

**THE EFFECT OF FARNESYLATED PRELAMIN A  
ACCUMULATION ON NUCLEAR MORPHOLOGY AND  
FUNCTION**

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**To my always supportive family Alison, Nicholas and Jennifer**  
**Goulbourne**

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## ABSTRACT

### THE EFFECT OF FARNESYLATED PRELAMIN A ACCUMULATION ON NUCLEAR MORPHOLOGY AND FUNCTION

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Failure to process prelamins A, by the enzyme ZMPSTE24, leads to the build up of farnesylated prelamins A, which has been implicated in causing the symptoms experienced in laminopathies and HIV therapy. A common feature to these conditions is the development of an irregular nuclear boundary, often including deep invaginations that form a nucleoplasmic reticulum. Additionally, dysregulated lipid synthesis is frequently associated with improper lamin A processing and I set out to address the molecular mechanisms behind these two features that could explain lipodystrophy experienced in patients. By using siRNA targeted against *Zmpste24* I utilised an array of biochemical, molecular and imaging techniques to uncover a mechanism that leads to the production of a nucleoplasmic reticulum that was dependent on both the farnesylated tail of prelamins A and the phosphatidylcholine synthesising enzyme CCT $\alpha$ . The morphology of this structure consisted of an invagination of both the inner and outer nuclear membranes with a cytoplasmic core or just invagination of the inner nuclear membrane. Serial section dual axis electron tomography provided a new insight into the ultrastructural changes at the nuclear periphery that revealed novel structural features. The dysregulation of lipid synthesis was assessed by investigating the effects farnesylated prelamins A has on the distribution and dynamics of the transcription factor SREBP-1 and assessment of the downstream consequences this has on its targets that regulate adipocyte differentiation potential. Finally, the metabolomic profile of an HIV protease inhibitor that leads to prelamins A build up was generated and revealed increases in lipolysis, glycolysis and mediators of inflammation. The research presented offers a new insight into the development of a convoluted nuclear boundary and nucleoplasmic reticulum, in the context of lamin A mutants and how dysregulated lipid synthesis, caused by farnesylated prelamins A, leads to lipodystrophy.

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**ABBREVIATIONS**

|        |                                                                                  |
|--------|----------------------------------------------------------------------------------|
| µg     | microgram                                                                        |
| µm     | micrometer                                                                       |
| µM     | micromolar                                                                       |
| CAAX   | Motif cysteine (C), aliphatic (A), aliphatic (A), any of several amino acids (X) |
| ChIP   | Chromatin Immunoprecipitation                                                    |
| CLSM   | confocal laser scanning microscopy                                               |
| DMSO   | dimethylsulphoxide                                                               |
| DNA    | deoxyribonucleic acid                                                            |
| dNTP   | deoxynucleotide triphosphate                                                     |
| EM     | electron microscopy                                                              |
| EMT    | electron microscopy tomography                                                   |
| ER     | endoplasmic reticulum                                                            |
| FCS    | fetal calf serum                                                                 |
| FTI    | farnesyl transferase inhibitor                                                   |
| HEPES  | (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid])                       |
| HIV    | human immunodeficiency virus                                                     |
| HIV-PI | HIV-protease inhibitor                                                           |
| ICMT   | isoprenyl cysteine methyl transferase                                            |
| IMS    | inter-membrane space                                                             |
| INM    | inner nuclear membrane                                                           |

|          |                                                  |
|----------|--------------------------------------------------|
| M        | molar                                            |
| mg       | milligrams                                       |
| mM       | millimolar                                       |
| NE       | nuclear envelope                                 |
| nm       | nanometre                                        |
| nM       | nanomolar                                        |
| NR       | nucleoplasmic reticulum                          |
| NPC      | nuclear pore complex                             |
| ONM      | outer nuclear membrane                           |
| PBS      | phosphate buffered saline                        |
| PFA      | paraformaldehyde                                 |
| PMSF     | phenylmethanesulphonylfluoride                   |
| PNS      | post-nuclear supernatant                         |
| qRT-PCR  | quantitative real time polymerase chain reaction |
| Rce1     | ras converting enzyme                            |
| RNA      | ribonucleic acid                                 |
| siRNA    | small inhibitory RNA                             |
| SREBP-1  | sterol regulatory element binding protein-1      |
| TX-100   | Triton X-100                                     |
| ZMPSTE24 | mammalian zinc metalloprotease Ste24p            |

# CHAPTER 1

## GENERAL INTRODUCTION

### 1.1 INTRODUCTION

The development of a nuclear envelope around genetic material in eukaryotes over two billion years ago lead to a greater degree of regulating gene expression and organising chromatin in the three dimensional environment of the nucleus. This was further enhanced in metazoans by the evolution of a proteinacious matrix underlying the inner nuclear membrane referred to as the nuclear lamina. Lamin A, which is encoded by the *LMNA* gene, is perhaps the most studied of all the components of the lamina and has been implicated in a diverse range of diseases referred to as nuclear laminopathies. The maturation pathway of lamin A from prelamin A is well documented but its exact function remains speculative. However, it is evident that improper processing of the precursor leads to laminopathies that display a wide spectrum of symptoms that affects different tissues.

One of the tissues affected is adipose tissue and the development of lipodystrophy syndrome is often a symptom of certain laminopathies as well as for patients on Highly Active Anti-retroviral Therapy to combat HIV replication. Lipodystrophy syndrome may develop partly as a result of prelamin A interference in adipogenesis but the exact mechanisms remain obscure. Additionally an aberrant nuclear architecture is often a sign of farnesylated prelamin A accumulation in the nuclei of cells from patients with

laminopathies, aged tissues and after various drug treatments. This invaginated state of the nuclear membrane is also present in normal cells and is termed a nucleoplasmic reticulum (NR). The exact role, mechanism of development and function of the NR, in the context of prelamin A accumulation, remains unknown. The structure and function of various components of nuclear architecture and how improper processing and structure of these diverse proteins lead to tissue-specific alterations is reviewed in this introduction before going on to address morphological and functional consequences of acute farnesylated prelamin A accumulation.

## **1.2 NUCLEAR ARCHITECTURE AND FUNCTION**

The nuclear boundary or nuclear envelope (NE) is a unique structure comprising two phospholipid bilayers (the inner nuclear membrane [INM] and outer nuclear membrane [ONM]), a 30-50 nm inter-membrane space (IMS) that is continuous with the lumen of the endoplasmic reticulum and a meshwork or lamina rich in intermediate filament proteins, which underlies the INM (Prunuske and Ullman, 2006). The NE is pierced at intervals by nuclear pores, highly structured focal continuities between the two membranes. The NE is not merely a fence between nucleoplasm and cytoplasm; it also contributes to nuclear structural integrity, selective bidirectional transport of ions and macromolecular cargo, regulation of gene expression, mechano-transduction and acts as a platform for signalling. Underlying the INM is the nuclear lamina, which over the last few decades has received increased research and understanding of the complex roles this proteinacious layer provides.

### 1.2.1 The Nuclear lamins

Humans and other mammals have three lamin genes (*LMNA*, *LMNB1* and *LMNB2*) that encode seven alternatively-spliced isoforms. A-type lamins include lamin A, A $\Delta$ 10, C and C2, which are all encoded by the *LMNA* gene (Fisher et al., 1986; Furukawa et al., 1994; Machiels et al., 1996; McKeon et al., 1986). The B-type lamins are lamin B1 and lamin B2-B3 encoded by genes *LMNB1* and *LMNB2* respectively (Biamonti et al., 1992; Furukawa and Hotta, 1993; Pollard et al., 1990; Stuurman et al., 1996). Lamins A, A $\Delta$ 10 and C are developmentally regulated and expressed principally in differentiated cells, while every vertebrate cell expresses at least one type of lamin B (Machiels et al., 1996; Rober et al., 1989). Only lamins C2 and B3 are expressed in germ-line cells (Alsheimer et al., 1999; Furukawa and Hotta, 1993; Furukawa et al., 1994).

During the evolution of metazoans the number and complexity of lamin genes increased. *Caenorhabditis elegans* has one lamin gene (*lmn-1*) that is expressed in all cells except for mature sperm (Liu et al., 2000). *Drosophila melanogaster* has one lamin that is expressed in essentially all cells throughout development (*Dm $\alpha$* ) and its only other lamin gene (*C*) is expressed late in embryonic development and in differentiated cells (Bossie and Sanders, 1993; Gruenbaum et al., 1988). Nuclear lamins are not encoded in the genomes of *Arabidopsis* and yeast, which suggests that these proteins evolved in animal cells during the transition from a closed to an open mitosis (Cohen et al., 2001).

Heptad repeats are found along the central rod domain of lamins, which are characteristic of  $\alpha$ -helical proteins (McKeon et al., 1986; Stuurman et al., 1998). These structures allow

lamin proteins to interact and form coiled-coil dimers, which is the basic structural unit of lamin assembly. The dimers then laterally associate via their rod domains with other dimers to form higher order structures that are essential to lamin polymerization (Stuurman et al., 1998). Conservation of the lamin rod domain with cytoplasmic intermediate filaments is very high except the nuclear lamins possess an additional six heptads and have no stutter residues that interrupt heptad repeats (Parry et al., 1986). Flanking the rod domain are non- $\alpha$ -helical domains at the amino and carboxy terminus, which are variable between the different lamins and are critical to proper lamin assembly (Moir et al., 1991).

A nuclear localization signal (NLS) is present in the carboxyl-terminal domain and enables nuclear import of the lamins. The lamins (apart from lamin C) also have a C-terminal CaaX motif that undergoes farnesylation by a farnesyltransferase (FTase) or geranylgeranylation by geranylgeranyl transferase I (GGTaseI) before it undergoes C terminal cleavage of the last three amino acids by either Ras Converting Enzyme 1 (RCE1) or Zinc metalloproteinase STE24 (ZMPSTE24). After this it undergoes methylation by Isoprenylcysteine Carboxyl Methyl Transferase (ICMT), which has been suggested to be important for targeting lamins to the nuclear envelope (Firmbach-Kraft and Stick, 1993; Hofemeister et al., 2000; Holtz et al., 1989; Kitten and Nigg, 1991). 74 kDa prelamins undergo an additional post-translational modification by ZMPSTE24, whereby the fifteen C-terminal amino acids are cleaved off, including the isoprenylated cysteine residue, yielding the 72kDa mature lamin A (Weber et al., 1989). It is interesting to note that synthesis of non-isoprenylated prelamins and direct production of mature



lamin A in mice still results in targeting of the protein to the INM (Coffinier et al., 2010 ; Davies et al. 2010), making the functional significance of the isoprenylation speculative.

### **1.2.2 Lamin-Binding proteins and their function**

The nuclear lamina is critical for the assembly, structure, shape and mechanical stability of the metazoan nucleus as well as regulating chromatin organization, gene expression and influencing multiple signalling pathways (Dechat et al., 2008; Gruenbaum et al., 2005). This requires many biochemical interactions between lamins, chromatin and a variety of partners including regulatory proteins responsive to external and intrinsic signals. The partners that can bind to the lamina include the lamina-associated polypeptides (LAPs), which are a group of nuclear proteins that co-fractionate with lamins and require high concentrations of monovalent salts and non-ionic detergents for their extraction from nuclei (Foisner and Gerace, 1993; Senior and Gerace, 1988).

The first LAPs identified were integral INM proteins and named LAP1 (Martin et al., 1995), LAP2 (Berger et al., 1996; Furukawa et al., 1995; Harris et al., 1994) and LBR (Worman et al., 1990). It was found that LAPs have widely differing and differentially regulated affinities for lamins, which influenced their biochemical extraction. Some LAPs bind to lamins very stably at the NE or interior while others associate more transiently as a means of regulating activity e.g. PCNA (Shumaker et al., 2008), c-Fos (Gonzalez et al., 2008), Retinoblastoma (Rb) (Johnson et al., 2004; Pekovic et al., 2007) and Oct-1 (Malhas et al., 2009).

Some of the LAPs contain an LEM (LAP2, Emerin, Man) domain that enables interaction with a conserved metazoan chromatin protein called Barrier to Autointegration Factor (BAF), which enables a bridge to form between the lamins and chromatin (Cai et al., 2001; Cai et al., 2007; Furukawa, 1999; Lee et al., 2001; Margalit et al., 2007; Shimi et al., 2004; Shumaker et al., 2001). Four LEM genes encoding integral INM proteins have been identified, which includes LAP2 ( $\alpha$ ,  $\beta$  and other isoforms), MAN1 (Lin et al., 2000), emerin and LEM2/NET25 (Brachner et al., 2005; Chen et al., 2006; Schirmer et al., 2005; Ulbert et al., 2006). Some of the LEM domain proteins have additional domains that can also directly interact with DNA or other chromatin proteins e.g. LAP2 (Cai et al., 2001), MAN1 (Caputo et al., 2006) and LEM2 (Caputo et al., 2006). The LEM-containing proteins can bind either A or B type lamins (Brachner et al., 2005; Clements et al., 2000; Lee et al., 2001; Sakaki et al., 2001), however the lamins have also been shown to directly interact with DNA (Luderus et al., 1992; Shoeman and Traub, 1990; Stierle et al., 2003) and histones (Mattout et al., 2007; Taniura et al., 1995).

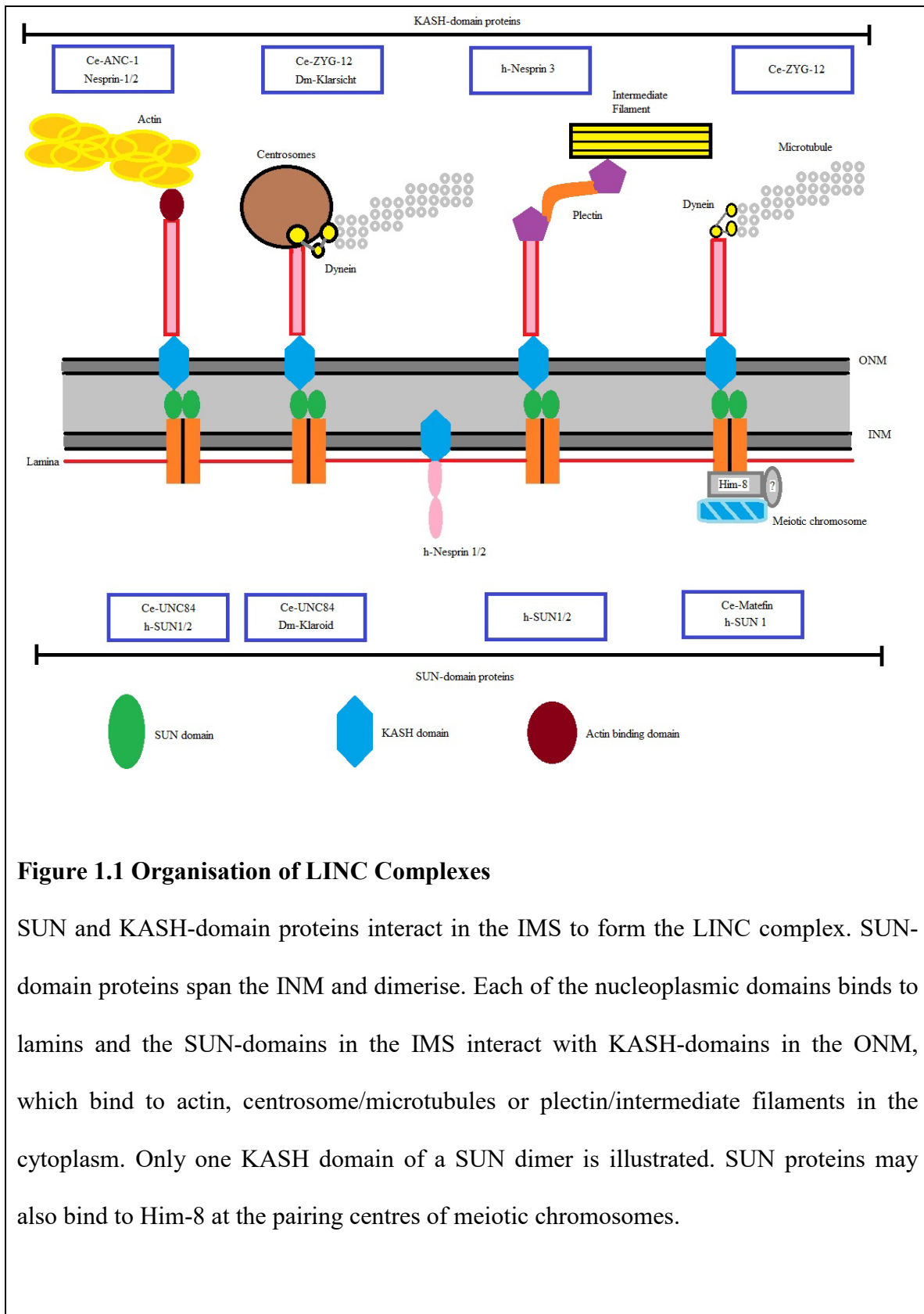
There appears to be a certain level of redundancy between LEM-containing proteins as single functional inactivation does not grossly disrupt chromatin organization. However, double knock down of two LEM proteins (Ce-emerin and Ce-lem2) resulted in embryonic lethality at the 100 cell stage, which is when embryonic gene expression usually begins in *C. elegans* (Liu et al., 2003). The nuclear phenotype consisted of aneuploidy, defective chromatin organization, nuclear structure defects and failure of nuclear assembly after mitosis. Clearly the lamina plays a critical role in genome organization and this was highlighted by the analysis of methylated DNA in cultured

*Drosophila* cells expressing lamin B1 fused to DNA methyltransferase (Dam), which revealed approximately 500 genes in close proximity with the lamina (Pickersgill et al., 2006).

The linker of nucleoskeleton and cytoskeleton (LINC) complex consists of a series of membrane proteins that span the NE and mechanically interlink the nucleoskeleton with the cytoskeleton (Figure 1.1) (Crisp et al., 2006). LINC complexes allow cytoplasmic-motor driven movement of an entire nucleus to new positions within the cell and movement of NE-tethered chromosomes to new positions along the nuclear periphery (Starr, 2009; Tzur et al., 2006; Wilhelmsen et al., 2006). Additionally, gene regulatory responses to external forces can be transmitted via mechanotransduction in which a force is transmitted along the cytoskeleton across the LINC complex and onto the nucleoskeleton where it may affect gene expression (Holaska, 2008).

The simplest composition of a LINC complex consists of lamin binding SUN (Sad1/UNC-84) domains located on the INM that interact in the IMS with KASH (Klarsicht/ANC-1/Syne-1 homology) domain proteins anchored in the ONM (Crisp et al., 2006; Haque et al., 2006; Hodzic et al., 2004; Padmakumar et al., 2005). KASH proteins bind either directly or indirectly to cytoplasmic actin, microtubules, centrosomes or intermediate filaments (McGee et al., 2006; Padmakumar et al., 2004; Roux et al., 2009; Wilhelmsen et al., 2005; Zhang et al., 2005; Zhen et al., 2002). LINC complexes are critical for determining the regular space of the IMS between the INM and ONM (Crisp et al., 2006; Zhang et al., 2007), positioning of NPCs (Liu et al., 2007), controlling

nuclear size and organization of nuclear architecture (Luke et al., 2008). It still remains to be determined how exactly the functions and interactions of SUN-KASH proteins are regulated (Starr, 2009). However, regulation may be performed by proteins located in the IMS, for example Torsin A, which is an AAA+ ATPase, located in the IMS and which is required to localize nesprin (Nery et al., 2008; Vander Heyden et al., 2009).



**Figure 1.1 Organisation of LINC Complexes**

SUN and KASH-domain proteins interact in the IMS to form the LINC complex. SUN-domain proteins span the INM and dimerise. Each of the nucleoplasmic domains binds to lamins and the SUN-domains in the IMS interact with KASH-domains in the ONM, which bind to actin, centrosome/microtubules or plectin/intermediate filaments in the cytoplasm. Only one KASH domain of a SUN dimer is illustrated. SUN proteins may also bind to Him-8 at the pairing centres of meiotic chromosomes.

The interaction of LAPs with the nuclear lamins also provides regulation of signalling and differentiation. For example, LAP2 $\beta$  binds to lamin B and interacts with transcription regulators, epigenetic modifiers and contributes to transcriptional repression (Nili et al., 2001; Somech et al., 2005). MAN1 directly binds to and inhibits receptor regulated SMADs, which controls the BMP and TGF- $\beta$  signalling in early vertebrate development (Cohen et al., 2007; Osada et al., 2003). Emerin has been shown to interact with more than ten partners, which suggests a high degree of biochemical complexity that would rival many cell surface structures. Partners of emerin include; structural proteins, INM proteins, proteins involved in signalling, transcription and mRNA splicing and BAF (Bengtsson and Otto, 2008; Haraguchi et al., 2004; Holaska et al., 2004; Holaska et al., 2003; Holaska et al., 2006; Lee et al., 2001; Mansharamani and Wilson, 2005; Markiewicz et al., 2006; Mislow et al., 2002; Wheeler et al., 2007). The large number of partners that emerin interacts with and their specific combination has been suggested to be regulated by phosphorylation, which may be mediated by different kinases and signalling pathways (Hirano et al., 2005; Luo et al., 2009; Roberts et al., 2006; Schlosser et al., 2006).

LAP2 $\alpha$  lacks a transmembrane domain and localizes to the nuclear interior and binds specifically to lamin A (Dechat et al., 2004; Dechat et al., 2000; Vlcek et al., 2002). This LAP is responsible for targeting and localizing a subset of lamins to the nuclear interior in proliferating cells (Naetar et al., 2007; Naetar et al., 2008). This nucleoplasmic lamin is stable but more dynamic than the lamin located at the periphery of the nucleus, which may be due to different assembly processes or its binding to chromatin (Moir et al., 2000).

Both LAP2 $\alpha$  and lamin A interact with the tumor suppressor Rb (Markiewicz et al., 2002; Ozaki et al., 1994; Pekovic et al., 2007) and repress it activating E2F/Rb target genes (Dorner et al., 2006) as well as controlling Rb mediated proliferation in tissue progenitor stem cells (Naetar et al., 2008). Cells that lack lamin A show destabilisation, proteolytic degradation (Johnson et al., 2004; Nitta et al., 2006; Nitta et al., 2007) and impaired phosphorylation-dependent repressor activity control of Rb (Van Berlo et al., 2005). This highlights the physiological relevance of intranuclear LAP2 $\alpha$ -lamin complexes in Rb regulation.

Other lamin binding proteins include cyclin D3 (Mariappan et al., 2007), transcription factors c-fos (Ivorra et al., 2006), Oct-1 (Imai et al., 1997), SREBP-1 (Capanni et al., 2005; Lloyd et al., 2002) and MOK2 (Dreuillet et al., 2008; Dreuillet et al., 2002), tumor suppressor ING1 (Han et al., 2008), 12 (S)-lipoxygenase (Tang et al., 2000), Protein Kinase C $\alpha$  (Martelli et al., 2002) and JIL-1 Kinase (Bao et al., 2005). Elimination of the interaction between these various proteins with the lamins has a detrimental effect on the physiology of the cell and nuclear function. Lamin B1 sequesters Oct-1 at the NE away from promoters but in lamin b1 hypomorphs genes that respond to oxidative stress and reactive oxygen species are dysregulated (Malhas et al., 2009). C-fos is sequestered at the NE by lamin A (Ivorra et al., 2006) and is activated by ERK1/2, during mitogen activated protein kinase signalling, resulting in the release of c-fos from the periphery facilitating cell proliferation (Gonzalez et al., 2008). Lamin A has also been shown to interact with SREBP-1 (Lloyd et al., 2002) and accumulation of prelamin A has been shown to sequester it at the periphery (Caron et al., 2003) resulting in decreased

adipogenesis that may underlie the mechanism of acquired lipodystrophy syndrome, which is further explored in this thesis.

### **1.2.3 Nuclear pore complexes**

The nuclear pore complex (NPC) is the site at which the INM and ONM fuse, permitting molecular transport across the nuclear membrane barrier. Nuclear imported cargo includes INM proteins and all the proteins found in the nucleoplasm. Exported cargo includes RNA and RNA associated proteins that are assembled into ribosomal subunits or messenger ribonucleoproteins. The NPC must then ensure that these cargoes are retained in their respective nucleoplasmic or cytoplasmic compartments. Additionally it must also prevent the transport of non-specific macromolecules, yet allow the free diffusion of ions, water and sugars. NPCs should also not just be viewed as transporters as certain nucleoporins are being increasingly implicated in regulating gene expression (Capelson et al., 2010; Ikegami and Lieb, 2010; Kalverda et al., 2010; Vaquerizas et al., 2010).

At its most fundamental level the NPC has octagonal symmetry with a diameter between 100-150 nm and a thickness of about 50-70 nm depending on the organism (Wente and Rout 2010). This general appearance seems conserved throughout all eukaryotes. The core of the NPC resides at the point where the INM and ONM fuse and consists of eight spokes that surround a central tube of ~30 nm diameter and ~50 nm long. This tube is the point at which the nucleoplasm and cytoplasm connect and where the exchange of macromolecules occurs. Filaments are attached to the core of the NPC and fill the central



hole as well as emanating into the nucleoplasm and cytoplasm. These filaments form a basket on the nucleoplasmic face of the pore.

The frequency and density of pores varies greatly between different cell and tissue types. *Saccharomyces cerevisiae* has a maximum density of NPCs at S-phase of  $\sim 14.6$  NPCs/ $\mu\text{m}^2$  (Winey et al., 1997) while cultured human HeLa cells contain  $\sim 11$  NPCs/ $\mu\text{m}^2$  (Maul and Deaven, 1977). These NPC frequencies are dwarfed by that of *Xenopus* oocytes, which have more than 50 NPCs/ $\mu\text{m}^2$  (Scheer, 1973). The distribution of the NPCs can be greatly influenced by the nuclear lamina as *Drosophila* lamin *Dm0* mutants show clustered NPCs (Lenz-Bohme et al., 1997), by perturbations in NPC components (Dawson et al., 2009; Doye and Hurt, 1997) or by varying heterochromatin state as a result of different developmental stages (Fawcett and Chemes, 1979). Human lamin mutant expressing cells have also been shown to affect the distribution of NPCs and this has a detrimental effect on nuclear transport of various fluorescently tagged cargoes (Busch et al., 2009).

### 1.3 THE NUCLEOPLASMIC RETICULUM

Initially viewed as a smoothly curved convex surface, this simple view of the NE was challenged by reports of intranuclear membranes (Bourgeois et al., 1979) (Dupuy-Coin et al., 1986; Malhas et al., 2011; Park and De Boni, 1992). Serial section TEM, 3D confocal microscopy and other powerful visualization techniques confirmed that the NE can contain convolutions including deep, branching invaginations (Fricker et al., 1997a; Fricker et al., 1997b; Pliss et al., 2010; Schermelleh et al., 2008; Schneider et al. 2010). It

is only using these 3D imaging techniques that the nucleoplasmic reticulum (NR) is observed, since in 2D it could appear morphologically similar to nucleoplasmic lamin foci.

Morphological comparisons with the endoplasmic reticulum (ER) suggested the name nucleoplasmic reticulum for this widespread intranuclear network (Echevarria et al., 2003b). A NR has now been reported in a wide variety of normal and abnormal cells from the plant and animal kingdoms. Moreover, NR is abundant in many tumour cell types, including brain, breast, kidney, bladder, prostate and ovary (Fischer et al., 2003; Sarkar et al., 2009). Since the NR is neither restricted to specific cell or tissue type, nor dependent upon a specific developmental state, these structures may have a general significance for normal function in eukaryotes (Clubb and Locke, 1998). Furthermore, NR morphology is altered in some pathological cases, suggesting that NR regulatory mechanisms are susceptible to pathological dysregulation. Although the existence of the NR is now widely accepted, its exact roles are still not fully understood. The existence of this structure in the nucleus offers an exciting prospect of the possibility of all of the functions of the peripheral NE being duplicated within the nucleoplasm providing further spatial control within the nucleus.

### **1.3.1 Structural Features**

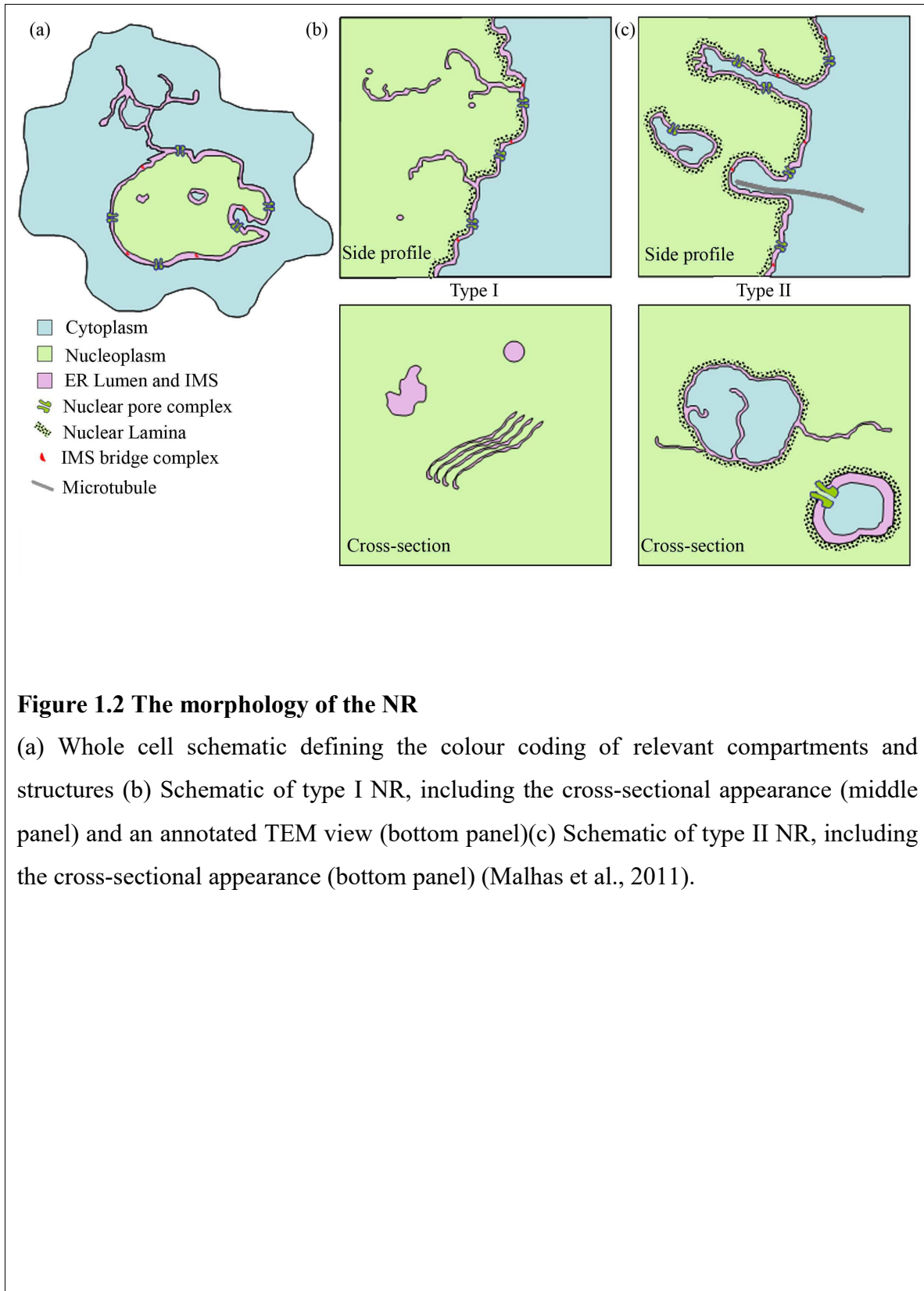
NR invaginations fall into two main classes, type I and type II, depending on whether the ONM is involved. Type I invaginations are those where the INM invaginates into the nucleoplasm, while type II involve the invagination of both the ONM and INM. NR

invaginations may be single or multiple and the two classes may co-exist in the same nucleus (Figure 1.2 and Figure 1.3). Indeed, the original serial section EM study identified type II invaginations with type I branched extensions, like the trunk and branches of a tree, as well as type II invaginations that made two contacts with the NE, (Gant and Wilson, 1997). Scrape-loading cytoplasmic tracers confirmed that the cores of many type II invaginations retain continuity with the cytoplasm. EM cross-sections show the characteristic double wall, IMS, and embedded NPCs, of the NE (Fricker et al., 1997b). Localisation of asymmetric NPC components confirmed the expected orientation; nucleoplasm facing NPC components (e.g. translocated promoter region; Tpr) lie to the nucleoplasmic side of the NPC in the NR, while cytoplasm facing components (e.g. nup214) are found adjacent to the cytoplasmic core of the NR (Shah et al., 1998).

The cytoplasmic core of type II NR may contain microtubules and microfilaments, as well as vesicles, multivesicular bodies and mitochondria (Bol'shakova et al., 2009; Brandes et al., 1965; Fricker et al., 1997b; Lui et al., 2003a). Mitochondria accumulate in the cytoplasm adjacent to the beginning of channels (Michelangeli et al., 2005) and shuttle in and out of invaginations (Lui et al., 2003b). LM-detected NR invaginations vary from none to more than ten per cell in the same cell type. Since this number is not directly linked to cell cycle stage (Broers et al., 2006), and invaginations are found in post-mitotic primary cells (Broers et al., 2006) and persist for days in cycle arrested cells (Fricker et al., 1997b) a simple role in nuclear envelope assembly or interphase growth

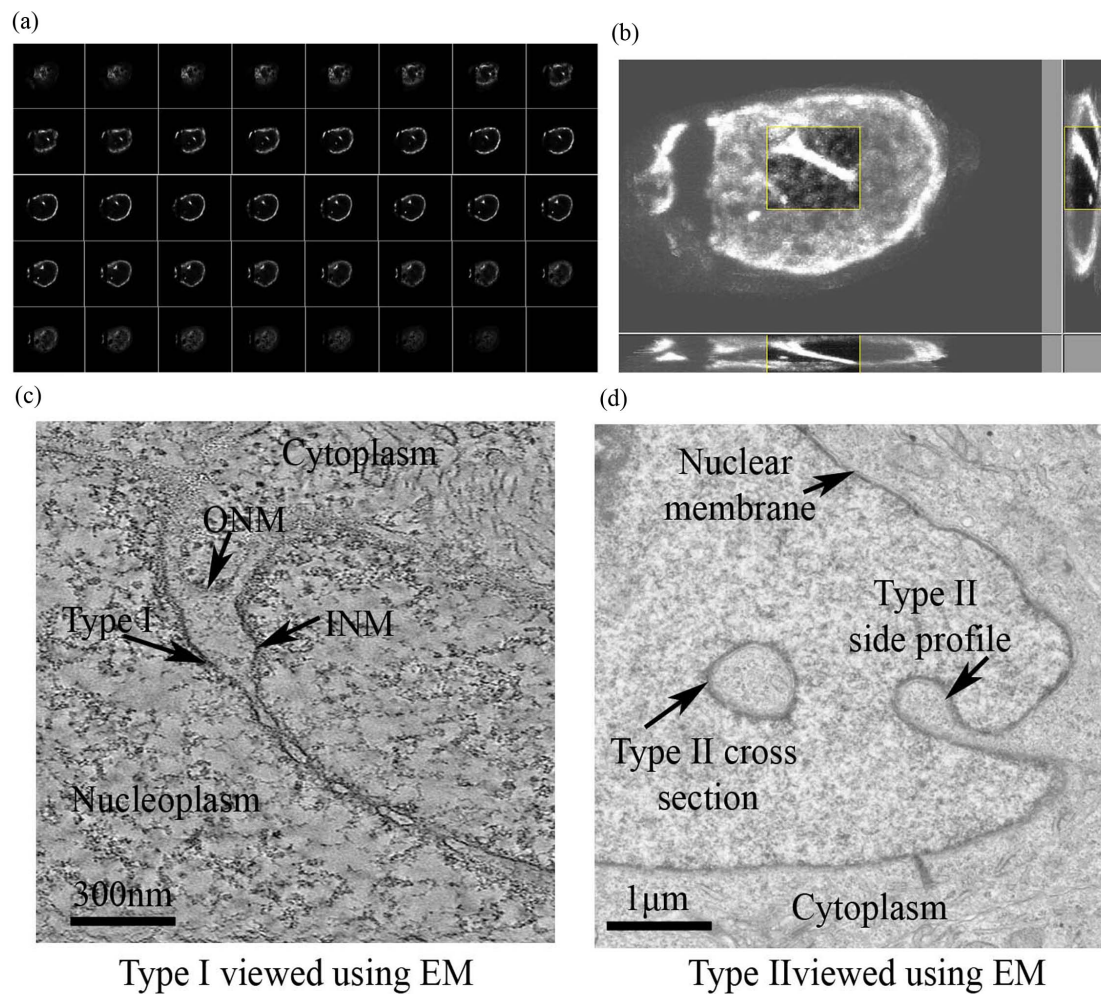
seems unlikely. Variation with state of differentiation has been described, however, which would be consistent with a role in regulated gene expression (Johnson et al., 2003).

Although the overall organization and structure of the NR described above has been described in the literature, the determining factor of invagination type and number within a particular nucleus and the mode of NR inheritance are all still not understood. A description of the composition of the NR and any differences between the NE, type I and type II invagination components would aid in answering some of the structural and ultimately functional questions.



**Figure 1.2 The morphology of the NR**

(a) Whole cell schematic defining the colour coding of relevant compartments and structures (b) Schematic of type I NR, including the cross-sectional appearance (middle panel) and an annotated TEM view (bottom panel)(c) Schematic of type II NR, including the cross-sectional appearance (bottom panel) (Malhas et al., 2011).



**Figure 1.3 Light and Electron Microscopy of NR**

(a) montage of serial optical sections through a single hepatocyte nucleus in a section of rat liver labelled with an antibody to lamin B1 (b) 3D reconstruction as XY, XZ and YZ planes of the data shown in (a) to reveal a major NR invagination connected twice to the NE (c) TEM of type I NR and (d) type II NR (Malhas et al., 2011).

### 1.3.2 Regulation

NR may arise during NE re-assembly after mitosis if synchrony between chromosome decondensation and fusion of recruited NE fragments is imperfect and chromatin-bound ER/NE cisternae become trapped in interstitial spaces, a process observed in tobacco cells (Gupton et al., 2006). However, NR can be formed *de novo* without mitosis (Fischer et al., 2003), persists throughout interphase (Fricker et al., 1997b) (Olins and Olins, 2009) and shows heritable patterns in specific cell types, strongly implying physiological regulation.

During cellularisation in *Drosophila* embryogenesis, a syncytium of synchronized cortical nuclei elongate whilst becoming enclosed by membranes. The rate-limiting factor for nuclear size increase during this step is a farnesylated nuclear protein called kugelkern (Brandt et al., 2006). Kugelkern resembles a mammalian B-type lamin, containing a putative coiled-coil domain, a nuclear localization signal and a C-terminal CxxM farnesylation motif. Over-expression of kugelkern promotes type II NR formation. Similarly, experimental over-expression of lamins A, B1, or B2, but not lamin C (which is not farnesylated), leads to NR proliferation in a number of cell types including HeLa and MEFs (Malhas et al., 2011). This is probably due to the close interaction between the farnesylated C-terminus of each of these proteins with the INM. A-type lamins lose their farnesylated C-termini during maturation and have less clear effects on NR formation; over-expression of fully processed GFP-lamin A may increase NR (Broers et al., 1999), other studies suggest that only non-processed lamin A causes NR proliferation (Prufert et al., 2004). There is agreement, however, that progerin, the

Hutchinson-Gilford progeria syndrome (HGPS) associated mutant lamin A isoform that lacks the maturation cleavage site (and therefore accumulates like a B-type lamin at the lamina), consistently causes NR proliferation (McClintock et al., 2006). Increased NR has also been observed in the presence of other *LMNA* mutations, such as those that lead to Emery-Dreifuss muscular dystrophy and Dunnigan-type familial partial lipodystrophy (Roblek et al. 2010).

Acute drug treatment may also result in NR development. Various HIV protease inhibitors have been shown to inhibit the enzyme responsible for removing the farnesylated tail of lamin A (Clarke, 2007; Coffinier et al., 2007; Coffinier et al., 2008; Hudon et al., 2008; Liu et al. 2010). Consequently an acute build up of farnesylated prelamin A at the periphery causes the nuclei to acquire a convoluted nuclear boundary with both types of invaginations being reported (Goulbourne and Vaux, 2010), a phenotype also exhibited in HGPS fibroblast nuclei.

A role for the condensation state of chromatin in regulating NR is suggested by the increased abundance of type II NR in mouse embryonic fibroblasts treated with the generic histone deacetylase inhibitor, trichostatin A (Galiova et al., 2008). This would increase the abundance of transcriptionally active chromatin, which may lead to more mRNA. The development of type II NR may consequently aid with increased export and translation of the upregulated mRNA transcription.



Membrane phospholipids also play a role in NR regulation. Phosphatidylcholine (PC) is the most abundant phospholipid in animal cell membranes, and thus a candidate for rate-limiting membrane synthesis. Nuclear activity of the three *de novo* PC synthesis enzymes, choline kinase (CK), CTP:choline phosphate cytidylyl-transferase alpha (CCT $\alpha$ ) and 1,2-diacylglycerol CDP:choline choline-phosphotransferase (CPT) has been reported, as has PC production by isolated nuclei (Hunt et al., 2001).

CCT $\alpha$  exists as an inactive soluble dimer that contains an amphipathic  $\alpha$  helix termed domain M. Products of PC catabolism, such as phosphatidic acid, diacylglycerol, and arachidonate cause activation of CCT $\alpha$  and domain M embeds the enzyme into anionic regions of membranes. The catalytic domain of CCT $\alpha$  then synthesises PC, which may increase positive curvature of the nuclear membrane to develop into a NR. (Gehrig et al., 2009). Over-expression of CCT $\alpha$  causes a proliferation of type II NR that is augmented by the presence of fatty acid substrates (oleate) that promote translocation of CCT $\alpha$  to existing NR and NE (Gehrig and Ridgway, 2010; Lagace and Ridgway, 2005a). Intriguingly, even catalytically-dead CCT $\alpha$  is recruited to the INM and has a significant effect on NR proliferation (Gehrig and Ridgway, 2011; Lagace and Ridgway, 2005a). CCT $\alpha$  may insert into a phospholipid leaflet to promote positive membrane curvature, as described for amphiphysin (Brandt et al., 2006; Peter et al., 2004), leading to tubulation of the INM and development of type I NR.

Type II NR could result if the tubulation includes the ONM through NPC and LINC complexes. If aberrant nuclear membrane proteins accumulate along the nuclear

envelope, as found in the laminopathies, then the architecture of the nuclear membrane may be altered. Consequently the distribution of NPCs or LINC complexes may change, which may lead to regions of the INM that can invaginate independently from the ONM when CCT $\alpha$  associates with it. For example mutations and siRNA mediated down regulation of nesprins results in separation of the INM and ONM leading to invaginations of just the INM (Zhang et al., 2007). siRNA targeted against some of the NPC components also leads to an aberrant nuclear morphology (Hawryluk-Gara et al., 2005; Mackay et al., 2009).

Development of the NR is dependent on the stoichiometric expression of the nuclear lamins (Gehrig et al., 2008). siRNA transfection against lamin A/C in CHOK1 cells markedly reduced NR development after the addition of oleate, an effect also seen after lamin B1 siRNA transfection. The farnesylated tail of lamin B1 is believed to increase the stability of the invagination by initiating the formation of the lamin scaffold as CCT $\alpha$  generates positive curvature on the nuclear membrane, enabling tubule formation.

However, formation of CCT $\alpha$  foci does not necessarily indicate NR development, as has been suggested (Favale et al. 2010). In MDCK cells subjected to 48h hypertonicity, CCT $\alpha$  foci colocalised with intranuclear lamin A/C signals. However, a marker for NR was absent from these foci and there were no invaginations present throughout the nucleus. This suggests that CCT $\alpha$  may have additional roles within the nuclei when cells are subjected to stressful conditions and foci of CCT $\alpha$  should not always be used as a marker for NR development.

NR regulation appears to be a dynamic process that is controlled by multiple processes. The formation of NR can be controlled by changing the composition of the phospholipid bilayer when CCT $\alpha$  is activated and binds to the INM. However, the formation of the invagination requires a scaffold to support its expansion and aid with the positive curvature of the membrane. The fact that there is an increased abundance of invaginations after histone deacetylase inhibition may support the notion of the NR involvement in aiding with increased expression of genes regulated by localised calcium release and increased export of mRNA for translation. Some of the functions of the NR have been investigated and are explored in the next section.

### **1.3.3 Function**

The functions of NR networks remain speculative, although there is mounting evidence for roles in transport, gene expression and calcium signalling. Nucleoli often associate with the NE by peripheral positioning, or by contact with the NR (Park and De Boni, 1992; Schneider et al. 2010). Type II NR have a cytoplasmic core offering tight spatial coupling of a transcriptionally highly active compartment with a potential transport channel (Bourgeois et al., 1979). Several transcription factors have been shown to interact with the NE (Malhas et al., 2009) therefore other spatial coupling functions of the NR may be in the form of focal delivery of transcription factors and nuclear enzymes to deep nucleoplasm.

It is becoming increasingly evident that certain nucleoporins are critical for transcription (Capelson et al., 2010; Ikegami and Lieb, 2010; Kalverda et al., 2010; Vaquerizas et al., 2010.) and the NPC is a dynamic structure that may act as a platform for regulating transcription (Mekhail and Moazed, 2010). It is possible that type II invaginations could bring NPCs to sites of gene transcription, deep within the nucleoplasm, potentially providing another level of transcriptional control. Furthermore, the NR can facilitate transport of nuclear products like mRNA from the deep interior to the cytoplasm. The cytoplasmic core of the type II NR would also provide far more rapid translation of mRNA that has been generated near to the invagination, as NPCs within it would be able to export the message to sites of translation within the core of the NR. The ABC50 protein, which is involved in translation initiation and control in mammalian cells was found inside the invagination of a cell, lending support to the notion that translation may occur inside the NR (Paytubi et al., 2009). Regulatory mechanisms involving NE-chromatin interactions may also operate deep within the nucleus on NR membranes (Malhas et al., 2007).

The existence of NR continuous with ER offers a potential deep nuclear calcium signalling source (Bootman et al., 2000). Calcium signalling is involved in a number of processes such as proliferation and apoptosis. It is thought that one of the factors determining the outcome of such signalling is the control of its location within the cell and hence some processes specifically require nuclear rather than cytosolic increases in calcium. The existence of the NR provides further specificity by allowing the focal delivery of calcium to specific sites within the nucleus. An example of nuclear calcium

signaling is the modulation of the transcription factor CRE binding protein (CREB) and its coactivator, CREB-binding protein (CBP), by changes in nuclear calcium (Chawla et al., 1998). Transcriptional activation of Elk-1 by EGF also depends on nuclear rather than cytosolic calcium (Pusl et al., 2002).

NR invaginations contain the high calcium concentration associated with the ER lumen and contain a range of calcium release mechanisms including inositol (1,4,5) trisphosphate receptors (IP3R) and ryanodine receptor (RyR) calcium channels (Bkaily et al., 2009; Bootman et al., 2009; Guatimosim et al., 2008; Irvine, 2006; Mellstrom et al., 2008). Functional activity of IP3R in NR of SKHep1 cells was confirmed in elegant studies using a two-photon uncaging approach to generate focal intranuclear IP3 transients (Echevarria et al., 2003). Following the uncaging of IP3, the resulting calcium signals begin sooner and rise faster in the vicinity of the NR, confirming that the NR is an IP3-gated calcium store that can give rise to local calcium signals in the nuclear interior. Evidence for a physiological function for such a nuclear calcium signalling pathway came from studies showing that nuclear IP3R activity in cardiac myocytes was associated with agonist mediated changes in gene expression that could be abrogated with specific IP3R inhibitors. This also provides evidence that localized calcium release in the nucleus can elicit responses i.e. gene expression changes, that are distinct from those elicited by cytosolic calcium release e.g. contraction (Garcia et al., 2004).

IP3-kinase isoform B (IP3KB) is important for further phosphorylating IP3 to inactivate this calcium releasing second messenger. IP3KB colocalises with type I and III IP3Rs in

vertical invaginations i.e. those perpendicular to the plane of the cell substrate, in H1299 cells (Nalaskowski et al., 2010). Once again these invaginations were seen to be closely associated with nucleoli, as described above. However, it should be noted that the NR is not essential for nuclear calcium signalling. For example, nuclei from neurons of *Aplysia californica* (a well-known model for studying the neuro-biology of learning) have no detectable NR invaginations and the non-invaginated nuclear membrane was sufficient for nuclear calcium signalling through RyRs in the NE (Bezin et al., 2008).

#### **1.3.4 Pathological Significance**

As part of the Bloom-Richardson grading system (Bloom and Richardson, 1957) nuclear phenotype is assessed and often found to be altered in malignant cells. This has been reported by changes in nuclear size, irregular nuclear membranes, changes in composition, enlargement and pleomorphism of nucleoli, thickening of the nuclear margin and budding or fragmentation of the nuclear membrane (Dey., 2010). Altered nuclear morphology is a common feature of many cancers and has adverse prognostic significance for cancers of the brain, breast, kidney, bladder, prostate and ovary (Bussolati et al., 2008; Fischer et al., 2003; Foster et al., 2010; Sarkar et al., 2009). The irregular nuclear margin often presents itself as invaginations of the nuclear membrane and this NR may be used in combination with other nuclear abnormalities as markers of a malignancy (Batistatou and Scopa, 2009).

A role for NR in viral access to the nuclear interior was suggested by pioneering single virus tracking imaging using Cy5-labelled adeno-associated virus (AAV) (Seisenberger et al., 2001). Directed, microtubule-dependent motion of intact viral capsids along restricted intranuclear trajectories was observed; the same trajectory was often followed by more than one virion. Confirmation that these tracks represent NR came from further experiments correlating motion of GFP-tagged AAV and anti-lamin B labeling (Lux et al., 2005).

Herpes virus nucleocapsids assemble in the nucleoplasm and escape via NE budding. Budding of capsids through INM has been described, as well as evaginations of the NE containing budded enveloped virion particles. Electron tomography studies implicate an extensive type I network in betaherpesvirus morphogenesis (Buser et al., 2007), although previous studies have identified both type I and type II NR accumulation in the nuclei of herpesvirus infected cells (Pignatelli et al., 2007), often showing extensive lamina disorganization, with prominent patches missing lamins A, B or C (Scott and O'Hare, 2001). The conserved herpesvirus UL34 protein localizes to INM (including NR), causes local dissolution of the nuclear lamina and promotes virion envelopment (Reynolds et al., 2001), although it is then lost from the virion during later maturation (Fuchs et al., 2002).

Invaginations of the nuclear envelope also seem to be a common feature of some of the laminopathies. In HGPS cells there is an increased abundance of invaginated nuclei (McClintock et al., 2006) which share similar morphological features to nuclei from normal aged cells (Scaffidi and Misteli, 2006). Progerin is the truncated form of lamin A

that lacks 50 internal amino acids, which includes the ZMPSTE24 cleavage site and so retains a farnesylated tail. Its farnesylated tail is likely to cause additional strain on the INM and cause invagination of the nuclear membrane. The fact that the invaginations can be ablated by treatment with a farnesyltransferase inhibitor (Wang et al., 2010) or morpholino targeted against the cryptic splice site of *LMNA* (Scaffidi and Misteli, 2005; Scaffidi and Misteli, 2006), suggests that the presence of the farnesylated tail causes invagination of the nuclear membrane. Blocking farnesylation alone does reduce HGPS symptoms in mouse models of the disease (Fong et al., 2006; Yang et al., 2010) but in other models of the disease it seems to have no effect on lifespan (Bar and Gruenbaum, 2010). Alternative routes of isoprenylation on lamin A may still occur that can only be circumvented by additional treatment with statins and aminobisphosphonates to hopefully extend the lifespan in HGPS (Varela et al., 2008).

It could be argued that NR is an artifact of additional strain on the nuclear envelope when cells are grown in 2D cell culture. The NR that develops may act as a structural support for the nucleus due to the increased compression it experiences in a 2D environment and have no functional role. However, the fact that NR is observed in nuclei from tissues (Fricker et al., 1997b; Langevin et al., 2010; Storch et al., 2007) and those grown in 3D cell culture (Chandramouly et al., 2007) supports the notion that it is a naturally occurring structure and not a side effect of the culture environment.

It is clear that the formation of NR can be induced in a pathological state. The reason why malignant cells seem to develop a higher frequency of invaginations is unclear. They may



be serving a functional role in which a change in nuclear architecture alters gene expression to aid cell survival. Alternatively they may be simply a morphological consequence of a break down in nuclear architecture as a cell adopts a cancerous state. It is clear that the use of NR is insufficient and sometimes misleading when used solely as a marker of malignancy and should instead be used as part of a combination of observations on changes to the nuclear morphology. The ability of viruses and other pathogens to manipulate the nuclear architecture in such a way to aid with infection and host cell colonization offers potential new therapeutic targets. If NR formation could be prevented in target cells then this may alter pathogen progression and manipulation of the host cell. It would be preferable to target factors that are produced by the pathogen that may induce and maintain NR formation. Targeting host cell components that generate NR could prevent pathogen colonization but as they are known to have multiple roles within the cell then inhibiting them may alter cellular homeostasis.

#### **1.4 THE LAMINOPATHIES**

The “one gene-one enzyme” hypothesis was first proposed by George Beadle and Edward Tatum (Beadle and Tatum, 1941). This was later modified to the “one gene-one polypeptide” hypothesis, which proposes that a single gene encodes one polypeptide that functions at one point of a metabolic pathway or cellular process. A corollary of this was formed by using positional cloning to identify disease genes, which led to the “one gene-one disease” principal. Today, we know that this is not the case and one of the best genes for highlighting this fact is *LMNA*.

The first human disease to be associated with *LMNA* mutations was autosomal dominant Emery-Dreifuss muscular dystrophy (EDMD) (Bonne et al., 1999). Some of the symptoms of the disease include slowly progressive muscle weakness in the upper arms and lower legs, dilated cardiomyopathy and early contractures of the elbows (Emery, 2000; Muchir and Worman, 2007). Mutations in *LMNA* were subsequently attributed to causing dilated cardiomyopathy type 1A (Fatkin et al., 1999) and limb girdle muscular dystrophy type 1B (Muchir et al., 2000). In these diseases striated muscle was affected and both lead to dilated cardiomyopathy with early onset atrioventricular conduction block. The difference between these two disease states is that skeletal muscle is minimally affected in dilated cardiomyopathy type 1A but the skeletal muscle around the shoulders and hips is affected in limb girdle muscular dystrophy type 1B.

The discovery of another *LMNA* monogenic disease that affected tissue other than striated muscle came as a surprising discovery. The genetic locus for Dunnigan-type familial partial lipodystrophy had been mapped by positional cloning to chromosome 1q21-22 (Jackson et al., 1998; Peters et al., 1998). Lipodystrophies are a group of disorders that are characterised by the absence or reduction of subcutaneous adipose tissue. At birth patients have a normal fat distribution but at the onset of puberty there is a regional loss of subcutaneous fat, which is associated with insulin resistance and diabetes mellitus (Dunnigan et al., 1974). As the disease phenotype of regional adipocyte degeneration was analogous to the EDMD regional muscle wasting and the fact that the genetic locus resided in the same area as *LMNA* (Cao and Hegele, 2000), it made this gene a prime candidate for causing the lipodystrophy syndrome. A novel mutation in exon 8 was

discovered that leads to a R482Q amino acid substitution (Shackleton et al., 2000; Speckman et al., 2000). Further mutations have been discovered that lead to lipodystrophy and some atypical forms also include muscle abnormalities (Vigouroux and Capeau, 2005).

By 2000 it had clearly been demonstrated that mutations in *LMNA* can cause two quite different diseases. However, the matter was further complicated when the genetic locus responsible for causing Charcot-Marie Tooth disease type 2, was also found to reside in the *LMNA* region (De Sandre-Giovannoli et al., 2002). This disease manifests itself with slight or absent nerve conduction velocities, loss of large myelinated fibres and axonal degradation (Chaouch et al., 2003). Mandibuloacral dysplasia (MAD-A) was also believed to be caused by a *LMNA* mutation (Novelli et al., 2002). Patients suffer from an underdeveloped clavicle, congenital bone abnormalities and lipodystrophy syndrome. Subsequently a mutation (R527H) was found in the *LMNA* gene (Novelli et al., 2002).

HGPS was initially reported over a century ago and results in segmental premature ageing (DeBusk, 1972; Mc, 1952). Individuals with this autosomal dominant syndrome often die in their second decade of life from myocardial infarction or stroke (DeBusk, 1972; Merideth et al., 2008). It was shown that eighteen out of twenty cases of HGPS had a *de novo* G608G (nucleotide 1824 C>T) mutation within exon 11 of *LMNA* (Eriksson et al., 2003). The mutation activates a cryptic splice donor site that results in the internal deletion of fifty amino acids from prelamin A and yields a protein called progerin. As a

result the removal of the farnesylated cysteine at the C-terminus of the protein is retained because the cleavage site for ZMPSTE24 has been removed.

Thus *LMNA* mutations can be broadly separated into diseases affecting (1) striated muscle, (2) adipose tissue, (3) peripheral nerve or (4) multiple tissues producing a progeroid phenotype. These tissue selective diseases occur even though A type lamins are present in virtually all differentiated somatic cells.

The laminopathies also include mutations in other genes that encode parts of the nuclear lamina. X-linked EDMD is caused by a mutation in the *EMD* gene that encodes emerin, which is expressed in virtually all cells (Bione et al., 1994). It is dependent on lamin A for localisation to the INM where it interacts directly with lamins and patients display a wider spectrum of disease than the classical EDMD phenotype (Astejada et al., 2007).

Lamin B receptor (LBR) is located on the INM and is an integral protein that binds to B-type lamins (Worman et al., 1988). Its N-terminus resides in the nucleoplasm and is basically charged, which enables it to bind to lamins, DNA and chromatin proteins (Worman et al., 1988; Ye and Worman, 1994; Ye and Worman, 1996). A heterozygous mutation in *LBR* causes Pelger-Huet anomaly, which is characterised by dysmorphic nuclei and altered chromatin organisation in blood neutrophils (Hoffmann et al., 2002). A homozygous *LBR* mutation is lethal *in utero* or leads to severe developmental abnormalities (Hoffmann et al., 2002; Oosterwijk et al., 2003).

MAN1 is also an integral INM protein and consists of two nucleoplasmic domains. One of these domains binds to both A and B type lamins as well as to emerin (Mansharamani and Wilson, 2005), while the other nucleoplasmic domain binds to regulatory SMADs and DNA (Caputo et al., 2006; Hellemans et al., 2004; Lin et al., 2005). Mutations in *LEMD3* (encoding MAN1) result in osteopoikilosis, Buschke-Ollendorff syndrome and skin abnormalities. These phenotypes are likely due to enhanced transforming growth factor  $\beta$  and bone morphogenic protein signalling, which are both mediated by regulatory SMADs.

*SYNE-1* encodes nesprin-1, which exists in several isoforms with the size of the protein determining whether it resides at the INM or ONM. Large nesprin-1 isoforms are located at the ONM and interact with SUN proteins in the perinuclear space, which bind to lamins forming the LINC complex (Crisp et al., 2006). Homozygous mutations in *SYNE-1* cause cerebellar ataxia, which affects a part of the brain, the cerebellum, involved in movement programmes (Gros-Louis et al., 2007).

### **1.5 HIV PROTEASE INHIBITORS AND ACQUIRED LIPODYSTROPHY**

According to the 2008 UNAIDS report, on the global AIDS pandemic, there are 33.2 million people in the world living with HIV (Este and Cihlar., 2010). Treatment for many of these individuals consists of a cocktail of anti retroviral drugs as part of Highly Active Antiretroviral Therapy (HAART), which interferes with the virus's replication (Perelson et al., 1997). One of the components of HAART is a protease inhibitor (PI) that inhibits the HIV protease (an aspartic protease) from generating structural proteins from

*gag* and *gag-pol* genes (Billich et al., 1988). Saquinavir was the first HIV PI approved by the US Food and Drug Administration (FDA) in 1995 (Nelson, 1995) whose administration remains prevalent in areas of Africa and Thailand (Dickinson et al., 2008). Its design was based on the observation that HIV proteases cleave Tyr-Pro or Phe-Pro sequences. The amide bonds of proline residues are not cleaved by mammalian endopeptidases, which would provide a higher degree of selectivity in the design of the inhibitor. Incorporation of a hydroxyethylamine moiety into the inhibitor mimics the transition state of the catalytic reaction, increasing affinity to the enzyme and replacement of a proline with a decahydroisoquinoline ring increased the potency of the inhibitor (Figure 1.2). The overall effect was the development of what appeared to be a highly selective PI that caused less than 50% inhibition to endogenous aspartic proteases by performing inhibition assays on the purified enzymes (Wlodawer and Vondrasek, 1998).

Most HIV PIs are taken in combination with another PI called ritonavir, which inhibits a PI metabolising enzyme called Cytochrome P450 3A4. (Zeldin and Petruschke, 2004). This allows the half life of HIV-PIs to increase and the anti retrovirals remain at a therapeutic concentration for longer. Other components of HAART include nucleoside and nucleotide reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, integrase strand transfer inhibitors and entry inhibitors. The combination of inhibitors used in a specific regimen is quite variable and should be individualised based on multiple factors such as resistance, toxicity and tolerability (Thompson et al., 2010). Currently many of the older PIs are being phased out due to the latest generation having a far greater affinity for the HIV protease. Darunavir was approved by the FDA in 2006

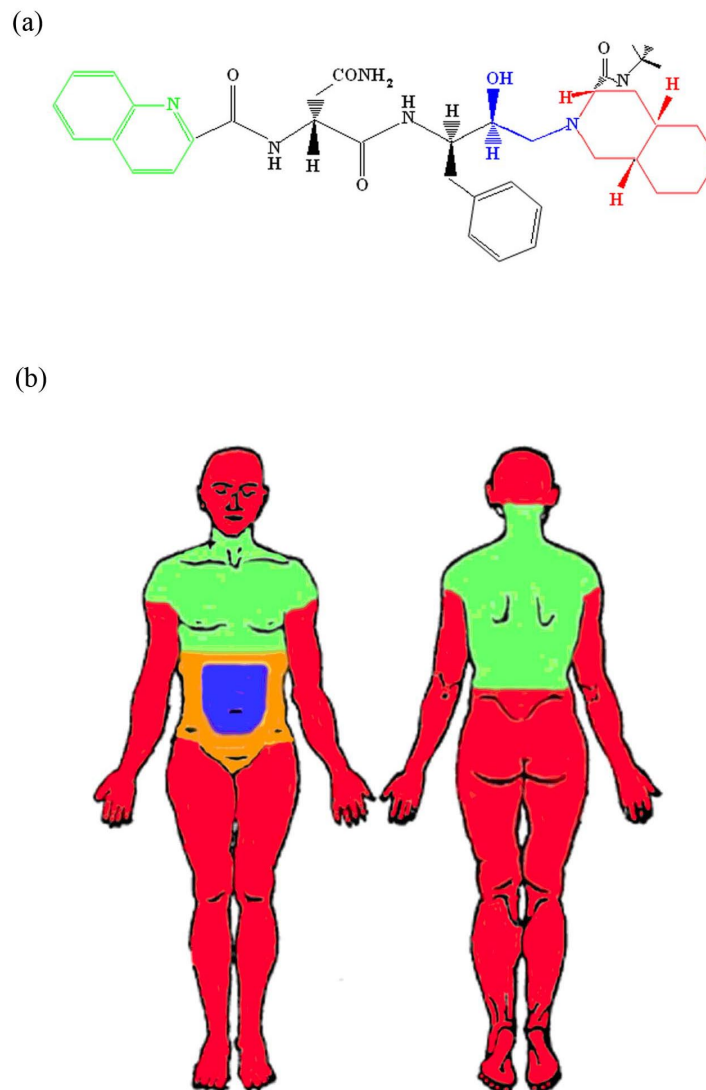
and has an affinity for HIV-1 protease 1000 times higher than saquinavir (Ghosh et al., 2007; McCoy, 2007).

Despite the benefits of the use of HIV PIs in HAART it became apparent that prolonged use of this combination of inhibitors could have many side effects. PIs have been implicated in dyslipidemia, insulin resistance, lipodystrophy and atherosclerosis (Capeau et al., 2010; Caron-Debarle et al., 2010; Green, 2002; Vidal et al., 2010; Vigouroux et al., 1999). The acquired lipodystrophy syndrome that develops in up to 40% of patients on HAART may appear after only 10 months of therapy (Vergel, 2007). It manifests itself with wasting of subcutaneous fat (lipoatrophy) in some areas of the body and accumulation of visceral fat (lipohypertrophy) in others (Figure 1.2). The development of a “buffalo hump” and other morphological changes can have psychological effects and has led to some of those patients affected abandoning therapy altogether (Collins et al., 2000; Vergel, 2007).

The fact that acquired lipodystrophy syndrome had a similar phenotype to lipodystrophy experienced in some of the laminopathies like MAD and RD, in which the ZMPSTE24/FACE1 enzyme was not functional (Agarwal et al., 2003; Moulson et al., 2005; Navarro et al., 2005), led some researchers to suspect that improper processing of lamin A might be involved. Caron et al were the first to show that two HIV PIs lead to altered lamin A processing and this impaired adipocyte differentiation potential (Caron et al., 2003). Other PIs were subsequently shown to elicit similar effects and shown to directly inhibit the ZMPSTE24 prelamins A processing enzyme leading to farnesylated

prelamin A accumulation and the development of a invaginated nuclear membrane (Capanni et al., 2005; Caron et al., 2003; Coffinier et al., 2007; Coffinier et al., 2008; Goulbourne and Vaux, 2010; Hudon et al., 2008; Liu et al., 2010). The effects are not permanent however as washout of inhibitor does reverse aberrant nuclear morphology (Liu et al., 2010). Prelamin A was also shown to retain the adipogenic factor SREBP-1 at the nuclear periphery and reduce adipogenic potential (Caron et al., 2003). This has been hypothesised to underlie the mechanism of lipoatrophy experienced in patients on HAART but was not investigated further at the molecular level and is addressed in this thesis.





**Figure 1.4. HIV acquired lipodystrophy syndrome**

(a) Molecular structure of saquinavir (green= Quinoline ring, blue = Hydroxyethylamine moiety, Red =Decahydroisoquinoline ring). (b) Up to 40% of people on HAART develop acquired lipodystrophy syndrome within 10 months of starting therapy. The diagram represents the distressing re-distribution of fat experienced by people on this anti-retroviral regimen (red = fat loss, green = varied fat excess, orange= varied fat loss, blue = intra abdominal fat excess).

## 1.6 AIMS OF THE THESIS

The NR is increasingly being recognised as a structure found in normal cells as well as forming in a pathological state. The fact that the accumulation of farnesylated prelamins A leads to an invaginated nuclear membrane reminiscent of the NR suggests that the mechanism of its formation may be dependent on the phosphatidylcholine synthesising enzyme CCT $\alpha$ . The morphology and effect on this developing NR as a result of acute prelamins A accumulation has never been fully investigated using dual axis electron tomography, which provides a new resolution and insight into this pathological phenotype. Improper processing of prelamins A also has an effect on the translocation of the activated SREBP-1 transcription factor to the nuclear interior but its downstream molecular consequences, which could potentially underlie the development of acquired lipodystrophy syndrome, have never been fully explored. Additionally, the effect of accumulating unprocessed lamin A on the metabolomics of the cell has never been addressed before and could potentially give new insights into symptoms experienced in the laminopathies and HAART.

The thesis therefore aims to:

1. Investigate the morphological consequences for the nuclear membrane of acute prelamins A accumulation and determine the mechanism surrounding the development of a nucleoplasmic reticulum in this pathological state.
2. Determine the consequences of acute prelamins A accumulation on SREBP-1 function and how this may underlie a potential mechanism of acquired lipodystrophy syndrome.
3. Characterise the metabolomic profile of cells treated with an HIV-PI that inhibits lamin A maturation and the effect it has on the pathways of metabolites.

## Chapter 2

### Materials and Methods

#### 2.1 REAGENTS

Sigma (Poole, UK) and BDH (Poole, UK) provided the chemicals used to make up buffers unless it is otherwise stated.

Plasmid pCSA-GFP, encoding the human active portion of the SREBP-1 transcription factor, was kindly provided by Dr Timothy Osborne (University of California, USA). Miss Susan Farrow generated the plasmid PcDNA4T0 PAGFP encoding GFP-lamin A. *Ccta*-mCherry plasmid was provided by Professor Neale Ridgway (University of British Columbia, Canada).

Professor Quentin Sattentau (University of Oxford, UK) provided the HIV PI, obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: (Saquinavir, cat 4658).

Professor H. P. Spielmann (University of Kentucky, USA) provided the farnesol analogue, 8-anilinogeraniol, and a mouse monoclonal antibody raised against it.

The Cells to cDNA II <sup>TM</sup> Kit was supplied by Ambion (London, UK) and contained the following reagents; Cell lysis II Buffer, DNase I, Random decamers, Oligo (dT)<sub>18</sub> primers, 10X reverse transcriptase buffer and RNase inhibitor. Superscript III reverse transcriptase was obtained from Invitrogen <sup>TM</sup> (Paisley, UK). Fast SYBR<sup>®</sup> Green Master Mix was supplied by Applied Biosystems (London, UK) and oligonucleotide primers were obtained from Invitrogen<sup>TM</sup> (Paisley, UK).

Products that were used for DNA purification and PCR purification were purchased from Qiagen (Crawley, UK).

Laboratory stocks of cells were used unless stated otherwise. Mouse embryonic fibroblasts from wild type and *Zmpste24*<sup>-/-</sup> animals were provided by Dr Stephen Young (UCLA, USA). Mouse pre-adipocytes were purchased from the Health Protection Agency (Porton Down, Salisbury, UK).

Sterile cell culture plastic ware was obtained from Appleton Woods (Birmingham, UK). All media and cell culture products were obtained from PAA (Pasching, Austria) unless stated otherwise.

## 2.2 DNA AMPLIFICATION, PURIFICATION AND CLONING

### 2.2.1 Bacterial cell culture and transformation.

Subcloning Efficiency™ DH5α™ Competent Cells (F-  $\phi$ 80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *deoR recA1 endA1 hsdR17* (rk<sup>-</sup>, mk<sup>+</sup>) *phoA*supE44 *thi-1 gyrA96 relA1*λ<sup>-</sup>) were purchased from Invitrogen (Paisley, UK) and used for all transformations and standard techniques. They were cultured in a rotary shaker at 225rpm at 37°C unless otherwise stated.

*E. coli* was cultured in autoclaved Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 170mM NaCl), which was inoculated using aseptic technique and flame sterilization of instruments. Cells successfully transformed were selected using kanamycin at 50 µg/ml.

### 2.2.2 Transformation of competent bacterial cells.

For each transformation 50 µl of Subcloning Efficiency™ DH5α™ Competent Cells were thawed on ice and gently resuspended. 1 to 5 µl (1-10ng) of DNA was added to the cells and mixed gently before being incubated for 30 minutes on ice. Cells were heat shocked for 20 seconds in a 42°C water bath without shaking before the tubes were placed on ice for 2 minutes. 950 µl of pre-warmed LB medium was then added and cultured for 1 hour. 150 µl from the transformation was spread on pre-warmed kanamycin plates and incubated at 37°C overnight.

### **2.2.3 Purification of Plasmid DNA from bacterial cells for transfection of mammalian cells**

The Qiagen Midiprep Kit was used for preparing DNA for transfection. Initially a single bacterial colony is picked from the selective plate and inoculated into 5ml of sterile LB with the appropriate antibiotic and cultured at 37°C at 225rpm overnight. 50 µl of the starter culture was then added to 50ml of sterile LB (1/100), with the appropriate antibiotic, and again cultured overnight at 37°C at 225rpm. The bacterial cells were then harvested by centrifuging in a JA-20 rotor (Beckman) at 6000g for 15 minutes at 4°C, before the supernatant was removed and the pellet of cells were placed on ice. Cells were then resuspended in 6ml of buffer P1 and then 6ml of buffer P2 was also added, before the suspension was vigorously shaken and incubated at room temperature for 5 minutes. This step lysed the cells and generated a homogenously coloured solution. 6ml of buffer P3 was then added to the lysate and vigorously shook so as to precipitate genomic DNA, proteins and cell debris. The lysate was then immediately poured into the barrel of a Qiafilter Cartridge and incubated at room temperature for 10 minutes, so as to separate the protein and genomic DNA precipitate from the solution.

A HiSpeed Midi tip was equilibrated with 4ml of buffer QBT by allowing it to empty by gravity flow. The lysate was then filtered through the equilibrated tip, which was then washed with 20ml of buffer QC. The DNA was then eluted by adding 5ml of QF to the tip and the DNA was precipitated by the addition of 3.5ml of isopropanol, mixed and incubated at room temperature for 5 minutes. The eluate/isopropanol mixture was then filtered through a QIAprecipitator Midi Module and the bound DNA was washed with

70% ethanol. The membrane was then dried to remove excess ethanol and the DNA was eluted by adding 1 ml of buffer TE. The eluate was then reapplied to the filter to maximize the yield of DNA. The concentration of DNA was determined by using a Nanodrop ND 1000 spectrophotometer at 260nm.

## **2.3 MAMMALIAN CELL CULTURE**

### **2.3.1 General cell culture technique**

Cell culture was performed in a category two laminar flow hood using sterile technique. Reagents and plastic ware were described previously. Antibiotics were not generally used in standard cell culture applications. Cultured monolayer cells were incubated at 37°C in the presence of 5% CO<sub>2</sub>.

### **2.3.2 Culture of individual cell lines**

HeLa cells, mouse embryonic fibroblast (MEF) and mouse-preadipocytes (MPad) were cultured in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine and non-essential amino acids. Upon reaching confluence MPad begin to differentiate into mouse adipocytes (Mad) and are then cultured in DMEM supplemented with 10% FCS, 2mM L-glutamine, non-essential amino acids and 5 µg/ml of insulin.

### **2.3.3 Passaging of cells**

Monolayer cells were passaged by trypsinisation with TrypLE (Gibco 12604-013). Trypsinisation was stopped by the addition of an equal volume of growth medium and the

cells were seeded at the appropriate density. Cells that were destined for frozen storage were trypsinated, centrifuged at 420 g at 4°C for 5 minutes and the supernatant was removed before the pellet was resuspended in 1 ml of freezing medium (FCS with 10% DMSO). Cells were frozen overnight to -80°C in a polystyrene holder to achieve an appropriate cooling rate and then in liquid nitrogen. MEFs and preadipocytes in all experiments were used at a low passage number (5-10).

Cells used for fixation and immunolabelling were grown on glass coverslips. Coverslips were sterilized by flame steriliastion in 100% ethanol. Cells were cultured for at least 24 hours before fixation.

#### **2.3.4 Drug treatment of cells in culture**

All drugs used in cell culture were diluted in CO<sub>2</sub> and temperature equilibrated growth medium to the desired concentration. Saquinavir stock was dissolved in DMSO and used at 20 µM for 48 hours for all experiments except where otherwise stated. Farnesyl transferase inhibitor FTI-277 (Calbiochem, Beeston, UK) was dissolved in DMSO and used at 10 µM for 48 hours unless otherwise stated. Hydroxyurea was dissolved in water and used at 1.5 mM for 24 hours, to arrest cells at the S phase of the cell cycle. To assess protein farnesylation cells were incubated with the farnesol analogue 8-anilinogeraniol, which was dissolved in DMSO and used at a final concentration of 50 µM for 48 hours (Troutman et al., 2005).



### **2.3.5 Transfection of mammalian cells in culture**

Cells were passaged the day before transfection and plated into 35mm culture dishes or on a glass coverslip. Transfection was performed with the lipophilic reagent Lipofectamine 2000 (Invitrogen, Paisley, UK). DNA and Lipofectamine were mixed separately into 200  $\mu$ l of DMEM serum free medium. The ratio of DNA to reagent used was 1  $\mu$ g DNA to 1mg Lipofectamine. The amount of DNA used per well was 2  $\mu$ g to 5  $\mu$ g. DNA in DMEM medium was combined with the Lipofectamine within four minutes of addition to the DMEM. DNA and reagent were allowed to complex for 20 minutes at room temperature. Finally, the transfection mixture was added into the wells containing DMEM and supplemented with 10% FCS and 1mM L-glutamine.

## **2.4 PROTEIN PURIFICATION AND ANALYSIS**

### **2.4.1 One dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE)**

SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli (Laemmli, 1970) using a Bio-Rad Miniprotean 3 apparatus (Bio-Rad). 13 cm isoelectric focussing strips were run on a larger gel format (The Sturdiar, Hoefer Scientific, USA). Gels were cast with a stacking gel (3.75% acrylamide/ bisacrylamide 37.5:1, 0.145 M Tris pH 6.8, 0.01% SDS (w/v), 0.3% TEMED (v/v)) overlaying the resolving gel (10% acrylamide/ bisacrylamide 37.5:1, 0.45 M Tris pH8.8, 0.1% glycerol (v/v), 0.1% SDS (w/v), 0.15% TEMED (v/v)). Polymerisation was allowed to proceed to for at least 30 minutes at room temperature for the resolving gel. The top and bottom buffer chambers were filled with SDS running buffer (25 mM Tris, 250 mM glycine,

0.1% SDS). Samples were run at a constant 80 volts through the stacking gel followed by a constant 150 volts through the resolving gel.

#### *2.4.1.1 Sample preparation for one dimensional protein electrophoresis*

Samples were prepared in Laemmli sample buffer (50 mM Tris-Cl pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and were boiled for five minutes immediately prior to loading onto the gel and centrifuged at 15000 g for 1 minute at room temperature in a bench top microfuge. Samples were loaded with gel loading tips to ensure minimal cross contamination of wells and accurate and reproducible volumes.

Cultured cells were washed three times with ice cold PBS in the well and lysed directly into hot 2X Laemmli sample buffer. The well was washed once with an equal volume of deionised water and combined with the rest of the sample to produce a whole cell lysate in Laemmli sample buffer for gel loading. Samples were sonicated for five seconds in a sonicator to break up high molecular weight DNA.

Where protein concentration of samples was required, cells were washed three times in the well in ice-cold PBS, scraped into PBS and pelleted at 1000g for two minutes at 4°C. Cells were resuspended in 50 µl of ice-cold lysis buffer (20 mM Tris-Cl pH 7.4, 0.2 mM EDTA, 1 mM PMSF). An aliquot of sample was used for determination of protein concentration as described.

#### **2.4.2 Determination of protein concentration**

The Bio-Rad protein assay (Bio-Rad laboratories, Munich, Germany) was used to determine protein concentration. Calibration of the assay was determined with the use of a bovine serum albumin standard solution provided by the supplier. Linear range of the assay was between 2 and 20 µg/ ml protein. Absorbance was measured at 594 nm in a spectrofluorometer in disposable plastic cuvettes with 1 cm path length. Dilutions of protein samples were performed to within the linear range of the assay.

#### **2.4.4 Transfer of proteins to nitrocellulose membranes**

Transfer of proteins from polyacrylamide gels to nitrocellulose membrane was performed using The Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Munich, Germany). The nitrocellulose membrane was overlayed onto the gel and sandwiched between two layers of Whatman 3 mm paper and foam pads on either side. The transfer was performed in transfer buffer (25 mM Tris, 145 mM glycine, 20% methanol) at 90 Volts for 60 minutes at 4°C with constant stirring of the transfer solution. After disassembly the membrane was removed and stained with Ponceau S solution (Sigma) and destained with transfer buffer. Membranes were washed extensively after destaining with PBS prior to immunolabelling.

#### **2.4.5 Western blot analysis of proteins on nitrocellulose membranes**

Western blotting was performed at room temperature in clean plastic ware and with constant agitation on a rocker table. Membranes were blocked with 5% dried milk in PBS

with 0.05% triton (PBST) for 30 minutes. Both primary and secondary antibodies were diluted in the blocking buffer at the recommended concentrations. Primary antibodies were left overnight at 4°C with rocking and secondary antibodies were incubated for one hour at room temperature. Membranes were washed with three changes of PBST over fifteen minutes between primary and secondary antibody incubations. Following incubation with the secondary antibody the membrane was washed with five changes of PBST over thirty minutes. The membrane was developed with the ECL chemiluminescence detection system (Amersham Pharmacia Biotech) for peroxidase conjugated secondary antibodies. Chemiluminescence was detected with ECL Hyperfilm (Amersham Pharmacia Biotech) and developed on a Kodak X-OMAT 2000 processor.

## **2.5 IMMUNOLABELLING OF CELLS FOR LIGHT MICROSCOPY**

### **2.5.1 Immunofluorescence labelling of cultured cells**

Cells grown as a monolayer on a coverslip were fixed immediately after aspiration of growth medium without any washing. Unless otherwise stated the fixation technique was employed using 4% paraformaldehyde (PFA) in 250 mM HEPES, pH 7.4 for 30 minutes at room temperature. All washing steps, unless otherwise stated, were performed with three changes of PBS. Free aldehyde groups were quenched with 20 mM glycine for 10 minutes before being permeabilised with 0.4% Triton X-100 and blocked with 0.4% fish skin gelatine (FSG) for 1 hour. All primary and secondary antibodies were diluted in the blocking buffer. Optimum concentrations of antibodies were determined by labelling optimally fixed cells at doubling dilutions of antibody. Primary and secondary antibody

incubation times were for 1 hour and 45 minutes at room temperature respectively.

Primary and secondary antibodies used in experiments are detailed in table 1.

**Table 1. Primary and secondary antibodies used in this thesis.**  
The table lists primary and secondary antibodies used in this thesis.

| <b><u>Antibody</u></b> | <b><u>Source</u></b> | <b><u>Supplier</u></b> | <b><u>Conjugate</u></b> |
|------------------------|----------------------|------------------------|-------------------------|
| Anti-lamin A/C         | Mouse monoclonal     | SantaCruz              | Unconjugated            |
| Anti-prelamin A        | Goat polyclonal      | SantaCruz              | Unconjugated            |
| Anti SREBP-1           | Rabbit polyclonal    | SantaCruz              | Unconjugated            |
| Anti CCT $\alpha$      | Mouse monoclonal     | R. Cornell             | Unconjugated            |
| Anti AG                | Mouse monoclonal     | H. Speilmann           | Unconjugated            |
| Anti Lamin B1          | Mouse monoclonal     | Own                    | Unconjugated            |
| Anti FABP-4            | Rabbit polyclonal    | Abcam                  | Unconjugated            |
| Anti- $\beta$ Actin    | Mouse monoclonal     | Abcam                  | Unconjugated            |
| Anti nup153            | Mouse monoclonal     | Abcam                  | Unconjugated            |
| Anti Mab414            | Mouse monoclonal     | Abcam                  | Unconjugated            |
| Anti calreticulin      | Rabbit polyclonal    | Abcam                  | Unconjugated            |
| Anti ZMPSTE24          | Rabbit polyclonal    | L. Fong                | Unconjugated            |
| Anti-mouse             | Donkey               | Molecular Probes       | Alexa 488               |
| Anti-mouse             | Donkey               | Sigma-Aldrich          | HRP                     |
| Anti-goat              | Donkey               | Molecular Probes       | Alexa 647               |
| Anti-goat              | Donkey               | Molecular Probes       | Alexa 488               |
| Anti-goat              | Donkey               | Jackson                | HRP                     |
| Anti-rabbit            | Donkey               | Jackson                | Cy3                     |
| Anti-rabbit            | Donkey               | Jackson                | Cy5                     |
| Anti-mouse             | Donkey               | Sigma-Aldrich          | HRP                     |

### **2.5.2 Light microscopy**

Immunofluorescence images were collected using a Plan Apo 63x 1.4na oil immersion lens and a Plan Neofluar 10x 0.3na lens on a Zeiss Axioplan 2 imaging microscope with fibre optic illumination (Sutter Instrument Company, Novato, CA, USA) and a Cool SNAP HQ 12 bit CCD camera (Photometrics, Marlow, UK), which was controlled by Metamorph software (Molecular Devices, Downingtown, PA, USA). The immunofluorescence was achieved by using a quad band dichroic reflector (Chroma Technology Corp, Rockingham, VT, USA) for DAPI (360nm exciter with 40nm bandwidth and 457nm emitter with 50nm bandwidth), FITC (490nm exciter with 20nm bandwidth and 528nm emitter with 35nm bandwidth), Cy3, TRITC and Con A (555nm exciter with 28nm bandwidth and 617nm emitter with 73nm bandwidth) and Cy5 (635nm exciter with 20nm bandwidth and 685nm emitter with 40nm bandwidth).

### **2.5.3 Image analysis and processing**

Images collected were either analysed in Metamorph, Zeiss LSM or Imaris software. All images were assembled in Adobe Photoshop v.9.0 (Adobe Systems UK, Uxbridge, UK).

## **2.6 LIVE CELL IMAGING**

### **2.6.1 Preparation of cells prior to imaging**

Cells were grown on a 25 mm coverslip and placed in an Attofluor® cell chamber (Invitrogen, Paisley, UK), which is then filled with 1 ml of culture medium. For cell tracking cells were cultured in a gridded MatTek dish (MatTek, Ashland, USA). Cells were kept at 5 % CO<sub>2</sub> and at 37 °C using the Zeiss environmental control (PeCon GmbH, Erbach, Germany) while imaging took place.

### **2.6.2 Fluorescence Loss in Photobleaching**

Fluorescence Loss In Photobleaching (FLIP) experiments were performed on a Zeiss 510 confocal laser scanning system. GFP was visualised using a laser excitation wavelength of 488nm and an emission filter of 505nm-long pass. A region of interest was selected and was photobleached at full laser power while scanning at 4% laser power elsewhere with 1 second intervals between scans over a period of 300 seconds. For quantitative analysis, background intensity was subtracted and intensities of a specific ROI outside the photobleached area were measured over time and normalized using intensities of an ROI in a transfected but nonbleached cell.

### **2.6.3 Time lapse imaging**

For cell cycle tracking in live cells a Zeiss inverted laser scanning microscope was used in a non confocal transmission phase contrast mode at 633nm using a Plan Neofluar 10x 0.3 na objective (for mitosis tracking) or a 555nm exciter mode using a Plan Apo 63x 1.4 na objective (CCT $\alpha$ -mCherry foci tracking) . An image was acquired every 15 minutes



for 48 hours. The Zeiss environmental control (PeCon GmbH, Erbach, Germany) was maintained at 37°C and 5% CO<sub>2</sub> while imaging the cells.

## **2.7 TRANSMISSION ELECTRON MICROSCOPY AND DUAL AXIS ELECTRON TOMOGRAPHY**

### **2.7.1 Sample preparation for routine TEM**

Cells were washed with PBS, trypsinated and centrifuged at 5000 rpm for five minutes into a pellet before being fixed in a mixture of 2.5% glutaraldehyde in 100 mM cacodylate buffer (pH 7.4) with 2 mM MgCl<sub>2</sub> overnight. This was followed by 1% osmium tetroxide in 100 mM cacodylate buffer (pH 7.4) for 1 hour and was then washed in distilled water four times at 10 minutes per wash. 1% aqueous uranyl acetate was then added to the sample and left at 4°C overnight in the dark. The next day the sample was washed, as before, with distilled water before being sequentially dehydrated in ethanol for 30 minutes per increase in concentration (20%, 30%, 50%, 70%, 90%, 100% and three times at 100% at 20 minutes each). After dehydration with ethanol the cells were infiltrated with increasing concentrations of epon resin (25% for 1 hour, 50% for 1 hour, 75% for 1 hour, 100% for 1 hour, 100% overnight). The sample was then placed in a resin mould and incubated in an oven at 70°C overnight.

### **2.7.2 Sectioning using the ultra microtome**

Resin embedded specimens were trimmed using a razor blade and glass knife so as to generate a trapezoid end containing the specimen. The block was then placed into a Reichert Ultracut Microtome (Reichert Technologies, Seefeld, Germany) and the sample

was sectioned using a 45 ° diamond knife (Diatome, Biel, Switzerland). For routine EM, 50 nm sections were cut and collected on copper grids. For tomography, 250 nm thick sections were collected on formvar-coated copper slot grids. Serial section dual axis electron tomography required multiple sections (up to ten) to come off the embedded samples block face sequentially. A human hair was secured diagonally across the trough of the diamond knife that rested on the surface of the water, which aided with aligning the serial sections when they were picked up by a formvar coated grid.

### **2.7.3 Formvar coated slot grids for tomography**

A 0.7% formvar solution was made up in chloroform and a clean slide was lowered into the solution. The slide is then quickly drawn out of the formvar and any excess solution is allowed to drain off. A razor blade is then used to cut a square into the formvar layer either side of the slide and it is then slowly lowered into a bowl of water allowing the square of formvar to float off the slide on the waters surface. A gold shimmer can be observed on the formvar sheet, indicating the layer to be at the correct thickness (approximately 90 nm). Slotted grids are then placed matt face down on to the floating formvar sheet and it is picked up on a slide with paper fixed to it, before allowing the coated grids to dry (Peters et al., 2006).

### **2.7.4 Reynolds lead citrate staining (Reynolds, 1963)**

The stain was prepared by dissolving 1.33 g  $\text{Pb}(\text{NO}_3)_2$  and 1.76 g  $\text{Na}_3(\text{C}_6\text{H}_5\text{O}_7) \cdot 2\text{H}_2\text{O}$  in 30 ml of distilled water. The suspension is then vigorously shaken for 1 minute and then allowed to stand for 30 minutes with intermittent shaking to ensure complete conversion

of lead nitrate to lead citrate. After 30 minutes 8.0 ml of 1 M NaOH is added and the suspension is diluted to 50 ml with distilled water and mixed by inverting. The final pH should be adjusted to 12.0  $\pm$  0.1. The grid with sections is then placed section side down on a drop of the lead citrate solution on parafilm and left for 5 minutes. The grid is then washed in distilled water and is then ready for viewing under the electron microscope. For tomography the staining procedure should be doubled due to the thicker sections.

### **2.7.5 10nm Gold Fiducial Labelling for Tomography**

For tomography sections, after the Reynolds lead citrate staining procedure, 10 nm gold fiducial particles are added to both sides of the grid. A drop ( $\sim$ 10  $\mu$ l) of 10 nm colloidal gold (BB international, Cardiff, UK) is placed on the grid for 10 minutes and then the other side of the grid is placed on a fresh drop of the solution and again incubated for the same time.

### **2.7.6 Dual axis Electron Tomography data collection**

The stained and gold-coated grid is placed on the holder and inserted into the vacuum chamber before adjustments are made for alignment, eucentricity, focus and exposure. The specimen is then tilted over a range between  $-65^\circ$  and  $+65^\circ$  and an image is acquired every  $1^\circ$  to complete a single tilt series using a Tecnai 12 operating at 120 kV. The grid is then rotated  $90^\circ$  in the specimen rod, with a rotating specimen holder, and another tilt series is collected to complete the data set for a dual axis tomogram. Serial section dual axis electron tomography involves the same process described above on sequential sections.

### **2.7.7 Immunogold labelling**

Grids were floated, section side down, on PBS (pH 7.4) containing 20 mM glycine and 1% BSA for 15 min. This inactivates any residual aldehyde groups present after fixation and block non-specific binding sites and hence lower any background labelling. The grids were then placed on separate drops of blocking solution, which consisted of PBS containing 1% BSA and 0.1% FSG supplemented with 5% normal serum (same species as the antibody in the second immuno incubation step) for 5-10 minutes. This step will block non-specific binding sites and hence lower any background labelling. The grids were transferred (while removing the excess liquid using a filter paper) on to drops (5-10  $\mu$ l each) of the primary antibody solution. The antibody is diluted using PBS containing 1% BSA and 0.1% FSG supplemented with 5% normal serum (same species as the antibody in the second immuno incubation step). The grids were covered and left for 1 hour at room temperature.

The grids were then rinsed by floating on droplets of PBS or dilution buffer; five washes for at least a total of 15 minutes. The grids were then transferred using forceps and removing excess liquid, to a droplet of the secondary antibody-gold probe (or a Protein A-gold solution) and incubated for 1 hour at room temperature. The secondary antibody-gold probe is diluted using PBS containing 1% BSA and 0.1% FSG supplemented with 5% normal serum (same species as the secondary antibody). It is then rinsed five times with PBS for a total of 30 min before being rinsed several more times with distilled water for 5 minutes. The grids are then stained and embedded in methyl cellulose, which is

performed on ice by mixing nine parts 2% methyl cellulose to one part 3% uranyl acetate, producing a final concentration of 1.8% methyl cellulose and 0.3% uranyl acetate. The grids are finally transferred through two large drops of methyl cellulose-uranyl acetate and then placed on a small, fresh droplet. After 10 minutes each grid is removed with forceps and excess methyl cellulose is removed with filter paper. The grids are allowed to dry and can then be examined in the TEM.

# **CHAPTER 3**

## **THE INDUCTION OF A NUCLEOPLASMIC RETICULUM BY FARNESYLATED PRELAMIN A ACCUMULATION REQUIRES CTP: PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE $\alpha$**

### **3.1 INTRODUCTION**

The consequences of accumulating farnesylated prelamins A, whether as a result of drug treatment or mutation, has sparked much interest in several areas of cell biology that includes HIV therapy (Capeau et al., 2006; Capeau et al., 2005; Caron et al., 2007; Clarke, 2007; Coffinier et al., 2007; Coffinier et al., 2008; Goulbourne and Vaux, 2010; Hudon et al., 2008; Rudich et al., 2005; Saillan-Barreau et al., 2008) and the normal ageing process (Ragnauth et al., 2010; Scaffidi et al., 2005; Scaffidi and Misteli, 2006; Scaffidi and Misteli, 2008). After farnesylated prelamins A accumulate the nucleus becomes dysmorphic with a highly convoluted nuclear envelope and develops a NR.

However, the relationship between these morphological changes and the increasingly well-recognised NR observed in different cell types and during various stages of cellular development remains obscure (Fricker et al., 1997a; Fricker et al., 1997b). Such structures are believed to increase the interface between nucleoplasm and cytoplasm to improve communication between the two environments as well as add structural support. It has also been reported that this NR regulates calcium signals in localised sub nuclear

regions, enabling control of gene transcription and cell growth (Chamero et al., 2008; Collado-Hilly et al., 2010; Nalaskowski et al., 2010).

NR development seems dependent on the enzyme CCT $\alpha$ , which is responsible for phosphatidylcholine synthesis in the biosynthesis and curvature of membranes (Gehrig et al., 2008; Gehrig et al., 2009; Lagace and Ridgway, 2005b). The retention of the farnesylated tail in prelamin A is believed to enhance NR development in cell nuclei by increasing stress on the nuclear membrane (McClintock et al., 2006; Roblek et al., 2010).

The current Chapter investigates the effects of acute farnesylated prelamin A accumulation on cells by assessing the morphology and organisation of the nucleus. It was found that farnesylated prelamin A accumulated in cells transfected for 48 hours with *Zmpste24* siRNA. This resulted in decreased ZMPSTE24 protein levels and a rise in farnesylated prelamin A accumulation that corresponded with dysmorphic nuclei containing a complex NR that sometimes colocalised with F-actin. Prelamin A, lamin B1 and calreticulin co-localise in the NR, which can form in interphase cells without an intervening mitosis. Treatment of farnesylated prelamin A accumulating cells with an siRNA of *Ccta*, leads to a reduction in NR but the nuclear phenotype is still dysmorphic when compared to control.

Further assessment of the morphology of treated nuclei at the electron microscope (EM) level led to the demonstration that the NR in these cells consisted of both double membraned invaginations of the nuclear envelope and invaginations of just the INM.

Furthermore, these structures contained regular fenestrations. EM tomography revealed that these fenestrations had dimensions similar to nuclear pore complexes (NPC). Immunolabelling at the LM and EM level confirmed the presence of Nup153 and mAB414 at these fenestrae. Quantitation of NPC, on the nuclear surface after prelamin A accumulation, revealed a reduction in NPC number. Taken together these results show that accumulation of prelamin A results in two forms of NR developing with many nuclear pores associated with them and development of the NR requires both CCT $\alpha$  and farnesylated prelamin A.

## 3.2 METHODS

**3.2.1 Cell culture:** Wild type MEFs, *Zmpste24*<sup>-/-</sup> MEFs (Stephen Young, UCLA) and HeLa cells were cultured as described in the methods section of Chapter 2.

**3.2.2 Cell transfections:** MEFs were grown to 80 % confluency on glass bottomed 35mm dishes (MatTek) and transfected with *Zmpste24* siRNA (Qiagen, SI00241633), control siRNA (Ambion, AM17012) or *Ccta* siRNA (Santa Cruz, sc-40395) using lipofectamine 2000 (Invitrogen). The *Zmpste24* siRNA sequence was AAC AAC AUA CAC CUU AGU CAA AGG G. The same procedure was carried out for HeLa cells that were transfected with *FACE1* siRNA (Santa Cruz, sc-45524) or a control siRNA (Santa Cruz, sc-37007). The *FACE1* siRNA sequence was CCC UUU GAC UAA GGU GUA UGU UGU U.



**3.2.3 Immunofluorescence microscopy:** Cells were prepared as described in the methods section of chapter 2. Primary antibodies used were a prelamin A specific antibody (Santa Cruz Biotechnology Inc sc-6214), anti-lamin B1 antibody (Maske et al., 2003), anti-calreticulin antibody (Abcam ab-2907), anti-CCT $\alpha$  antibody (R. Cornell), anti-mAB414 (Abcam ab024609) or an anti- Nup153 antibody (Abcam ab-24700). All primary antibodies were used at a dilution of 1 in 100 in 0.4 % FSG and ConA was used at a concentration of 100  $\mu$ g/ml. Phalloidin conjugated to TRITC was used for F-actin detection at a concentration of 1 in 2000. The cells were then incubated with Alexa Fluor 488 donkey anti mouse IgG (Invitrogen A-11029), Alexa Fluor 647 donkey anti goat IgG (Invitrogen A-21447) and Cy3 conjugated donkey anti rabbit IgG (Jackson ImmunoResearch 711-166-152). All secondary antibodies were incubated at 1 in 300 in 0.4 % FSG. Finally the cells were mounted in Mowiol supplemented with DAPI. Immunofluorescence images were collected as described in Chapter 2.

**3.2.4 Time-lapse imaging:** For monitoring whether cells had gone through mitosis the conditions and set up of the microscope were followed as described in Chapter 2. Cells were treated for 24 hours with 1.5  $\mu$ M HU to synchronize them. They were then placed in fresh medium and transfected with the appropriate siRNA. At the end of 48 hours the cells were prepared as for immunofluorescence and labelled with lamin B1 primary antibody (1 in 100) and Alexa Fluor 488 donkey anti mouse IgG secondary antibody (1 in 300) before being mounted in Mowiol with DAPI. The final image of the live cell imaging could then be overlayed with the fixed lamin B1 cells to determine whether cells had developed NR that had not passed through mitosis. To image changes of CCT $\alpha$ -

mCherry nuclear intensity HeLa cells were transfected with the construct 24 hours prior to imaging in a Martek dish. The cells medium was subsequently changed and either *FACE1* or control siRNA was transfected. Live cell imaging took place for 48 hours at 15 minute intervals using excitation at 555nm and 617nm emitter with the environmental conditions described above.

**3.2.5 Western Blotting:** The protocol for western blotting was carried out as described in Materials and Methods Chapter. Nitrocellulose membranes were bathed in ZMPSTE24 antibody at 1:200 (L. Fong, UCLA), lamin A antibody at 1:200 (Abcam ab26300),  $\beta$ -actin antibody at 1:5000 (Abcam 8226), prelamin A antibody at 1:100 (SantaCruz sc-6214) and CCT $\alpha$  antibody at 1:200 (R. Cornell) overnight. The secondary antibodies used were peroxidase AffiniPure Goat Anti-Mouse IgG (Sigma A4416), peroxidase Affinipure Goat Anti-Rabbit IgG (Sigma A6154) and peroxidase Affinipure Donkey Anti-Goat IgG (Jackson ImmunoResearch Europe Ltd 705-035-147) at a dilution of 1:5000, 1:6000 and 1:20,000 respectively.

**3.2.6 CCT $\alpha$  pixel and foci quantification:** Pixel intensity of a nucleus stained with CCT $\alpha$  was quantitated in Metamorph software to generate a histogram of intensities. Ten nuclei per transfection had their intensities averaged to produce the mean intensity of CCT $\alpha$  inside a nucleus. Foci of CCT $\alpha$  were counted and averaged over fifty separate nuclei for each condition. Intranuclear CCT $\alpha$  foci were determined as nucleoplasmic signals that had an intensity that corresponded with intensities found at the nuclear periphery. The boundary of individual foci was marked out by intensity that matched the

nucler peripheral intensity of the enzyme. The reason for choosing peripheral intensity was because this is where the enzyme localizes and concentrates in its active form and serves as a reliable indicator of membrane association.

**3.2.7 Cell cycle analysis:** Cells were treated with 1.5 mM hydroxyurea (HU) (Sigma-Aldrich, H8627) for 24 hours before being transfected with *Zmpste24* or control siRNA. Images of DAPI stained nuclei were acquired using a SNAP HQ 12 bit CCD camera with a Plan Neofluar 10x 0.3na lens. The phase of the cell cycle was assessed by using the cell cycle application in Metamorph software that assesses DAPI intensity to correlate with different stages of the cell cycle.

**3.2.8 NPC count:** Cells were treated with 1.5 mM hydroxyurea (HU) for 24 hours before being transfected with *Zmpste24* or control siRNA. The cells were then fixed and processed for immunofluorescence as described earlier. Images of grazing sections of anti-Nup153 and anti-mAB414 labelled nuclei were acquired and processed in Metamorph software. Processing involved thresholding the image, binarising the thresholded features, median filtering the binary image (3 x 3 kernel) to remove single pixels and ultimate eroding the resulting binary image such that each NPC is represented by a single pixel. Over a 25  $\mu\text{m}^2$  area the numbers of NPCs were counted for twenty different nuclei in each treatment.

**3.2.9 Farnesylation assays:** Farnesyl transferase inhibitor FTI-277 (Calbiochem, Beeston, UK) was dissolved in DMSO and used at 10  $\mu\text{M}$  for 48 hours. To assess protein

farnesylation cells were incubated with the farnesol analogue 8-anilinogeraniol (H. Spielmann, University of Kentucky, USA), which was dissolved in DMSO and used at a final concentration of 50  $\mu$ M for 48 hours. Level of farnesyl analog was then detected by western blot using a mouse monoclonal antibody (1 in 5000) against the analogue (Troutman et al., 2005).

**3.2.10 EMT, TEM and Immunogold:** Processing of cells and data acquisition for EMT, TEM and immunogold results are all described in Chapter 2. Nup153 primary antibody concentration was used at 1 in 100 and mAB414 primary antibody was used at 1 in 20.

### 3.3 RESULTS

#### 3.3.1 Farnesylated Prelamin A produces a dysmorphic nuclear perimeter independent of CCT $\alpha$

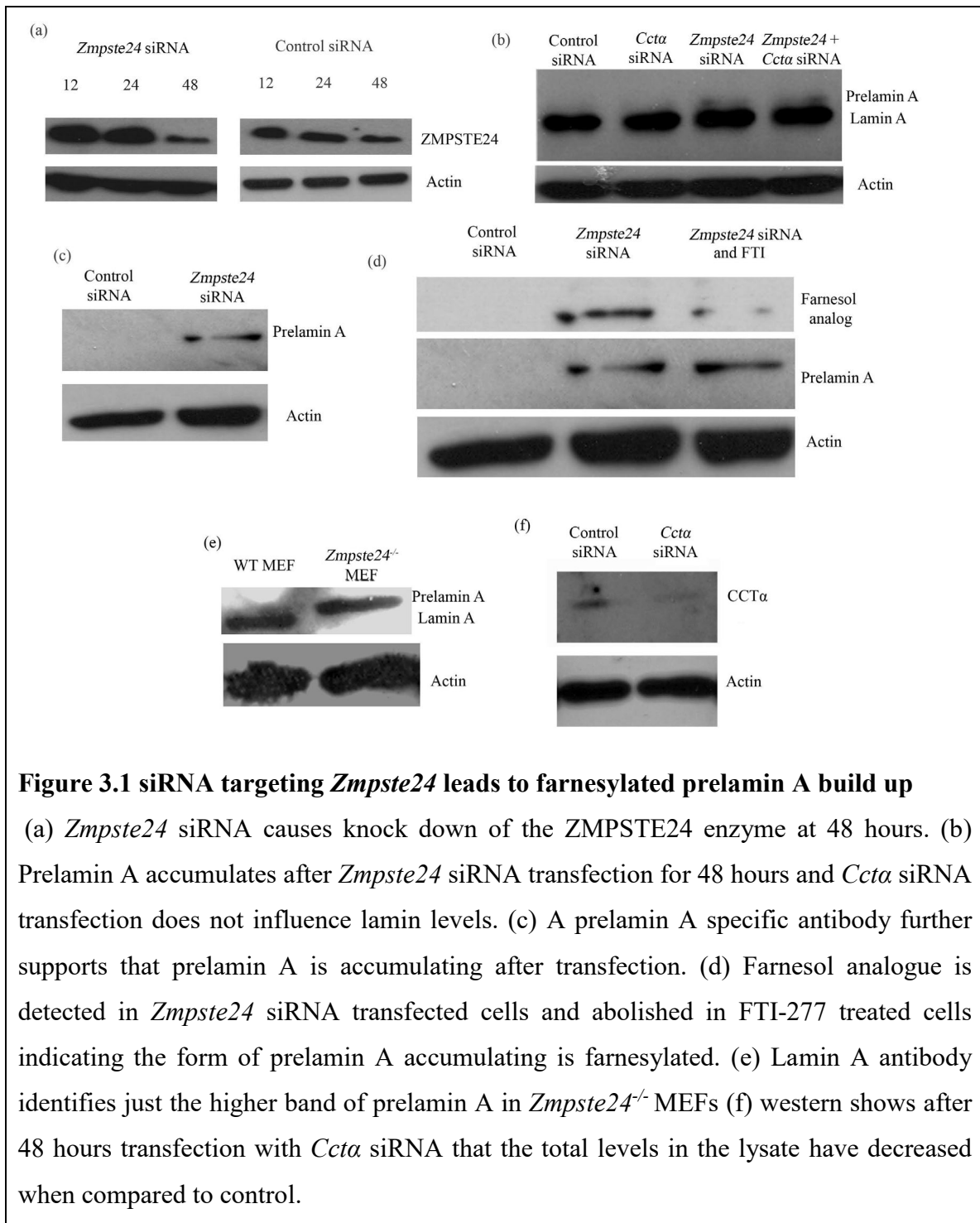
Upon transfection with *Zmpste24* siRNA in MEFs the ZMPSTE24 enzyme had reduced abundance at 48 hours (Figure 3.1a). The earlier time points of 12 and 24 hours post transfection showed no noticeable reduction in band intensity. The control siRNA showed no alteration of *Zmpste24* protein levels over the three time points (Figure 3.1a). Based on this initial observation subsequent experiments were assessed at the 48 hour time point, as this was when levels of the ZMPSTE24 enzyme were being down regulated.

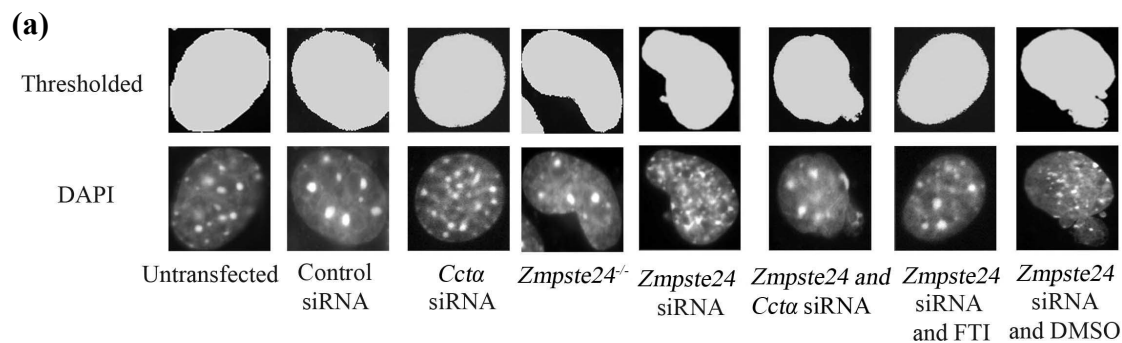
At 48 hours post transfection a band with a molecular weight that is higher than lamin A was observed by western blotting, which is indicative of the unprocessed precursor

accumulating (Figure 3.1b). This was further supported by a signal being detected when using a prelamin A specific antibody, which was absent in control siRNA treated cell lysates (Figure 3.1c). However, the prelamin A specific antibody detects between amino acids 645 and 664 and does not distinguish whether the form is farnesylated or not (Mattioli et al., 2011). Consequently cells were incubated with a farnesol analogue, 8-anilinogeraniol, which is incorporated into anilinogeranyl diphosphate (AGPP). AGPP is used as a substrate by protein farnesyltransferase for the modification of CaaX containing proteins, which includes prelamin A (Troutman et al., 2005). An antibody raised against the analogue detected prelamin A after *Zmpste24* siRNA transfection but this band was absent in control transfected cells and reduced after *Zmpste24* siRNA transfection in combination with FTI-277 treatment (Figure 3.1d). The prelamin A specific antibody detected a band in both the *Zmpste24* transfected and *Zmpste24* transfected with FTI-277 treatment (Figure 3.1d).

The *Zmpste24*<sup>-/-</sup> MEFs were shown to only accumulate prelamin A using a Lamin A/C antibody and none of the mature lamin A was detected (Figure 1e). Co-transfection of *Zmpste24* siRNA with *Ccta* siRNA into wild type MEFs did not affect the quantity of accumulating prelamin A or lamin A levels (Figure 3.1b). Transfection with *Ccta* siRNA alone did not cause unprocessed protein formation and did not affect the levels of lamin A (Figure 3.1b). The *Ccta* siRNA also significantly reduced *Ccta* expression at the protein level as indicated by the western blot, which was detected using an anti-CCT $\alpha$  antibody (Figure 3.1f).

The effect that farnesylated prelamin A has on nuclear morphology appears to result in a dysmorphic phenotype with blebs and invaginations developing. Two hundred DAPI stained nuclei were analysed by thresholding and binarising the images, before determining individual nuclear perimeters and areas (Figure 3.2a). These parameters were used in the contour ratio (CR) formula ( $4\pi \times \text{area}/\text{perimeter}^2$ ) to establish how dysmorphic nuclei had become, with lower CR values being increasingly dysmorphic (Figure 3.2b). It was found that *Zmpste24* siRNA transfection lead to CRs of  $0.63 \pm 0.14$ . *Zmpste24*<sup>-/-</sup> nuclei seemed to be significantly more dysmorphic than the acute farnesylated prelamin A accumulating cells with a CR of  $0.52 \pm 0.14$ . This may be due to the *Zmpste24*<sup>-/-</sup> cells constantly accumulating farnesylated prelamin A as they were originally isolated from mice lacking ZMPSTE24, which led to the significantly lower CR. Nuclei, after control siRNA transfection, had significantly higher CRs of  $0.79 \pm 0.04$ , indistinguishable from control untreated nuclei. Transfection with *Ccta* siRNA also did not lead to a dysmorphic nuclear appearance and produced a CR of  $0.76 \pm 0.08$ . Co-transfection of this siRNA with *Zmpste24* siRNA generated a CR of  $0.61 \pm 0.27$ , which was not significantly different to the other treatments accumulating farnesylated prelamin A. This would suggest that the perimeter of the nucleus becomes highly convoluted in the presence of farnesylated prelamin A without involving CCT $\alpha$ . Indeed when cells transfected with *Zmpste24* siRNA were additionally treated with an FTI it caused an increase of CR to  $0.70 \pm 0.18$  whereas treatment with the DMSO vehicle control produced no significant difference to the CR when compared to *Zmpste24* siRNA transfection alone.





**(b)**

| Treatment                             | Mean Contour Ratio | Standard Deviation | P Value |
|---------------------------------------|--------------------|--------------------|---------|
| Untransfected                         | 0.78               | 0.05               |         |
| Control siRNA                         | 0.79               | 0.04               | >0.05   |
| <i>Ccτα</i> siRNA                     | 0.76               | 0.08               | >0.05   |
| <i>Zmpste24</i> <sup>-/-</sup>        | 0.52               | 0.14               | <0.01   |
| <i>Zmpste24</i> siRNA                 | 0.63               | 0.14               | <0.01   |
| <i>Zmpste24</i> and <i>Ccτα</i> siRNA | 0.61               | 0.27               | <0.01   |
| <i>Zmpste24</i> siRNA and FTI         | 0.70               | 0.18               | <0.01   |
| <i>Zmpste24</i> siRNA and DMSO        | 0.64               | 0.12               | <0.01   |

**Figure 3.2 Farnesylated prelamins A produces dysmorphic nuclei**

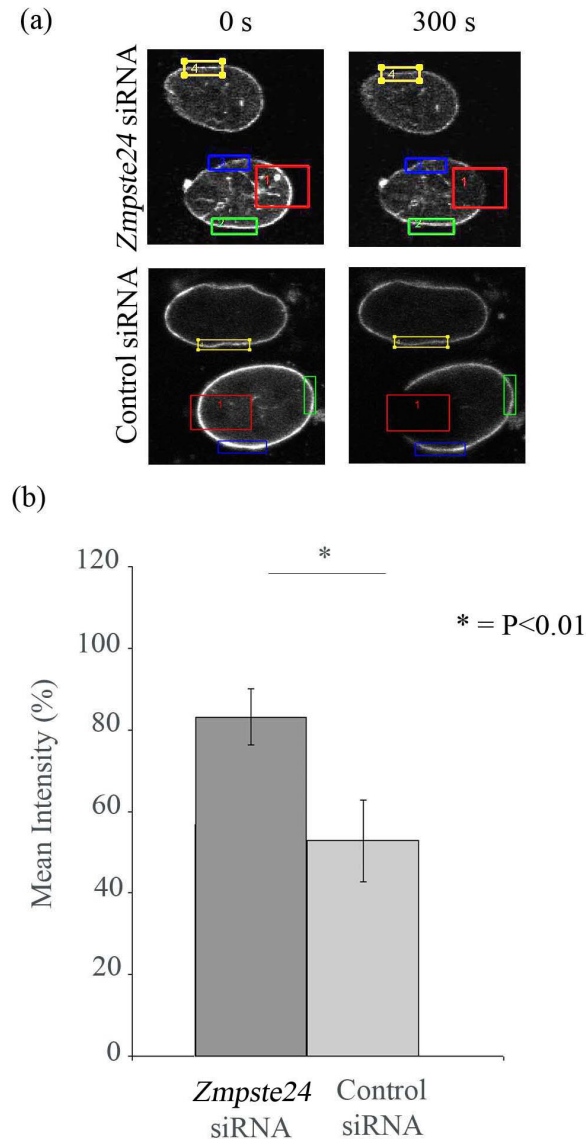
(a) DAPI stained nuclei were thresholded and binarised in Metamorph to generate perimeters and areas for each nucleus (200 nuclei per condition were processed).

(b) A contour ratio was assigned to each nucleus by using the formula  $CR = 4 \pi \times (\text{area}/\text{perimeter}^2)$ . The lower the contour ratios the more dysmorphic the nucleus had become. Significance was determined using the Mann-Whitney U Test.



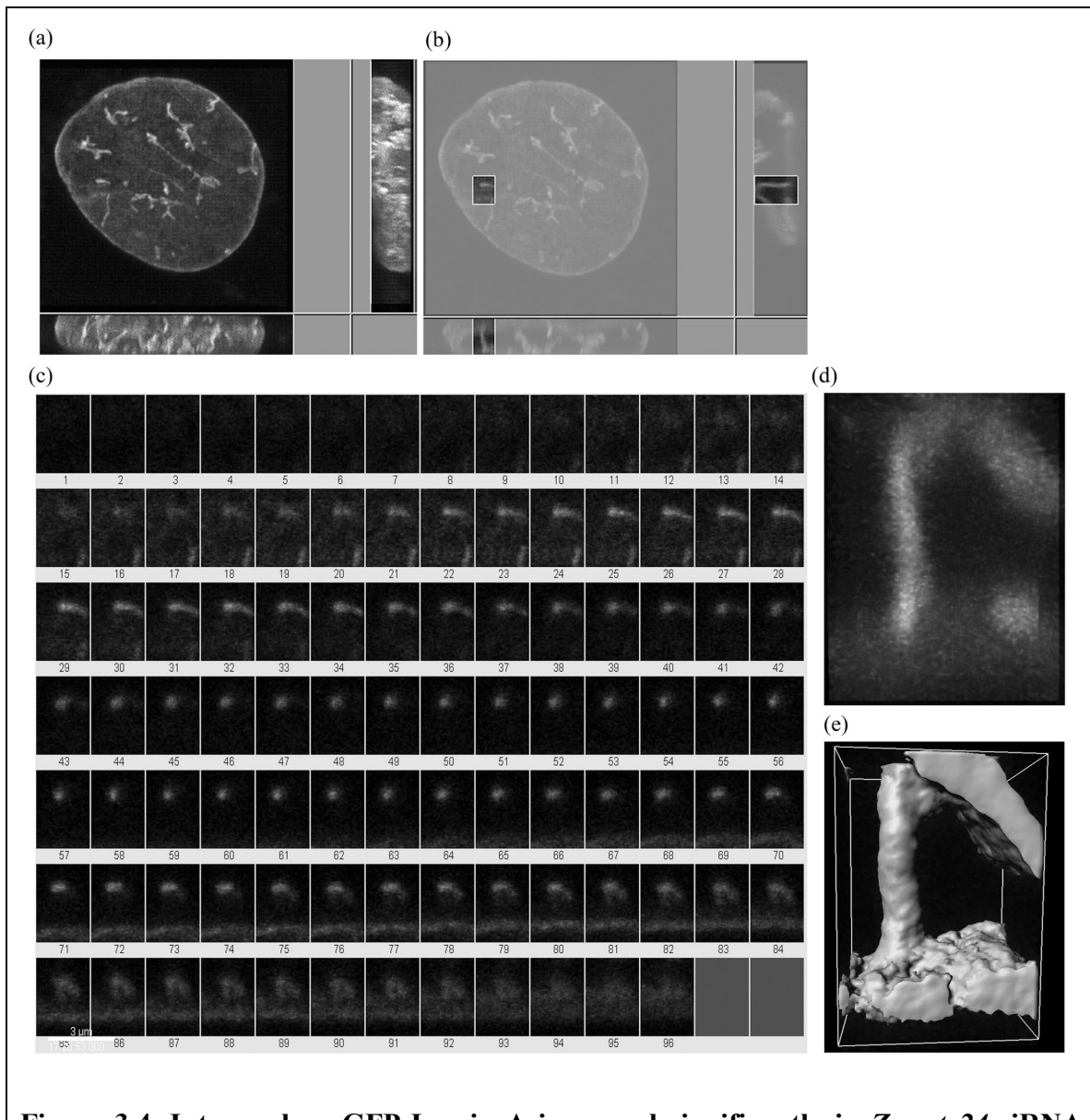
### 3.3.2 Acute *Zmpste24* siRNA transfection leads to a less dynamic lamina

A retained farnesylated tail on prelamin A is believed to hydrophobically interact with the INM and therefore reduce the mobility of lamin A at the periphery. To further validate that the *Zmpste24* siRNA was indeed leading to farnesylated prelamin A accumulation FLIP was performed at the nuclear periphery of GFP lamin A expressing cells (Figure 3.3a). For 5 minutes a region of the fluorescently labelled lamina was photobleached and adjacent regions of interest were monitored for changes in fluorescent intensity. The control siRNA transfected cells showed a decrease in intensity down to 52.8%  $\pm$  10.1 (Figure 3.3b). This suggests that under control conditions GFP lamin A is dynamic and able to move around the periphery to areas that have been photobleached in a relatively short space of time. However, when the cells were transfected with *Zmpste24* siRNA it had a dramatic effect on the dynamics of GFP-lamin A. After 5 minutes of photobleaching the regions of interest only dropped to 83.1 %  $\pm$  6.9. This indicates that in the environment where *Zmpste24* is down regulated the laminas dynamics are severely hindered. It is likely that the retention of the farnesylated tail on prelamin A enables hydrophobic interaction with the INM, which reduces GFP-lamin A's ability to move as freely at the periphery. In the live cells many more GFP lamin A intranuclear signals could be detected that were often connected to the nuclear periphery and are likely to be associated with nuclear membrane invaginations due to the retention of the farnesylated tail embedding itself in the INM (Figure 3.4). The fact that these structures could be observed in live cells suggests that the structures seen in immunofluorescence are not a consequence of fixation or processing.



**Figure 3.3 FLIP reveals *Zmpste24* siRNA transfection leads to increased lamin stability**

(a) An ROI was photobleached (red square) for 300 seconds and adjacent areas were monitored for changes in intensity (green and blue squares). Background photobleaching was normalised by measuring the change of intensity of an adjacent GFP-lamin A transfected nucleus that was not being photobleached (yellow square). (b) At 300 seconds the mean intensity of the *Zmpste24* siRNA transfected nuclei ( $n = 10$  per condition) was significantly higher than in the control siRNA transfected nuclei, suggesting a less dynamic peripheral lamina.



**Figure 3.4. Intranuclear GFP-Lamin A increased significantly in *Zmpste24* siRNA transfected cells.**

(a) MEFs transfected with *Zmpste24* siRNA had a far greater abundance of intranuclear GFP-lamin A that was often continuous with the nuclear membrane. (b) Cropped XY, ZX, YZ view of a GFP lamin A intranuclear signal that traverses both sides of the nucleus. (c) Z-stack of the GFP-lamin A intranuclear column taken at 0.5 μm intervals. (d) 3d volume and (e) isosurface of the GFP-lamin A nucleoplasmic signal making connection with both sides of the nucleus.

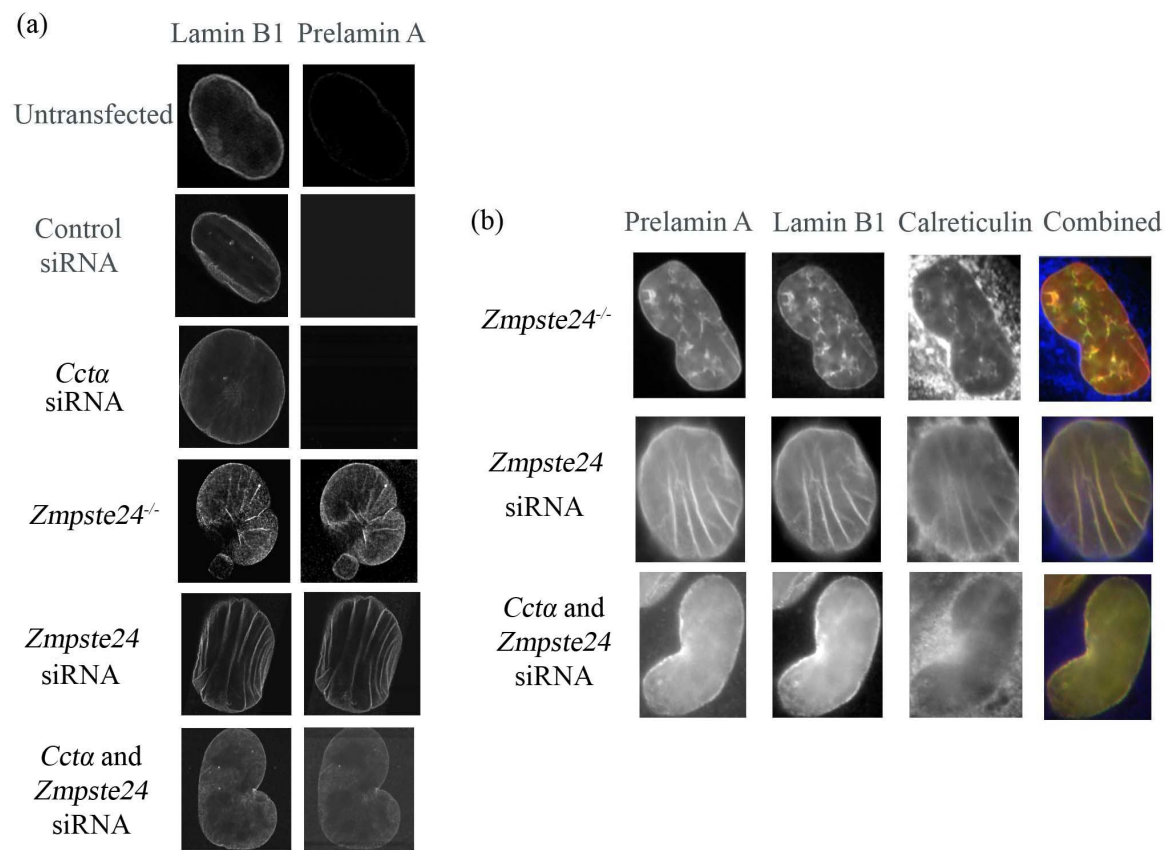
### 3.3.3 Colocalisation of prelamin A and lamin B1 with a developing NR is dependent on CCT $\alpha$

Immunofluorescence microscopy was used to examine the organisation of the nuclear architecture in cells accumulating prelamin A (Figure 3.5). The proteins stained for were prelamin A, lamin B1 and calreticulin. Lamin B1 is a farnesylated protein, which has a high affinity for the INM due to its farnesylated tail and serves as a good marker for the outline of the inner INM. Calreticulin, an ER luminal protein that is also found within the perinuclear space, would also serve as a good marker of whether the nuclear membrane was invaginating (Echevarria et al., 2003a).

There was little staining of prelamin A in untransfected nuclei, while lamin B1 often had a very strong association with the nuclear periphery with very little intranuclear signal (Figure 3.5a). Calreticulin labelled ER throughout the cytoplasm and around the nuclear membrane with very little signal detected inside the nucleus (Figure 3.5b). *Zmpste24* siRNA transfection leads to strong staining of prelamin A at the periphery and within intranuclear structures that colocalised with lamin B1 and calreticulin (Figure 3.5b). *Zmpste24*<sup>-/-</sup> nuclei had a very similar appearance to the siRNA knock down nuclei, containing an extensive NR that colocalised with prelamin A and lamin B1 (Figure 3.5a and b). The staining observed could be as a result of the INM folding in, with it just having an ER luminal core or both the INM and ONM folding in with a cytoplasmic core surrounded by a ring of ER lumen. The fact that prelamin A also colocalises with these two proteins further supports the fact that it is the farnesylated form of prelamin A accumulating, as the unfarnesylated form is reported as forming isolated intranuclear foci

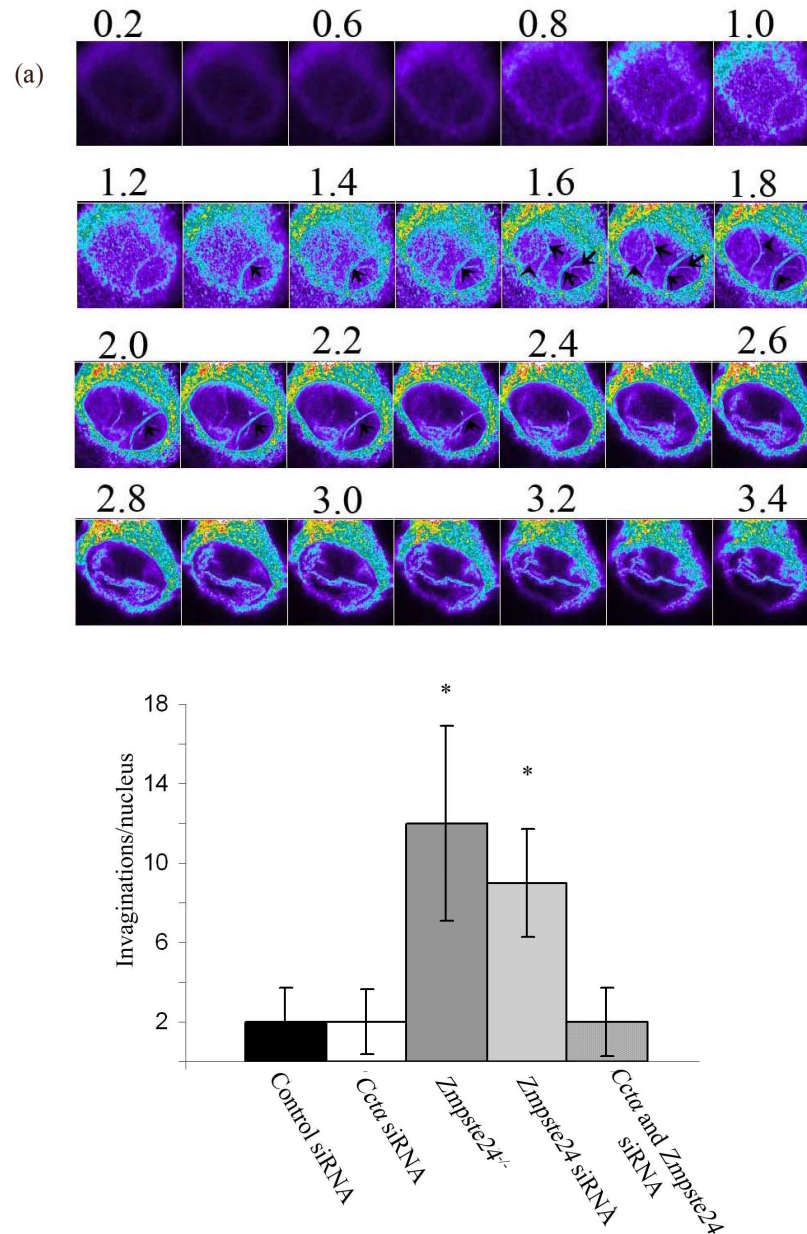
due to reduced affinity with the INM rather than these nucleoplasmic structures representing NR (Toth et al., 2005).

Quantification of invaginations was achieved by acquiring z stacks of nuclei and counting the number of calreticulin positive intranuclear structures that were continuous with the nuclear membrane (Figure 3.6a). It was found that in control siRNA transfected nuclei there was an average of two invaginations per nucleus. In *Zmpste24* siRNA transfected and *Zmpste24*<sup>-/-</sup> nuclei the number had increased significantly with respective counts per nucleus being nine and twelve (Figure 3.6b). Co-transfection with *Zmpste24* and *Ccta* siRNA did reduce intranuclear staining of both lamins and the presence of NR to three calreticulin positive intranuclear signals per nucleus. However, even though the invagination count was reduced the nuclear morphology still had a dysmorphic phenotype, as supported by the CR (Figure 3.2b).



**Figure 3.5 NR colocalisation with prelamin A and lamin B1 requires CCTα**

(a) Prelamin A and lamin B1 colocalise around the nuclear envelope and at invaginations within the nucleoplasm and *Cctα* siRNA transfection ablated intranuclear signals. (b) Presence of invaginations is supported by the fact that prelamin A and lamin B1 colocalise with calreticulin at the intranuclear signals.



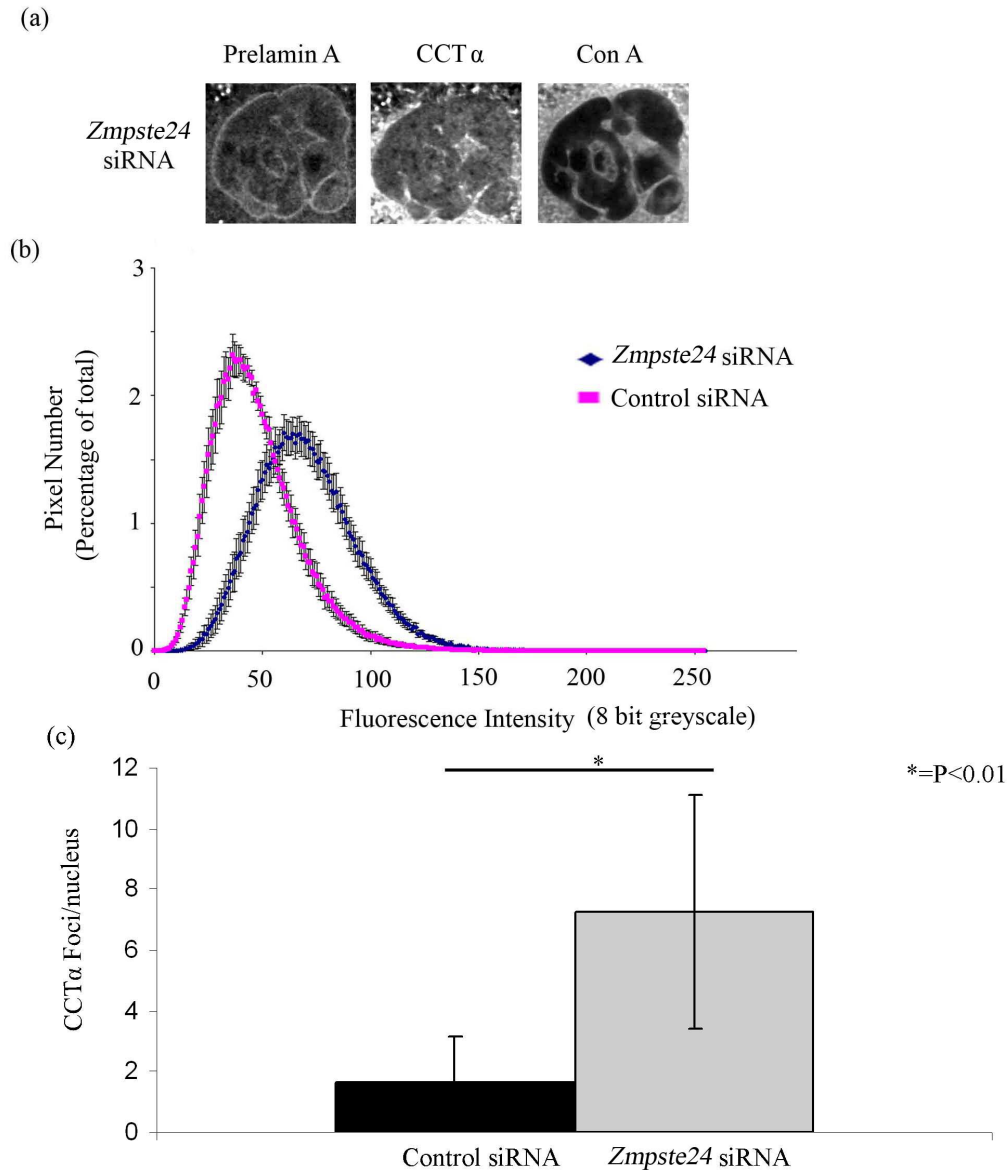
**Figure 3.6. NR quantification using calreticulin and z stacks**

(a) Quantification of invaginations was achieved by taking z stacks of nuclei and counting the number of calreticulin positive intranuclear signals that connected with the periphery of the nucleus (arrows indicate invagination). N=25 per condition (b) Nucleoplasmic reticulum was significantly more abundant in prelamins A accumulating cells and transfection with *Ccta* siRNA significantly reduced their frequency. Asterisk represents a p value (Two tailed t test) of less than 0.01 when compared to control siRNA.

### 3.3.4 CCT $\alpha$ forms intranuclear foci that colocalise with invaginations in prelamin A accumulating cells

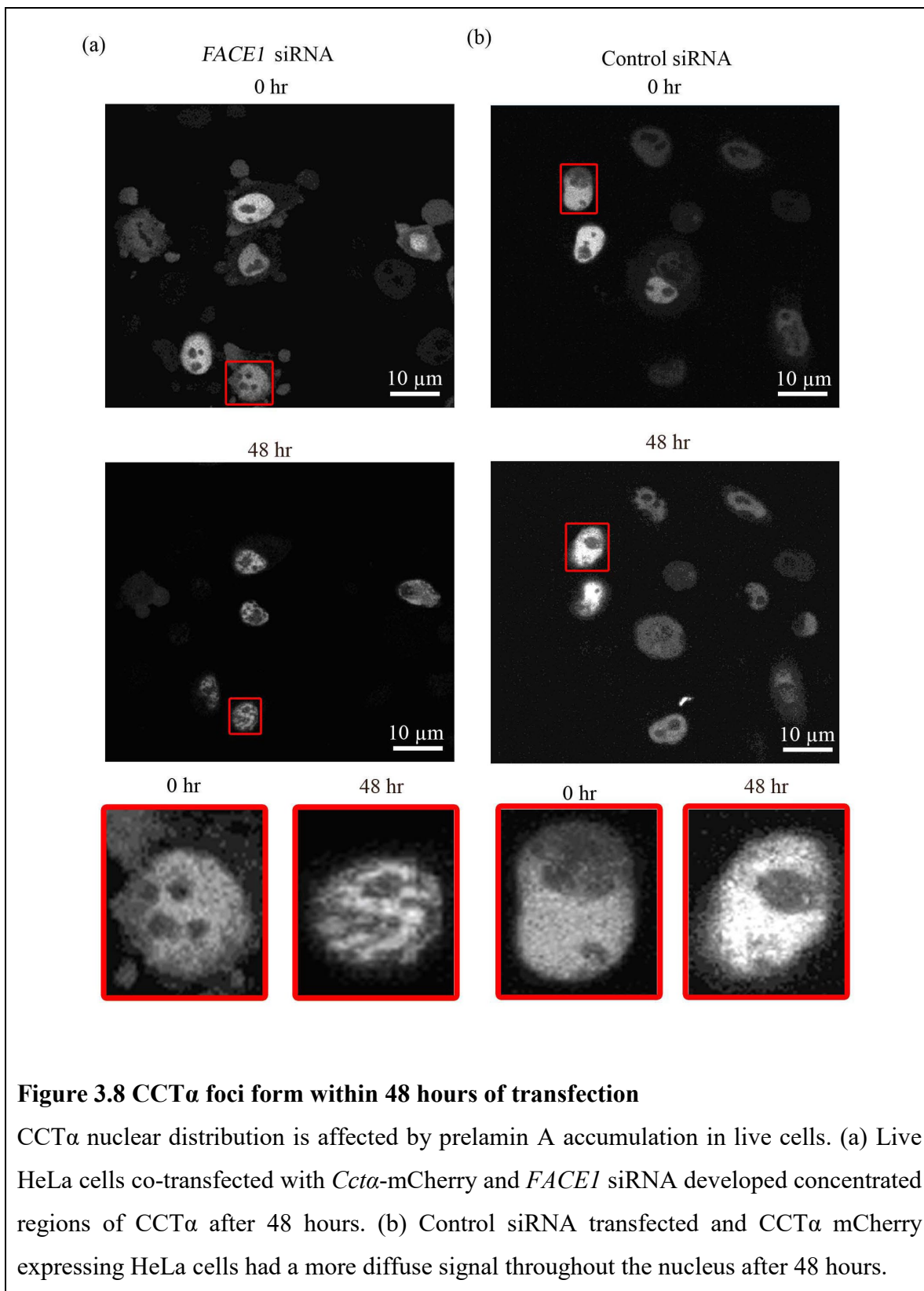
Cells transfected with *Zmpste24* siRNA were stained against prelamin A, CCT $\alpha$  and concanavalin A (ConA) (Figure 3.7a). Con A is a lectin that binds to high mannose forms of N-linked oligosaccharide side chains on glycoproteins in the ER, IMS and NR interior (Fricker et al., 1997b). ConA revealed invaginations present in the nucleus that colocalised with the prelamin A. Overall pixel intensity for CCT $\alpha$  labelling, within the nuclei of *Zmpste24* siRNA transfected cells, resulted in a shift to a greater abundance of higher intensity pixels (Figure 3.7b). CCT $\alpha$  appeared as intense foci along the invaginations, as has previously been observed (Gehrig et al., 2008) Quantification of these foci revealed that the abundance of CCT $\alpha$  foci increased when nuclei acutely accumulated prelamin A (Figure 3.7c). Live cell imaging was performed on HeLa cells that had been transfected with *Ccta*-mCherry and subsequently transfected with either *FACE1* siRNA (Figure 3.8a) or control siRNA (Figure 3.8b) for 48 hours. It revealed that the control siRNA transfected cells had a more diffuse signal of the nuclear CCT $\alpha$  mCherry after 48 hours, while the *FACE1* siRNA transfected cells had regions that had a greater intensity and developed intranuclear foci, as had been observed in the fixed cells for immunofluorescence.





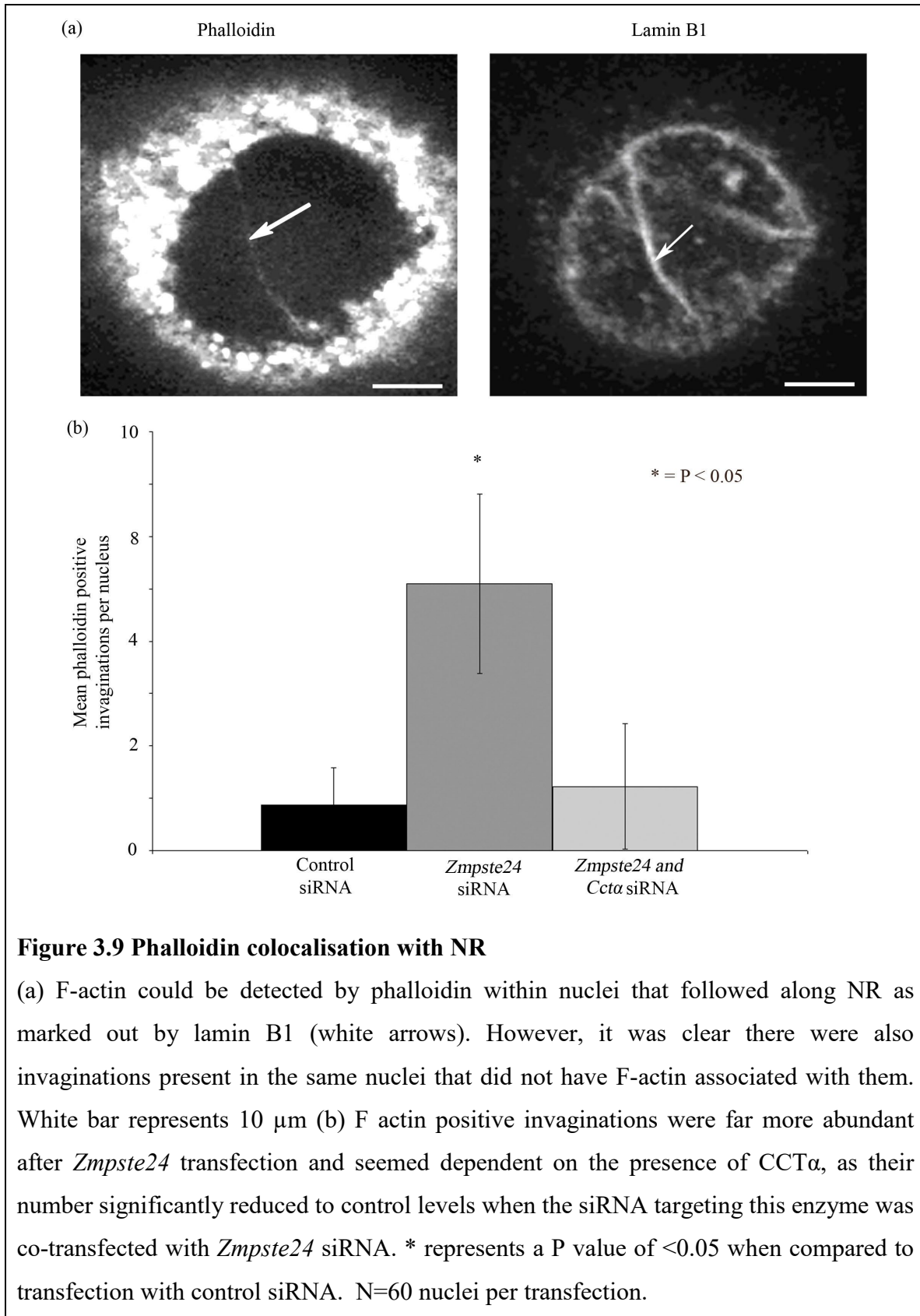
**Figure 3.7 CCTα forms foci along NR**

(a) After *Zmpste24* siRNA transfection CCTα was found as foci along the invaginations that partly colocalised with prelamin A. (b) A histogram of CCTα intensity frequency was calculated for each nucleus (fifty nuclei per treatment) and *Zmpste24* siRNA transfection resulted in a shift to the right, indicating a greater abundance of higher intensity CCTα pixels. (c) CCTα foci were quantified (30 nuclei per condition) and showed a significantly (two tailed t test) increased abundance after *Zmpste24* siRNA transfection.



### 3.3.5 F-actin is associated with parts of the NR

It has been reported that within type II NR of many different cell types cytoskeletal components are observed. The exact function of these structures remains speculative but certainly at the EM level vesicles can be seen associated with these tracks running along the cytoplasmic core of the NR (Malhas et al., 2011). To investigate whether the NR developing after acute *Zmpste24* siRNA transfection contained F-actin phalloidin-TRITC was used to assess its distribution (Figure 3.9). It was clear that the more abundant NR in *Zmpste24* siRNA transfected cells also colocalised with phalloidin for many but not all of the invaginations (Figure 3.9a). The mean number of invaginations positive for phalloidin after *Zmpste24* siRNA transfection was five while the total number of invaginations was nine (Figure 3.9 b and Figure 3.6b). The distribution of the phalloidin often ran along the length of the invagination, sometimes connecting two sides of the nucleus. They seemed to be associated with the NR as co-transfection with *Ccta* siRNA significantly reduced their abundance down to levels observed in the control siRNA transfected cells. It was also evident that not the entire NR signal had Phalloidin associated with it. This may be simply due to the F-actin not extending down these particular invaginations or that the NR lacking phalloidin signal was due to these invagination being of the type I form and therefore lack the cytoplasmic core for actin to polymerise into.



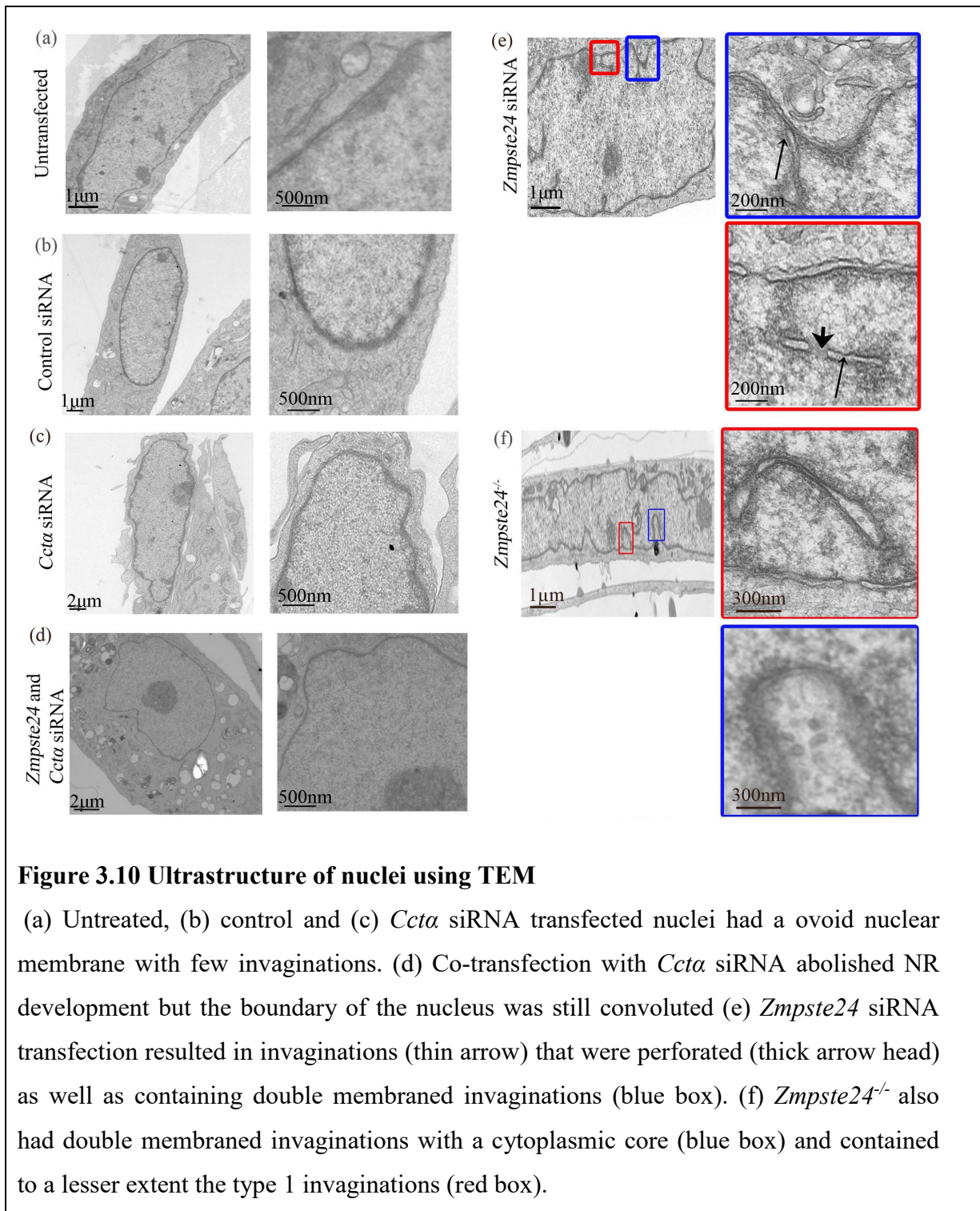
### 3.3.6 The NR contains nuclear pore complexes

TEM was used to assess the morphology of nuclei developing NR, which revealed two types of membranous structure that formed within the nucleus when farnesylated prelamin A accumulates. Untransfected cells or those transfected with a control siRNA often contained nuclei with an ovoid shape with very few nuclear aberrations or intranuclear structures (Figure 3.10a, b). However, after *Zmpste24* siRNA transfection and in *Zmpste24*<sup>-/-</sup> cells it became apparent that the nuclei contained many membrane-bound, flattened cisternal structures containing membrane-lined fenestrations (Figure 3.10e, f). These structures are often perpendicular to the nuclear membrane and can vary from several hundred nanometres in length up to stretching the diameter of the nucleus. An electron dense signal is often found in close association along the membrane, which may be heterochromatin or the lamina scaffold. They were also frequently found to terminate close to nucleoli. Co-transfection with *Zmpste24* and *Ccta* siRNA lead to a convoluted perimeter but no intranuclear membranous structures could be observed (Figure 3.10d). Transfection with the *Ccta* siRNA alone resulted in a morphology similar to that observed in untreated and control siRNA transfected nuclei (Figure 3.10c).

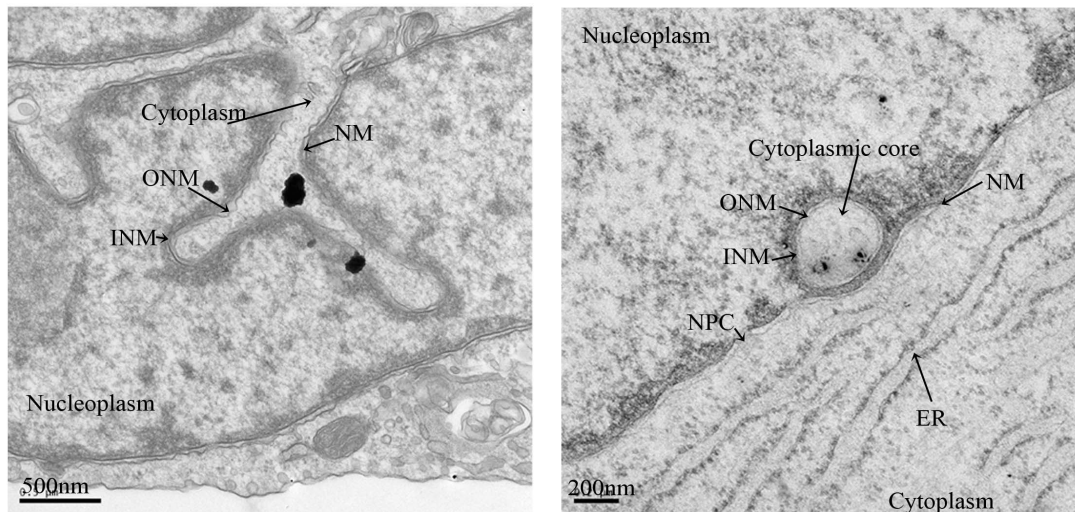
The fenestrations could be seen to regularly appear along the length of these structures and upon further examination, using dual axis EM tomography on 250nm sections, it could be seen that these fenestrations were actually pores that had very similar dimensions to NPCs but had a nucleoplasmic to nucleoplasmic orientation (Figures 3.10 e red box and 3.11c). A 3D model could be generated by using iMOD software that revealed pores with a diameter of approximately 100nm, which correlates well with

current estimates of NPC diameters (Figure 3.13). Serial section dual axis electron tomography revealed that these structures are sheet-like and have many pore structures along them (Figure 3.14). EMT also revealed that these structures were in fact invaginations of the INM (Figure 3.15), which explains the presence of an intranuclear calreticulin signal observed with immunofluorescence. The region of continuity between the NE and NR may be very short, measuring only a few tens of nanometres.

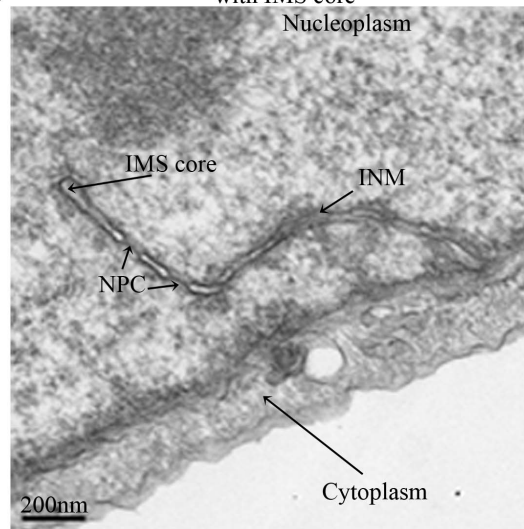
Another type of invagination observed in prelamin A accumulating nuclei consisted of an in-folding of both the INM and ONM, which contained a cytoplasmic core (Figure 3.10e, f blue boxes and Figure 3.11a). These structures could either be single or branching and entered the nucleus anywhere from several micrometres up to spanning its entire length. Cross sections of these structures gave the appearance of double-walled vesicles within the nucleoplasm (Figure 3.11b), with NPCs clearly visible bridging the nuclear membrane between the cytoplasmic core and nucleoplasm. There appear to be two types of invaginations that develop after prelamin A accumulation, which are represented in the schematic (Figure 3.12).



(a) Side profile of invagination with cytoplasmic core (b) Cross section of invagination with cytoplasmic core



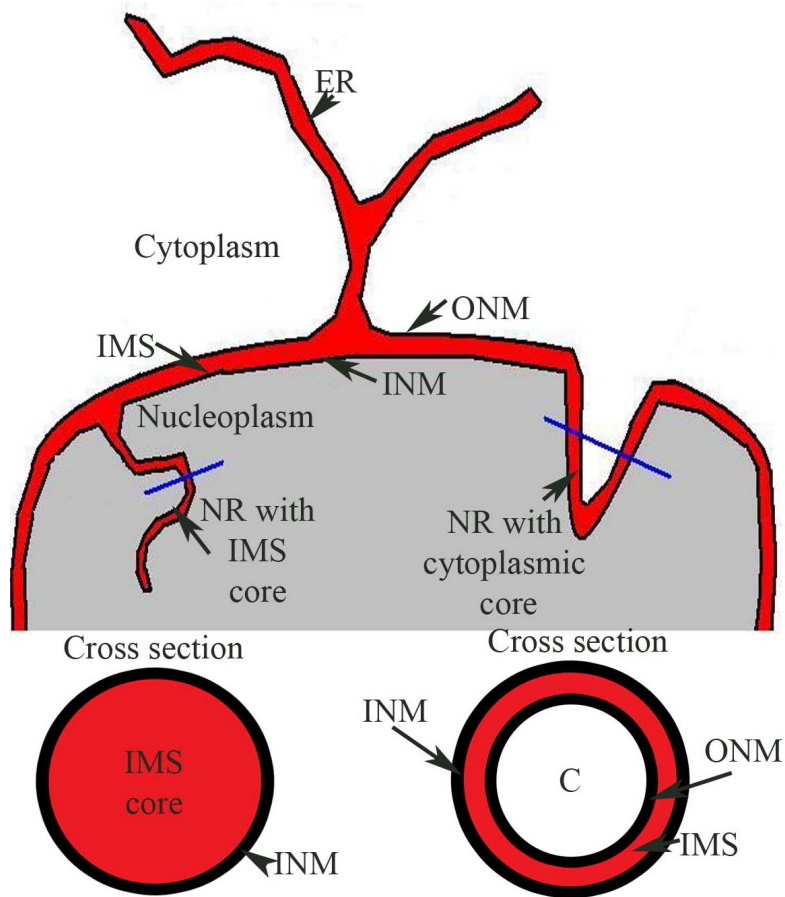
(c) Side profile of invagination of INM with IMS core  
Nucleoplasm



### Figure 3.11 Different types of NR revealed by TEM

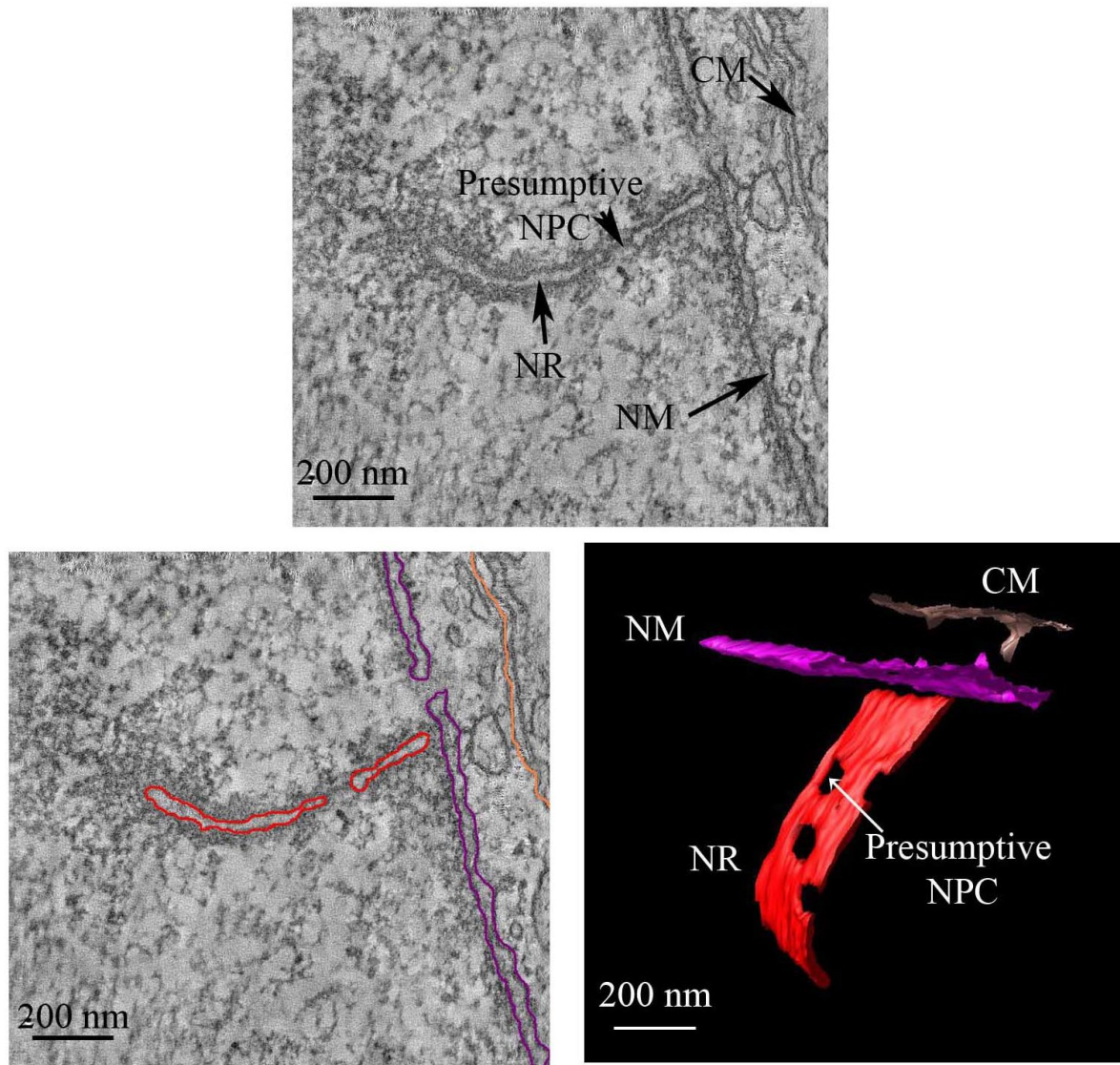
(a) Invaginations could exist as double membraned invaginations of both the INM and ONM with a cytoplasmic core. (b) Cross section of a double membrane invagination appears like a double membraned vesicle with a cytoplasmic core. (c) Invaginations of just the INM usually had pores associated with them, which were orientated in a nucleoplasmic to nucleoplasmic manner. The NPC in this case is presumptive as it is not known if it is complete like those found at the periphery.





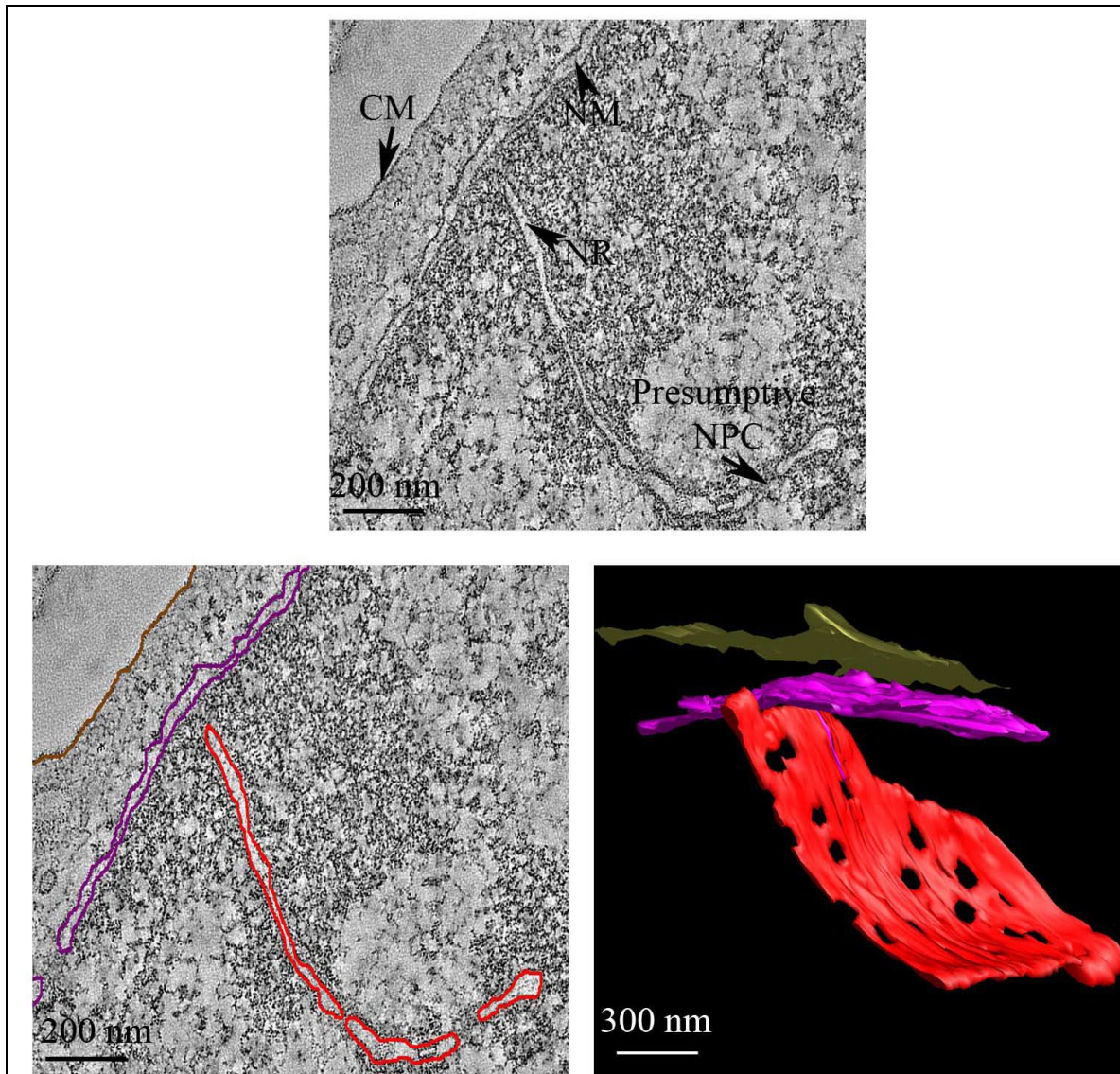
**Figure 3.12 Schematic illustrating the two types of invaginations that develop as part of the NR when farnesylated prelamin A accumulates.**

Type I invaginations consist of just an invagination of the INM and contain an IMS core continuous with the ER lumen. Type II invaginations have both the INM and ONM invaginating into the nucleoplasm and have a cytoplasmic core (C).



**Figure 3.13 Dual axis electron tomography of NR structure**

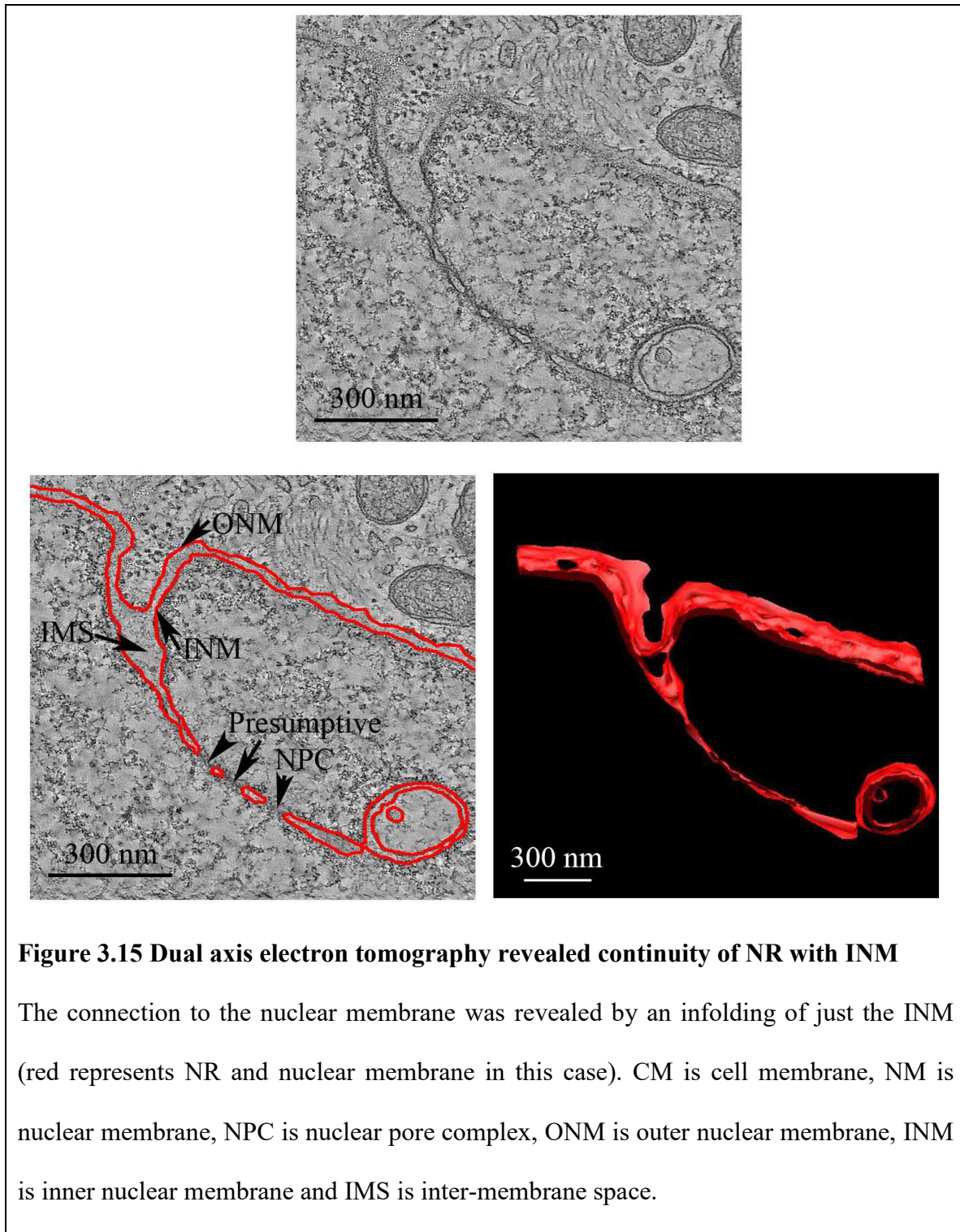
Dual axis electron tomography of a 250 nm thick section revealed that the NR contained regularly sized pores, with similar dimensions to NPCs. Red is NR, purple is nuclear membrane and brown is cell membrane. CM is cell membrane, NM is nuclear membrane, NPC is nuclear pore complex, ONM is outer nuclear membrane, INM is inner nuclear membrane and IMS is inter-membrane space.



**Figure 3.14 Serial section dual axis electron tomography of NR**

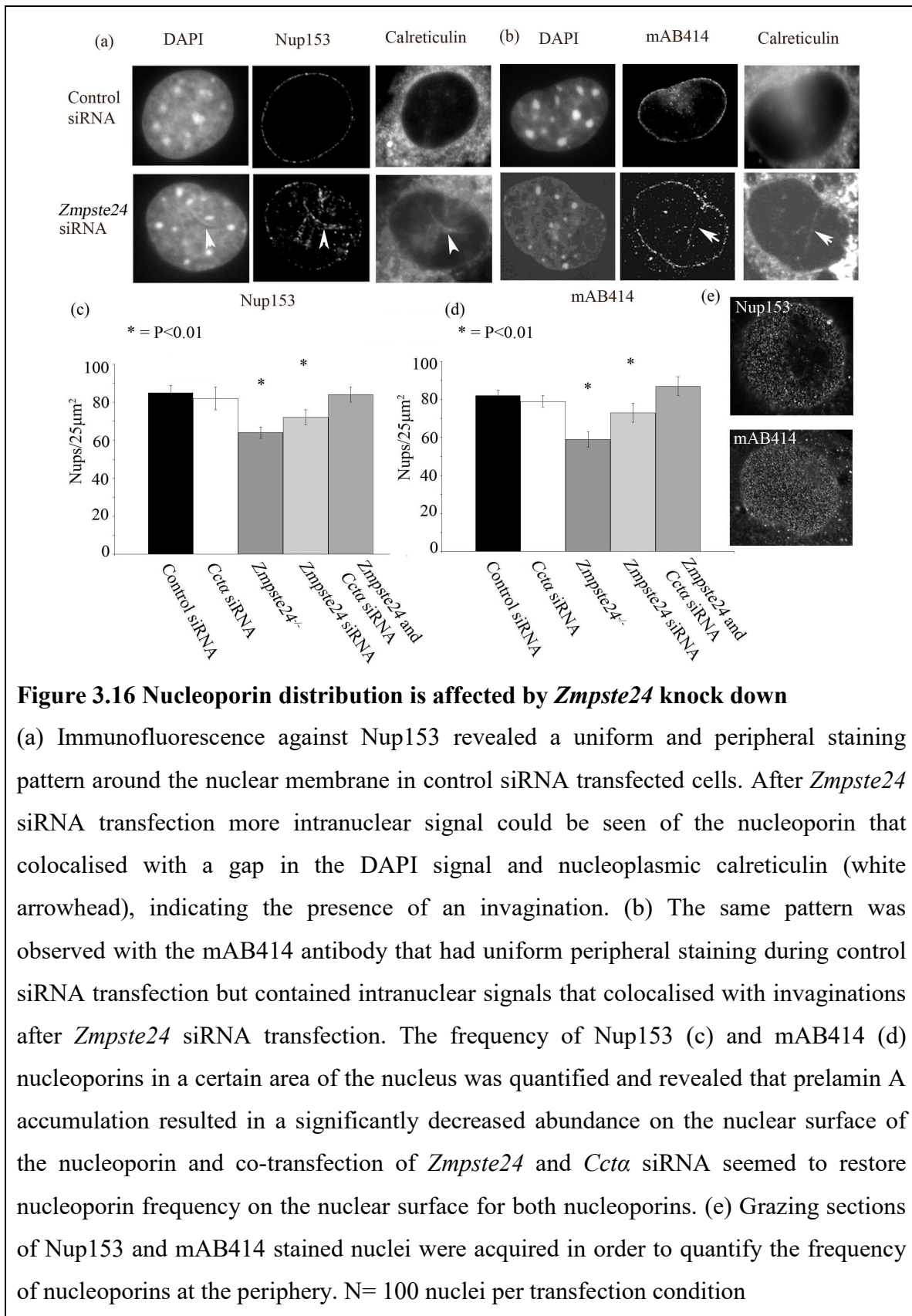
Serial section dual axis electron tomography of seven 120 nm thick sections revealed that the NR was sheet like and contained many presumptive NPCs. Red is NR, purple is nuclear membrane and brown is cell membrane. CM is cell membrane, NM is nuclear membrane, NPC is nuclear pore complex, ONM is outer nuclear membrane, INM is inner nuclear membrane and IMS is inter-membrane space.

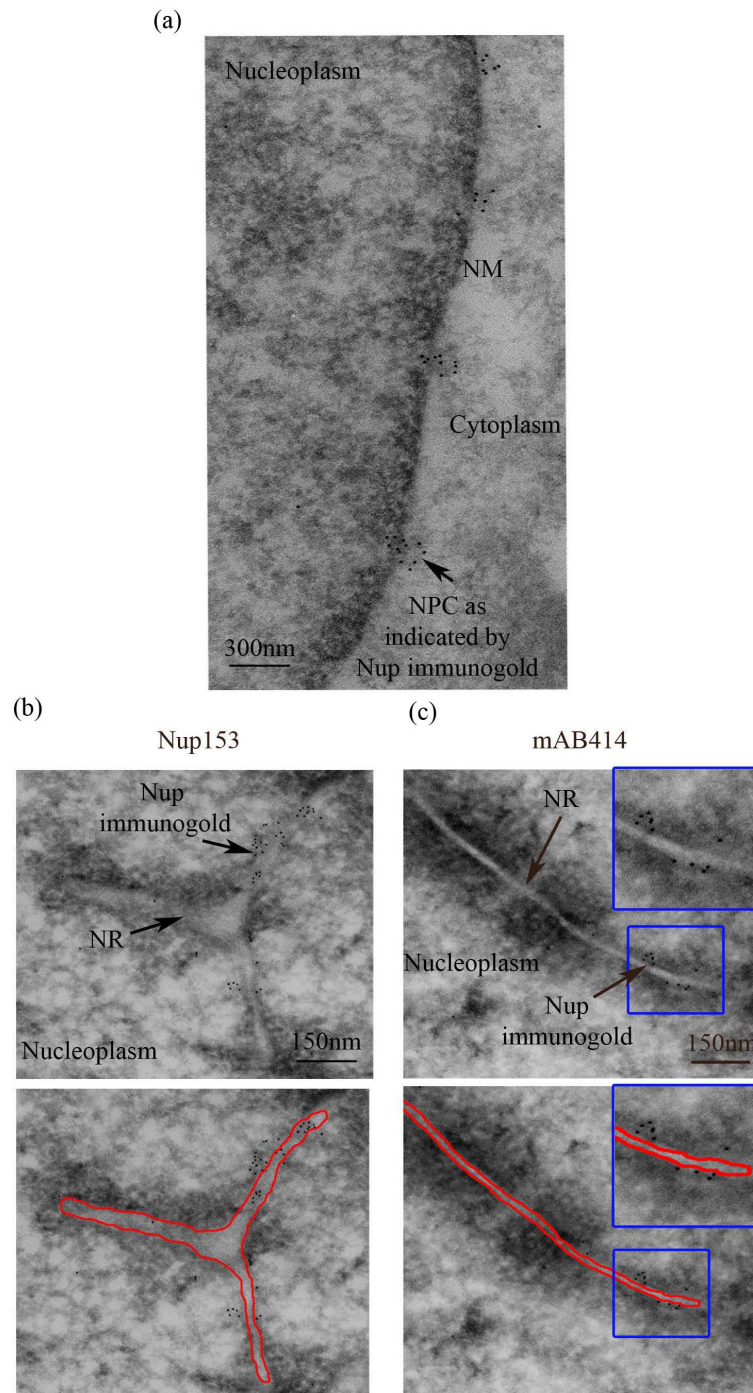




Nuclei of MEFs were subsequently labelled for nucleoporin153 (Nup153), which is found within the nuclear pore basket and mAB414 that identifies FG regions in the core nucleoporins (Figure 3.16). A recent study investigated the effect on the distribution of Nup153 in cells expressing nuclear envelopathy-causing lamin A mutants and found it to be highly disorganised (Busch et al., 2009). The problem with using only Nup153 as a marker of NPCs is that it is located in the basket and is known to be quite a dynamic nucleoporin (Daigle et al., 2001). As a result any Nup153 signal associated with NR may simply be just the nucleoporin rather than a marker of NPC. mAB414 recognises the conserved domain FXFG in the core and is stably associated with the NPC, which would give a greater indication if a NPC was present or not.

It was found after *Zmpste24* siRNA transfection of HU treated cells there was an intranuclear distribution of Nup153 and mAB414 labelling (Figure 3.16 a, b). Quantification of the number of NPCs on the nuclear surface was done by processing images of Nup153 and mAB414 labelled grazing sections and determining the number per 25  $\mu\text{m}^2$  (Figure 3.16 c, d, e). It was clear that there was a significant reduction in the number of NPCs on the surface of prelamin A accumulating nuclei compared to control. Additionally immunogold labelling revealed association of the Nup153 antibody (Busch et al., 2009) and mAB414 with the NR, supporting the notion that they contain at least certain components of a NPC (Figure 3.17).





**Figure 3.17 Immunogold EM of nucleoporin distribution**

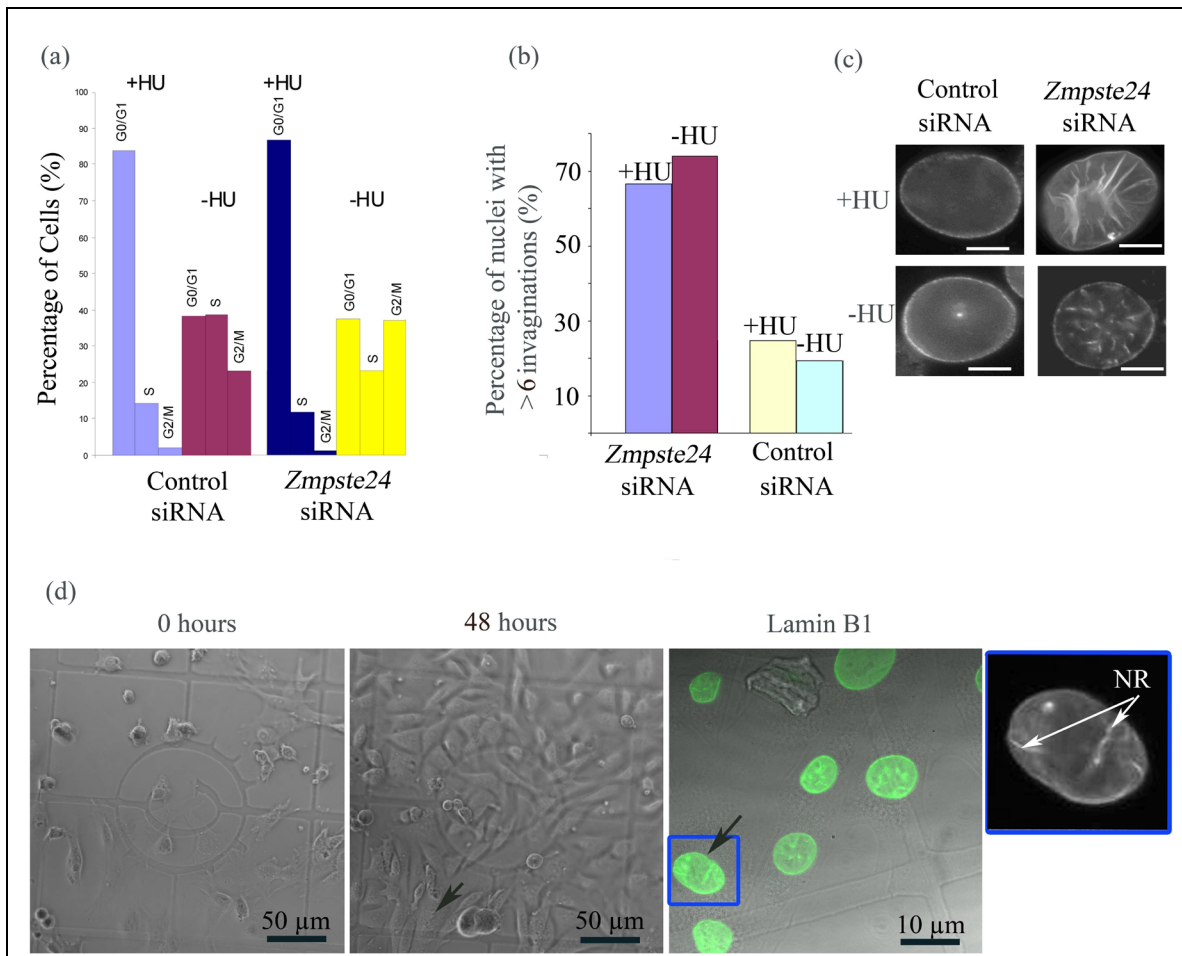
(a) Immunogold EM of Nup153 antibody indicated regular peripheral staining at nuclear pore complexes. (b) Nup153 and (c) mAB414 gold particles could also be seen to associate with NR of cells transfected with *Zmpste24* siRNA.

### 3.3.7 Mitosis is not required for the development of NR

The mechanism by which an NR is produced remains unknown. One model requires a mitosis accompanied by disordered NE assembly in the daughter nuclei; the presence of excess prelamins A might effect such a disorganisation. In order to investigate this question we used both a cell cycle inhibitor and time lapse phase contrast imaging. MEFs were treated with HU for 24 hours, which arrests the cells in the S phase of the cell cycle (Figure 3.18). They were then either transfected with *Zmpste24* siRNA or control siRNA before being fixed and labelled with an anti lamin B1 antibody. The antibody was chosen as it was shown to colocalise with the NR marker calreticulin and was a reliable indicator of NR developing. Determining cells were actually in the S phase of the cell cycle was done by using a cell cycle application in Metamorph and it was found that the majority of the cells treated with HU did remain in the S phase (Figure 3.18a). Quantification of the number of invaginations was done by counting the number of intranuclear lamin B1 intranuclear signals, with greater than six lamin B1 positive labels being counted as an invaginated nucleus (Figure 3.18b). It was clear that the HU treated nuclei compared to no HU treatment had no difference in the number of invaginations between the three different treatments (Figure 3.18b, c)

To further support this observation, synchronised *Zmpste24* siRNA transfected MEFs were cultured on a gridded Mattek dish and were time-lapse imaged for 48 hours, with an image acquired every 15 minutes. After this time the cells were fixed and labelled with lamin B1. It was clear that a complex NR formed both in cells that did and did not divide confirming that NR formation is independent of mitosis (Figure 3.18d).





**Figure 3.18 NR is not an artefact of mitosis**

(a) Treatment with HU arrests cells in the S phase of the cell cycle, preventing them passing through mitosis. (b) and (c) The treatment with HU had no significant effect on the number of invaginations (white bars represent 5  $\mu$ m). (d) Live cell imaging of cells that had been transfected with *Zmpste24* siRNA showed examples of cells that had not passed through mitosis but still developed an NR. Twenty cells were tracked that did not pass through mitosis and an NR could be seen to develop.

### 3.4 DISCUSSION

The development of an NR in a variety of different cell types is being increasingly reported and recognised (Malhas et al., 2011). The presence of an NR has been documented under normal cellular conditions (Fricker et al., 1997b) and pathological states (Bussolati et al., 2008). However, the exact function of such structures remains speculative and the current study is the first to thoroughly interrogate these structures in the context of farnesylated prelamin A accumulation and determine a potential mechanism for their formation.

The observation of an irregular nuclear boundary developing, after the accumulation of unprocessed lamin A, has been frequently reported, particularly in the context of laminopathies (McClintock et al., 2006; Roblek et al., 2010), ageing (Scaffidi and Misteli, 2006) and HIV therapy regimens (Coffinier et al., 2007). The invagination of the nucleus along the periphery has been suggested to be due to prelamin A putting additional strain on the nuclear envelope (Prufert et al., 2004; Ralle et al., 2004). However, in healthy cells CCT $\alpha$  has been implicated in the production of NR by binding to the INM, leading to the generation of positive curvature and an infolding of the nuclear membrane (Gehrig et al., 2008; Gehrig et al., 2009; Gehrig and Ridgway, 2011).

A consequence of reduced expression of *Zmpste24* is that there is an accumulation of prelamin A (Figure 3.1), which has retained 15 C-terminal amino acids including an isoprenylated terminal cysteine. The effects on the nucleus must be highly dominant as a small quantity of the prelamin A is able to elicit its effect. The presence of this

isoprenylated tail has been well documented in increasing the affinity of the protein to the INM (Toth et al., 2005); furthermore, prevention of farnesylated prelamin A accumulation, by using an FTI, (Fig 2a, b) displaces prelamin A to the nuclear interior as foci and restores the dysmorphic nuclear shape to normal (Fong et al., 2006; Toth et al., 2005). The CR of nuclei from co-transfected *Zmpste24* and *Ccta* siRNA cells reveals that the presence of farnesylated prelamin A and a reduced amount of CCT $\alpha$  was enough to produce a convoluted nuclear boundary but not an NR (Figure 3.2a, b). The appearance of a convoluted nuclear boundary has also been reported in HeLa cells that have been transfected with *FACE1* siRNA (Gruber et al., 2005). The importance of lamin A being responsible for this morphological alteration was supported when lamin A deficient HeLa cells or cells that had lamin A acutely knocked down with siRNA, were subsequently transfected with *FACE1* siRNA. This resulted in the number of nuclear aberrations being far lower when compared to the wild type cells transfected with *FACE1* siRNA.

However, immunofluorescence revealed that the co-transfection of *Zmpste24* and *Ccta* siRNA led to a significantly reduced amount of NR, further supporting the notion that CCT $\alpha$  is necessary for its formation (Figure 3.6). The fact that the frequency of invaginations is significantly lower in non prelamin A accumulating cells suggests that the presence of farnesylated prelamin A is acting in concert with CCT $\alpha$  to generate the NR and that the reduced presence of the CCT $\alpha$  enzyme means the improperly processed lamin accumulates at the periphery and causes the membrane to become dysmorphic without the generation of NR.

The presence of the uncleaved isoprenylated C-terminal fragment is clearly important for initiating NR development, due to the lack of this structure in the untransfected cell nuclei. However, the fact that NR is not present after co-transfection with *Zmpste24* and *Ccta* siRNA would suggest that the isoprenylated tail is sufficient for generating a convoluted nuclear envelope but is insufficient for NR production. The farnesylated tail of prelamin A may act as a nucleation site for CCT $\alpha$  activity. The initial positive curvature induced by the farnesylated tail interacting with the INM may attract CCT $\alpha$  to this site.

CCT $\alpha$  was observed to form foci along the NR that colocalised with prelamin A and there was an increased abundance of the nucleoplasmic foci after prelamin A accumulation (Figure 3.7). The production of these foci is due to the inactive form of the CCT $\alpha$  enzyme becoming activated and localising at the nuclear membrane where it may synthesise PC and extend the envelope into the nucleoplasm to form the NR (Gehrig et al., 2008; Gehrig et al., 2009; Lagace and Ridgway, 2005b). However, it has recently been revealed that the catalytic activity of CCT $\alpha$  is not necessarily required for the development of the NR (Gehrig and Ridgway, 2011; Lagace and Ridgway, 2005b) and the M domain of CCT $\alpha$  may act like the amphipathic ENTH domain found in epsin (Itoh and De Camilli, 2006). The epsin ENTH domain causes membrane deformation by inserting itself into the membrane by directly binding to phosphoinositide and pushes the phospholipid head groups apart and reduces the energy to curve the membrane, which is supported and maintained by a clathrin lattice (Ford et al., 2002; Itoh and De Camilli, 2006). CCT $\alpha$  may act in a similar way but instead of the clathrin lattice supporting invagination it is

supported by the nuclear lamina, which is perhaps why the NR is ablated by lamin siRNA (Gehrig et al., 2008).

During live cell imaging and in fixed cells it was apparent that, unlike prelamin A, CCT $\alpha$  did not fully colocalise with the NR but instead was found along sections of it. This may be due to the enzyme localising at points where the NR is expanding and further invagination is happening. Cells treated with a fatty acid substrate develop CCT $\alpha$  foci, which colocalise with the NR whose formation is dependent on the presence of the nuclear lamins (Gehrig et al., 2008). Recently it was revealed that cells expressing GFP-Progerin also develop nuclear invaginations that colocalise with CCT $\alpha$  (Gehrig and Ridgway, 2011), which means a similar mechanism of nuclear membrane deformation may be occurring in HGPS patients. The common feature between the data presented here and this recent publication is the retention of the farnesylated tail on improperly processed lamin A. It will be interesting to investigate, in the context of progerin accumulation in cell nuclei, whether *Ccta* siRNA alters CCT $\alpha$ 's distribution and the frequency of NR development.

The NR that forms during prelamin A accumulation had two principal morphologies (Figure 3.11). The first consists of an infolding of both the INM and ONM, with a cytoplasmic core that often contains nuclear pore complexes. The purpose of the double membraned invagination has been studied and it is proposed to act as a conduit for localised calcium release within the nucleus to regulate transcription of genes controlled by calcium (Bkaily et al., 2009; Bootman et al., 2009; Chamero et al., 2008; Collado-

Hilly et al., 2010; Guatimosim et al., 2008; Lui et al., 1998; Marius et al., 2006; Nalaskowski et al., 2010). This may help the cell to adapt during the pathological accumulation of prelamin A.

By shortening the distance between intranuclear expressing genes and the cytoplasm it could also increase the rate of translation of proteins to aid with the cellular shift to a pathological state. The fact that these invaginations contain nuclear pore complexes and have been reported to contain translational machinery (Paytubi et al., 2009) further supports the notion of increased export and generation of proteins for active genes deep within the nucleus. Treatment with a histone deacetylase inhibitor actually increases the frequency of invaginations, which may be due to the increased abundance of transcriptionally active genes (Galiova et al., 2008). NR invaginations are also frequently reported as terminating at nucleoli (Fricker et al., 1997b; Schneider et al., 2010), which again may serve as tight spatial coupling between a transcriptionally active compartment with a potential transport channel.

If the positive curvature is being applied to just the INM by CCT $\alpha$  then why do you get double membrane invaginations? A reason may be that the LINC complexes (Crisp et al., 2006; Houben et al., 2007), bridging the INM and ONM, hold the two membranes together, so when the INM invaginates it brings the ONM with it. In the case of just the INM invaginating, as was frequently observed, the accumulation of the farnesylated prelamin A may disrupt nuclear organisation at the INM and therefore affect the distribution of LINC complexes. Consequently, the uniform distance between the two

nuclear membranes breaks down and infolding of just the INM occurs. This has been observed in siRNA targeting of nesprin isoforms, leading to the nuclear membranes separating and the INM invaginating (Zhang et al., 2007).

F-actin was also found to be present in some of the nuclear invaginations induced by *Zmpste24* siRNA, whose abundance was dependent on the CCT $\alpha$  enzyme (Figure 3.8). Actin filaments in invaginations of cells grown in culture and from tissue samples have been reported but their exact function is speculative (Abe et al., 2004; Storch et al., 2007). It could purely be by chance that polymerising actin fibres in the cytoplasm extend down the core of type II invaginations and serve no functional role. However, they could be serving as an internal scaffold for the developing NR, which provides support as the nuclear membrane extends into the nucleoplasm. The LINC complexes found along the nuclear membrane could be connected to the actin filaments in these invaginations. Mechanotransduction might then play a role to genes proximal to the invagination and provide another level of controlling gene expression as the cell shifts to a pathological state. The invaginations that do not contain an actin signal may be of the type I variety and therefore cannot facilitate actin entry, as the ONM does not invaginate to provide a cytosolic core for actin to gain access.

The presence of NPCs in the INM-only invaginations presents a topological conundrum, especially as these structures can form in an intact interphase nucleus. The structures observed cannot represent fragments of complete nuclear envelope trapped in the nucleoplasm after mitosis. Perhaps these features are not a complete pore but simply the

nucleoplasmic half of the NPC, which has been torn apart during invagination of the INM. However, tomography reveals that these pores do have symmetry of electron dense material either side of the pore but whether they are complete complexes is yet to be determined. They certainly cannot be normally functioning pores as both their sides face the nucleoplasm. The fact that there is a significantly reduced number of Nup153 and mAB414-positive structures at the nuclear surface and an increased abundance of these nucleoporins localised inside the nucleus (Busch et al., 2009), in association with the NR, suggests that they are being drawn in by the invaginations. Their function may be a transcriptional one, as components of the NPC are being increasingly implicated as necessary components for initiating transcription (Capelson et al., 2010; Ikegami and Lieb, 2010; Kalverda et al., 2010; Vaquerizas et al., 2010).

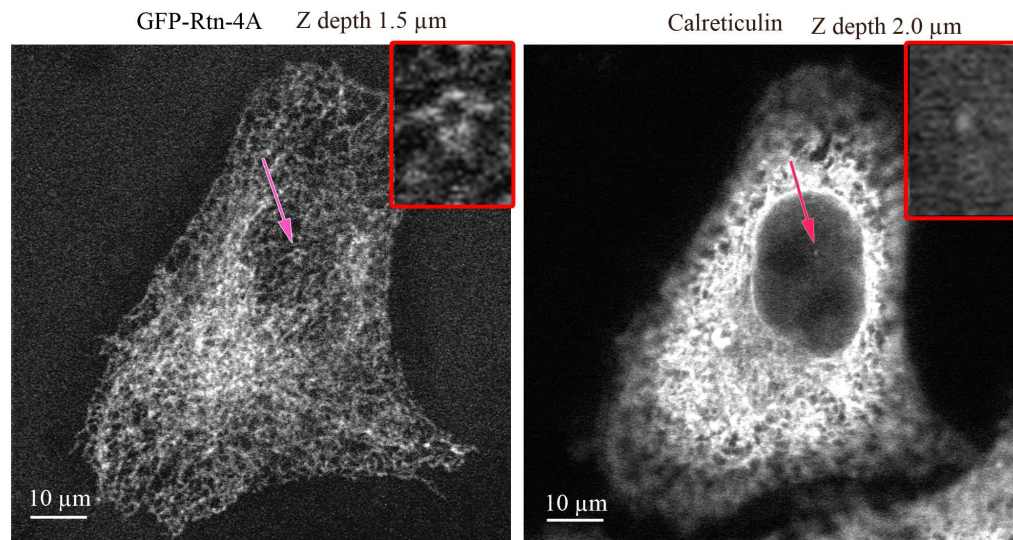
NR has been suspected of simply being an artefact of cells grown in 2D culture or is the consequence of the nuclear envelope not reforming completely after the cell passes through mitosis. The fact that we observed development of these structures in live cells that had not passed through mitosis and those inhibited at the S phase of the cell cycle indicates that the developing NR was a consequence of the build up of farnesylated prelamin A and not an artefact (Figure 3.16). It will be interesting to assess the heritability of these invaginations in daughter nuclei. If these structures were simply forming randomly as a consequence of the build up of the unprocessed lamin A protein then no pattern of inheritance would be expected. However, if they serve a functional role then it is likely the daughter nuclei of a cell that is invaginated will display a similar pattern of NR production. Live cell imaging of wild type MEFs passing through mitosis



has already revealed a significant heritability of NR between related nuclei (Vaux Lab, unpublished data) and it will be interesting to explore this further in a pathological state.

How an NR forms may involve other membrane curvature-inducing components such as the reticulons (Rtns). The Rtns are a group of proteins that have been found to be responsible for maintaining the tubular state of parts of the ER (West et al., 2011). Overexpression of these proteins leads to longer unbranched tubes while depletion results in a more cisternal ER. Certain reticulons have been found at the nuclear periphery (RTN-4A) and have been shown to be critical for nuclear envelope reformation and growth (Kiseleva et al., 2007). Preliminary research in the Vaux laboratory has found RTN-4A to form a ring like structure that overlays NR (Figure 3.19). The exact role of this protein in NR production remains to be explored but offers a new and exciting avenue of research in the regulation of NR development.

The current chapter illustrates for the first time the connection between the acute accumulation of farnesylated prelamin A and involvement of CCT $\alpha$  in generating an NR. The exact function of the NR in this pathological state remains speculative but is likely to be an adaptive response of the cell to the toxic build up of farnesylated prelamin A.



**Figure 3.19 Rtns may play a role in NR proliferation**

GFP-Rtn-4A formed a ring like structure (pink arrow) at the nuclear periphery that overlayed a calreticulin positive NR (red arrow) in HeLa cells. The exact role of the Rtn's in NR development requires further research but the association between the two offers potential new mechanisms of formation.

## **CHAPTER 4**

# ***ZMPSTE24* KNOCK DOWN SEQUESTERS SREBP-1 AT THE NUCLEAR PERIPHERY LEADING TO REDUCED ADIPOGENESIS**

### **4.1 INTRODUCTION**

Lipoatrophy involves wasting of subcutaneous fat due to a combination of decreased adipocyte differentiation potential and increased lipolysis of lipid droplet stores, with symptoms including wasting of adipose tissue in the face, legs and buttocks. Lipohypertrophy is generally associated with visceral fat and is the opposite of lipoatrophy with symptoms resulting in accumulation of fat in the torso and around the internal organs (Capeau et al., 2011; Caron-Debarle et al., 2011).

A surprising link to a potential cause of lipoatrophy was found in one of the nuclear lamina components. In the case of Dunnigan Type Familial Partial Lipodystrophy a mutation was found in the gene encoding lamin A/C (Cao and Hegele, 2000; Shackleton et al., 2000; Speckman et al., 2000), which leads to the accumulation of the unprocessed form of the protein farnesylated prelamin A. Sufferers of another rare *LMNA* mutation, HGPS (Eriksson et al., 2003), generate a truncated version of the lamin that retains the farnesylated C-terminus, due to removal of a cleavage site for FACE1. Consequently, one of the symptoms includes lipoatrophy in patients and mouse models of the disease (Merideth et al., 2008; Yang et al., 2006).

Interestingly, it has been reported that ZMPSTE24 is inhibited by HIV PIs, which are one of the components of HAART. Patients taking this cocktail of drugs often develop lipodystrophy syndrome (Capeau et al., 2006; Caron-Debarle et al., 2010; Pushpakom et al., 2010). Farnesylated prelamin A was shown to accumulate in cells treated with HIV PI's (Caron et al., 2007; Clarke, 2007; Coffinier et al., 2007; Coffinier et al., 2008; Goulbourne and Vaux, 2010; Hudon et al., 2008; Liu et al., 2010) and has been shown to directly interact with and sequester the SREBP-1 transcription factor (Capanni et al., 2005; Caron et al., 2003; Caron et al., 2001; Lloyd et al., 2002; Maraldi et al., 2008). These PIs have also been implicated in reducing adipogenesis (Caron et al., 2003; Caron et al., 2009; Prot et al., 2006; Rivas et al., 2007) and underlie a potential mechanism for lipodystrophy.

The literature has often suggested a relationship between acute prelamin A build up, tethering of the SREBP-1 transcription factor and reduced adipogenic potential but no direct proof of dysregulated SREBP-1 targets has been shown in this scenario. This chapter reveals that within 48 hours of reduced expression of *Zmpste24* there is an increased abundance of this transcription factor at the nuclear periphery, without any change in total SREBP-1 levels. This results in a decreased peripheral dynamic of SREBP-1 and a reduced ability to bind to its target genes. The consequence of this is a dysregulation of SREBP-1 target genes and a decrease in markers of adipogenesis. Additionally nuclear morphological changes occur to the nuclear periphery in mouse pre-adipocytes that resemble the ultrastructural changes in MEF's. These data show that

farnesylated prelam A build-up can contribute to the lipotrophy experienced in laminopathies and HIV therapy due to its interaction with SREBP-1.

## 4.2 METHODS

**4.2.1 Cell Culture:** Cell lines used include *Lmna*<sup>-/-</sup> MEFs and mouse pre-adipocytes that were cultured in conditions described in Chapter 2. Mouse pre-adipocytes differentiated into adipocytes upon reaching confluence and were cultured in medium supplemented with insulin as described in Chapter 2.

**4.2.2 Immunofluorescence microscopy:** Cells were prepared as described in the methods section of chapter 2. Primary antibodies used were a prelam A specific antibody (Santa Cruz Biotechnology Inc sc-6214) and a SREBP-1 specific antibody (Santa Cruz Biotechnology Inc, sc-8984). All primary antibodies were used at a dilution of 1 in 100 in 0.4 % FSG. The cells were then incubated with Alexa Fluor 647 donkey anti goat IgG (Invitrogen A-21447) and Cy3 conjugated donkey anti rabbit IgG (Jackson ImmunoResearch 711-166-152). Finally the cells were mounted in Mowiol supplemented with DAPI. Immunofluorescence images were acquired as described in Chapter 2.

**4.2.3 Western blotting:** Western blotting was carried out as previously described. The nitrocellulose membranes with transferred proteins were bathed in antibodies against lamin A at 1:200 (Abcam ab26300),  $\beta$ -actin at 1:5000 (Abcam 8226), prelam A at 1:100 (SantaCruz sc-6214), SREBP-1 at 1:200 (SantaCruz sc-8984) and lamin B1 at 1:500 (C. Maske) overnight. The secondary antibodies used were peroxidase AffiniPure

Goat Anti-Mouse IgG (Sigma A4416), peroxidase Affinipure Goat Anti-Rabbit IgG (Sigma A6154) and peroxidase Affinipure Donkey Anti-Goat IgG (Jackson ImmunoResearch Europe Ltd 705-035-147) at a dilution of 1:5000, 1:6000 and 1:20,000 respectively for 45 minutes.

**4.2.4 Measuring nuclear SREBP-1 and total FABP-4 fluorescent intensity:** The nuclear intensity of SREBP-1 was compared to the total cellular intensity of the transcription factor to generate relative nuclear percentage intensity. Nineteen cells were analysed in Metamorph software for each transfection. Total FABP-4 intensity in a cell was measured using the same software and fifty nuclei under each condition were assessed.

**4.2.5 Sequential Nuclear Extraction** The method for subcellular fractionation was adapted from previous reports (Gonzalez et al., 2008; Schreiber et al., 1989).  $5 \times 10^6$  cells were trypsinized and pelleted at 1000 g for 5 minutes at 4°C. The pellet was then washed in 1 ml of PBS and pelleted again as before. The supernatant is then removed and 800 µl of buffer A (10mM HEPES pH 7.9, 10mM KCl, 1mM DTT and protease inhibitor cocktail from Roche) is added. The solution was placed on ice for 15 minutes before 80 µl of 100% NP-40 is added and vortexed for 10 seconds. It is then centrifuged at 11,000 g for 30 seconds at 4°C and the supernatant containing the cytoplasmic fraction and RNA is kept for analysis while the pellet contains the nuclear lysate. The pellet is resuspended in 100 µl of buffer B (20mM HEPES pH 7.9, 0.4M NaCl, 1mM DTT and protease inhibitor cocktail) and agitated at 4°C for 15 minutes. The solution is then centrifuged at 11,000 g

for 45 minutes at 4°C and the supernatant is the soluble nuclear fraction (SNF) while the pellet is the extraction resistant nuclear fraction (ERNF).

**4.2.6 TEM and EMT of adipocytes:** The cells were processed and imaged as described in Chapter 2.

**4.2.7 Fluorescence Loss In Photobleaching of nuclear GFP-SREBP-1:** GFP-SREBP-1 was transfected into cells 24 hours before transfecting with either *Zmpste24* or control siRNA twice at two 24 hour time points. 72 hours after the initial transfection a region at the periphery or nucleoplasm was photobleached for 5 minutes and adjacent regions were monitored for loss in fluorescent intensity as described previously. 10 nuclei were selected for each condition.

#### **4.2.8 qRT-PCR of SREBP-1 target genes**

*4.2.8.1 Cell Lysis, DNase I Treatment and Inactivation:* The protocol followed that of the Cells-to-cDNA™ II kit (Applied Biosystems) In brief; cell lysis buffer was added to mouse pre-adipocytes and incubated at 75 °C for 10 minutes. 2 µl of DNase I is added per 100 µl of lysis buffer and then incubated at 37 °C for 15 minutes. DNase is subsequently inactivated at 75 °C for 5 minutes and the total RNA is generated.

*4.2.8.2 Two step qRT-PCR using the StepOnePlus™ system:* 10 µl of cell lysate is added to 4 µl of dNTP and 2 µl of first strand primers before being heated to 70°C for 3 minutes, placed on ice for 1 minute and 2 µl of 10x RT buffer, 1µl of MMLV reverse transcriptase

and 1  $\mu$ l of RNase I is added and incubated at 42 °C for 1 hour and 95°C for 10 minutes. For 1  $\mu$ l of cDNA then 50.6  $\mu$ l of the forward and reverse primers is added to 379.5  $\mu$ l of the SYBR green master mix and 227.7  $\mu$ l of nuclease free water and the samples are amplified using the Step One Plus RT-PCR system (Applied Biosystems). This consisted of 40 cycles of amplification, which included 21 secs at 95 °C and 20 secs at 60 °C. Forward (F) and reverse (R) primers for each gene were ordered through Invitrogen and are as follows: *Acs1* F 5'-(TCCATGAATTGTTCCGGTAT)-3', *Acs1* R 5'-(CGTACATGGTTCTGACATCG)-3', *Klotho* F 5'-(TGTTTATCCATGGCCATTTT)-3', *Klotho* R 5'-(CTTCTTAATGGGCTGCTCTG)-3', *Fasn* F 5'-(ATTCGGTGTATCCTGCTGTC)-3', *Fasn* R 5'-(GGCTTGTCCTGCTCTAACTG)-3', *Mttp* F 5'-(GAGCGGCTATACAAGCTCAC)-3', *Mttp* R 5'-(AACACAACGTCCACATCAGA)-3', *Ppar $\gamma$*  F 5'-(AGCTTTCCTGGACCATCTCT)-3', *Ppar $\gamma$*  R 5'-(ACATGCTTTCCTCTCCCTTT)-3'. Fold change for qRT-PCR was subsequently determined by subtracting the mean  $\Delta$ Ct of each gene for transfected samples from the mean  $\Delta$ Ct of each gene for untransfected samples to yield  $\Delta\Delta$ Ct, which could then be used to establish fold change for gene expression.

#### 4.2.9 Chromatin Immunoprecipitation of SREBP-1 promoters

*4.2.9.1 Cross linking, cell harvesting and sonication:*  $\sim 10^6$  mouse pre-adipocytes were cultured and DNA was cross linked to proteins by adding 1% formaldehyde for 10 minutes followed by 125 mM glycine for 5 minutes at room temperature. All subsequent washes and lysis steps were performed in the presence of complete protease inhibitor cocktail (Roche). Cells were washed with ice-cold PBS, scraped, pelleted and lysed with



10 mM Tris, 10 mM KCl and 1% NP-40, pH 8 on ice for 10 minutes. Nuclei were pelleted and lysed with nuclear lysis buffer (50 mM Tris, 1 % SDS, pH 8) on ice for 10 minutes. Micrococcal Nuclease was used to digest chromatin and was incubated at 37 °C for 20 minutes to produce DNA fragments that were ~150-900 bp. The reaction was stopped upon addition of 50 mM EDTA and the nuclei were pelleted again before being resuspended in ChIP dilution buffer (0.1 % SDS, 2 mM EDTA, 1 % Triton X-100, pH 8) and incubated on ice for 10 minutes. They were then sonicated to break the nuclear membrane and incubated on wet ice for 30 seconds.

*4.2.9.2 Immunoprecipitation, elution and reversal of cross links:* The lysate was cleared by centrifugation and approximately 10 µg of chromatin DNA was diluted in ChIP buffer. 1 % of this was kept as the input samples. 2 µg of SREBP-1 antibody was added to the samples along with protein G agarose beads and the samples were rotated at 4 °C for 2 hours. Negative control consisted of no addition of antibody. Precipitated samples were washed three times with low salt washing buffer (20 mM Tris, 50 mM NaCl, 2 mM EDTA, 0.1% SDS, and 1% Triton X-100, pH 8) and once with high salt washing buffer (low salt buffer with 500 mM NaCl), all washes were done with rotation at 4°C for 5 minutes. Protein–DNA complexes were eluted using 0.1 mM NaHCO<sub>3</sub> and 1% SDS (which was also added to the input samples) and were incubated at 65°C for 30 minutes with gentle vortexing. Cross links were reversed by addition of 0.2 M NaCl, 40 µg/ml proteinase K and incubated for 2 hours at 65 °C. DNA was recovered using spin columns and ethanol precipitation. All immunoprecipitated DNA samples were resuspended in 50 µl DNA elution buffer.

**4.2.9.3 qRT-PCR of immunoprecipitated DNA:** qRT-PCR was performed on A StepOnePlus system (Applied Biosystems) with the Fast SYBR Green Master Mix (Applied Biosystems). PCR mixtures contained 4 µl immunoprecipitate or input samples and a final primer concentration of 200 nM. Relative gene expression values were determined using the  $2^{-\Delta\Delta C_t}$  method (Livak and Schmittgen, 2001). The  $C_t$  values from qRT-PCR were normalized using those of the input samples and were used to calculate the fold enrichment of SREBP-1 binding in *Zmpste24* siRNA transfected cells compared with control siRNA transfected cells.

**4.2.9.4 Primer and oligonucleotide design:** Promoter sequences of *Acs1*, *Ppar $\gamma$* , *Mttp* and *Fasn* were obtained using BioMart. MAPPER (Marinescu et al., 2005) was used to identify potential SREBP-1 binding sites within these sequences, and appropriate primers for qRT-PCR were designed using Oligo Perfect Designer (Invitrogen). Primers for the detection of *Actb* have been described previously (Zhao et al., 2004).

**4.2.10 Oil red O assay of lipid droplets in adipocytes:** Monolayers of cells were cultured on 25 mm coverslips before media was aspirated off and washed in PBS. The cells were then fixed in 4% paraformaldehyde in PBS for 1 hour at room temperature before 60% isopropanol was added for 5 minutes. Oil Red O solution was added to the cells and incubated for 5 minutes and were then rinsed gently with distilled water and visualized using a DD1 Coolscope Slide Scanner (Nikon). To quantify the amount of Oil Red O after the cells are rinsed in water 100 % isopropanol is used to elute the stain for 10 minutes. The OD was then measured at 500 nm using a spectrophotometer.

## 4.3 RESULTS

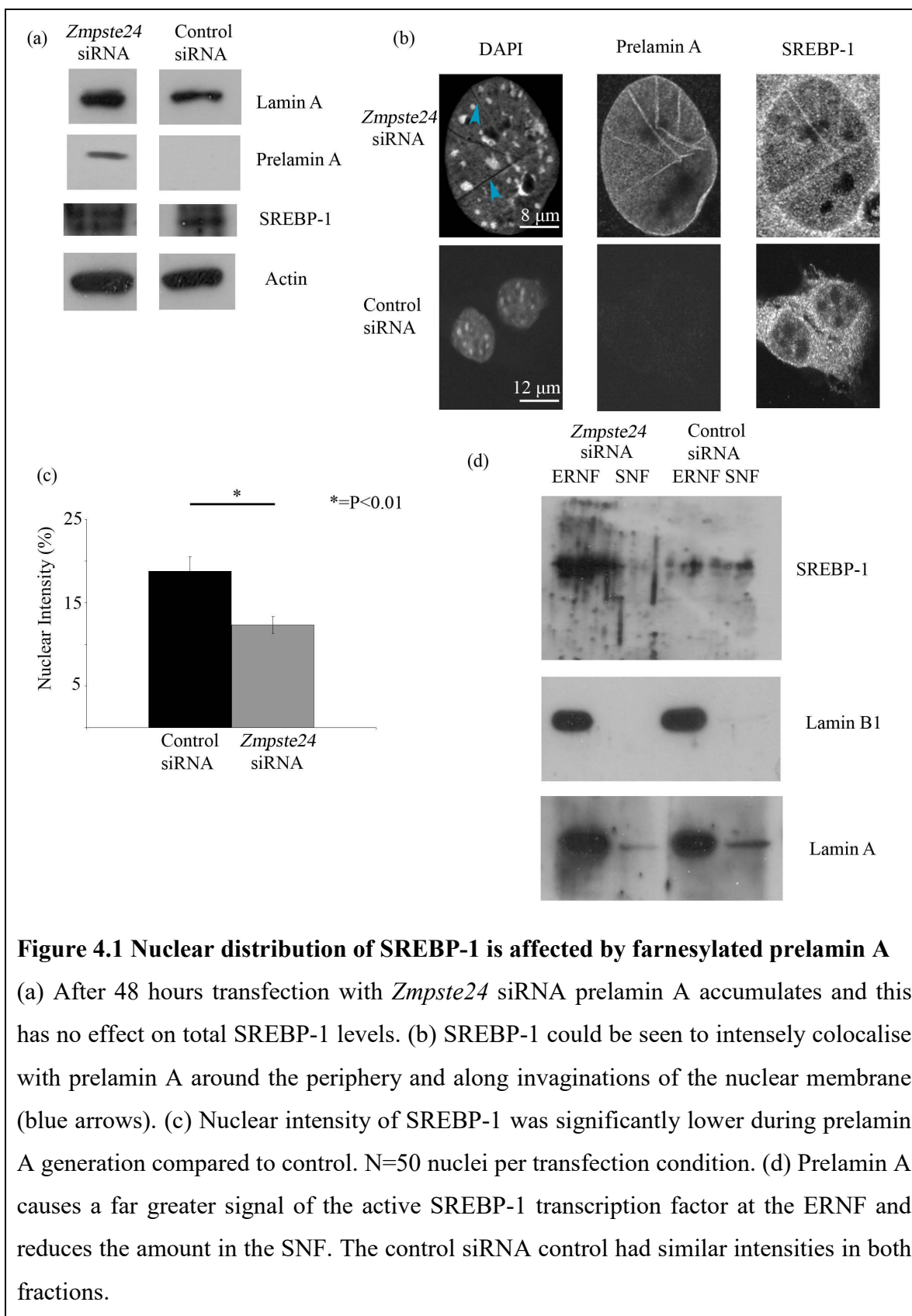
### 4.3.1 SREBP-1 is localised at the nuclear periphery during prelam A accumulation

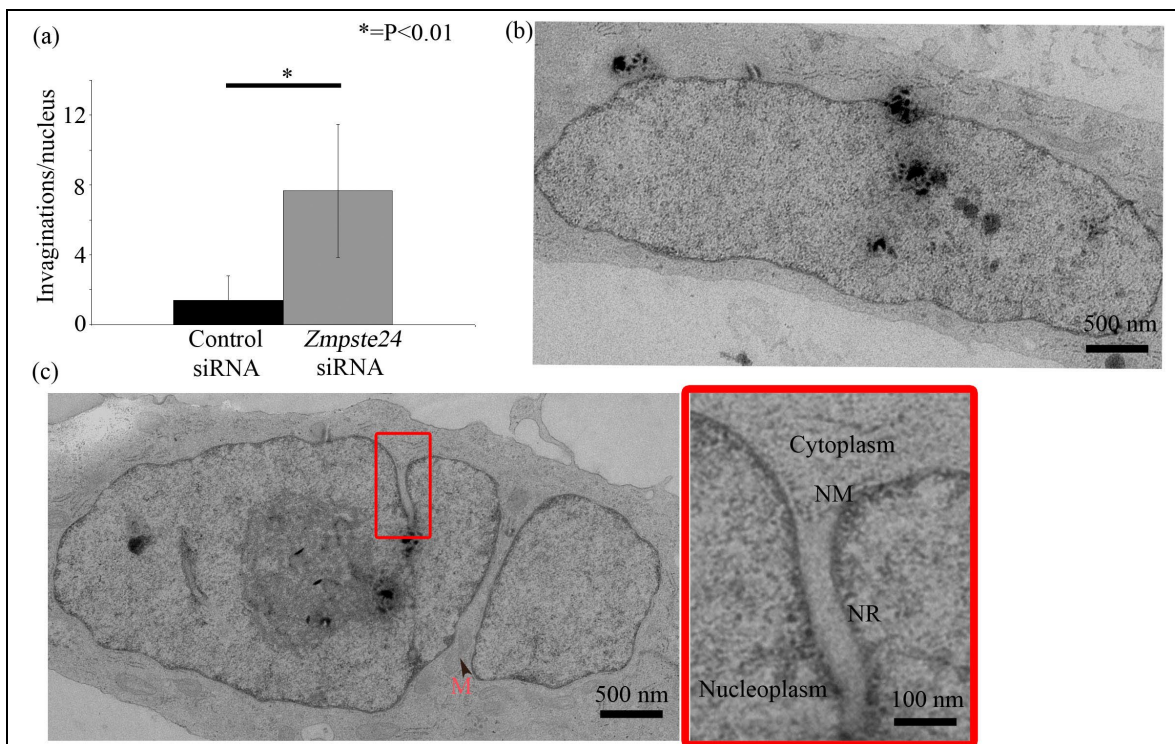
After 48 hour transfection of mouse pre-adipocytes with *Zmpste24* siRNA prelam A was detected, without having an effect on total SREBP-1 levels (Figure 4.1a). The distribution of the SREBP-1 was particularly intense around the nuclear membrane and along invaginations of the nucleus (Figure 4.1b). In the control siRNA transfected cells the distribution of SREBP-1 was more diffuse and there were significantly fewer invaginations in the nucleus (Figure 4.2a). Nuclear intensity of the SREBP-1 transcription factor was significantly reduced in cells accumulating prelam A (Figure 4.1c).

Sequential nuclear extraction yielded two fractions; a soluble nuclear fraction (SNF), associated with the nuclear interior and an extraction resistant nuclear fraction (ERNF), associated with the nuclear periphery. It was clear that there was a far greater signal of SREBP-1 in the ERNF of prelam A accumulating cells when compared to the control (Figure 4.1d). Lamin B1, with its farnesylated tail, was exclusively found in the ERNF, due to strong association with the INM. Mature lamin A was predominantly found in the ERNF as most of it is incorporated into the nuclear lamina, which underlies the INM. However, a small fraction was found in the SNF, which is recognised as a nucleoplasmic pool of the lamin that has been assigned functions in transcriptional regulation (Dorner et al., 2007).

### **4.3.2 Invaginations of the nuclear membrane were present after acute prelamin A accumulation**

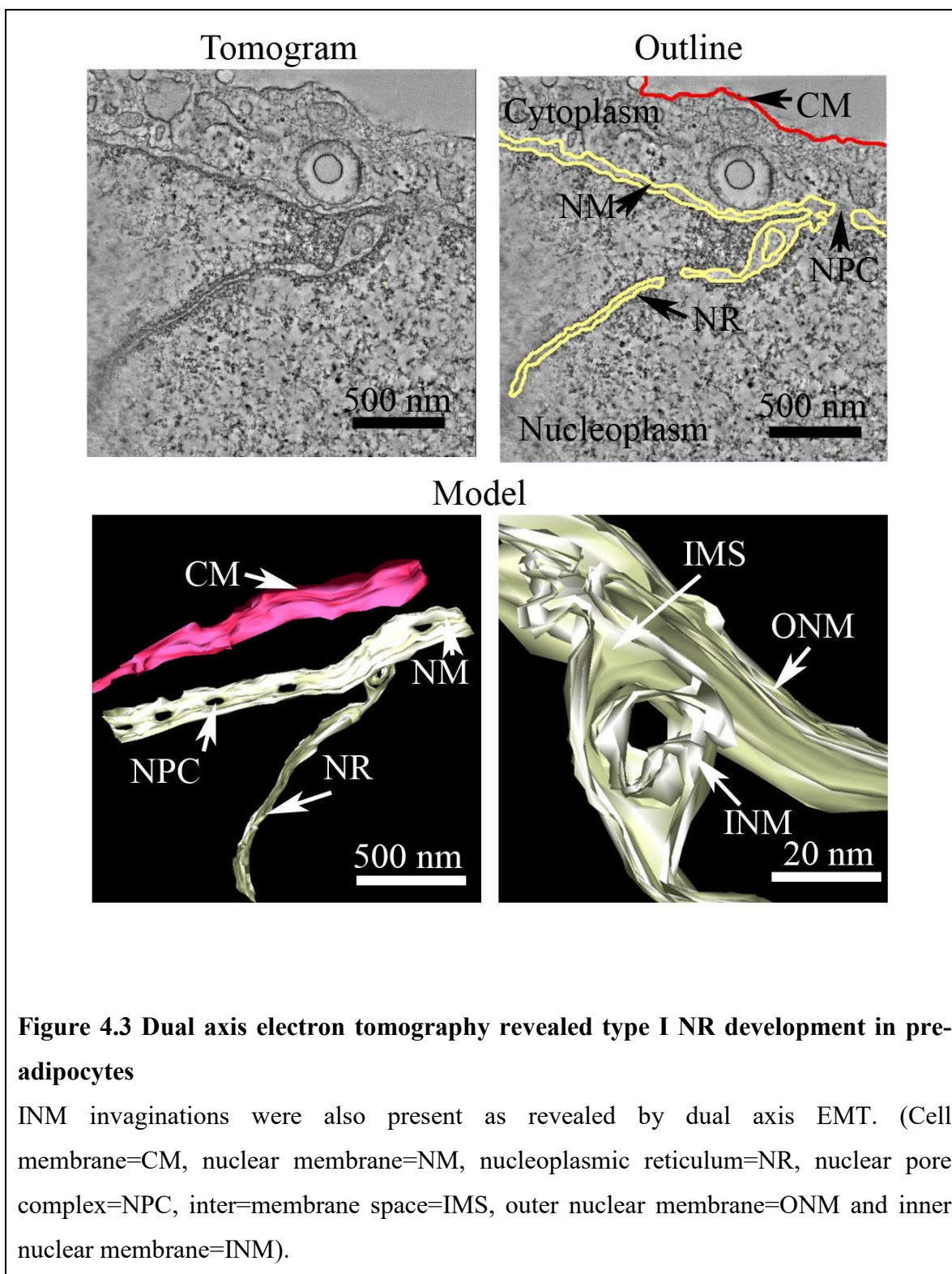
Dual axis electron tomography and TEM were used to assess the ultrastructure of pre-adipocytes exposed to *Zmpste24* siRNA (Figure 4.2). It was clear that there were significantly more invaginations of the nuclear envelope of pre-adipocytes that had been transfected with the *Zmpste24* siRNA (Figure 4.2a). Control transfected pre-adipocytes often had a smooth nuclear periphery with few nuclear invaginations (Figure 4.2b). Invaginations that developed after acute prelamin A accumulation tended to fall into the two morphological categories described in the previous chapter. Additionally some of the NR actually contacted both sides of the nuclear membrane providing an internal column of cytoplasm that entirely stretched across the nucleus and was large enough to accommodate mitochondria (Figure 4.2c). Dual axis EMT once again revealed the morphology of the NR and presumptive NPCs seemed to be present across the structure (Figure 4.3).





**Figure 4.2 NR development is observed in mouse preadipocytes**

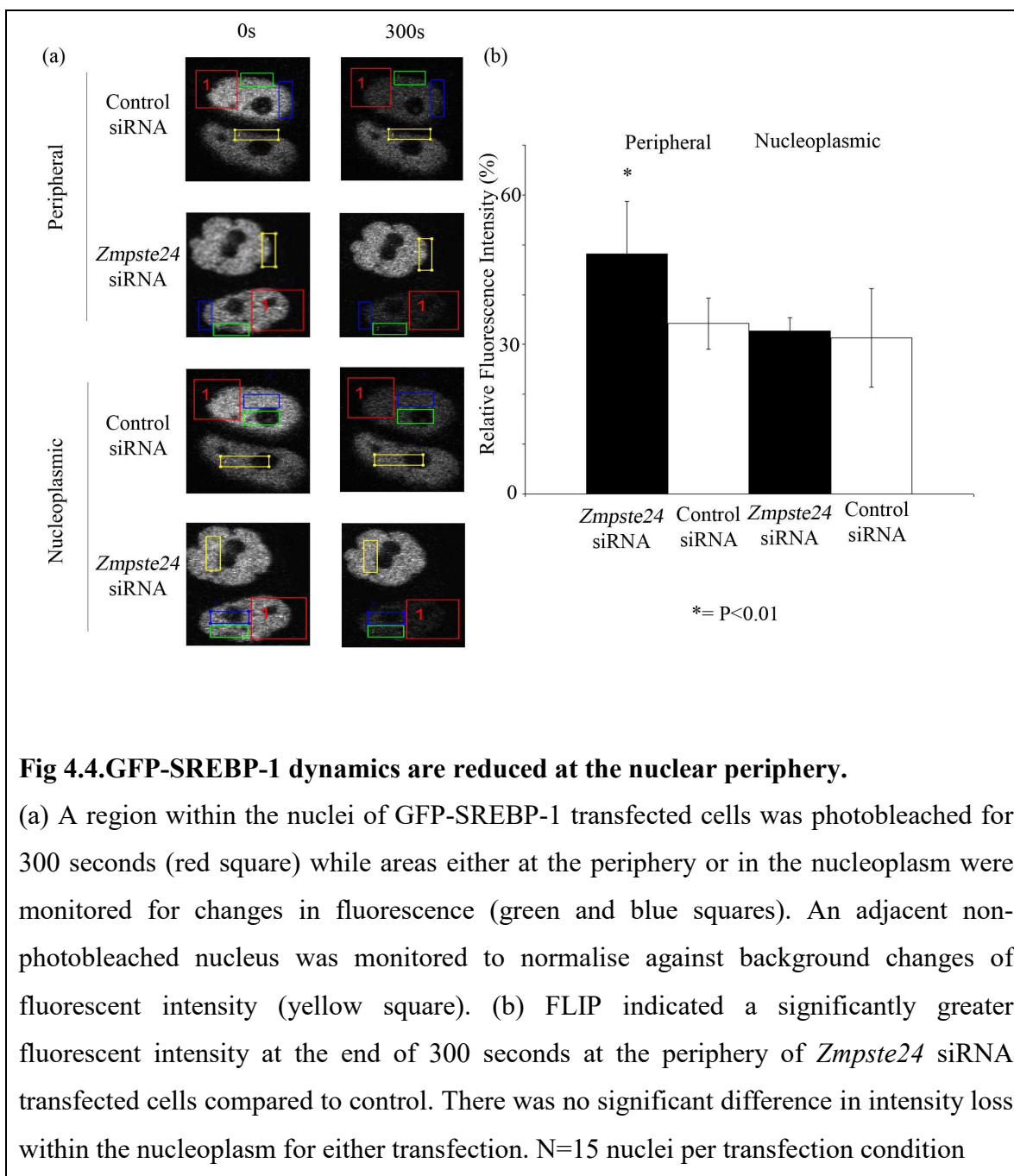
(a) There were significantly more nuclear invaginations after *Zmpste24* siRNA transfection in pre-adipocytes, which were quantified at the light level. N= 100 nuclei per transfection condition. (b) Generally the control siRNA transfected nuclei had a uniform nuclear membrane with relatively few invaginations. (c) The nuclear invaginations induced by the presence of prelamin A could have a cytoplasmic core with an infolding of both nuclear membranes and occasionally traversed both sides of the nucleus that may also contain mitochondria (M).



### 4.3.3 The dynamic nature of SREBP-1 is reduced at the periphery

Fluorescence Loss in Photobleaching (FLIP) was used to assess the dynamics of a GFP tagged transcriptionally active version of SREBP-1 (Figure 4.4). A region was photobleached and adjacent areas at the periphery or nucleoplasm were monitored for decreases in intensity over 300 seconds (Figure 4.4a). There was no significant difference in the dynamics of the transcription factor in the nucleoplasm between the *Zmpste24* and control siRNA transfected cells which had intensities of 31.3 %  $\pm$  2.6 and 32.8 %  $\pm$  9.9 respectively (Figure 4.4b). However, when a comparison was made at the nuclear periphery it was clear that the dynamics of SREBP-1, in an environment where prelamin A accumulates, had a reduced mobility in *Zmpste24* siRNA transfected cells of 48.2 %  $\pm$  10.5 compared to 34.2 %  $\pm$  5.1 in control (Figure 4.4b).





#### 4.3.4 SREBP-1 gene targets are dysregulated due to prelamins A accumulation.

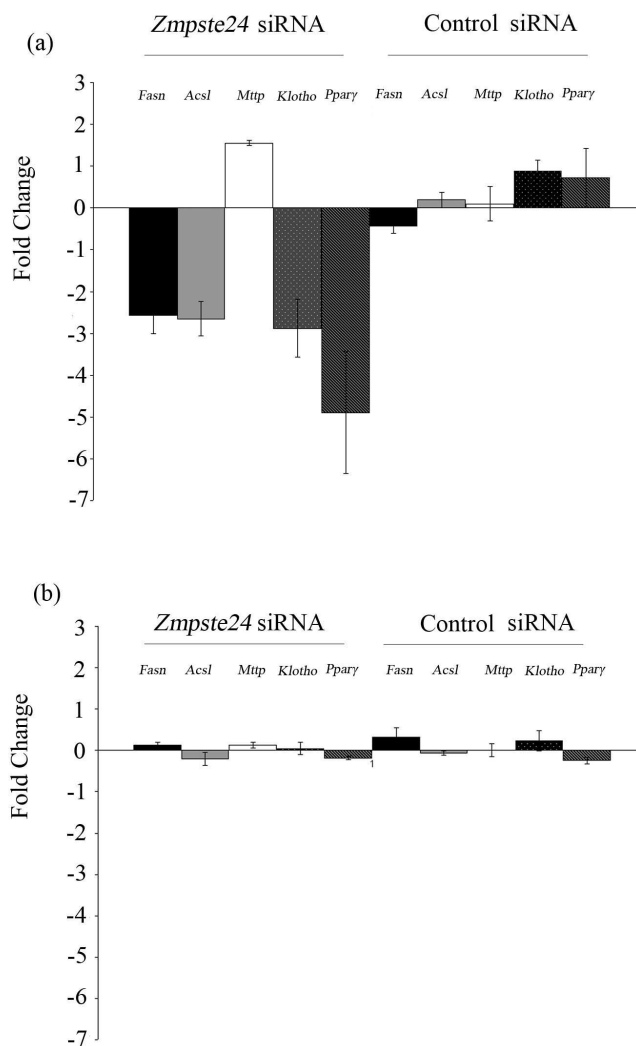
Upon activation by low levels of sterols in the cytoplasm mature SREBP-1 is liberated from the endoplasmic reticulum and golgi apparatus before translocating to the nucleus (Brown and Goldstein, 1997). Upon entry into the nucleoplasm the transcription factor binds to genes involved in fatty acid and sterol biosynthesis. Fatty acid synthase (*Fasn*) and Acetyl-CoA synthetase (*Acs1*) are responsible for synthesising fatty acids in response to decreased concentrations and they are positively regulated by the SREBP-1 transcription factor (Ikeda et al., 2001; Kim et al., 1998a; Latasa et al., 2000). Acute accumulation of farnesylated prelamins A led to a significant down regulation of these genes (Figure 4.5a) with *Fasn* being reduced by 2.57 $\pm$ 0.43 fold and *Acs1* being lowered by 2.65  $\pm$ 0.41 fold. Microsomal triglyceride transfer protein (*Mtp*) is critical for assembling and secreting very low density lipoproteins and chylomicrons and is negatively regulated by SREBP-1 (Sato et al., 1999). The reverse was observed for this gene as there was a 1.56  $\pm$ 0.06 fold increase in expression (Figure 4.5a).

Another target of SREBP-1 is the promoter of the adipocyte differentiation factor peroxisome proliferator-activated receptor  $\gamma$  (*Ppar $\gamma$* ) (Fajas et al., 1999). This protein is critical during adipogenesis (Tontonoz et al., 1994a; Tontonoz et al., 1994b) and down regulation of the receptor would impair the transition of pre-adipocytes to mature adipocytes. The gene did show reduced expression after *Zmpste24* siRNA transfection compared to control and was lowered by 4.89  $\pm$ 1.47 fold (Figure 4.5a). Further confirmation that *Ppar $\gamma$*  was being down regulated was the fact that a PPAR $\gamma$  regulated

gene, *Klotho* (Zhang et al., 2008), had a reduced expression level by  $2.88 \pm 0.70$  fold (Figure 4.5a).

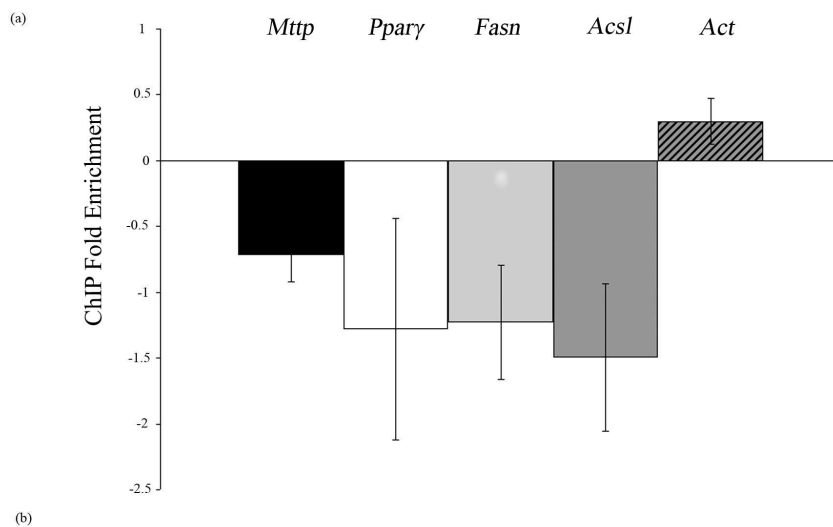
The control siRNA showed no significant alteration of SREBP-1 and PPAR $\gamma$  regulated genes, suggesting that the presence of prelamin A was the causative factor (Figure 4.5a). To further validate the expression changes were dependent on lamin A, all of the same genes were examined in *Lmna*<sup>-/-</sup> cells, which had either been transfected with *Zmpste24* or control siRNA (Figure 4.5b). No significant alteration in gene expression was observed, which further supported the notion that it was the presence of the unprocessed lamin A that caused the alteration in expression.

To further prove that the dysregulation of the genes was actually because SREBP-1 was not reaching its targets chromatin immunoprecipitation (ChIP) was performed on the genes known to be regulated by this transcription factor (Figure 4.6). In all cases there was a significant decrease of transcription factor enrichment at the promoter sites.



**Fig 4.5 Genes regulated by SREBP-1 are dysregulated by farnesylated prelamins A.**

(a) *Fasn*, *Acsl*, and *Pparγ* are all positively regulated by SREBP-1 and show downregulation after prelamins A accumulates. *Mtp* is negatively regulated by SREBP-1 and after prelamins A accumulates its expression increases. *Klotho* is positively regulated by PPAR $\gamma$  and also shows downregulation. (b) In *Lmna*<sup>-/-</sup> cells siRNA targeted against *Zmpste24* had no significant effect on any of the genes regulated by SREBP-1 or PPAR $\gamma$ .



| Gene         | Expression Fold Change |               | ChIP Fold Enrichment | Mapper Score | Mapper E Value | Mapper Model |
|--------------|------------------------|---------------|----------------------|--------------|----------------|--------------|
|              | <i>Zmpste24</i> siRNA  | Control siRNA |                      |              |                |              |
| <i>Fasn</i>  | -2.570                 | -0.424        | -1.227               | 3.6          | 18             | M00776       |
| <i>Mttp</i>  | 1.555                  | 0.0990        | -0.715               | 3.4          | 20             | M00776       |
| <i>Pparγ</i> | -4.890                 | 0.732         | -1.280               | 1.4          | 19             | T01556       |
| <i>Acsl</i>  | -2.648                 | 0.191         | -1.495               | 3.4          | 20             | M00776       |

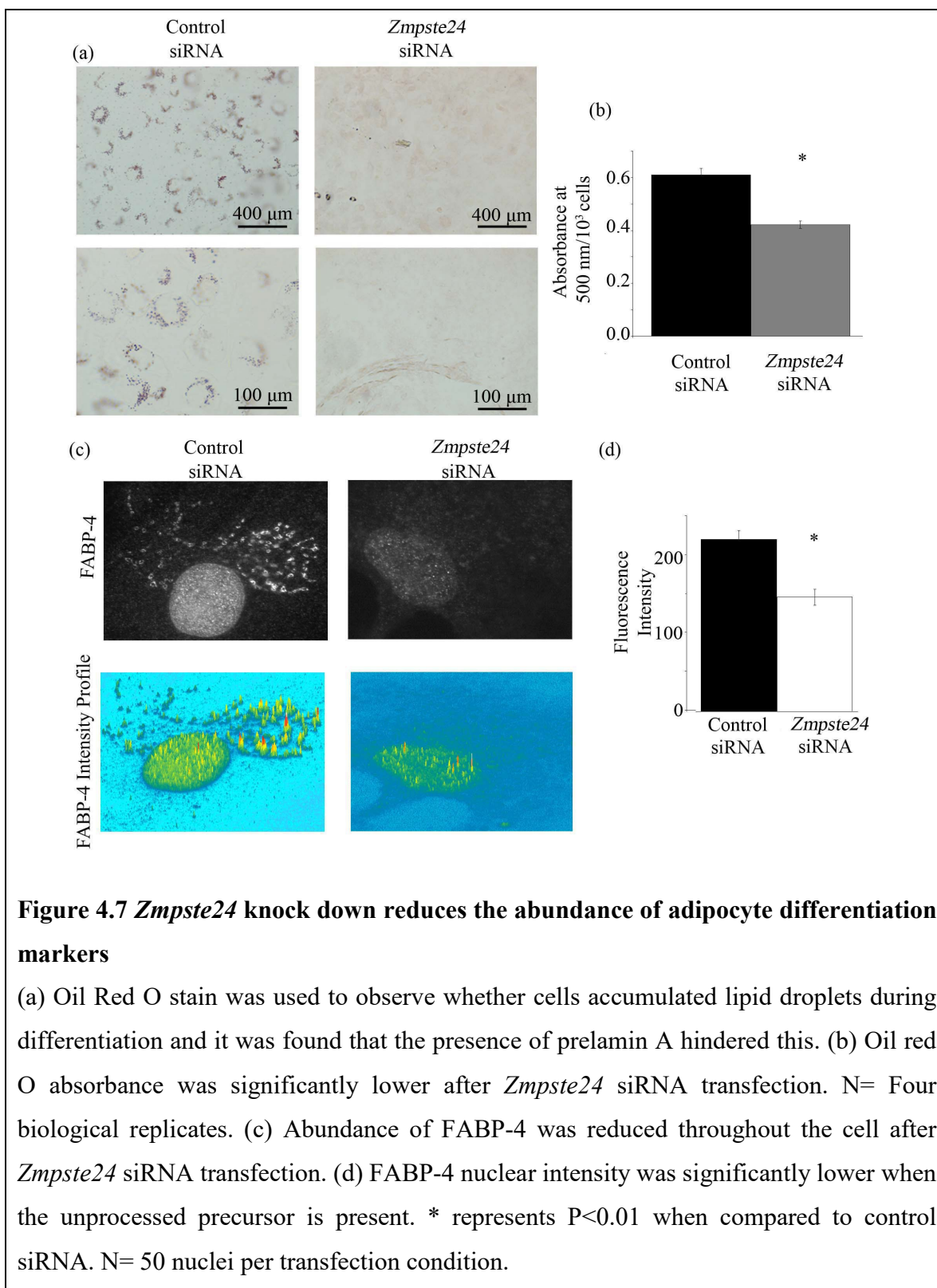
**Fig 4.6 Reduced SREBP-1 enrichment at promoters of genes regulated by SREBP-1**

(a) Both the positively regulated genes (*Pparγ*, *Fasn*, *Acsl*) and the negatively regulated gene (*Mttp*) showed reduced binding of SREBP-1 at promoter sites identified by the MAPPER search engine. No change was seen for a SREBP-1 independent gene, actin. (b) Table to compare the alterations in gene expression with the reduced SREBP-1 enrichment at promoters.

#### 4.3.5 *Zmpste24* knock down reduces adipocyte differentiation markers

As a preadipocyte differentiates into an adipocyte its ability to store fatty acids as triglycerides and lipids increases as more of the genes responsible for lipid storage are upregulated. A mature adipocyte has many lipid droplets, which can be identified by the Oil Red O stain, which stains red upon association with lipids. Pre-adipocytes mature into adipocytes upon reaching confluency in culture and are changed to new medium supplemented with insulin to maintain this differentiated state as described in Chapter 2. After two days at full confluency and addition of the new medium the cells were transfected for 48 hours. After this time the control transfected cells contained many lipid droplets that could easily be seen, while in the *Zmpste24* siRNA transfected cells there were significantly fewer (Figure 4.7a). This was supported by the fact that absorbance for the stain was significantly lower in prelamina A accumulating cells (Figure 4.7b).

Fatty Acid Binding Protein (FABP-4) is a key regulator in adipocyte differentiation. It resides in the cytoplasm until it is activated by a fatty acid ligand, which leads to a conformational change to FABP-4 and it translocates to the nucleus, where it directly binds to and activates PPAR $\gamma$ , leading to more FABP-4 expression and upregulation of other genes involved in adipocyte differentiation. FABP-4 level were assessed by fixing the cells after the conditions described above and the protein was labeled against for immunofluorescence. It was found that the nuclear intensity of FABP-4 was reduced significantly ( $P < 0.01$ ) in prelamina A accumulating cells (Figure 4.7c, d).



## 4.4 DISCUSSION

Lipoatrophy is a symptom experienced with certain laminopathies and is a debilitating side effect of up to forty percent of people on HAART (Caron-Debarle et al., 2010; Worman et al., 2010). The exact mechanism is unclear and is likely to involve a variety of cellular processes, but it is evident that the presence of prelamin A can influence lipid storage and the potential of adipocytes to differentiate. The identification that the *LMNA* gene is mutated in Dunnigan type Familial Partial Lipodystrophy (Shackleton et al., 2000) paved the way for suggesting prelamin A influences fat storage around the body. The fact that other laminopathies suffer with lipoatrophy, such as HGPS, MAD and RD, further supported the involvement of this unprocessed lamin (Worman et al., 2010).

*In vitro* lamin A does interact with the N-terminus of SREBP-1 (Lloyd et al., 2002). It was found that one of the lipodystrophy causing mutations within *LMNA* (R482) actually lead to a decrease of SREBP-1 binding and it was proposed that the reduced affinity for the transcription factor leads to dysregulation of fatty acid metabolism effecting lipid storage (Lloyd et al., 2002). However, it has also been shown that *in vivo* only the prelamin A precursor can interact with SREBP-1 and this effects its distribution within the nucleus (Capanni et al., 2005).

The sequential nuclear extraction and immunofluorescence presented here indicate that under normal conditions there is an equal presence of the active form of SREBP-1 at the periphery and within the nucleoplasm (Figure 4.1). Upon prelamin A accumulation, however, this distribution is drastically altered with the majority residing in the lamina



associated fraction. Prelamin A would therefore appear to affect SREBP-1's ability to dissociate from the periphery and migrate to the nuclear interior. This may be because the additional fifteen C-terminal amino acids of prelamin A retain it at the nuclear lamina. It was apparent that the nucleus developed significantly more invaginations as a result of prelamin A accumulation. SREBP-1 colocalised with prelamin A intensely around these invaginations, whose function remains speculative but their morphology once again resembled what was reported in chapter 3 (Figure 4.2).

The dynamics of a GFP tagged version of SREBP-1 indicated a reduced mobility at the periphery (Figure 4.3). As prelamin A is interacting with the transcription factor then its ability to move around the nucleus to its targets will be reduced. This was further confirmed by the fact that positively regulated genes were down regulated and negatively regulated genes were up-regulated (Figure 4.5). The fact that the *Lmna*<sup>-/-</sup> cells showed no significant alteration in gene expression, after *Zmpste24* siRNA transfection, indicates that it is the presence of the unprocessed lamin A that is causing dysregulation of gene expression and not any off-target effect. ChIP further supported the observation that there was reduced binding of the transcription factor at promoters of SREBP-1 regulated genes (Figure 4.6).

The fact that *in vitro* lamin A has been observed to interact with SREBP-1 (Lloyd et al., 2002) but *in vivo* it appears not to (Capanni et al., 2005), casts doubt on the physiological role of SREBP-1 binding at the nuclear periphery. A variety of transcription factors use lamin A as a platform, such as cFos, which is activated by phosphorylation from ERK1/2,

enabling its release from lamin A to activate genes involved in various key cellular processes such as proliferation (Gonzalez et al., 2008). SREBP-1 also possesses many sites that can be post translationally modified, meaning it could operate under a similar mechanism (Bengoechea-Alonso et al., 2005; Kotzka et al., 2010). However, if lamin A is a binding partner for SREBP-1 then it would be anticipated that knock out of lamin A should release any sequestered SREBP-1 and cause upregulation of SREBP-1 positively regulated genes (*Fasn*, *Acs1*, *Pparγ*) and down regulation of the negatively regulated genes (*Mttp*). The fact that the *Lmna*<sup>-/-</sup> expression data reveals no dysregulation of SREBP-1 genes in combination with a failure to co-immunoprecipitate with lamin A (Capanni et al., 2005) means that signals of SREBP-1 at the nuclear periphery under normal conditions may be just the protein caught translocating the nucleus and it may make only temporary associations with the lamina to aid its passage to the nucleoplasm.

*Pparγ* downregulation, due to SREBP-1 retention, influences the ability of cells to undergo adipogenesis. PPARγ is one of the first proteins expressed for the commitment of pre-adipocytes to differentiate into mature adipocytes. One of the markers of adipocyte differentiation is increased storage of triglycerides in the form of lipid droplets, which can be detected by an oil red O stain. *Zmpste24* siRNA did lead to significantly reduced levels of oil red O being detected, indicating reduced adipogenesis (Figure 4.7). PPARγ agonists have been suggested as overcoming impaired adipogenesis (Capanni et al., 2005); however, if the gene is down regulated then these would have minimal effect on its expression and so adipogenesis should still be impaired. This was found when the

PPAR $\gamma$  agonist Rosiglitazone was used to treat lipoatrophy in patients on HAART but had no effect (Carr et al., 2004; Sutinen et al., 2003).

KLOTHO is a protein that has been implicated in the ageing process as mutations in the gene result in accelerated ageing (Kuro-o et al., 1997) and over expression of it leads to increased longevity in mice (Kurosaki et al., 2005). It has been shown to be regulated by PPAR $\gamma$  (Zhang et al., 2008) and consequently *Klotho* may be down regulated in patients accumulating prelamins A, which perhaps underlies a mechanism for the increased ageing phenotype experienced by people on HAART (Guaraldi and Baraboutis, 2009; Guaraldi et al., 2009; Lo et al., 2011) and sufferers of accelerated ageing laminopathies.

Another marker for adipogenesis is FABP-4, which again had a reduced abundance after prelamins A accumulate (Figure 4.7). FABP-4 is usually activated by fatty acids enabling its translocation to the nucleus where it activates PPAR $\gamma$ . However, as genes involved in fatty acid synthesis are down regulated then there will be less activated FABP-4 and consequently less activated PPAR $\gamma$ . As well as FABP-4 activation PPAR $\gamma$  is also directly activated by fatty acids (Kim et al., 1998b), so any disruption in their synthesis will further lead to an inactive pool of nuclear receptor that manages to get expressed.

This chapter has shown that the acute knock down of *Zmpste24* by siRNA causes the SREBP-1 transcription factor to be sequestered at the nuclear periphery that leads to it having a reduced dynamic at the nuclear membrane. The downstream consequences of this are a dysregulation of many of the SREBP-1 targets, with reduced binding of the

transcription factor at the promoters of these genes. This culminates in reduced differentiation potential of pre-adipocytes into mature adipocytes that may underly the lipoatrophy experienced in patients accumulating improperly processed lamin A. Further confirmation that farnesylated prelamin A is affecting the biochemical make up of the cell can be explored by using metabolomics, which is discussed in the next chapter.

## **CHAPTER 5**

# **METABOLOMIC PROFILING OF PRELAMIN A ACCUMULATING CELLS**

### **5.1 INTRODUCTION**

The study of the entire set of small molecules in a biological sample was first described as metabolomic profiling in 1998. It rapidly became evident that more than one detection platform was required to assess the wide array of different metabolite classes and obtaining reproducible data could take years of instrument optimization. These different platforms include capillary electrophoresis–mass spectrometry, liquid chromatography–mass spectrometry, gas chromatography–mass spectrometry and nuclear magnetic resonance; all paired with various enrichment and separation technologies. However, once optimised the platform provides a very powerful snapshot of a cell's metabolic state and a measure of the pathway flux of various biochemicals. This can be used in conjunction with other omic platforms (e.g. genomic, proteomic, interactomic), to get a better understanding of the phenotypes experienced at the cellular and organismal level in various disease states.

Metabolomics has thus far not been used to assess the effect prelamins A has on metabolite levels and whether this relates to biochemical and phenotypic observations reported in lipodystrophy syndromes. One of the principal causes of acquired

lipodystrophy syndrome is the administration of HIV-PIs for people on HAART regimens. There are indications that some of these drugs block ZMPSTE24 from processing farnesylated prelamin A, which may lead to acquired lipodystrophy syndrome by perturbing adipocyte function and differentiation potential that is supported by the results presented thus far. Further confirmation of this can be achieved by examining the metabolomic profile of cells treated with the HIV PI saquinavir. Lipodystrophy syndrome has been reported in patients taking saquinavir as part of HAART, yet there has been no study to indicate whether it causes the accumulation of prelamin A.

During the early part of the first decade of the 21<sup>st</sup> century the emergence of PIs with better bioavailability saw saquinavir lose market share in the developed world and its use seemed to be diminishing. However, the advent of ritonavir boosted PI regimens (which prolong the half life of PIs *in vivo*) has seen saquinavir receive renewed interest. In 2009 the GEMINI trial showed that compared to boosted lopinavir (the most widely prescribed HIV-PI), ritonavir-boosted saquinavir treatment had a similar efficacy and a better triglyceride profile (Walmsley et al., 2009). Furthermore saquinavir has consistently been used in resource limited settings, particularly Thailand and parts of Africa (Dickinson et al., 2008). This use in the developing world looks set to continue; in 2006 the manufacturers of saquinavir (Roche) announced a technology transfer initiative, offering local drug companies in Africa and Asia help in producing the drug.

The fact that its use is once again increasing highlights the importance of a thorough understanding of the molecular actions this drug has on the cell and whether this affects

prelamin A processing that might lead to a metabolomic profile indicative of developing lipodystrophy syndrome.

## 5.2 MATERIALS AND METHODS

**5.2.1 Cell culture:** MEFs were cultured as previously described in Chapter 2.

**5.2.2 Drug treatment:** Saquinavir was dissolved in DMSO (Sigma 472301) to make a 5 mM stock solution and was used at a concentration of 20  $\mu$ M for 48 hours, as this represented a therapeutic concentration. After the initial 24 hours of treatment the medium was changed and there was a fresh addition of saquinavir for the remaining 24 hours. The re-application of the drug was because of the metabolism of HIV-PIs by cytochrome P450.

**5.2.3 TEM and dual axis EMT:** EM techniques were carried out as described in Chapter 2.

**5.2.4 Western Blot:** The protocol for western blotting was carried out as described in the Materials and Methods chapter. Nitrocellulose membranes were bathed in  $\beta$ -actin antibody at 1:5000 (Abcam 8226) and lamin A antibody at 1:200 (Abcam ab26300) overnight. The secondary antibodies used were peroxidase AffiniPure Goat Anti-Mouse IgG (Sigma A4416) and peroxidase Affinipure Goat Anti-Rabbit IgG (Sigma A6154) at a dilution of 1:5000 and 1:6000 respectively.

**5.2.5 Immunofluorescence:** Cells were prepared as described in the methods section of chapter 2. Primary antibodies used were a prelamin A specific antibody (Santa Cruz Biotechnology Inc sc-6214), anti-lamin B1 antibody (Maske et al., 2003) and an anti-calreticulin antibody (Abcam ab-2907). All primary antibodies were used at a dilution of 1 in 100 in 0.4 % FSG. The cells were then incubated with Alexa Fluor 488 donkey anti mouse IgG (Invitrogen A-11029), Alexa Fluor 647 donkey anti goat IgG (Invitrogen A-21447) and Cy3 conjugated donkey anti rabbit IgG (Jackson ImmunoResearch 711-166-152). All secondary antibodies were incubated at 1 in 300 in 0.4 % FSG. Finally the cells were mounted in Mowiol supplemented with DAPI. Immunofluorescence images were collected as previously described.

**5.2.6 Fluorescence Loss In Photobleaching:** The technique was carried out and data collected in GFP-Lamin A expressing MEFs as described in Chapter 3. 72 hours prior to photobleaching the cells had been transfected with GFP-lamin A. 24 hours after initial GFP-lamin A transfection the cells were treated for 48 hours with saquinavir or DMSO.

### **5.2.7 Metabolomic profiling:**

*5.2.7.1 Cell line culture:* Six replicates of DMSO, saquinavir and untreated MEFs were cultured in DMEM supplemented with 10% foetal calf serum and 1% non essential amino acids at 37 °C with 5% CO<sub>2</sub>. Cell cultures were seeded into flasks two days before harvesting, at 10<sup>7</sup> cells per sample. Cells were harvested by washing with PBS before trypsination; subsequently, an equal volume of culture medium was added and the cell



suspension was centrifuged at 2500 rpm for 5 minutes at 4 °C to obtain a cell pellet which were flash frozen, and stored at -80 °C prior to shipment to Metabolon.

*5.2.7.2 Metabolite analysis:* Metabolic profiling analysis of samples was carried out in collaboration with Metabolon as previously described (Gao et al., 2009; Louis et al., 2007; Vander Heiden et al., 2009). Sample processing is briefly described below.

*5.2.7.3 Sample-accessioning:* Every sample was accessioned into the Metabolon Laboratory Information Management System (LIMS) and was given a unique identifier that was associated with the original source identifier. This identifier was used to track sample handling, tasks, results and other data. The samples were bar-coded and tracked by the LIMS system. Any divisions of samples were automatically given unique identifiers by LIMS when a new task was produced and the relationship of these samples was also tracked. All samples were kept at -80 °C until processed.

*5.2.7.4 Sample preparation:* On the day of extraction, samples were thawed on ice and the cell pellet was resuspended in 100 µl of water. Proteins were precipitated and small molecules recovered by performing a series of methanol and aqueous extractions using an automated system called MicroLabSTAR (Hamilton Company). The methanol contained four standards that allowed the monitoring of extraction efficiency. The extract was then divided into fractions for analysis by liquid chromatography (LC+, LC-) and gas chromatography (GC). To remove the organic solvent, the samples were briefly placed on

a TurboVap (Zymark). Each sample was subsequently frozen and dried under vacuum. Samples were then prepared for either LC-MS or GC-MS analysis.

*5.2.7.5 Quality assurance/QC:* For QC purposes additional samples were run with each day's analysis. These included a pool of an aliquot from each experimental sample, an ultrapure water process blank, a well characterised pool of human plasma and an aliquot of solvents used in extraction to segregate contamination sources in the extraction. QC compounds, which will not interfere with the measurement of the endogenous compounds, were added to every sample.

*5.2.7.6 LC-MS and LC-MS/MS:* The LC-MS portion of the platform was based on an ACQUITY ultra-performance liquid chromatography (UPLC) system (Waters) and a linear trap quadrupole (LTQ) mass spectrometer (Thermo-Finnigan), consisting of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. Sample extracts were split into two aliquots, dried and then reconstituted in acidic (formic acid) or basic (ammonium bicarbonate) LC-compatible solvents that each contained eleven or more injection standards at known concentrations. One aliquot was analyzed using acidic positive ion-optimized conditions and the other using basic negative ion-optimized conditions in two independent injections using separate dedicated columns (2.1 mm x 100 mm Waters BEH C18 1.7  $\mu$ m particle columns heated to 40 °C). Extracts reconstituted in formic acid were gradient eluted at 350  $\mu$ l/min using water and methanol, both containing 0.1 % formic acid. The basic extracts contained 6.5 mM ammonium bicarbonate and were eluted at the same rate and also used water and methanol. The MS analysis

alternated between MS and data-dependent MS/MS scans using dynamic exclusion. Dynamic exclusion is a process that allows greater MS/MS coverage of ions present in the MS scan because the instrument will not trigger an MS/MS scan of the same ion repeatedly.

*5.2.7.7 GC-MS:* Samples were re-dried under vacuum dessication for a minimum of 24 hours before being derivatized under dried nitrogen using Bis(trimethylsilyl)trifluoroacetamide (BSTFA). The GC column was 5% phenyl and the temperature ramp was from 40° to 300 °C over a 16 minute period. A Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron-impact ionization was used to analyse samples. On a daily basis this instrument was calibrated and tuned for mass resolution and accuracy.

*5.2.7.8 Instrument and process variability:* Technical differences in the performance of the LC-MS/MS or GC-MS/MS in each sample run (instrumental variability) and differences in the preparation of individual samples (process variability) inevitably cause differences between samples that are unrelated to the biological variable of interest. Information of instrument variability was acquired by using internal standards that were added to each sample before injection into the mass spectrometers. Then the median relative SD (RSD) of ion counts of the standards for all samples in each batch was calculated. Overall process variability was calculated by the median RSD for all endogenous metabolites (i.e. non-instrument standards) present in 100% of the cell lysate

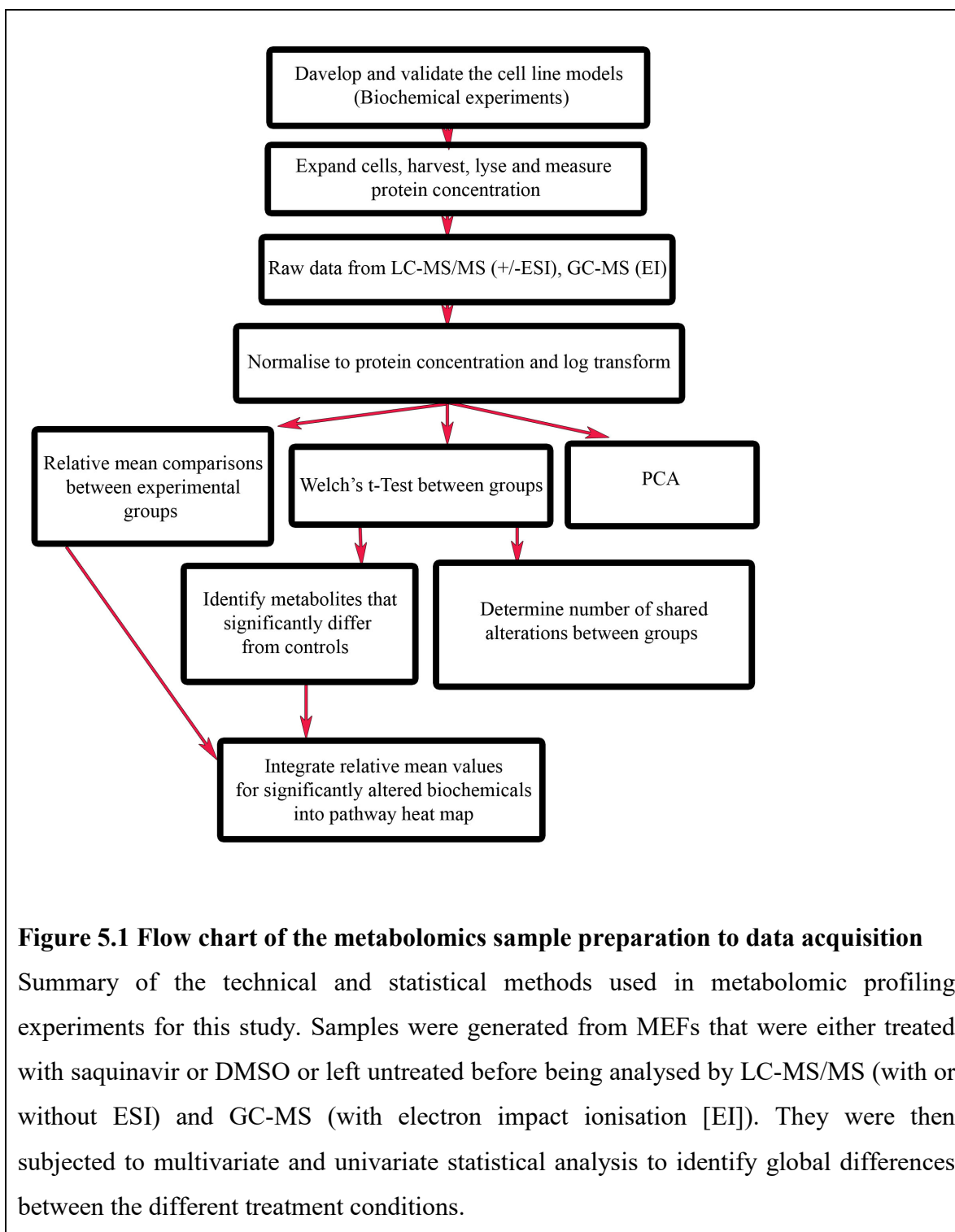
samples in each batch. The median RSD values for all MEF cell lysates was 5% for internal standards and 10 % for endogenous metabolites.

*5.2.7.9 Statistics. Imputation and normalisation:* Certain biochemicals were detected in some, but not all samples in an experiment. Biochemicals detected in all samples from one or more groups but not in samples from other groups were assumed to be near the lower limit of detection in the groups in which they were not detected. In this case the lowest detected level of these biochemicals was imputed for samples in which that biochemical was not detected. Quantification values then were normalized to protein concentrations obtained using the Bradford assay. KEGG release 41.1 (<http://www.genome.jp/kegg>) was used to map biochemicals to pathways (Kanehisa, 2009).

*5.2.7.10 Univariate Statistics:* In order to determine if the mean levels of a biochemical in one experimental group differed from the mean levels in another group Welch's t test was used.

*5.2.7.11 Multivariate Statistics:* Heat maps of fold changes were plotted using non log<sub>2</sub>-transformed data. PCA plots were generated within Array Studio version 4.1.1.20 (Omicsoft). The analysis was generated using Array Studio software. Array Studio,

Array Viewer and Array Server and all other Omicsoft products or service names are registered trademarks or trademarks of Omicsoft Corporation., Research Triangle Park, NC, USA



## 5.3 RESULTS

### 5.3.1 Saquinavir causes prelamin A accumulation that produces NR development and a less dynamic lamina

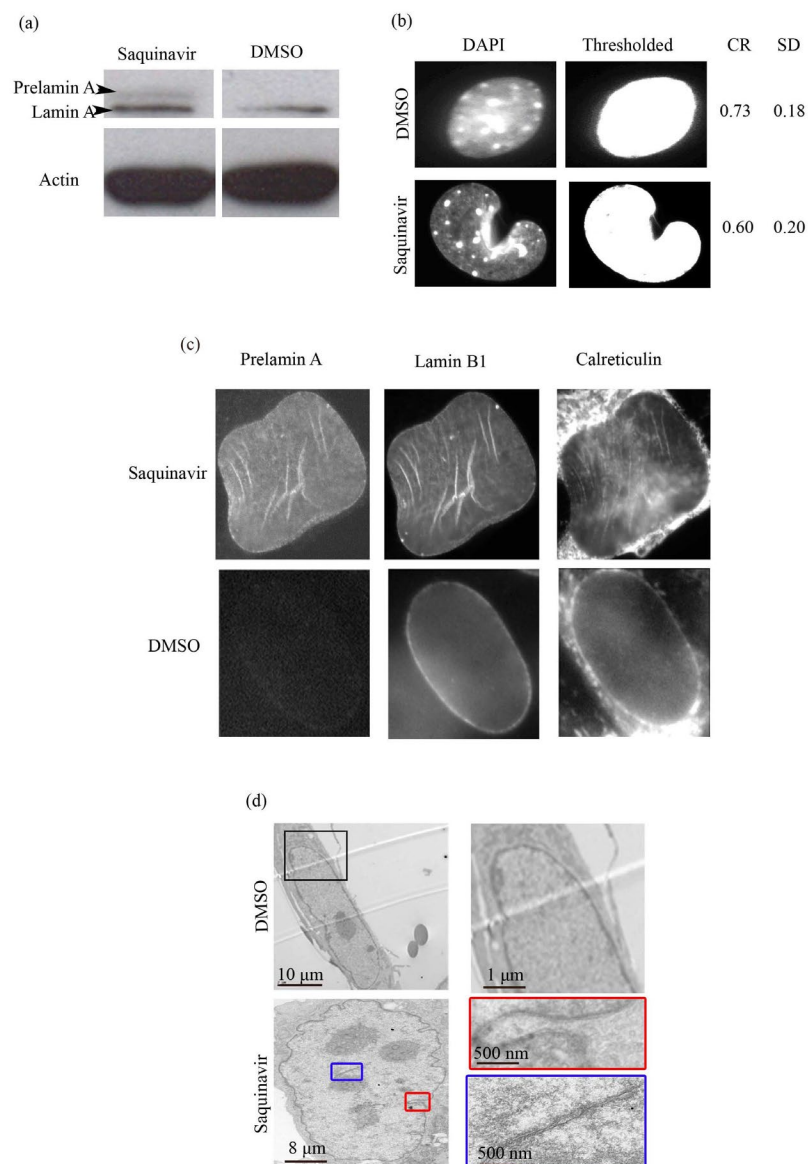
Therapeutically relevant concentrations of saquinavir were added to MEFs for 48 hours and western blot revealed the accumulation of a faint prelamin A band above the more prominent 72kDa mature lamin A band (Figure 5.2a). This prelamin A signal could not be detected in the DMSO control. A contour ratio was again used to assess the effect the acute prelamin A accumulation was having on the nuclear morphology. As with the *Zmpste24* siRNA transfection there was a significantly reduced ( $P < 0.01$ ) contour ratio to  $0.60 \pm 0.20$  when compared to the control treated nuclei of  $0.73 \pm 0.18$  (Figure 5.2b). Further morphological similarities with the *Zmpste24* siRNA transfection experiments could be observed when assessing the distribution of the prelamin A by immunofluorescence (Figure 5.2c). There was intense staining of the prelamin A around the nuclear periphery and at intranuclear structures that colocalised with lamin B1. These structures were found to be both type I and II NR that had almost identical morphology to the NR that develops after *Zmpste24* siRNA transfection (Figures 5.2 d and 5.3a, b).

FLIP was used to assess whether prelamin A accumulation was having an effect on GFP-lamin A dynamics at the periphery of the nucleus (Figure 5.4). A region of interest was photobleached for 5 minutes and the GFP-lamin A was found to be significantly less dynamic than the DMSO control (Figure 5.4a, b). After 300 seconds the mean intensity of DMSO treated nuclei in the ROI's had dropped to  $57\% \pm 2.41$  while for the saquinavir treated nuclei the lamina appeared significantly more stable with only a drop to  $93\% \pm$

4.08. This had very similar characteristics to the dynamics of the lamin after *Zmpste24* siRNA transfection. Also the increased abundance of intranuclear GFP-lamin A imaged in live cells once again confirmed that the structures observed in fixed samples for light and electron microscopy were unlikely to be side effects of the fixation process (Figure 5.4a).

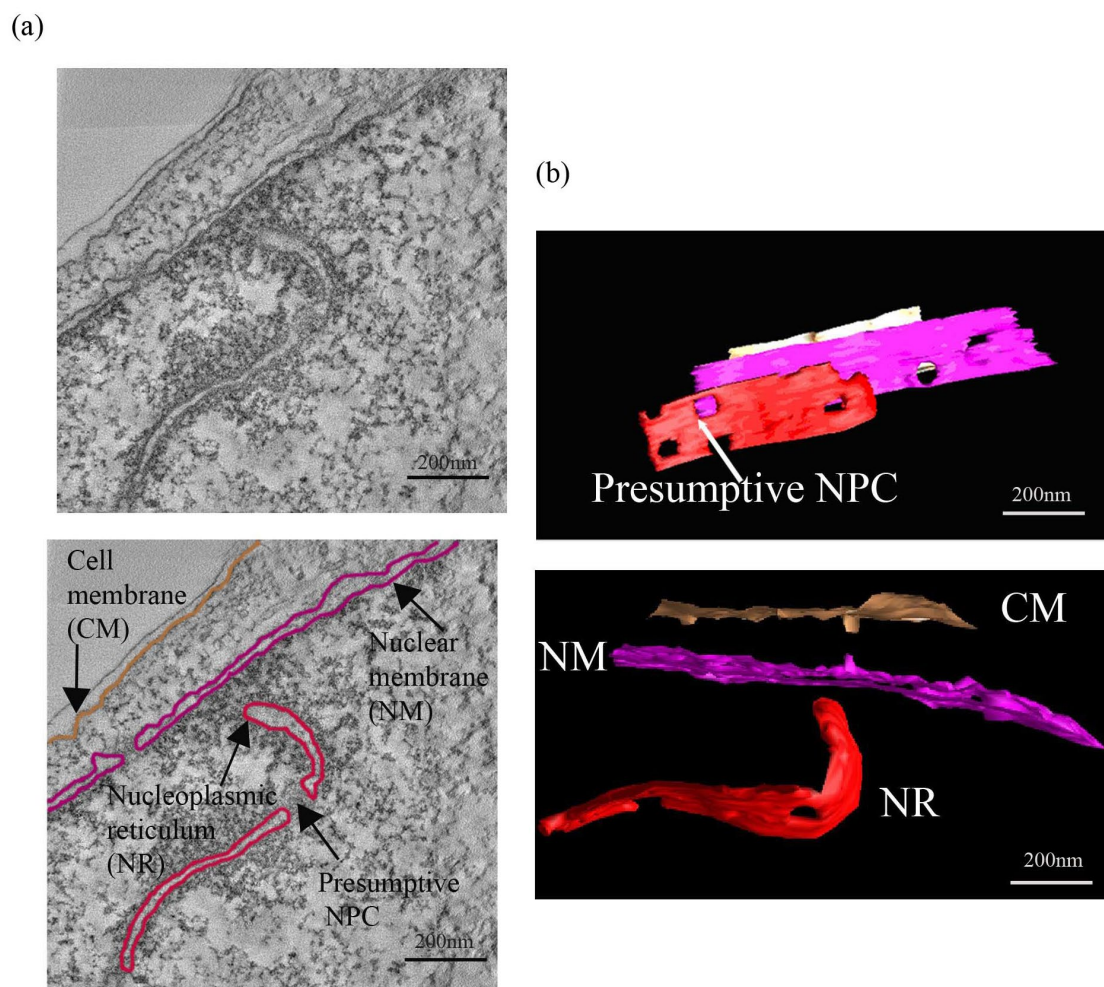
The biochemical, nuclear morphological and dynamic effects that saquinavir was having suggests that it was perturbing the lamin A processing pathway and that the prelamins A being generated had similar features and effects as was observed when acutely knocking down ZMPSTE24 using siRNA.





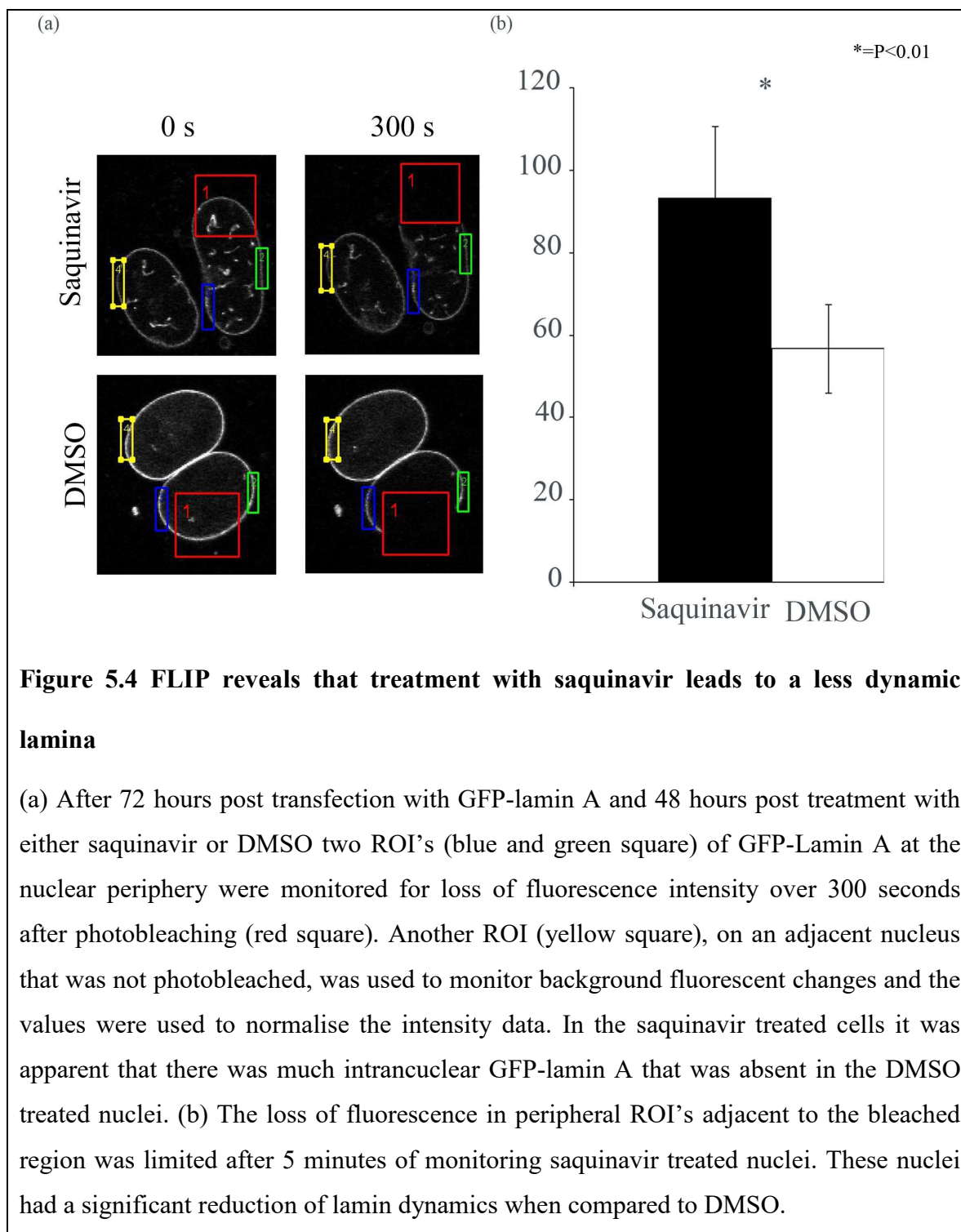
**Figure 5.2 Saquinavir leads to the build up of prelamins A**

(a) After 48 hours of drug treatment a faint band of prelamins A was seen above the predominant lamin A band. (b) This treatment leads to a dysmorphic nuclear phenotype as indicated by the significantly lower CR. (c) Invaginations of the nuclear envelope could be seen as indicated by a calreticulin signal colocalizing with intranuclear prelamins A and lamin B1. (d) These structures were further validated using TEM that showed type I (blue box) and type II (red box) NR development. (e) Dual axis EMT also revealed that the type I NR had similar ultrastructure to the cell nuclei after *Zmpste24* siRNA transfection and contained regular fenestrations.



**Figure 5.3. Saquinavir treatment leads to a complex NR as revealed by EMT**

(a) Dual axis EMT also revealed that the type I NR had similar ultrastructure to the cell nuclei after *Zmpste24* siRNA transfection and contained regular fenestrations. (b) model of the tomogram generated in IMOD revealed a striking similarity to what was observed previously and the presumptive NPCs was once again present in the NR.



### 5.3.2 Metabolomic profiling of saquinavir treated cells

To assess the metabolic consequences of saquinavir treatment unbiased metabolic profiling was performed on MEFs that were treated with saquinavir or DMSO vehicle and were compared to the wild type. Lysates were analysed from cells growing in the logarithmic growth phase by using three mass spectrometry platforms, liquid chromatography (LC)-MS/MS with or without electrospray ionisation (ESI) and gas chromatography (GC)-MS [ with electron ionisation (EI)], in six replicates per sample. The analysis revealed MS ion counts that corresponded to 208 known metabolites. The data were normalised to protein concentration and the Kyoto Encyclopaedia of Genes and Genomes (KEGG) was used to map the mean level of biochemicals to pathways. In order to determine the global metabolic profile features shared between the different treatments univariate comparisons, correlation analysis and principal component analysis (PCA) were used. PCA is a dimension reduction strategy that transforms a large number of variables (metabolites) into a small number of variables (variation among groups).

The heat map and z-score plot illustrate the effect these conditions had on all 208 identified biochemicals and the effect the treatments have on the various major metabolic pathways (Figure 5.5a, b). The saquinavir treated cells had fifty seven biochemicals significantly (cut-off value for significance:  $P < 0.01$ , Welch's t-Test) upregulated and nineteen biochemicals downregulated compared to DMSO, while the DMSO vehicle also had significant effects on metabolism with fifty metabolites showing upregulation and sixty seven with significant down regulation (Figure 5.5c). When comparing the effects that saquinavir and DMSO have on metabolism they only shared seven upregulated and

six downregulated metabolites (Figure 5.5c). This illustrated the dramatically different effect the presence of the HIV-PI in the vehicle has on the metabolism of the cell. PCA revealed that both DMSO and saquinavir were distinguished from the untreated cells by their first principal component (PC1) value (26 % of variance) (Figure 5.5 d). The PCA analysis reveals that the separation between the treatments and untreated cells is caused by a shared set of metabolic alterations (Figure 5.5 e).

In order to integrate the findings and identify biochemicals that were most altered in cells treated with DMSO or saquinavir the biochemicals that were two fold or greater in their dysregulation were analysed further to elucidate pathways affected by either treatment (Figures 5.6 and 5.7). For saquinavir and DMSO there were forty four and seventy metabolites respectively that were significantly and reproducibly altered by at least two fold.

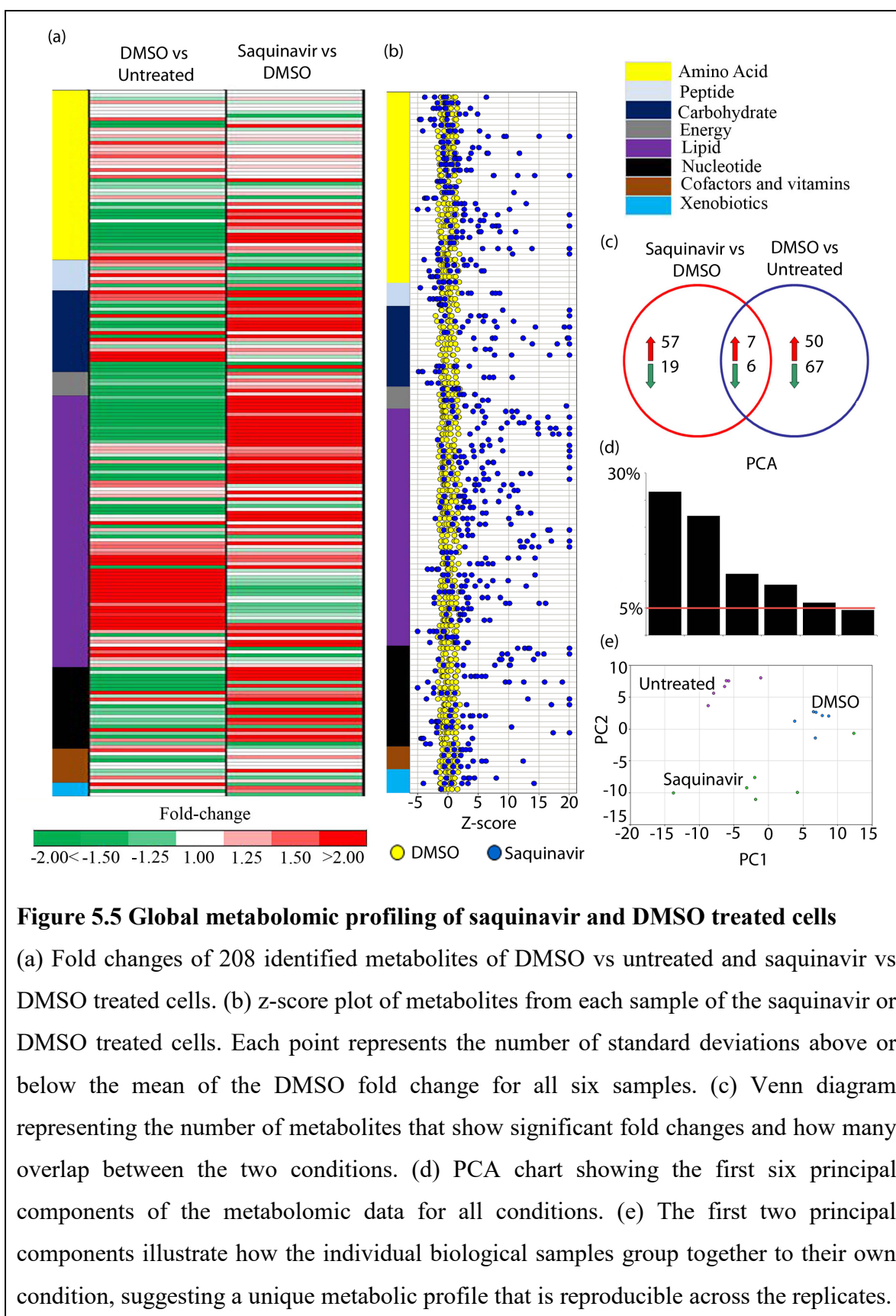
### **5.3.3 Fatty Acid, prostaglandin and glycolytic metabolite levels are altered in saquinavir treated cells**

KEGG subpathways that had significant or reproducible alterations in >50 % of biochemicals from that subpathway in cells treated with saquinavir were determined and mapped into simplified versions of these pathways with their levels. The analysis revealed that many fatty acids had increased (range 1.50 to 5.64 fold;  $P < 0.05$ ) (Figure 5.8). These included essential fatty acids (e.g. linoleate 2.94  $\pm$  0.98 fold, linolenate 4.87  $\pm$  2.07 fold, eicosapentaenoate 5.39  $\pm$  2.20 fold), long chain fatty acids (e.g. myristate 3.17  $\pm$  1.21 fold, palmitoleate 2.82  $\pm$  1.05 fold, oleate 2.40  $\pm$  0.81 fold) and

monohydroxy fatty acids (2-hydroxypalmitate 5.92  $\pm$  1.76 fold and hydroxyoctadecadienoic acid 5.50  $\pm$  2.13 fold). This would initially seem contrary to the findings reported in Chapter 4, as fatty acid synthesis should be reduced in prelamina A accumulating cells due to SREBP-1 being sequestered at the periphery. However, the lipid storage molecule diacylglycerol, which is a precursor to triglyceride synthesis, had decreased (3.00  $\pm$  0.81 fold;  $P < 0.01$ ). This suggests that the fat stores within the cell are undergoing lipolysis and releasing fatty acids to compensate for the lack of fatty acid synthesis.

Interestingly the long chain fatty acid, arachidonate, had increased levels (3.44  $\pm$  2.29 fold;  $P < 0.01$ ) and is a lipolytic product of phospholipid breakdown by phospholipase A2S (Figure 5.9). The fact that diacylglycerol has lower levels (3.00  $\pm$  0.81;  $P < 0.01$  fold) suggests the mechanism of arachidonate production is mediated by phospholipase A2S rather than by phospholipase C. Another product of this reaction are lysophospholipids that showed an increase, which included 1-stearoylglycerophosphoethanolamine (1.69  $\pm$  0.52;  $P < 0.01$  fold) and 1-oleoylglycerophosphoethanolamine (1.64  $\pm$  0.43;  $P < 0.01$  fold). Increased levels of arachidonate lead to a rise in downstream metabolites such as the prostaglandins. Initially arachidonate is converted to prostaglandin H<sub>2</sub> by prostaglandin H<sub>2</sub> synthase, which is a common metabolite precursor for both prostaglandins D<sub>2</sub> (4.43  $\pm$  1.28;  $P < 0.01$  fold) and E<sub>2</sub> (3.01  $\pm$  0.92;  $P < 0.01$  fold) that are synthesised by prostaglandin D<sub>2</sub> and E<sub>2</sub> synthases respectively.

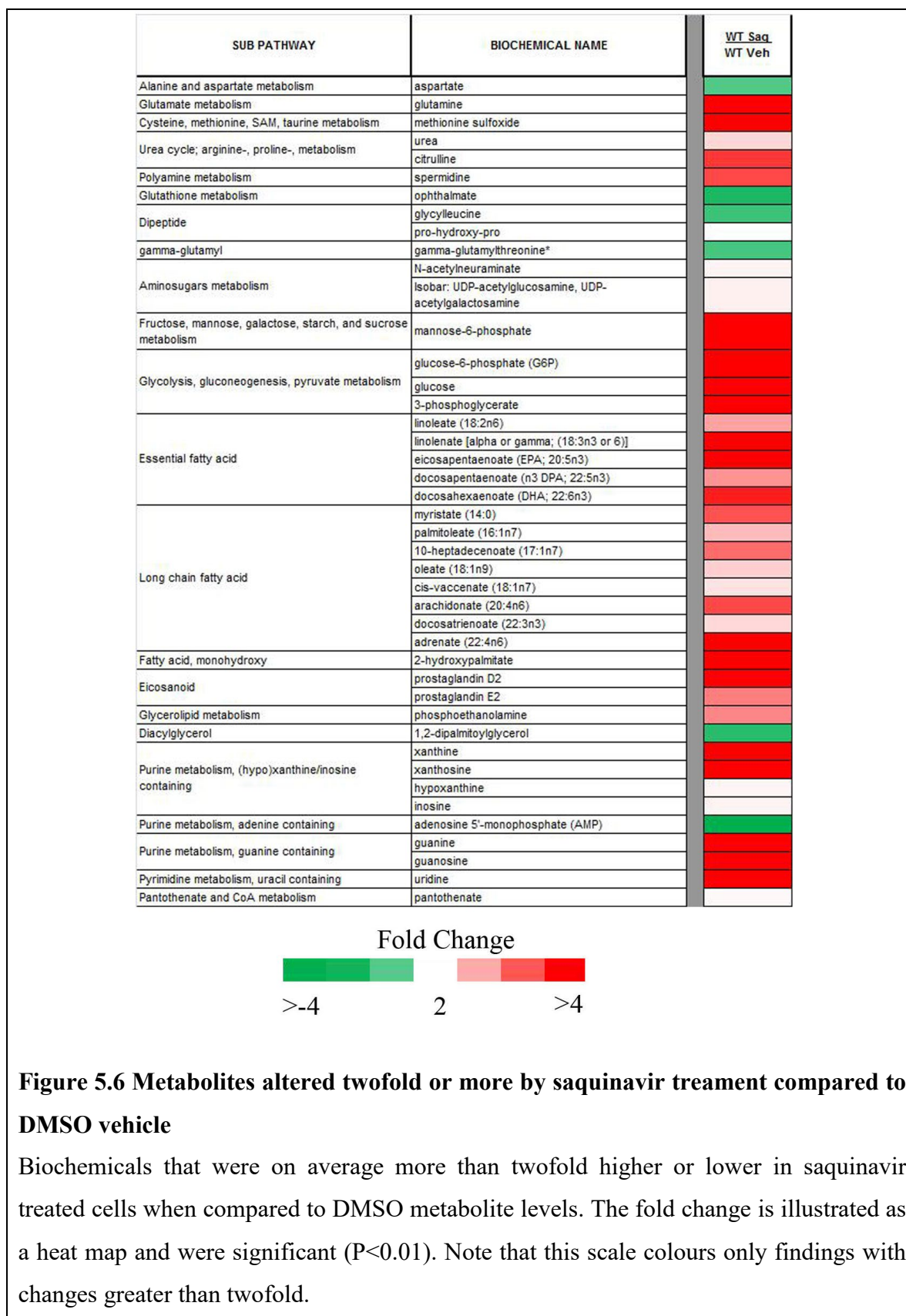
Glycolytic metabolites had increased levels that include glucose (11.29  $\pm$  2.64;  $P < 0.01$  fold), glucose-6 phosphate (8.42  $\pm$  2.12;  $P < 0.01$  fold), fructose-6-phosphate (3.33  $\pm$  0.78;  $P < 0.01$  fold), dihydroxyacetone phosphate (DHAP) (1.35  $\pm$  0.21;  $P < 0.01$  fold) and 3-phosphoglycerate (13.41  $\pm$  2.29;  $P < 0.01$  fold) (Figure 5.10). The increase in glucose suggests that there is increased import of the biochemical into the cell. The increase in DHAP can be produced through breakdown of triglycerides to yield glycerol that is subsequently converted to glycerol-3-phosphate by glycerol kinase. Dehydrogenation of this metabolite yields DHAP, which can then be fed into the glycolytic pathway. This would further support that there is increased lipolysis occurring within prelamina A accumulating cells.

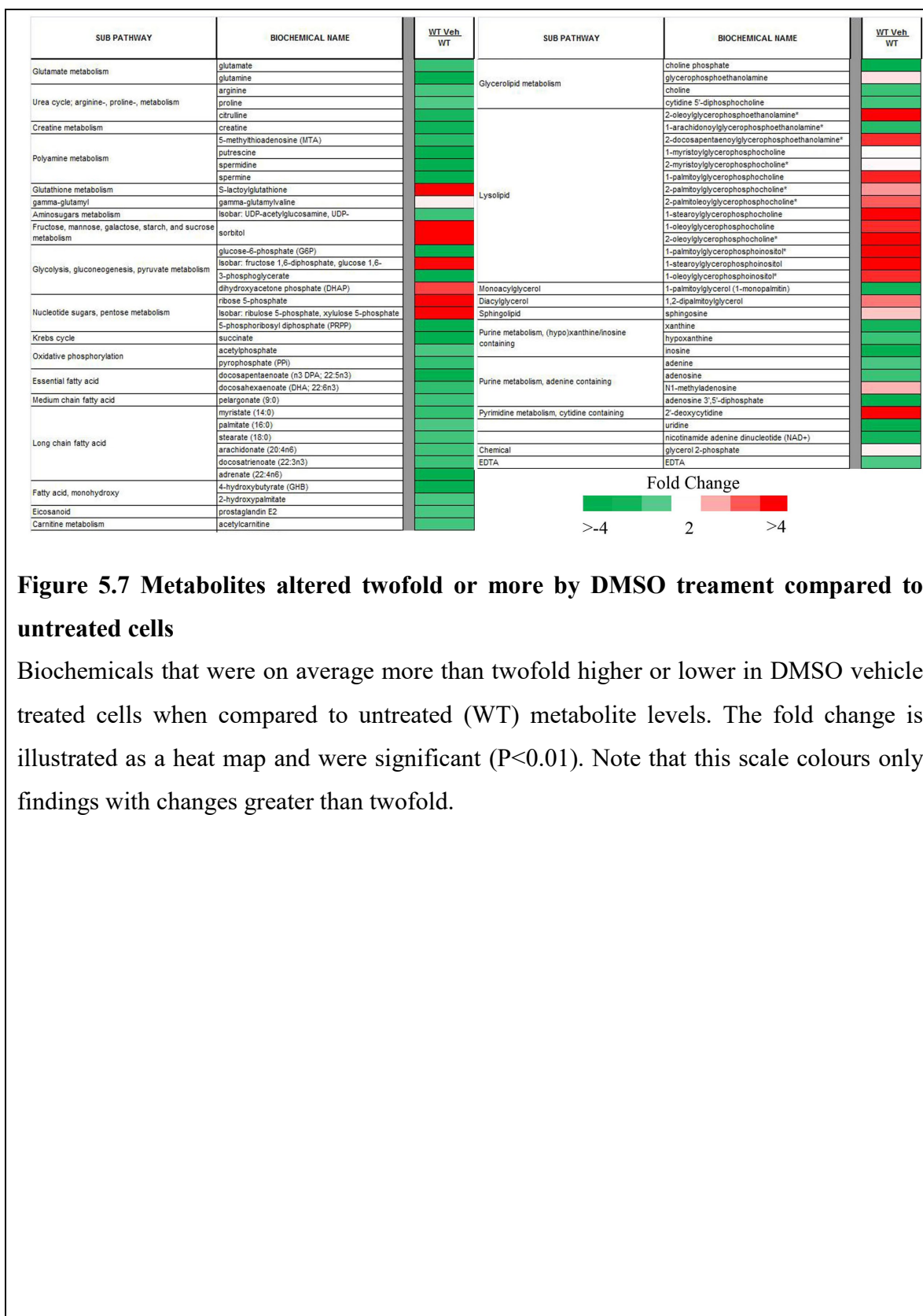


**Figure 5.5 Global metabolomic profiling of saquinavir and DMSO treated cells**

(a) Fold changes of 208 identified metabolites of DMSO vs untreated and saquinavir vs DMSO treated cells. (b) z-score plot of metabolites from each sample of the saquinavir or DMSO treated cells. Each point represents the number of standard deviations above or below the mean of the DMSO fold change for all six samples. (c) Venn diagram representing the number of metabolites that show significant fold changes and how many overlap between the two conditions. (d) PCA chart showing the first six principal components of the metabolomic data for all conditions. (e) The first two principal components illustrate how the individual biological samples group together to their own condition, suggesting a unique metabolic profile that is reproducible across the replicates.

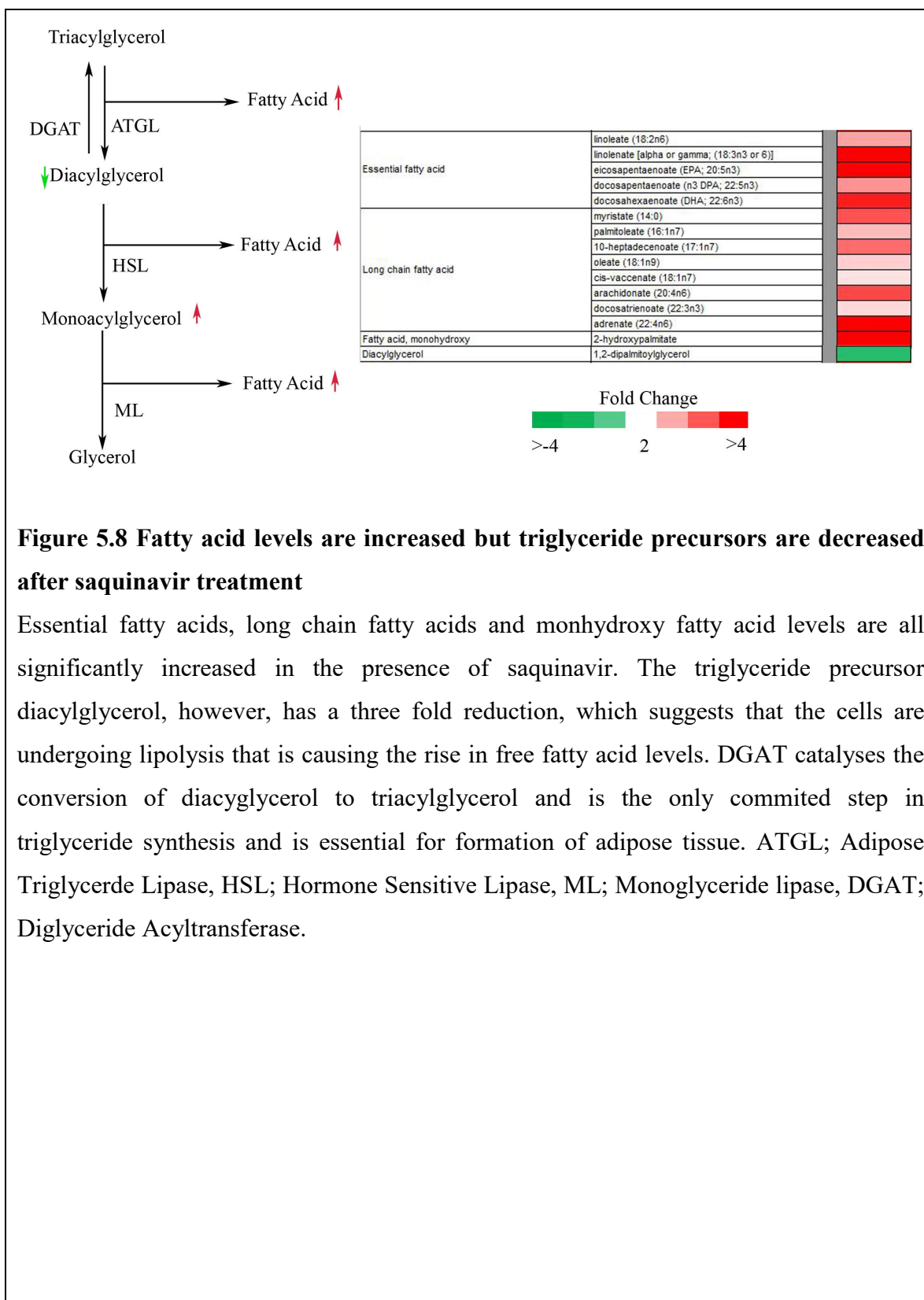






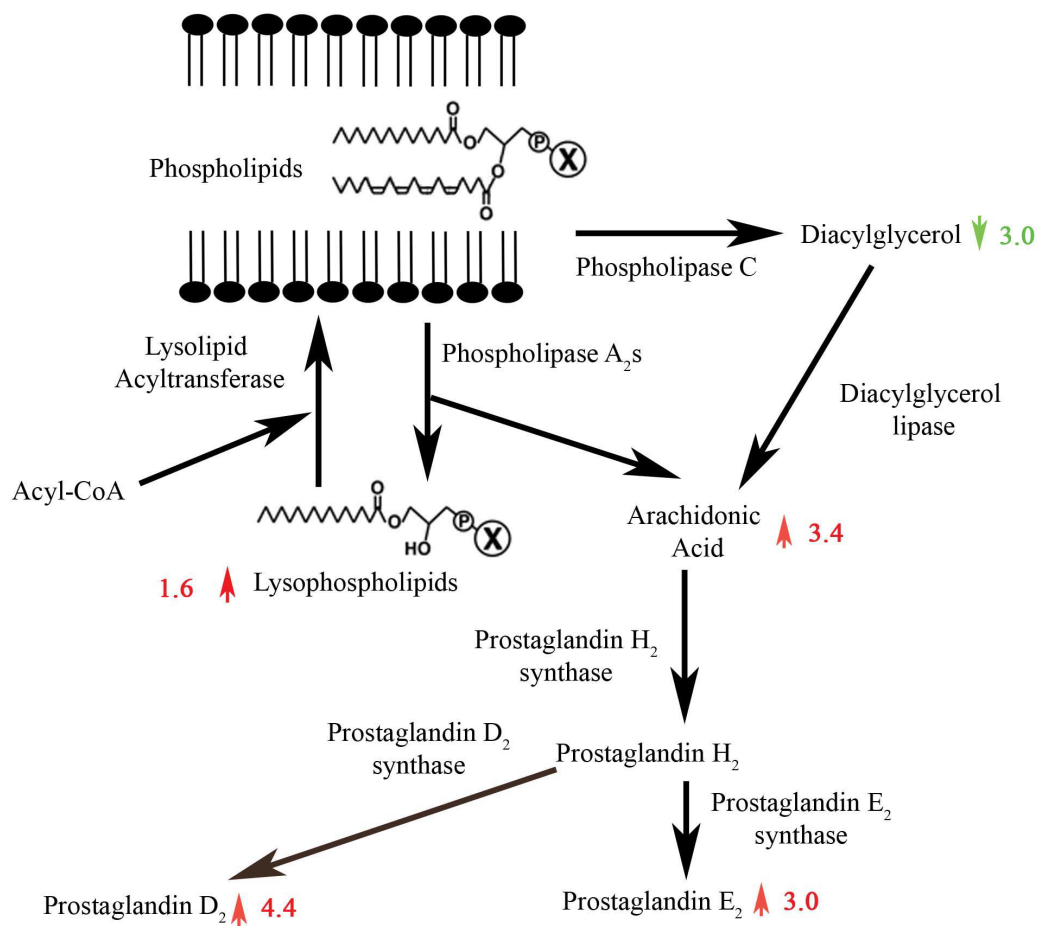
**Figure 5.7 Metabolites altered twofold or more by DMSO treatment compared to untreated cells**

Biochemicals that were on average more than twofold higher or lower in DMSO vehicle treated cells when compared to untreated (WT) metabolite levels. The fold change is illustrated as a heat map and were significant ( $P < 0.01$ ). Note that this scale colours only findings with changes greater than twofold.



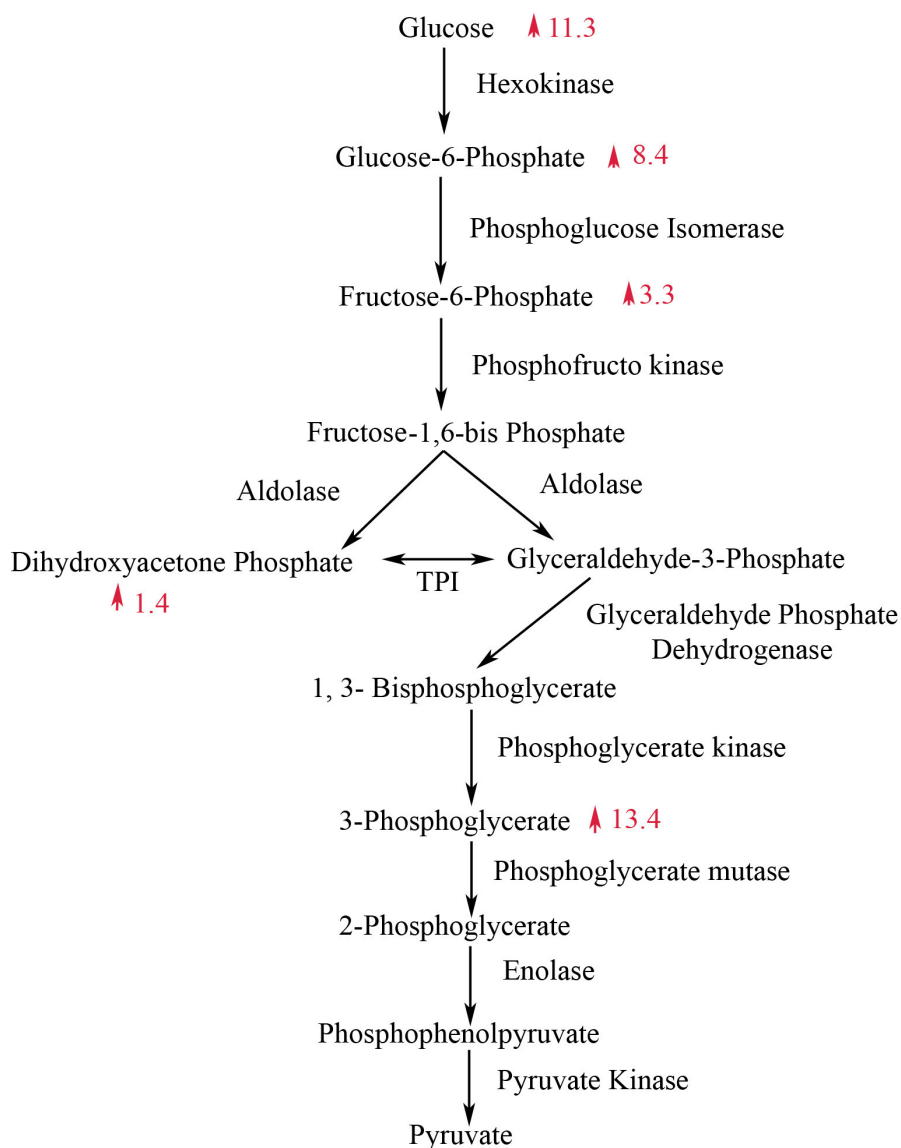
**Figure 5.8 Fatty acid levels are increased but triglyceride precursors are decreased after saquinavir treatment**

Essential fatty acids, long chain fatty acids and monhydroxy fatty acid levels are all significantly increased in the presence of saquinavir. The triglyceride precursor diacylglycerol, however, has a three fold reduction, which suggests that the cells are undergoing lipolysis that is causing the rise in free fatty acid levels. DGAT catalyses the conversion of diacylglycerol to triacylglycerol and is the only committed step in triglyceride synthesis and is essential for formation of adipose tissue. ATGL; Adipose Triglyceride Lipase, HSL; Hormone Sensitive Lipase, ML; Monoglyceride lipase, DGAT; Diglyceride Acyltransferase.



**Figure 5.9 Lipolysis of phospholipids leads to increased prostaglandin levels**

Phospholipase A<sub>2</sub>S breaks down phospholipids into lysophospholipids and arachidonic acid (arachidonate). Arachidonic acid is the precursor for prostaglandin synthesis and in the presence of saquinavir prostaglandins D<sub>2</sub> and E<sub>2</sub> are upregulated.



**Figure 5.10. Glycolysis metabolites are significantly increased after saquinavir treatment**

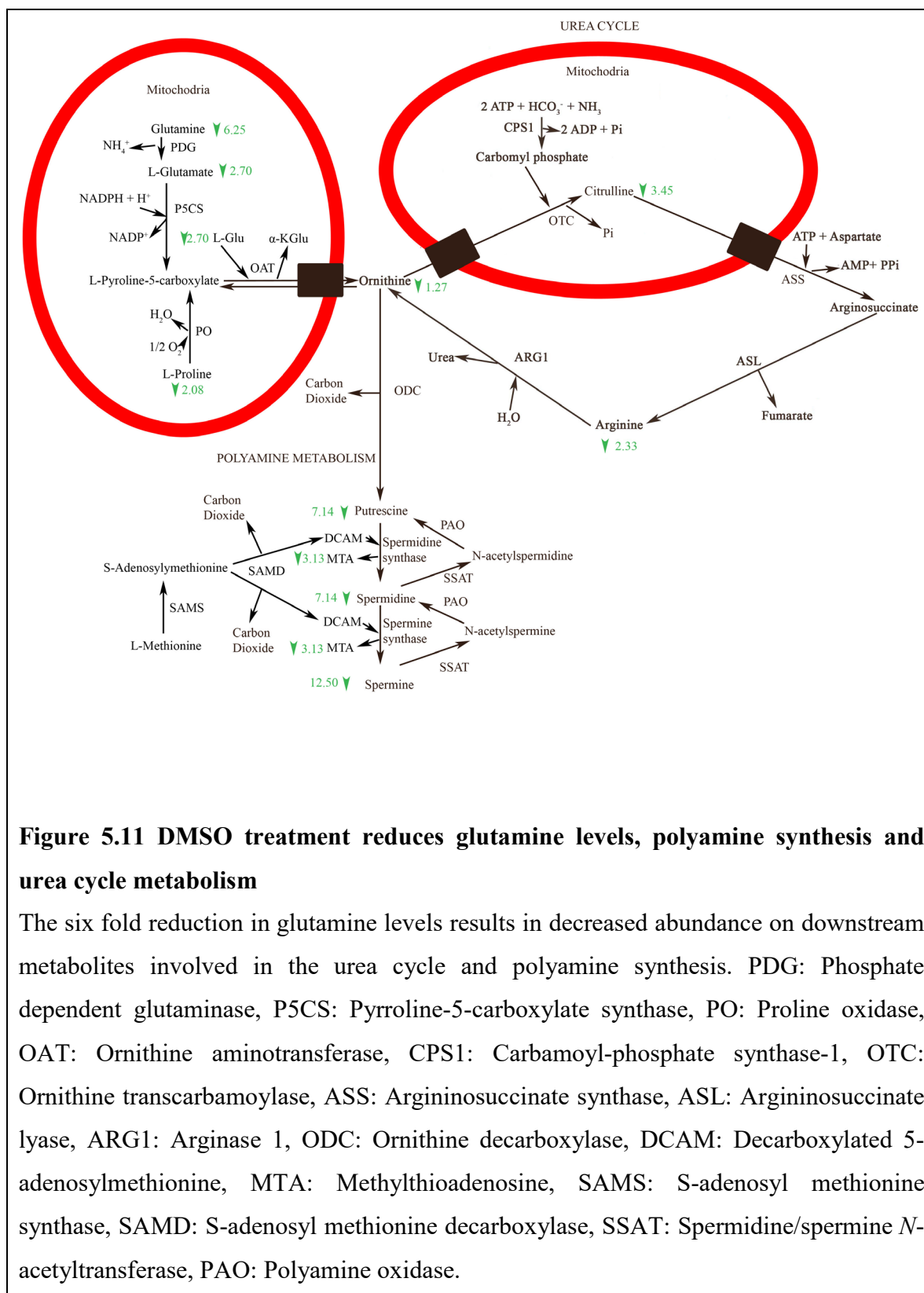
Certain biochemicals in the glycolytic pathway were significantly increased after 48 hours treatment with saquinavir when compared to the DMSO vehicle. The increase in DHAP is a marker of increased lipolysis. TPI; Triose Phosphate Isomerase.

#### **5.3.4 DMSO treatment leads to decreased levels of glutamine, which reduces levels of Urea cycle and polyamine metabolites.**

DMSO is frequently used as a vehicle control in many pharmacological studies at the cellular and organismal level and it was a surprise to see a large number of metabolites with such significant fold changes. The PCA chart clearly illustrated that the six sample replicates tightly group together and are quite independent from the two other conditions, suggesting a unique metabolic profile (Figure 5.5e). This was further supported by such a low number of shared metabolites going in the same direction when compared to saquinavir treatment. There is a considerable amount of literature that suggests DMSO has varied effects on the metabolism of the cell (Santos et al., 2003), but there has been no study so far exploring the effect on a global metabolomic level as is presented here.

Glutamine feeds into several pathways within the cell that includes the Urea cycle, and polyamine synthesis (Figure 5.11). It can eventually be converted into the amino acid ornithine, which can then be incorporated into polyamines or enter the urea cycle to remove excess ammonia by liberating urea. Proline may be converted to pyrroline-5-carboxylate by proline oxidase, which is then converted to ornithine as well. Either pathway is not dependent on ornithine being synthesised from glutamine, as the urea cycle can generate it from arginine, using the enzyme arginase but this is dependent on several metabolite precursors. The metabolomic data reveals that DMSO treatment results in reduced ornithine levels ( $1.27 \pm 0.10$ ;  $P < 0.01$ ) that can be accounted for by the significantly reduced amounts of glutamine ( $6.25 \pm 1.93$ ;  $P < 0.01$ ), glutamate ( $2.70 \pm 0.55$ ;  $P < 0.01$ ) and proline ( $2.08 \pm 0.49$ ;  $P < 0.01$ ). This has an effect on the urea cycle as

citrulline and arginine levels are also reduced ( $3.45 \pm 0.72$ ;  $P < 0.01$ ,  $2.33 \pm 0.46$ ;  $P < 0.01$  respectively). Polyamine synthesis appears severely affected by the reduced ornithine levels as all of the main metabolites of this pathway have significantly reduced levels. These include putrescine ( $7.14 \pm 3.96$ ;  $P < 0.01$ ), spermidine ( $7.14 \pm 2.32$ ;  $P < 0.01$ ), spermine ( $12.50 \pm 6.61$ ;  $P < 0.01$ ) and 5-methylthioadenosine ( $3.13 \pm 0.71$ ;  $P < 0.01$ ).





## 5.4 DISCUSSION

Administration of HIV PIs clearly results in multiple effects on the human body whose symptoms resemble that found in some of the laminopathies, which include lipodystrophy syndrome, cardiovascular disease and accelerated ageing. The fact that these symptoms have a striking resemblance to individuals that have *LMNA* mutations lead investigators to suspect that HIV-PIs were somehow affecting lamin A processing. Indeed multiple HIV-PIs were found to inhibit ZMPSTE24 leading to the build up of farnesylated prelamin A and saquinavir is another drug that can be added to this growing list of compounds that effect lamin A processing.

The effect the drug had on nuclear morphology and lamin stability had a striking resemblance to the acute knock down of *Zmpste24* by siRNA reported in Chapter 3 (Figures 3.3, 3.5, 3.10, 5.2, 5.3 and 5.4). Farnesylated prelamin A has been shown to lead to sequestration of the SREBP-1 transcription factor and effect target genes involved in fatty acid synthesis in Chapter 4. Metabolomics could be used to investigate if the biochemical profile of cells, treated with an HIV-PI, resembled a shift to a lipolytic state. This is the first ever global metabolomic screen of cells treated with an HIV-PI that leads to the accumulation of prelamin A. It revealed both known and novel pathways that may give a greater appreciation of some of the symptoms experienced by individuals on HAART and those suffering with laminopathies.

The large differences of metabolic levels that were induced after only 48 hours of treatment with saquinavir or DMSO were striking (Figure 5.5). Saquinavir lead to

increased levels of many different fatty acids that seemed to counter the conclusions suggested in Chapter 4. However, the fact that diacylglycerol was significantly lower in saquinavir treated cells indicates that lipolysis of lipid stores was occurring and fatty acids were being released. The increased lipolysis may be the cells response to decreased fatty acid synthesis due to farnesylated prelamins A preventing SREBP-1 reaching its target genes. Adipose triglyceride lipase (ATGL) is responsible for lipolysis and is not just confined to adipose tissue, as revealed by ATGL knock out mice that developed increased fat deposition in virtually all tissues (Haemmerle et al., 2006; Lass et al., 2010). ATGL is regulated by interacting with the regulator protein comparative-gene-identification 58 (CGI-58) (Lass et al., 2006), whereas the protein G0S2 is involved in inhibiting ATGL (Yang et al., 2011). The exact mechanism behind controlling either of these ATGL regulators is unknown, but clearly in the presence of saquinavir the activity of this lipase is increased to hydrolyse lipid stores within the cell that may be used to compensate for a lack of fatty acid synthesis mediated by SREBP-1 regulated genes.

As DHAP levels in the glycolytic pathway are also increased, it further supports the notion that lipolysis is occurring (Figure 5.10). This is because glycerol generated from triglyceride breakdown, is ultimately converted into DHAP, which then enters the glycolytic pathway. The increased lipolysis and higher levels of glycolytic metabolites suggests that the cells are increasing energy production perhaps because of a shift to a more stressful state by the presence of saquinavir and the effects it is having on the cell. A potential explanation for the increased glycolysis is the damaging effect HIV-PIs play on mitochondria, which has been observed in both human fibroblasts and adipocytes

(Caron et al., 2007). This was monitored by alterations in the expression of respiratory chain proteins and a decrease in the mitochondrial membrane potential. Interestingly, treatment with an FTI in cells also treated with HIV-PIs actually reversed the incidence of mitochondrial damage, as indicated by a drop in reactive oxygen species (ROS). ROS hyperproduction has been linked to the free radical theory of ageing, which occurs by mitochondrial oxidative phosphorylation that activates cellular stress pathways (Balaban et al., 2005; Dufour and Larsson, 2004). Perhaps the increased mitochondrial damage leads to the upregulation of glycolysis as a means to compensate for reduced energy production. Even though the shift to energy production via glycolysis is inefficient when compared to mitochondrial energy production (2 ATP per glucose as opposed to 36 ATP per glucose), it has been reported in various cancer cells (Warburg effect), where the mitochondria contain mutations in their DNA that encodes components of the respiratory chain (Pelicano et al., 2006). It will be interesting to repeat the metabolomics study by administering HIV-PI treated cells with an FTI to see if this reverses the increased glycolysis.

The significantly higher glucose levels could also be attributed to increased facilitated diffusion by a greater abundance of GLUT transporters at the plasma membrane. GLUT-4 is a glucose transporter mainly associated with adipocytes and muscle cells that is transported from the cytosol to the plasma membrane upon activation by insulin signalling that increases glucose import by up to 15 fold (Blot and McGraw, 2008). GLUT-4 is also present in fibroblasts (Lampson et al., 2000) but does not undergo translocation to the plasma membrane upon insulin activation and maintains a more

constant presence compared to the membranes of lipid and muscle cells. GLUT-4 transports between the plasma membrane and the endosomal recycling compartment with increased abundance at the plasma membrane being mediated by insulin (Lampson et al., 2001). The presence of the HIV PI may somehow increase the effects of insulin mediated signalling, resulting in a rise of GLUT-4 abundance at the plasma membrane. Alternatively, the direct effect of the HIV-PI on the GLUT-4 transporter can increase the rate of glucose transport as has been shown for ritonavir, whether insulin was present or not (Germinario et al., 2003). A similar mechanism may occur for saquinavir but further research will have to be done in order to understand how this PI alters GLUT-4 mediated glucose transport.

The increased levels of arachidonate lead to a rise in prostaglandin synthesis (Figure 5.9). Arachidonate production is regulated by Phospholipase A2S, which is in turn controlled by phosphorylation from mitogen activated protein kinases (MAPKs)(Leslie, 1997). MAPKs are successively acting phosphorylases that function as regulators of cell growth, differentiation and transformation that have been implicated in many pathological and physiological processes (Garrington and Johnson, 1999). Mutations in *LMNA* that lead to EDMD have been attributed to increased MAPK activity, as indicated by upregulation of target genes (Muchir et al., 2007), which regulate additional genes involved in sarcomere structure, cardiomyofiber organisation and other aspects of heart function (Gillespie-Brown et al., 1995; Thorburn et al., 1995). Dysregulation of these genes can lead to cardiomyopathy. The accumulation of farnesylated prelamin A by HIV-PI treatment may lead to increased MAPK activity, which is indicated by the increased

levels of arachidonate that are controlled by MAPK regulated phospholipase A2S. It may explain why some of the HARRT patients also develop cardiovascular disease (Mehta and Reilly, 2005).

The increase in prostaglandin production is a novel feature in the context of prelamin A accumulation and may underly the increased ageing phenotype experienced in some patients. Retinoic acid inducible gene I (RIG-1) mediates senescence-induced expression of pro-inflammatory cytokines such as Interleukin 6 (IL-6) (Liu et al., 2011). IL-6 has been shown to positively regulate prostaglandin E<sub>2</sub> production (Hinson et al., 1996). The secreted form of KLOTHO directly interacts with this interleukin, leading to decreased levels of inflammation that is often associated with cellular senescence. As discussed in Chapter 4, *Klotho* expression is regulated by PPAR $\gamma$ , which in turn is regulated by SREBP-1. If SREBP-1 is prevented from increasing *Ppar $\gamma$*  expression, due to its interaction with prelamin A at the nuclear envelope then *Klotho* levels will decrease, leading to an increased abundance of IL-6. This may be the cause of the three fold rise in prostaglandin E<sub>2</sub> production revealed by the metabolomic data. Inflammation mediated by IL-6 has been described in HIV-therapy patients and this can culminate in the production of atherosclerotic plaques (Chan et al., 2009). It may also be a mechanism by which atherosclerosis develops in progeria sufferers and the normal ageing process. Upregulation of *Klotho* in these conditions may be a way of minimising the side effects of the drug and prolonging the patient's lifespan as well as offering another therapeutic target.

The biological properties of DMSO were only discovered in 1960 and since then it has been frequently used as a solvent in biological studies and used as a vehicle for drug therapy. However, the side effects are often overlooked and the current metabolomic data highlights the importance of recognizing the effect the solvent can have on the regulation of many biochemicals within the cell. DMSO has been reported to have an effect on cell cycle, differentiation, apoptosis, inflammation and lipid metabolism (Santos et al., 2003). Polyamine metabolism and biochemicals within the Urea cycle were adversely affected by DMSO treatment (Figure 5.11). The metabolomic data allowed the causative factor for this reduced abundance to be traced back to the six fold reduction of glutamine levels.

Glutamine is an essential metabolic precursor in nucleotide, glucose and amino sugar biosynthesis, glutathione homeostasis and protein synthesis (Neu et al., 1996). Glutamine transporters in the plasma membrane can be broadly classified into sodium dependent ( $ASC$ ,  $B^{0,+}$ ,  $Y^{+L,A}$  and  $N$ ) and independent ( $L$ ,  $b^{0,+}$  and  $n$ ) (Bode, 2001). The sodium dependent channels use the potential energy in the transmembrane sodium electrochemical gradient, maintained by the  $Na^+/K^+$ -ATPase, which drives the uptake of amino acids against their concentration gradient. This mechanism enables cytoplasmic amino acid levels to be maintained above their transmembrane equilibrium distribution. The latter type of transporter facilitates the selective movement of amino acids across the plasma membrane independent of sodium. DMSO has been shown to have an inhibitory effect on sodium pumping in multiple cell types (Jourdon et al., 1986; McLarnon et al., 1986; Nakahiro et al., 1992; Ogura et al., 1995) and is believed to do so by directly blocking these transporters and preventing sodium transport. As the sodium dependent

glutamine transporters require this ion to be present in order to shuttle the amino acid across the membrane it could help explain why there is such a significant reduction of cytoplasmic glutamine.

The physiological consequences for the cell as a result of reduced glutamine levels will be diverse as highlighted by the wide array of cellular processes it is involved in. Metabolomically one of the downstream effects was on polyamine metabolism (Figure 5.10). Ornithine is the metabolite precursor for this pathway and had decreased levels due to the metabolites that synthesise it from the urea cycle and glutamine metabolism having reduced abundance. Polyamines appear to be regulators of the cell cycle with increases in ODC and polyamines occurring at the G1/S and G2 phases (Wallace et al., 2003). Their exact role remains speculative but have been suggested to regulate cyclin degradation (Thomas and Thomas, 2001). Clearly dysregulation of polyamine synthesis will have an effect on the progression through the cell cycle and decreased levels may lead to premature senescence. It is important to take these metabolomic alterations into consideration when using DMSO as a control in pharmacological studies, which includes much of the research surrounding HIV-PIs on cell culture. The multiple effects it appears to have may explain some of the phenotypes thought to be solely responsible for the action of the HIV-PI and farnesylated prelamins A effect.

Metabolomics is a powerful tool for understanding how the flux of biochemicals is being dysregulated in drug treated cells. The effects seen as a result of saquinavir treatment may not solely be down to farnesylated prelamins A build up, as the drug may have other off

target effects that dysregulates other components of the cellular machinery. The study could be repeated in *Lmna*<sup>-/-</sup> cells treated with saquinavir to see if the identified changes are still affected. However, many of the alterations in metabolism support the literature in the effect these drugs are having on lipid metabolism, glycolysis and inflammation. The parallel between the symptoms displayed by both HAART patients and sufferers of certain *LMNA* mutations suggests that improperly processed lamin A may be the root cause of the symptoms common to both conditions.



## CHAPTER 6

### GENERAL DISCUSSION

Nuclear structure, organisation and function are areas of cell biology that received increasing amounts of attention as molecular and cell biology matured and the tools to fully assess these areas were developed. The nucleus is a highly organised structure that coordinates many levels of regulation in order to determine rates of gene expression and subsequent protein translation. It contains both proteins with redundant functions as well as proteins that are specific and critical for proper functioning of the nucleus, with improper activity of these more specific proteins potentially having detrimental consequences for the cell and organism.

The nuclear lamina is gaining increasing recognition as a critical player in many aspects of metazoan cell biology. The evidence of its multiple roles for maintaining cellular homeostasis offers exciting new frontiers of research and a more complete understanding of how the genome is organised and regulated. Lamin A is just one component of the nuclear lamina, yet the laminopathies have revealed how mutations along *LMNA* can present severe phenotypic changes that may be specific to one tissue or affect multiple tissues, depending on where the mutations reside along the gene.

The accumulation of full length farnesylated prelamin A, as a result of improper processing by ZMPSTE24/FACE1, has been reported in normal ageing, HIV therapy and certain laminopathies (Coffinier et al., 2007; Moulson et al., 2005; Scaffidi and Misteli, 2006). One common phenotypic feature between these conditions is the development of an NR that is used as a diagnostic feature for the accumulation of this unprocessed protein. The fact that dysmorphic nuclear shape can be restored to a smoother perimeter after FTI treatment (Fong et al., 2006) initially suggested that the development of NR is dependent on the farnesylated tail of prelamin A. However, the research conducted in this thesis reveals for the first time that in the context of acute *Zmpste24* down regulation the development of a NR requires both farnesylated prelamin A and the phosphatidylcholine synthesising enzyme CCT $\alpha$ . This suggests that altered nuclear shape and the development of an NR are separate events.

CCT $\alpha$  has been shown to be critical for the development of a NR in the nuclei of cells under normal conditions and requires a lamin scaffold to produce the invaginations. The catalytic activity of the enzyme appears not to play a significant role in the production of a NR and it may function similarly to epsin during invagination of the plasma membrane (Gehrig et al., 2008). This has been further validated recently when GFP-progerin expressing cells were shown to develop NR that colocalises with CCT $\alpha$ , yet there was no change in phosphatidylcholine synthesis (Gehrig and Ridgway, 2011). The retention of the farnesylated tail on prelamin A may elicit initial membrane curvature that does produce a convoluted boundary, as indicated by the low nuclear contour ratio of *Zmpste24* and *Ccta* siRNA cotransfected cells. However, for complete NR development

CCT $\alpha$  must also be present at the point of invagination. It was clear from the immunofluorescence that CCT $\alpha$  did not colocalise along the entire length of the invaginations but was instead localised to certain regions of the NR. These foci may maintain the leading edge of the NR into the nucleus as well as form at points along the NR where further branches can develop.

The fact that these structures develop during acute accumulation of low levels of farnesylated prelamins A highlights how sensitive the nuclear membrane and its architecture are to changes in lamina composition. This compositional alteration significantly reduced the dynamic characteristic of lamin A at the INM, which can have multiple effects on the functioning of the cell, especially as a nucleoplasmic pool of lamin A has been shown to be critical for regulating cell proliferation (Pekovic et al., 2007). There was also a significant change of NPC distribution, as some were found to be incorporated into nuclear invaginations, which reduced their frequency at the periphery. The presence of the NPCs bridging the INM and ONM in type II nuclear invaginations would still enable them to fulfill its functional role as their environment and organisation is essentially the same as the NPCs found at the periphery. However, the presence of presumptive NPCs in type I invaginations is a topological conundrum as how does the cytoplasmic face of the NPC end up in the INM with a nucleoplasmic to nucleoplasmic orientation?

The presence of two of the nucleoporins, the dimensions of the fenestrations and the symmetry of electron density revealed from the tomography data all indicate that an NPC

is present, however how complete it is can only be fully answered by doing extensive immunogold labelling against nucleoporins specific to the cytoplasmic or nucleoplasmic face. One could envisage that expansion of the INM, in an area deficient of LINC complexes, could put sufficient strain on the nucleoplasmic side of the NPC and pull the two faces apart, leading to just the nucleoplasmic half residing in these INM invaginations. NPCs present in these type I invaginations will clearly be unable to carry out their role of transport. They could be there as a pathological consequence of farnesylated prelamin A production and the reorganisation of the NE as NR develops. However, they could serve a transcriptional role as there is increasing evidence of nucleoporin involvement in regulating gene expression (Kalverda et al., 2010). The sheets of type I invaginations that are studded with NPCs could regulate the expression of certain genes, while the type II invaginations, with their cytoplasmic core and NPCs bridging both nuclear membranes, could enable more rapid export of the mRNA and translation in the core. However, much more research will have to be conducted around the role of the NR in mRNA export and translation in future experiments.

The presence of type I invaginations would also enable a larger calcium pool to be delivered to the nucleoplasm for calcium mediated gene expression. IP3 and RyR receptors have been localised to the INM and calcium stores are found in the IMS due to its continuity with the ER lumen. The presence of a type II invagination would provide localised calcium release into the nucleoplasm. However, as it also contains a cytoplasmic core there is less volume for high levels of calcium ions to build up if the equivalent space was just occupied by the IMS and infolding of the INM, as found with

the type I invaginations. Localised calcium release by NR has received much research but the potential of one type of invagination being favoured over the other is yet to be explored functionally and mechanistically.

At the transcriptional level acute farnesylated prelamins A accumulation does reduce expression of SREBP-1 target genes by tethering the transcription factor at the nuclear periphery, reducing its dynamics and preventing it from reaching binding sites at the promoters. It was clear that genes critical for generating precursors for triglyceride synthesis as well as the PPAR $\gamma$  adipocyte differentiation factor were significantly down regulated, which lead to reduced levels of markers of adipocyte differentiation. The metabolomic data also supported this observation as there were increased levels of lipolytic products. The downstream consequences of this may underly the mechanisms associated with lipoatrophy in patients receiving HAART as well as in certain laminopathies.

It is perhaps encouraging that the HIV PI darunavir has been reported to not inhibit ZMPSTE24. This offers an insight into the properties of HIV protease inhibitors that allow certain types to affect the active sites of both the HIV aspartate protease and the Zinc metalloprotease 24. However, all HAART regimens also require ritonavir to be co-administered to prolong the half life of HIV PIs. As this drug has been shown to lead to prelamins A accumulation an alternative must be developed to completely remove the effect of prelamins A associated lipoatrophy. There is currently no FDA or EMEA approved alternative to ritonavir, but another PI booster, called Cobicistat (Gilead

Sciences), is currently in phase III trials that could be just as effective as ritonavir and may not inhibit ZMPSTE24, due to the different structure of the compound.

Despite a potential mechanism for lipoatrophy being elucidated in the regions of the body containing subcutaneous fat there is yet to be one proposed for lipohypertrophy (fat deposition). This condition is also reported in the same individuals suffering with lipoatrophy but in different regions of the body; commonly affecting brown adipose tissue and visceral fat, which are located at the upper back (buffalo hump) and the torso respectively. The condition is prevalent enough in HAART patients that late last year Tesamorelin (Theratechnologies) was the first and only drug approved by the FDA to reduce regions of excess fat deposition (Grunfeld et al., 2011; Traynor., 2010). The different environment that these fat tissues occupy within the body, compared to subcutaneous fat, may be the reason why prelamin A build up doesn't elicit its lipotrophic effect. The exact mechanism behind lipohypertrophy, however, remains to be determined.

*Klotho* has been receiving increasing attention as a gene involved in the normal ageing process. Recently it was shown *in vitro* and *in vivo* that KLOTHO suppressed IL-6 mediated ageing related inflammation. An unexplored avenue of lamin research is a potential link between KLOTHO, ageing and the nuclear lamina. The fact that prelamin A build up indirectly down regulates *Klotho* gene expression and that the metabolomic data revealed a significant up regulation of prostaglandin E2, which positively regulates IL-6 levels, suggests another mechanism and potential therapeutic target for both normal and accelerated ageing.

The metabolomic data are the first of their kind in the context of HIV PI therapy. This kind of high throughput large scale screening of many different metabolites will become an increasing feature of research into many diseases, so as to gain a greater understanding of the molecular pathology that may reveal novel therapeutic targets. The metabolomic data reinforced many of the biochemical and molecular results obtained surrounding the effects of HIV-PIs and prelamins A accumulation. The observation that there was increased lipolysis in these cells could offer a potential new target against either ATGL or its activator CGI-58. Alternatively upregulation or increased activation of the ATGL inhibitor G0S2 may act as another therapeutic target. Inhibition of ATGL could alleviate the lipodystrophy experienced in subcutaneous adipose tissue but would have the detrimental effect of exacerbating the lipohypertrophy in the visceral fat regions. More research will have to be done into regulating fatty acid levels that could aid with the symptoms experienced in HAART and laminopathies.

The increased level of glycolytic metabolites suggests both increased import of glucose by GLUT transporters and a greater dependency of the cell to use this pathway as an energy source. If HIV-PI action is damaging the function of the mitochondria, as has frequently been reported, then the cell will become more dependent on glycolysis as a mechanism of energy generation as observed in various cancer cell types. If FTI treatment reverses the mitochondrial damage then one would expect glycolysis flux to be decreased if co-administered with saquinavir. Why farnesylated prelamins A should have an effect on mitochondrial viability is an avenue of future research. However, the FTI

treatment is not specific to prelamin A as it will also affect other farnesylated proteins in the cell. As a result the restoration of mitochondrial activity may be due to unfarnesylating other proteins in the cell and have nothing to do with prelamin A. Treating *Lmna*<sup>-/-</sup> cells with saquinavir would truly elucidate the involvement of prelamin A in mitochondrial damage after HIV-PI treatment, as lamin A is the only known substrate for the enzyme. Additionally the large effects that DMSO has on metabolite levels acts as a reminder of the dysregulation this solvent causes, which should be remembered when it is used as a control in many of the HIV-PI studies.

The data presented in this thesis have covered the morphological, biochemical and molecular alterations that occur as a result of farnesylated prelamin A accumulation and elucidated potential mechanisms that underly these changes. An understanding of the mechanisms that lead to the devastating symptoms of the laminopathies and acquired lipodystrophies will help with paving the way for developing therapeutics for patient's suffering with these diseases as well as give a greater understanding of the regulation of normal cellular processes.



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