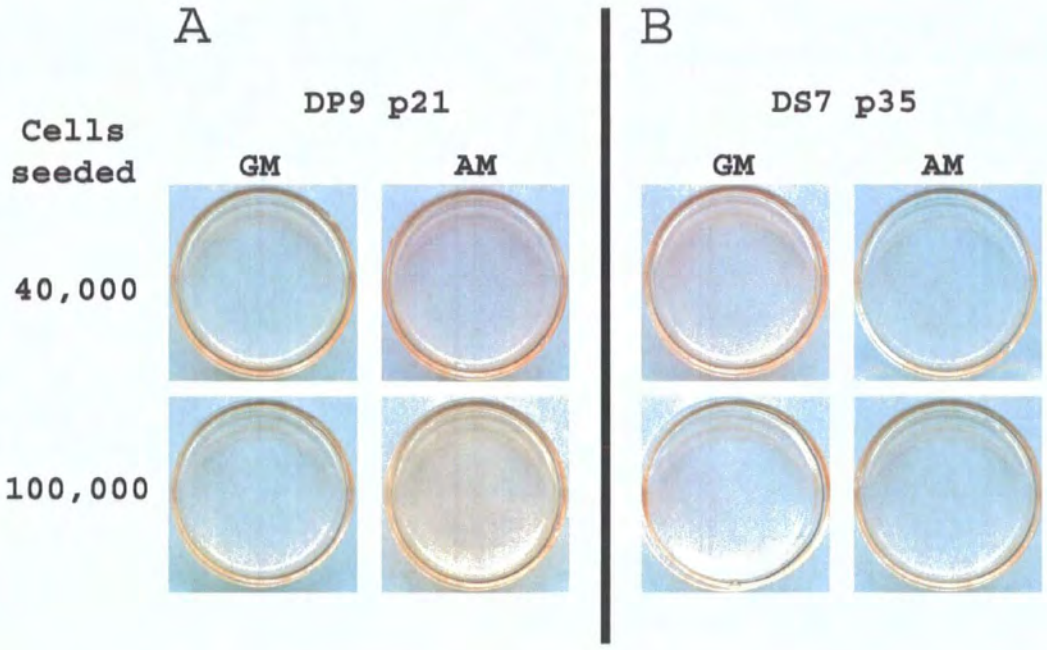


Figure 19. DP9 and DS7 cells fail to differentiate under different adipogenic conditions. When exposed to a different adipogenic medium, DS7 cells (*B*) failed completely to accumulate intracellular lipids at two initial seeding densities, while DP9 cells (*A*) accumulated a very low but detectable level. No spontaneous differentiation occurred after six days of control growth medium.

Adult stem cells that overexpress either wild type lamin A or two lamin A mutants inhibits their ability to accumulate intracellular lipids.

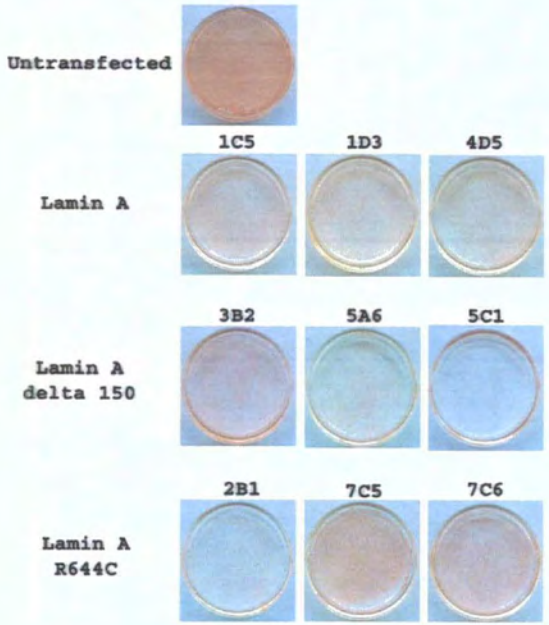
It has been suggested that adult stem cells may be implicated in laminopathies by failing to differentiate and thus ultimately failing to regenerate new tissue (Hutchison and Worman 2004; Markiewicz *et al.*, 2005). To test this hypothesis, three clones of DP9 cells overexpressing either FLAG-prelamin A, FLAG-prelamin AΔ50, or FLAG-prelamin A(R644C) were subjected to adipogenic conditions for six days, fixed and stained for intracellular lipid accumulation with oil red-O. All nine clones failed to accumulate the same level of intracellular lipids as the untransfected DP9 cells (Figure 20*A*). DP9 cells overexpressing wild type FLAG-prelamin A all had similarly low levels of intracellular lipid accumulation. In contrast, DP9 cells overexpressing FLAG-prelamin AΔ50 or FLAG-prelamin A(R644C) were more variable, albeit at a level still far below the untransfected DP9. Interestingly, one clone overexpressing FLAG-prelamin AΔ50 failed to accumulate any intracellular lipids at all (Figure 20*A 5C1*).

Semi-quantitative analysis of the nine clones by spectrophotometric measurement of the extracted oil red-O revealed a significant reduction in the ability of cells to differentiate, as compared to untransfected DP9 cells (Figure 20*B*). DP9 cells stably expressing FLAG-prelamin AΔ50 showed the greatest reduction with an 80% decrease in differentiation potential (Mean 20%; SD ± 17.4% [n=3]). The large standard deviation for the pooled FLAG-prelamin AΔ50 data may be the accounted for by clone 5C1, which failed to differentiate at all (Figure 20*A 5C1*). DP9 cells stably expressing FLAG-prelamin A wild type had a 77% reduction in differentiation potential (Mean 23%, SD ± 2.3% [n=3]), whilst cells stably expressing FLAG-prelamin A(R644C) had a 64% decrease in differentiation potential (Mean 36%, SD ± 3.9% [n=3]). All three stable lines showed a statistically significant difference in differentiation potential compared to untransfected DP9 cells, based on a Student's t-test: WT (P<0.0001); Δ50 (P<0.01); R644C (P<0.001).

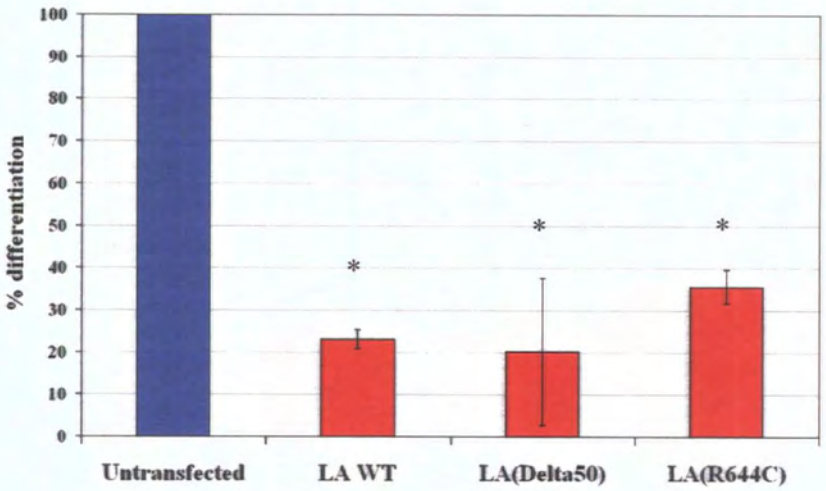
Intracellular lipid accumulation followed a similar distribution to that observed for untransfected DP9 and DS7 cells, with non-uniform localised areas of lipid accumulation, whilst some areas showed no accumulation of lipids at all (Figure 20*C*). Taken together, these results show that intracellular lipid accumulation is inhibited in stably-expressing cells, which suggests that continued overexpression of wild type or mutant lamin A inhibits the ability of adult stem cells to differentiate *in vitro* towards an adipogenic lineage. This result confirms an earlier report whereby wild type and mutant lamin A FLAG-tagged constructs that were overexpressed in 3T3-L1 preadipocytes led to the inhibition of intracellular lipid accumulation, although the mutant lamins were based on mutations found in FPLD patients (Boguslavsky *et al.*, 2006).

Figure 20. Overexpression of lamin A and two mutants associated with premature aging inhibits the ability of adult stem cells to accumulate intracellular lipids. Stable DP9 cells overexpressing wild type FLAG-LA, FLAG-LAΔ50 or FLAG-LA(R644C) were grown for six days under adipogenic conditions and stained for intracellular lipid accumulation with oil red-O (*A*). Oil red-O was extracted and the percentage of differentiation of three clones for each construct was expressed as a percentage of the total differentiation attained by untransfected DP9 (*B*). * Significantly reduced differentiation potential: WT ($P<0.0001$); Δ50 ($P<0.01$); R644C ($P<0.001$). Non-uniform distribution of oil red-O staining after six days of adipogenic conditions reveals localised areas of differentiation in all but one of the nine clones tested (*C*). (Scale 200μm)

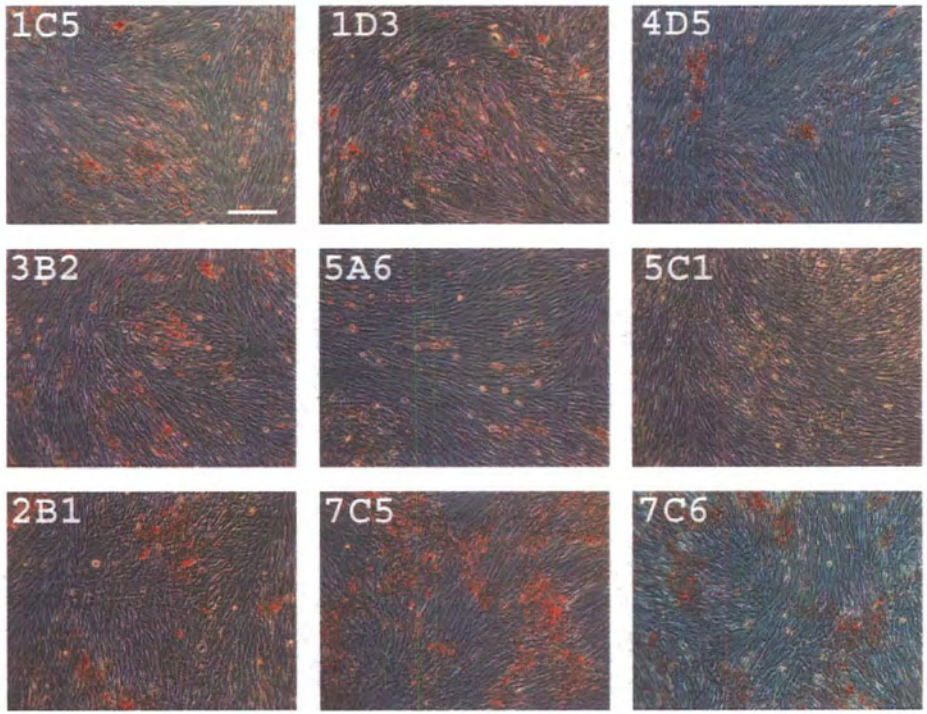
A


Untransfected
1C5
1D3
4D5
Lamin A
3B2
5A6
5C1
Lamin A
delta 150
2B1
7C5
7C6
Lamin A
R644C

B


% differentiation
100
90
80
70
60
50
40
30
20
10
0
*
*
*
Untransfected
LA WT
LA(Delta50)
LA(R644C)

C



Establishment of an antibiotic-inducible gene expression system for the controlled expression of FLAG-tagged lamin A, lamin AΔ50 and lamin A(R644C).

As DP9 cells are transformed, their number should remain constant with serial passage; indeed, growth potential results confirm this (Figure 17). Therefore, DP9 cells were seen as an ideal model for an adult stem cell system in which to introduce a Tet-On Advanced gene expression system. This is a valuable tool with which to modulate expression levels and is particularly useful when the protein of interest is toxic to the cell, i.e. progerin, and can be used to follow cell cycle phenotypes in a single clone expressing different levels of the protein (Glynn and Glover, 2005; Dorner *et al.*, 2006; Shumaker *et al.*, 2006). Following a double-stable transfection and selection process, the system itself permits gene expression to be tightly regulated in response to varying concentrations of antibiotic.

In order to determine the concentration of doxycycline with which to induce gene expression, a luciferase assay was carried out to measure activity over a range of concentrations. The cell line used was U2-OS Luc Tet-On Advanced (as supplied by Clontech), an osteosarcoma cell line containing wild type p53 but with the overexpression of the p53-inhibitor Mdm2 (Nelissen *et al.*, 2000), that has been previously stably transfected with the Tet-On Advanced luciferase construct. The lowest concentration of doxycycline required to induce expression in these cells after 48 hours was 10ng/ml (Figure 21). Additionally, prior to beginning the first stable transfection, a fixed number of DP9 cells were subjected to various concentrations to hygromycin B and G418 in order to determine the optimal concentration for selection of DP9 cells (data not shown). The optimal concentration of hygromycin B was found to be 100μg/ml and G418 400μg/ml. Thereafter, the optimal plating density for which cells should be plated for stable selection was then determined and judged to be 5×10^5 (Figure 22). Further preliminary work included cloning ATG-FLAG-prelamin A wild type, Δ50 and R644C into the pTRE-Tight vector to be used for the second stable transfection.

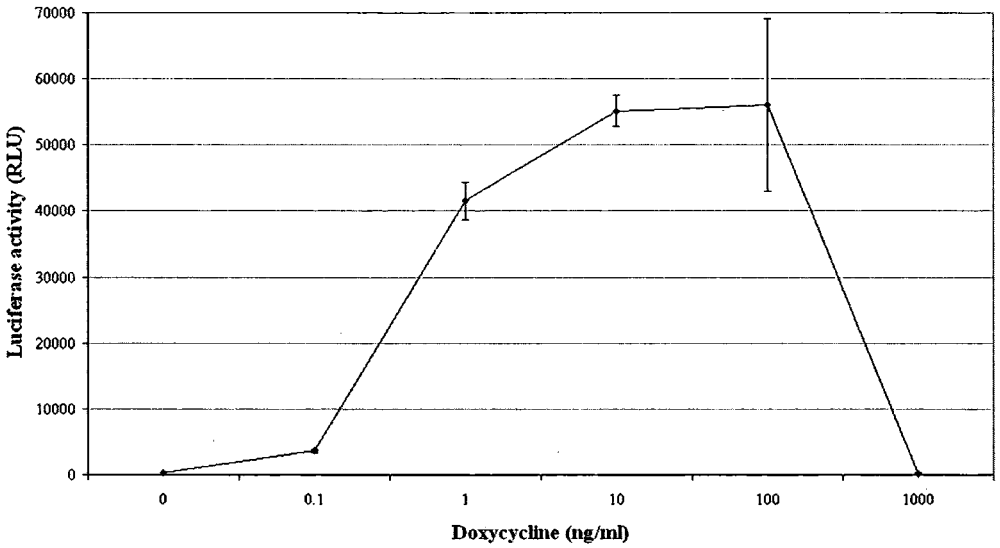


Figure 21. DP9 dose-response curve. Increasing concentrations of doxycycline was added to U2-OS Luc Tet-On Advanced cells and then assayed for luciferase activity after 48 hours.

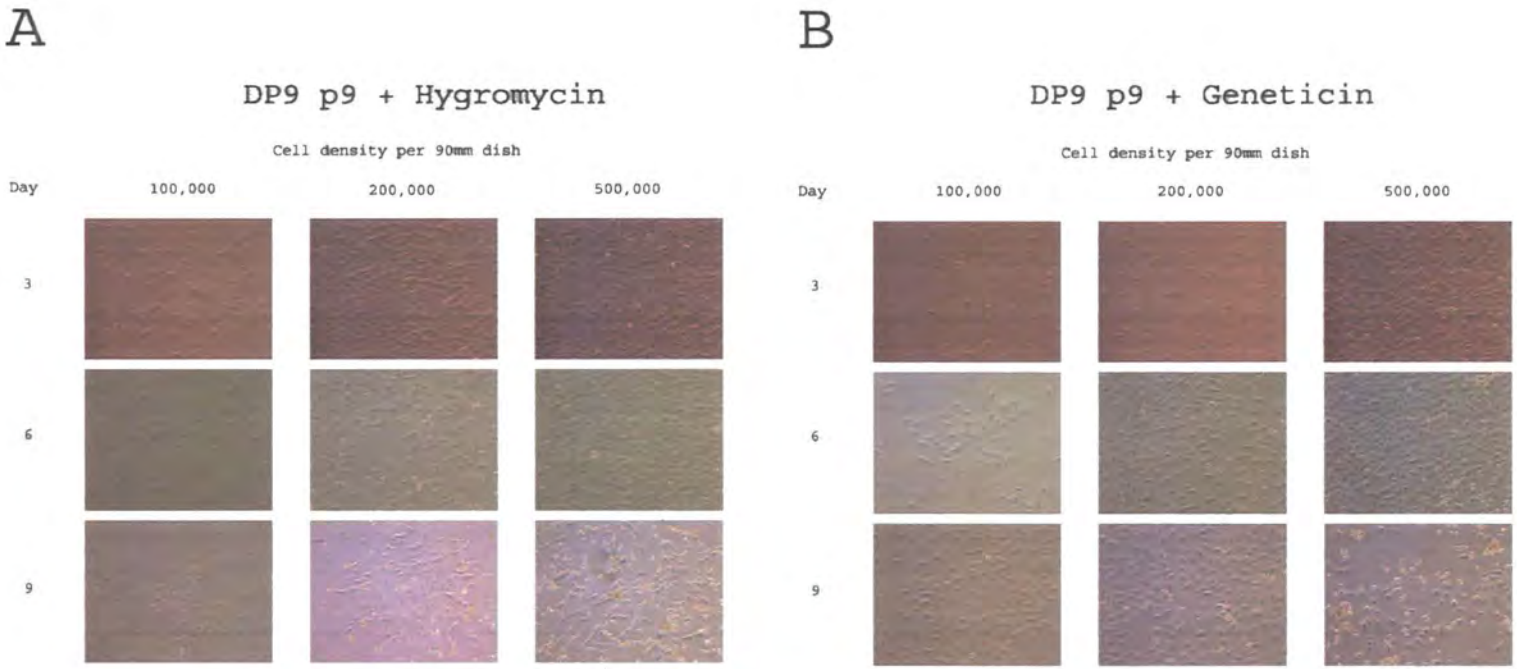


Figure 22. Determination of DP9 optimal plating density. After an optimum concentration of hygromycin B and geneticin were found, 100μg/ml and 400μg/ml respectively, DP9 cells were plated at three different cell densities and grown for a number of days to determine the number to be plated for stable selection.

The first stable transfection was then performed with the pTet-On Advanced construct, selected in G418, with 24 clones propagated. Expression levels of the rtTA-Advanced protein were assayed by Western blot analysis with a monoclonal antibody specific to the rtTA-Advanced protein. A single clone (D3) was then identified that expressed the highest levels of the rtTA-Advanced protein and chosen for the second stable transfection (Figure 23). Due to time restraint, the second stable transfection was not started and the cells frozen down. In time, these cells will provide a great opportunity to assess the pathology of overexpressing mutant lamin A in adult stem cells.

Figure 23. DP9 Tet-On Advanced clonal selection. DP9 cells were transfected with pTet-On Advanced and selected in 400μg/ml geneticin. Single clones were propagated and then assayed for the production of the pTet-On Advanced protein rtTA with the TetR monoclonal antibody at a dilution 1:2000. Lane 2, clone D3, showed the highest levels of rtTA expression and will be used for the second stable transfection for the Tet-On Advanced gene expression system.

Chapter 4. Discussion & Conclusion

A functional lamina is required to maintain nuclear envelope structure in adult stem cells

Here, it has been shown that transient overexpression of two mutants associated with typical and atypical HGPS in adult stem cells causes a variety of nuclear abnormalities (Figures 6 and 7). This supports a vast body of evidence whereby a host of laminopathy mutations give rise to nuclear abnormalities when primary cells are grown *in vitro* or cells are transiently transfected with expression plasmids harboring laminopathy mutations (Östlund *et al.*, 2001; Raharjo *et al.*, 2001; Vigouroux *et al.*, 2001; Novelli *et al.*, 2002; Bechert *et al.*, 2003; Capanni *et al.*, 2003; Chen *et al.*, 2003; De Sandre-Giovannoli *et al.*, 2003; Eriksson *et al.*, 2003; Favreau *et al.*, 2003; Holt *et al.*, 2003; Mounkes *et al.*, 2003; Csoka *et al.*, 2004b; Goldman *et al.*, 2004; Muchir *et al.*, 2004; Navarro *et al.*, 2004; Reichart *et al.*, 2004, Scaffidi and Misteli, 2005). How these nuclear envelope structural defects manifest themselves as the phenotypes associated with the various laminopathies is an area of ongoing research. The complexity of the INM has recently been shown to be greater than previously imagined and thus it is likely that different laminopathy mutations result in different disease mechanisms that govern the phenotype (Schirmer *et al.*, 2003). The observed nuclear abnormalities found in laminopathy cells has prompted the suggestion that one such disease mechanism is based on the role of lamins as a tensegrity element, a vital underlying structural support of the nucleus (Hutchison *et al.*, 2001; Wilson *et al.*, 2001; Burke and Stewart, 2002; Goldman *et al.*, 2002; Hutchison and Worman, 2004). This 'structural' hypothesis is supported by evidence that cardiomyctes from *LMNA*-null mice leads to progressive cytoskeletal disorganisation with detachment of desmin filaments from the nuclear surface (Nikolova *et al.*, 2004). Furthermore, mutations within nesprins are associated with EDMD, with cells exhibiting mislocalisation of emerin and SUN2 (Zhang *et al.*, 2007). The implication of this is that the LINC complex, connecting the nucleus to the cytoskeleton, may be disrupted by A-type lamin mutations, leading to increased sensitivity to mechanical stress, especially

those cells that undergo physical forces such as cardiomyctes (Hutchison and Worman, 2004; Burke and Stewart, 2006; Worman, 2006). Underlining this, *LMNA*-null MEFs show a decreased mechanical stiffness with increased nuclear deformation, defective mechanotransduction, impaired cellular viability under mechanical strain and an overall increase in cellular weakness (Broers *et al.*, 2004; Lammerding *et al.*, 2004). The enhanced nuclear fragility may help explain the cardiac and skeletal pathologies as the forces generated during muscle contraction may lead to the preferential breakage of nuclei containing a defective nuclear lamina. However, this structural hypothesis cannot underlie the dramatic phenotype seen in patients with typical and atypical HGPS, indeed, that *LMNA*-null MEFs show attenuated NF-κB signaling suggests that other mechanisms are involved in disease pathogenesis (Lammerding *et al.*, 2004).

Evidence suggests that the incomplete processing of progerin and the retention of the farnesyl group may be a significant factor in the development of HGPS (Goldman *et al.*, 2004; Glynn and Glover, 2005; Gruber *et al.*, 2005; Toth *et al.*, 2005; Yang *et al.*, 2005). Indeed, the number of misshapen nuclei in fibroblasts from typical HGPS increases with passage number, which is correlated with the accumulation of progerin along the nuclear envelope (Goldman *et al.*, 2004). Similarly, *Zmpste24*-null mice also exhibit misshapen nuclei and HGPS-like disease phenotypes, caused by accumulation of unprocessed farnesylated and carboxymethylated prelamin A (Bergo *et al.*, 2002; Pendas *et al.*, 2002; Fong *et al.*, 2004). This has led to the suggestion that progerin exerts a dominant negative effect in the nucleus and that prelamin A is toxic to cells, directly causing misshapen nuclei (Gruber *et al.*, 2005; Scaffidi and Misteli, 2005; Toth *et al.*, 2005; Yang *et al.*, 2005). The current work underpins this 'prelamin A toxicity' hypothesis, and shows for the first time that adult stem cells also exhibit nuclear abnormalities upon transient transfection with expression plasmids. However, the extent to which these nuclear abnormalities reflect the *in vivo* situation and contribute to the disease phenotype, rather than as an artefact of *in vitro* culture methods, remains to be seen.

Nuclear aggregates of lamins have been widely reported previously for various lamin mutations (Schmidt *et al.*, 1994; Izumi *et al.*, 2000; Östlund *et al.*, 2001; Raharjo *et al.*,

2001; Vaughan *et al.*, 2001; Markiewicz *et al.*, 2002b; Capanni *et al.*, 2003; Holt *et al.*, 2003). In this study, transient overexpression of plasmids harbouring two premature aging mutants caused a degree of intranuclear aggregate formation. The observation that transient overexpression of LA(R644C) in DP9 cells caused the formation of more intranuclear aggregates than LAΔ50 is puzzling. The mutant protein formed by the LA(R644C) mutation is expected to be both farnesylated and carboxymethylated, therefore rendering it likely to associate with the INM due to its lipid anchor, as is the case for LAΔ50. Studies using farnesyl transferase inhibitors (FTIs) to treat primary HGPS fibroblasts and MEFs from a HGPS mouse model have shown that blocking lamin A biogenesis at the first stage, i.e. blocking farnesylation, leads to the relocalisation of progerin away from the nuclear envelope into nucleoplasmic aggregates, normalising nuclear envelope shape (Toth *et al.*, 2005; Yang *et al.*, 2005). This is in accordance with previous work showing that in mevinolin treated HEp-2 cells the blocking of farnesylation causes prelamin A to accumulate in intranuclear foci, with the suggestion that these foci may be the site of lamin A-heterochromatin formation before subsequent targeting of prelamin A to the nuclear envelope that would help to reorganise the three dimensional structure of chromatin (Sasseville and Raymond, 1995). Furthermore, it has been suggested that farnesylation occurs at nucleoplasmic foci (Lutz *et al.* 1992, Moir *et al.* 1995). Lamin A microinjected into 3T3 cells accumulates into intranuclear foci before gradually integrating into the nuclear lamina (Goldman *et al.*, 1992). In the current study, it would be predicted that these intranuclear aggregates would have undergone the farnesylation processing step, however, their location in the nucleoplasm suggests otherwise. One might postulate that the overexpression of prelamin A, which exists only transiently in wild type cells with a half-life of 90-100 minutes, might accumulate by binding to itself (Gerace *et al.*, 1984; Beck *et al.*, 1990). Taken together, if mutant prelamin A were to follow a similar spatial processing pathway then the intranuclear aggregates may arise by failure to be shuttled to the nuclear periphery for integration into the nuclear lamina. Support of this comes from FRAP studies, suggesting that progerin is less mobile in the nucleus (Dahl *et al.*, 2006). However, a more detailed study using a panel of prelamin A antibodies would clarify the nature of these intranuclear aggregates. Some insight may be gained from a previous report that has shown that overexpression of

dominant negative lamin A/C mutant mutants causes the redistribution of lamin B1 and endogenous lamin A/C into intranuclear foci together with the mutant lamin A/C, yet there still remained traces of lamin B1 and endogenous lamin A/C at the nuclear periphery (Östlund *et al.*, 2001). Moreover, emerin, an A-type lamin binding partner, was mislocalised away from the INM (Östlund *et al.*, 2001). The current study has shown that the formation of intranuclear lamin aggregates does not mislocalise emerin away from the INM (Figure 11). This suggests that endogenous levels of lamin A/C are not perturbed by the presence of these aggregates, although further work is required to reveal the distribution of endogenous lamin A/C and lamin B1 in cells with intranuclear lamin aggregates.

In this study, it has been shown that LAΔ50 and LA(R644C) were targeted to the nuclear envelope in the majority of cells as they are both expected to retain their farnesyl anchor due to the deletion of, or mutation in, respectively, the second endoproteolytic Zmpste24 cleavage site (Figure 7) (Goldman *et al.*, 2004; Dechat *et al.*, 2007). The most common nuclear abnormality observed was a transnuclear lamin structure (Figure 8). Analysis of these structures revealed that these are deep extensions of the nuclear envelope rather than accumulation of lamin protein within the nucleoplasm. Further evidence suggesting these are continuous extension of the nuclear envelope is supported by the colocalisation with the INM protein emerin (Figure 11). The observation that they are extensions of the nuclear envelope may be accounted for by the presence of the farnesyl motif, which is expected to locate progerin to the INM during interphase (Goldman *et al.*, 2004). Previous reports have shown that over-expression of prenylated lamins in cells induces excessive nuclear membrane formation and growth (Prufert *et al.*, 2004; Ralle *et al.*, 2004). Based on this, it has since been suggested that the continued presence of progerin at the INM is responsible for the characteristic nuclear blebbing due to excessive nuclear membrane growth (Goldman *et al.*, 2004; Capell *et al.*, 2005). Work presented here supports this, as nuclear blebbing was observed in some cells and, additionally, the transnuclear lamin structures observed could conceivably be the precursor for more pronounced nuclear blebbing over time. However, the majority of cells retained a normal nuclear envelope

structure with the mutant prelamin A being incorporated into the nuclear envelope, which suggests that nuclear blebbing may not be the major determinant in disease pathogenesis.

Premature aging mutations inhibit the self-renewal capacity of adult stem cells

The present work has shown that the continued overexpression of mutant prelamin A has a negative effect on the growth potential of DP9 cells *in vitro* (Figure 17). This implies that self-renewal of adult stem cells may be inhibited in somatic tissues in laminopathy patients. Before discussing the potential implication for this, it is important to underline that further work is required to clarify this finding, either through a suitable apoptotic or senescence assay. Furthermore, the level of overexpression in the nine cell lines (three each of LA, LAΔ50 and LA(R644C)) used needs to be quantified, either at the mRNA or protein level. Despite this, it is tempting to speculate as to the effect that a diminished number of adult stem cells may have on tissue regeneration. A previous report of a mouse model for HGPS has shown that continued culture of $LMNA^{L530P/L530P}$ adult fibroblasts resulted in apoptosis rather than senescence with no differences in telomere length between wild type and $LMNA^{L530P/L530P}$ mice (Mounkes *et al.*, 2003). Routine passage of primary human HGPS fibroblasts leads to increased proliferation rate during early passage numbers followed by a dramatic decrease as a result of apoptosis and/or senescence (Bridger and Kill, 2004; Goldman *et al.*, 2004). These results suggest a loss of cell cycle control in early passage number HGPS fibroblasts, probably via Rb-dependent pathways (Mancini *et al.*, 1994; Ozaki *et al.*, 1994; Markiewicz *et al.*, 2002b; Bridger and Kill, 2004; Nitta *et al.*, 2006). With an increase in DNA damage in HGPS, it could be suggested that genomic instability as a result of deregulated cell cycle control may promote entry into senescence (Liu *et al.*, 2005). This premature entry into senescence is inextricably linked to the presence of the farnesylated prelamin A mutant (Bridger and Kill, 2004; Goldman *et al.*, 2004; Caron *et al.*, 2007). Strengthening this link, Rb has been shown to be involved in cellular senescence as it is upregulated by the tumour suppressor $p16^{INK4A}$ and believed to form repressive heterochromatin loci containing E2F targets

(Campisi, 2005). It is also feasible that senescence may be achieved via a p53-dependent pathway (Campisi, 2005). Underlining this, lamin A/C is activated in a p53-dependent manner in cells responding to DNA damage, and *Zmpste24*-null MEFs show upregulation of p53 (Varela *et al.*, 2005; Rahman-Roblick *et al.*, 2007). Although there is a putative p53-binding site in intron 1 of lamin A/C, it has been suggested that downstream effectors such as $p21^{CDKN1}$ may be involved in the senescence process of HGPS (Cox and Faragher, 2007; Rahman-Roblick *et al.*, 2007).

On the other hand, as opposed to either Rb or p53-dependent senescence, replicative senescence is linked to telomere shortening (Faragher and Kipling, 1998). One report has shown that telomere length is reduced in primary HGPS fibroblasts (Allsop *et al.*, 1992). In addition, whilst a subset of dermal fibroblasts from HGPS patients may be immortalised by telomerase expression, more detailed analysis showed that some clones retrovirally infected with hTERT fail to immortalise (Ouellette *et al.*, 2000; Wallis *et al.*, 2004). This is in contrast to reports of $LMNA^{L530P/L530P}$ adult fibroblasts where no such telomere shortening was observed (Mounkes *et al.*, 2003). This apparent difference may be due to fundamental differences between mouse/rat and human cells, underlined by the mouse model of HGPS whereby a homozygous L530P mutation causes disease pathogenesis, rather the classical heterozygous G608G mutation found in humans. Thus, it is conceivable that senescence may by driven by telomere shortening in HGPS patients but is unlikely in mice.

An important derivation from the above, and in line with the current work, might then implicate the senescence of stem cells not only in premature aging, but also in the normal aging process. Indeed, $p16^{INK4A}$ expression limits the regenerative capacity of pancreatic β-cells with aging (Krishnamurthy *et al.*, 2006). Evidence also suggests that $p16^{INK4A}$ expression increases with age in HSCs, which causes cellular and molecular hematopoietic stem cell aging (Beausejour and Campisi, 2006; Janzen *et al.*, 2006; Rossi *et al.*, 2007). Furthermore, skeletal muscle satellite cells show an age-related decrease in differentiation potential (Lees *et al.*, 2006). The proposed effect of age-related changes in adult stem cell acts at both the self-renewal and differentiation level. The current work

highlights that prelamin A mutations inhibit the *in vitro* replication of adult stem cells, which suggests that their ability to self-renew is diminished. As well as proliferation data, transient overexpression of prelamin A mutants resulted in structural defects, such as binucleated cells and micronuclei. The colocalisation of emerin with mutant lamin A in the micronuclei suggests that the mutant lamin A was first incorporated into the lamina before subsequently being 'expelled' out of the parent nuclei. It seems likely that complications during mitosis may cause these defects. Recent reports from two groups have elucidated the defects associated with HGPS during mitosis (Cao *et al.*, 2007; Dechat *et al.*, 2007).

Progerin perturbs the cell cycle at multiple stages. Whilst lamin A solubilises freely during mitosis, progerin assembled into insoluble cytoplasmic aggregates and associated with membrane-like network structures, as evidenced by its FLIP (fluorescence loss in photobleaching) data and its colocalisation with emerin and lamin B (Cao *et al.*, 2007; Dechat *et al.*, 2007). Progerin also caused an increase in lagging chromosomes at anaphase and this was reflected by the increased time the cells spent in late mitosis. This delay in resolving lagging chromosomes also delays cytokinesis and the targeting of nuclear envelope components to daughter cell nuclei in late telophase/early G1, which in turn may cause binucleated cells (Cao *et al.*, 2007; Dechat *et al.*, 2007). It was also suggested that an absence of phosphorylated Rb in highly lobulated HGPS cells might be due to the inhibition of cyclin D-cdk 4,6 by $p16^{INK4A}$, which is an attractive hypothesis as $p16^{INK4A}$ expression limits the regenerative capacity, as described previously. Therefore, combined with mitotic defects, this will invariably drive cells towards either apoptosis or senescence (Cox and Faragher, 2007). The potential consequence of this is damage to the microenvironment, which may then contribute to age-related decline in tissue function (van Zant and Liang, 2003; Campisi, 2005, Carlson and Conboy, 2007). The current work showed reduced levels of Rb phosphorylated at serine 807/811 in DP9 cells transfected with prelamin A R644C constructs, and may reflect the induction of a cell cycle arrest, resulting in loss of phosphorylated Rb. Intimately linked to this, the role of LAP2α in the self-renewal of adult stem cells also requires further investigation, as previous reports have shown that lamin A/C-LAP2α-Rb complexes exist in the nucleoplasm and act to

control cell cycle progression (Dechat *et al.*, 2000a; Markiewicz *et al.*, 2002b; Dorner *et al.*, 2006; Pekovic *et al.*, 2007). The current report showed that LAP2α distribution is not affected by either premature aging mutant at the confocal microscopic level (Figure 12). Previous studies have shown that overexpression of lamin mutants can cause LAP2α mislocalisation into intranuclear aggregates, however, there were no such observations here (Dechat *et al.*, 2000a; Markiewicz *et al.*, 2002b). This may be accounted for by the type of construct used. Previous studies have used lamin B1 mutations, and since A-type lamins are dependent on functional B-type lamins for nuclear lamina incorporation, then the intranuclear aggregates formed by these mutations have disrupted endogenous lamin A/C and caused the redistribution of LAP2α into these aggregates (Dyer *et al.*, 1999; Dechat *et al.*, 2000a; Izumi *et al.*, 2000; Markiewicz *et al.*, 2002b). As no obvious effect was seen here, further *in vitro* binding assays might clarify the nature of the LAΔ50/LA(R644C)-LAP2α interaction, whilst it might be of note to observe its localisation with serial passage and perhaps its ability to maintain lamin binding with passaging.

Prelamin A inhibits the differentiation potential of adult stem cells

The current work shows that stable overexpression of lamin A and two mutants associated with premature aging causes the inhibition of multipotent adult stem cells to differentiate *in vitro*. Before discussing how this might occur in the context of this and other work, there are some noteworthy points regarding these results. Firstly, that the use of a different adipogenic medium may affect the ability of adult stem cells to accumulate intracellular lipids (Figure 18 and 19). Although this has no significant bearing on the validity of the results obtained with a different adipogenic medium, it does suggest that the *in vitro* medium is crucially important and serves as a potential warning for future work and for other reports. Indeed, it has been shown that the percentage of nuclear abnormalities found in AD-EDMD fibroblasts vary significantly depending on the culture medium used (Holt *et al.*, 2006). Taken together, these results suggest that *in vitro* results may not always accurately represent the *in vivo* situation. The difference between the two adipogenic

media was rabbit serum and the concentration of dexamethasone, which was ten times less in the medium with rabbit serum (Jahoda *et al.*, 2003; Boguslavsky *et al.*, 2006). Whether it was the presence/absence of the rabbit serum or concentration of dexamethasone that affected lipid accumulation remains to be seen. The current results are supported by previous data showing that overexpression of lamin mutations causes the inhibition of differentiation in satellite cells and preadipocytes (Favreau *et al.*, 2004; Markiewicz *et al.*, 2005; Boguslavsky *et al.*, 2006; Frock *et al.*, 2006). However, these results are directly contradicted by one paper suggesting that overexpression of both wild type lamin A and lamin A harboring FPLD mutations had no effect on the ability of 3T3-L1 cells to differentiate (Kudlow *et al.*, 2005). During the differentiation assays this group removed the selective agent against the expression plasmid (Kudlow *et al.*, 2005). However, the counter argument against these findings is that differentiation assays were carried out in multiple clonal populations of 3T3-L1 cells, obtaining similar results with these and an MEF model (Boguslavsky *et al.*, 2006). To add further transparency to these results, it might be prudent to examine whether after six days in growth medium stably transfected DP9 cells still express FLAG-prelamin A, Δ50 or R644C to the same extent as when grown for six days in the presence of the selection agent, hygromycin B. If they do, then the cells could be subjected to an adipogenic medium for six days without the presence hygromycin B, which would then clarify if the presence or absence of the antibiotic plays any importance in the differentiation process. More importantly, the immediate questions that need to be addressed then is why overexpression of both wild prelamin A and prelamin A harboring premature aging mutants results in the inhibition of adult stem cell differentiation, whether they work by the same mechanism and what is the likely effect *in vivo* for HGPS patients.

Adipogenesis is initiated by the rapid and transient increase of two transcription factors, C/EBP-β and -δ, which are members of the bZIP family of CCAAT/enhancer binding proteins (C/EBPs) (Darlington, 1998; MacDougald and Mandrup, 2002; Fève, 2005; Rosen, 2005; Ailhaud, 2006; Bengoechea-Alonso and Ericsson, 2007). This is followed rapidly by the expression of C/EBPα, and by the nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ) (Spiegelman *et al.*, 1997). These two factors

remain elevated for the rest of the differentiation process activating their own and the other's expression (Rosen *et al.*, 2002). Furthermore, the helix-loop-helix (HLH) factor SREBP1c has been shown to be highly expressed in adipocytes, and to promote differentiation when over-expressed (Tontonoz *et al.*, 1993; Kim and Spiegelman, 1996; Brown and Goldstein, 1997; Kim *et al.*, 1998). This may occur in part through direct actions of SREBP1c on target promoters including that of PPARγ and also indirectly by inducing the generation of a ligand for PPARγ (Fajas *et al.*, 1999). It has been suggested that the lipodystrophy associated with HGPS may be explained by comparisons to another laminopathy that shows adipose tissue defects, FPLD. Mutations found in FPLD are located in the C-terminal near a solvent-accessible area of the immunoglobulin-like fold, suggesting that these mutations may alter an interaction with a specific binding partner (Dhe-Paganon *et al.*, 2002; Krimm *et al.*, 2002). MADA and atypical WS patients also have an associated lipodystrophy, and it has been shown that a percentage of cells from these patients show an accumulation of prelamin A (Capanni *et al.*, 2005). This is important because it has been shown that SREBP1c binds to prelamin A, but not mature lamin A or C *in vivo* (Capanni *et al.*, 2005). Therefore, a mechanistic basis for the pathology of lipodystrophy found in FPLD, MADA and atypical WS is suggested whereby activation of SREBP1c causes its subsequent translocatation to the nucleus, but with accumulation of prelamin A at the nuclear envelope its causes the sequestration of SREBP1c away from the nuclear interior, in turn impairing the PPARγ-dependent adipogenesis (Brown and Goldstein, 1997; Capanni *et al.*, 2005; Filesi *et al.*, 2005; Maraldi *et al.*, 2006a,b; Maraldi *et al.*, 2007a,b). This mechanism for disease pathogenesis is an attractive proposal for FPLD and indeed for the lipodystrophy associated with HGPS, however, at present there is no other evidence that the accumulation of prelamin A affects lamin binding partners of other tissues affected in HGPS, and thus does not serve as a general theorem to explain the far more drastic phenotype of HGPS as compared to FPLD. Cell lines from other tissues affected by HGPS might be examined in a yeast two hybrid screen with the C-terminal of progerin as bait, similar to previous work that first identified SREBP1c as a lamin A binding partner (Lloyd *et al.*, 2002).

In contrast to this hypothesis, the involvement of Rb in adipogenesis and myogenesis implicates a different pathogenic mechanism for cellular differentiation defects in laminopathies. Terminal differentiation requires the permanent withdrawal from the cell cycle and the expression of specific genes. Early in adipogenesis, Rb is hyperphosphorylated, corresponding to the period of clonal expansion, and soon thereafter it becomes hypophosphorylated, accumulating to prevent reentry into the cell cycle and promote terminal differentiation (Puigserver *et al.*, 1998; Classon *et al.*, 2000). Heterodimeric cyclin and cyclin-dependent kinases (CDKs) complexes phosphorylate RB, allowing G1-S phase transition, and cyclin-dependent kinase inhibitors (CKIs) modulate CDK activity. At the onset of adipogenesis, as well as activating adipogenic-specific transcription, C/EBP-β and –δ proteins stimulate CDK inhibitor p21 expression by directly binding its the promoter region, leading to the inhibition of CDK-mediated retinoblastoma protein (Rb) phosphorylation, resulting in cell cycle exit (Chen *et al.*, 1996; Timchenko *et al.*, 1996; Puigserver *et al.*, 1998; Johnson, 2005). Rb can also inhibit adipogenesis via recruiting HDAC to PPARγ promoters, helping to regulate temporal expression of adipogenic genes and also acts as a molecular switch, determining white versus brown adipose tissue formation (Fajas *et al.*, 2002; Hansen *et al.*, 2004).

Rb is also critical for myogenesis, during which nucleoskeleton remodeling is required for effective adult muscle satellite stem cell differentiation (Novitch *et al.*, 1996; Novitch *et al.*, 1999; Mariappan and Parnaik 2005; Markiewicz *et al.*, 2005). A previous report has shown that lamin A and lamin C is relocated from the nucleoplasm to the nuclear periphery during myogenesis (Markiewicz *et al.*, 2005). The authors suggest that expression of mutant lamin A inhibits C2C12 differentiation because it may maintain or enhance lamin A/C-LAP2α associations, therefore causing inhibition of a fully functional Rb that is required for entry into a postmitotic state (Markiewicz *et al.*, 2005). Lamin A/C has been shown to interact with chromatin at the periphery and the nucleoplasm, thus the consequence of remodeling the nuclear lamina during differentiation may lead to the relocation of specific chromatin to the periphery of the nucleus, a location associated with gene silencing, and thus silencing genes necessary to maintain the differentiated cell state (Moir *et al.*, 1995; Goldman *et al.*, 2002). Therefore, applying this to the current work

suggests that premature aging mutants inhibit remodeling of the nucleoskeleton in adult stem cells, causing a failure to differentiate. In this study, transient transfection revealed no obvious LAP2α defects, although further work might seek to elucidate properties of endogenous lamin A/C, lamins B1/B2 and LAP2α during adipogenic differentiation, as it has previously been shown that overexpression of LAP2α causes cell cycle exit and enhances differentiation by binding hypophosphorylated Rb (Dorner *et al.*, 2006). Therefore, it could be suggested that premature aging mutants may have a dominant negative effect in this lamin A/C-LAP2α-Rb complex, leading to inefficient cell cycle arrest required for differentiation. Moreover, if this was the case it may help to explain the senescent phenotype as the lamin A/C-LAP2α-Rb complex is required for correct progression into S-phase, and if this complex is affected by premature aging mutants then it is possible that cells prematurely enter into S-phase, with a concomitant accumulation of DNA damage, resulting in eventual cellular senescence. Taken together, the finding that premature aging mutants cause the inhibition of adult stem cell differentiation suggests that ineffective regeneration of tissue in HGPS patients may be an underlying and important cause of the disease pathogenesis.

A recent paper has shown that the farnesylated and carboxymethylated human prelamin A C-terminal peptide that is normally cleaved by Zmpste24 in the last step in prelamin A processing induces mitotic arrest, muscle-specific gene expression, and myotube formation, thereby acting as a signal for differentiation (Brodsky *et al.*, 2007). Furthermore, it is involved in physically separating DNA into distinct globular chromatin domains (Brodsky *et al.*, 2007). Taken together, these results suggest for the first time that the prelamin A processing by-product has a function within the nucleus and may help to explain the conserved mechanism of prelamin processing steps. Moreover, lamin A mutants that cannot undergo this last processing step cannot release the peptide and therefore are inhibited to differentiate, which is in line with the work presented here as both LAΔ50 and LA(R644C) are expected to remain farnesylated and carboxymethylated without the final Zmpste24 cleavage step (Brodsky *et al.*, 2007).

Pathogenic mechanisms for laminopathies: potential role of adult stem cells

In light of previous reports that have shown that there exists a population of human multipotent adipose-derived stem cells (hMADs), it may be suggested based on the data presented here that the lipodystrophy associated with HGPS may be attributed to the failure of these hMADs to effectively regenerate new tissue (Zuk *et al.*, 2001; Zuk *et al.*, 2002; Rodriguez *et al.*, 2004). Secondly, the results may be applied to the alopecia associated with HGPS patients to suggest that hair follicle stem cells fail to regenerate in this highly cycling appendage. Hair follicle development is not affected during development and alopecia presents during early childhood in patients with HGPS (Sarkar and Shinton, 2001). In line with this, A-type lamins are developmentally regulated and are most highly expressed in differentiated cells (Hutchison and Worman, 2004). Therefore, the expression of mutant lamin A coincides with hair follicle decline, which is associated with adult stem cell failure. Although this report has shown that overexpression of A-type lamin mutants inhibits the growth potential of adult stem cells, and possibly promotes subsequent entry into apoptosis and/or senescence, it could be suggested that the greater factor in tissue decline is likely to be made by the failure of adult stem cells to regenerate rather than to proliferate, as adult stem cells rarely undergo mitosis *in vivo* (Ma *et al.*, 2004; Nystul and Spradling, 2006; Waters *et al.*, 2007).

The tissues affected in laminopathies are mesodermal in origin (Hutchison and Worman, 2004). Therefore, it is plausible that adult stem cell niches may be affected by the mutations found in other laminopathies at the level of differentiation and proliferation, and may exhibit phenotypes that a resultant from stem cell failure to regenerate new tissues (Wagers and Weissman, 2004; Nystul and Spradling, 2006). However, as there is no specific genotype-phenotype relationship of the locations of the mutation within lamin A/C, i.e. not all may affect adult stem cells, then the disease phenotypes are likely to arise via a number of disease mechanisms, which may be attributed to defects in the main roles of lamins, namely, genomic caretaker, tensegrity element, transcriptional regulator and chromatin scaffold (Broers *et al.*, 2006; Verstraeten *et al.*, 2007).

The study of HGPS has been used to draw comparisons to the normal aging process. HGPS fibroblasts show a loss of peripheral heterochromatin and a loss of epigenetic modification regulation (Goldman *et al.*, 2004; Shumaker *et al.*, 2006). This loss of epigenetic regulation is comparable to nuclei from aged individuals, which also show nuclear abnormalities that are characteristic of HGPS cells, suggesting a link between premature and normal aging (Scaffidi and Misteli, 2005). Further studies revealed that the cryptic splice site that is activated in HGPS via the G608G mutation is actually activated in fibroblasts from normal control fibroblasts (Scaffidi and Misteli, 2006). It was also shown that the amount of progerin did not accumulate over time with serial passaging or between young and old individuals, in contrast to HGPS fibroblasts (Goldman *et al.*, 2004; Scaffidi and Misteli, 2006). The implication of this is that there may be a minimum progerin tolerance, and this prolonged exposure over time may contribute to the normal aging process (Scaffidi and Misteli, 2006).

Various reports have shown that it is the farnesylation of progerin that makes it fundamentally toxic to cells (Capell *et al.*, 2005: Glynn and Glover, 2005; Gruber *et al.*, 2005; Mallampalli *et al.*, 2005: Toth *et al.*, 2005; Yang *et al.*, 2005). Previous work with *Zmpste24*-null mice drew the same conclusion, and mice were bred to reduce the prelamin A production in half by creating a *Zmpste24*$^{-/-}$ *LMNA*$^{+/-}$ genotype, which in turn reduced the number of cells with nuclear defects, unequivocally implicating farnesyl prelamin A as a toxic agent (Bergo *et al.*, 2002; Pendas *et al.*, 2002; Fong *et al.*, 2004). Since then several reports have sought to reverse the cellular phenotype in the hope of eventual holistic disease amelioration. The first approach is based on blocking the farnesylation of progerin by treating cells with a farnesyl transferase inhibitor (FTI), which has been shown to reduce nuclear abnormalities by targeting progerin away from the nuclear envelope and into nucleoplasmic aggregates, and also improved whole organism phenotypes in two mouse models (Capell *et al.*, 2005: Glynn and Glover, 2005; Mallampalli *et al.*, 2005; Toth *et al.*, 2005; Yang *et al.*, 2005; Fong *et al.*, 2006; Yang *et al.*, 2006). Despite the doubts that still remain as to the effect that prolonged FTI exposure will have on cells, clinical trials with HGPS patients began in May 2007 and represents a great and rapid success since *LMNA* was first identified as the causative gene in 2003

(Young *et al.*, 2005; Meta *et al.*, 2006; Rusinol and Sinensky, 2006; Young *et al.*, 2006; Stewart *et al.*, 2007).

As HGPS fibroblasts show both total and focal loss of peripheral heterochromatin, attempts have been made to simultaneously block prelamin A farnesylation and to rescue heterochromatin formation (Columbaro *et al.*, 2005; Maraldi *et al.*, 2006a,b; Maraldi *et al.*, 2007a,b). The combination of mevinolin, to block farnesylation, and trichostatin A, which inhibits histone deacetylase causing decondensation of chromatin domains, dramatically lowers progerin levels leading to rescue of heterochromatin organisation. Furthermore, intranuclear lamin A associated with splicing speckles was restored upon this dual treatment, which resulted in the reorganisation of transcripts in HGPS fibroblasts (Jagatheesan *et al.*, 1999; Columbaro *et al.*, 2005).

In an alternative to drug treatment, the use of morpholino antisense oligonucleotides, designed to target the cryptic splice site activated in the *de novo* G608G mutation leads to a reversal of the cellular phenotype normally found in HGPS patients (Scaffidi and Misteli, 2005). Moreover, the use of the morpholino caused the correct expression of several misregulated genes discovered by microarray experiments (Ly *et al.*, 2000; Csoka *et al.*, 2004a; Scaffidi and Misteli, 2005).

Use of short hairpin RNAi (shRNAi) designed to target the progerin mRNA for degradation also led to reduced progerin levels, reduced senescent staining, and significantly improved the replicative potential and nuclear morphology of HGPS fibroblasts (Huang *et al.*, 2005). However, as yet there is no effective delivery mechanism that may allow shRNAs to reach the numerous tissues affected in HGPS, especially the arterial wall cells, which may play an important role in the pathogenesis of the accelerated atherosclerosis (Huang *et al.*, 2005; McClintock *et al.*, 2006).

In light of the current work, future stem cell therapies should also be considered for the treatment of HGPS. This could be by replenishment with younger stem cells from healthy individuals. A potential problem to this method is underlined by evidence that an aged

microenvironment has a dominant negative effect on satellite stem cells, rather than intrinsic stem cell aging (Conboy *et al.*, 2005; Rando, 2006; Rossi *et al.*, 2007). Therefore, transplantation with healthy stem cells may be a futile exercise as the microenvironment of HGPS patients is already prematurely aged.

One possible avenue that may be explored for future stem cells therapies to treat HGPS is nuclear reprogramming, or de-differentiation, to generate an ESC-like cell from a somatic stem cell (Gurdon *et al.*, 1958; Tada *et al.*, 2001; Takashi and Yamanaka, 2006). The generation of new stem cells could be used to replenish the tissues in HGPS patients. Together with a low success rate for this method, the problem for HGPS patients would be finding a suitable somatic stem cell to reprogramme. Moreover, choice of a wrong one could actually deteriorate the tissue (Rosenthal, 2005). However, correction of nuclei from HGPS patients by drug treatment may reverse their cellular phenotype before initiating reprogramming.

Despite potential problems, stem cell therapies are currently being employed to try and treat patients with a range of diseases, including vascular disease, myocardial dysfunction and *osteogenesis imperfecta* (Ho *et al.*, 2005; Mauney *et al.*, 2005; Bobis *et al.*, 2006; Devogelaer and Coppin, 2006; Giordano *et al.*, 2007; Pichinich *et al.*, 2007). HGPS patients are now being treated with FTIs, yet there remains the possibility that this treatment will not reverse all cellular and ultimately tissue defects, and as such, stem cell therapies directed towards treating HGPS might represent a realistic alternative.

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